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DE LOS MECANISMOS MOLECULARES A LA MORFOGÉNESIS, EL DESARROLLO Y LA EVOLUCIÓN: EL CASO DE LA FLOR HOMEÓTICA DE *LACANDONIA SCHISMATICA*

TESIS

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RESUMEN

El estudio de *Lacandonia schismatica* (Lacandoniaceae/Triuridaceae) es relevante dentro del contexto de la evolución del desarrollo de las angiospermas dado que esta especie pertenece al único género conocido donde se ha documentado una heterotopía floral única: la presencia de estambres centrales y carpelos laterales en una flor bisexual. En esta investigación doctoral se abordaron las bases genético-moleculares relacionadas con el papel de los genes homeóticos *LsAPETALA3 (LsAP3) LsPISTILLATA* y *LsAGAMOUS* en la formación de estambres centrales, así como la regulación en *cis* de *LsAP3*. A su vez, se llevó a cabo un análisis filogenético al interior del orden Pandanales con el fin de establecer escenarios de evolución floral en Triuridaceae. En el capítulo 1 se presenta un resumen de los antecedentes más importantes sobre estudios de evolución del desarrollo de las flores; en el capítulo 2 se analizó la base molecular que subyace al desarrollo de estambres centrales en *L. schismatica*, en donde se ha podido determinar que el desplazamiento de uno de los genes de la función B al centro del meristemo floral *(LsAP3*) aunado al traslape con la función C, es suficiente para explicar la heterotopía floral de esta especie. A su vez, se abordó el papel de la regulación en *cis* de *LsAP3*, presentando aquí datos preliminares. En el capítulo 3 se resumen los análisis filogenéticos del orden Pandanales realizados mediante el uso de marcadores morfológicos y moleculares, donde Triuridaceae está más cercanamente emparentado con Velloziaceae que con otras Pandanales. En este contexto se describen hipótesis alternativas sobre las posibles transiciones florales ocurridas en el ancestro de *L. schismatica*. En el último apartado de este documento (capítulo 4) se presentan las conclusiones generales y perspectivas sobre investigaciones complementarias a las realizadas para esta tesis.

ABSTRACT

The study of *Lacandonia schismatica* (Lacandoniaceae/Triuridaceae) is relevant within the context of the study of the evolutionary developmental biology of angiosperms, as this species is part of the only known genus where a unique floral heterotopy has been documented: the development of central stamens surrounded by carpels. In this doctoral research the molecular bases of this phenotype were addressed through the analysis of the role of homeotic genes *LsAPETALA3 (LsAP3) LsPISTILLATA* and *LsAGAMOUS* in the formation of central stamens. The *cis* regulation of *LsAP3* was also investigated. In a second part of this thesis a phylogenetic analysis of the Pandanales was elaborated with the aim of establishing different scenarios of floral evolution within Triuridaceae. Chapter 1 reviews the most relevant studies on the evolution of floral development; in chapter 2 the molecular basis of central stamen formation in *L. schismatica* was addressed, where it was determined that the displacement of the domain of expression of B function (*LsAP3*) to the central whorl of the floral meristem together with the C function is the explanatory mechanism of the floral heterotopy of this species. Additionally, the role of *cis* regulation of *LsAP3* was analyzed and preliminary data is presented. In chapter 3 a review of the phylogenetic analyses based on molecular and morphological chapters was undertaken; the main findings in this section is that Triuridaceae are more closely related with the flower-baring family Velloziaceae than with other Pandanales. In this context, alternative hypotheses of the floral transitions that could have occurred in the ancestor of *L. schismatica* are discussed. The last section of this work (chapter 4) presents general conclusions as well as perspectives on complimentary investigations to this thesis.

1. INTRODUCCIÓN

La presente tesis se circunscribe dentro del marco conceptual de la evolución del desarrollo (cuyo apelativo en inglés es *evo-devo*), con énfasis en la evolución del desarrollo de la flor. La evolución del desarrollo tiene como objetivo central el entender la evolución de los mecanismos de desarrollo en un marco conceptual de biología evolutiva, para estudiar la evolución morfológica. En evo-devo se integran conocimientos e información generados en diferentes áreas de la biología como la embriología, la genética, la biología molecular, la fisiología, la evolución y la paleontología, entre otros, con el fin de entender los patrones de desarrollo y su papel en la diversificación morfológica de diferentes linajes de seres vivos. La evolución del desarrollo considera tanto los aspectos proximales o mecanísticos del desarrollo, como las explicaciones últimas o evolutivas de los patrones de diversificación morfológica resultantes.

Con esta perspectiva en s mente, esta tesis aborda el tema de la evolución del desarrollo floral de *Lacandonia schismatica* a partir de diferentes aproximaciones complementarias. En este capítulo (1) se considera un artículo conceptual e integrativo previamente publicado (Piñeyro-Nelson et al., 2010) sobre el estado del arte del conocimiento disponible sobre *L. schismatica* hasta 2010, apuntando a los debates generados a partir de la descripción de esta planta, así como las preguntas pendientes de ser abordadas e, idealmente, resueltas. Como preámbulo a este artículo, se presenta una introducción general sobre la construcción del conocimiento en botánica y taxonomía, así como sus impactos en estudios posteriores de morfología floral. La última sección trata sobre las teorías recientes sobre el origen evolutivo de la flor y los fenómenos de homeosis floral, fenómenos que cobran relevancia en esta tesis por ser la investigación sobre la evolución de la flor de *L. schismatica* el tema central de esta tesis. En el capítulo 2 se presenta la investigación en torno a los mecanismos proximales moleculares del desarrollo floral único de *Lacandonia schismatica,* y en particular se centra en los posibles efectos de la regulación diferencial del promotor del gen *LsAP3* en el desplazamiento del dominio de expresión del RNAm de *LsAP3* al centro del meristemo floral. Como antecedente a esta investigación, se presentan tres publicaciones anexas en las que participé de manera importante durante el doctorado. En

la primera publicación se integra el conocimiento disponible hasta 2010 en torno a la genética del desarrollo floral a partir de estudios realizados en la especie modelo *Arabidopsis thaliana* (Álvarez-Buylla et al., 2010a), así como la caracterización molecular y funcional de los genes involucrados en la formación de estambres en *L. schismatica* (*LsPI* y *LsAP3*; Álvarez-Buylla et al., 2010b). El tercer artículo anexo resume la información morfogenética disponible para *L. schismatica* y ahonda en los escenarios moleculares alternativos que ayuden a explicar el desarrollo de estambres centrales en *L. schismatica* (Garay-Arroyo et al., 2012). En el capítulo 3 se investiga el contexto histórico en el que se originó el fenotipo homeótico de las flores de *L. schismatica*. Este estudio permite un estudio comparativo con base en hipótesis filogenéticas que se plantean a partir de un análisis de marcadores moleculares y morfológicos para investigar las afinidades de Triuridaceae dentro del orden Pandanales (Piñeyro-Nelson et al., en preparación). El último capítulo (4) integra los conocimientos fundamentales de los primeros tres capítulos dentro del marco de la *evo-devo* y presenta las perspectivas de investigación que se vislumbran para complementar las investigaciones que resumen en esta tesis doctoral.

1.1. Antecedentes al estudio de la evolución del desarrollo en plantas: esquemas clasificatorios, taxonomía y morfología comparada.

El origen del estudio de los procesos de desarrollo en las plantas, tiene como antecedente fundamental el establecimiento de esquemas clasificatorios, de morfología comparada y eventualmente, de sistemática. El primer ámbito de estudio del ciclo de vida y desarrollo de algunas plantas partió de las observaciones realizadas para poder establecer la agricultura en las diferentes sociedades humanas. Durante este proceso, necesariamente se generaron esfuerzos de ordenamiento y categorización de los seres vivos guiados por la observación de las variaciones entre diferentes especies de plantas y animales, dando paso a los primeros sistemas de clasificación, basados en el parecido externo entre individuos o en características útiles para el hombre. Este fenómeno produjo sistemas de clasificación particulares en diferentes culturas donde un aspecto central ha sido el énfasis en el valor de uso (Smyth, 2005). Dado lo anterior, los criterios de lo considerado "importante" *vs* lo no importante estuvieron estrechamente relacionados con si una planta o animal cumplía la función de proveer a los seres humanos con abrigo, alimento, combustible, medicinas,

materia prima para construcción y elaboración de artefactos y vivienda, entre otros criterios (Pavord, 2005).

En las plantas, el primer ejercicio de categorización que intentó establecer las partes mínimas de algo que pudiera llamarse "planta", así como un ordenamiento en función de su parecido y lo que ahora podríamos llamar nicho ecológico, fue el trabajo hecho por el filósofo griego Teofrasto, alrededor del 300 a. C. Si bien autores posteriores retomaron parte de la lógica de Teofrasto en la elaboración de los compendios de plantas útiles (*herbals*) y autores como Plinio, cuyo trabajo influenció grandemente a los estudiosos de la edad media en Europa, retomaron muchos de los conocimientos vertidos ahí, el énfasis continuó siendo en documentar e ilustrar aquellas plantas que eran útiles al ser humano. Conforme se fueron estableciendo universidades y jardines botánicos en diferentes partes de Europa (el primer jardín botánico de occidente estuvo en Padua, Italia, en el S.XV), el interés clasificatorio fue cambiando gradualmente a clasificar todos los seres vivos en general (Pavord, 2005).

Este énfasis en clasificar a los objetos naturales (tanto seres vivos como rocas, compuestos químicos, etc.) se potenció durante el Renacimiento, cuando el descubrimiento de nuevos territorios por parte de diferentes imperios europeos implicó el contacto con plantas y animales exóticos que no cabían en los esquemas de clasificación disponibles (muchas de las especies encontradas no tenían un referente medianamente parecido en el contexto europeo), lo que obligó a muchos estudiosos de la época a evaluar la pertinencia de los sistemas clasificatorios disponibles (Walters, 1961; Pavord, 2005). Posteriormente, durante la Ilustración, los especímenes llevados a Europa a través de las numerosas expediciones científicas realizadas en América, África y Asia, en conjunto con el énfasis en la organización y compilación del conocimiento "universal" en enciclopedias, favoreció la elaboración de nuevos esquemas de clasificación que por un lado intentaban reflejar las relaciones entre los diferentes seres vivos dentro de un orden jerárquico y místico ("la gran cadena del ser"), mientras que por otra parte se buscaban esquemas que fueran prácticos para ser utilizados por los diferentes naturalistas involucrados en el estudio de los seres vivos (Walters, 1961, Stevens, 1997).

Si bien varios esquemas de clasificación fueron propuestos para organizar la diversidad de formas existentes en la naturaleza, el sistema que fue rápidamente acogido en su tiempo y que sentó las bases del sistema clasificatorio actual, es aquel propuesto por Carolus Linneaus en su obra *Systema Naturae* (1735) donde se sentaron las bases para la taxonomía moderna. En el caso de las plantas, los fundamentos epistémicos y filosóficos de su esquema clasificatorio serían ampliados y refinados en su monografía *Species Plantarum* (1753).

El sistema de clasificación de Linneaus propuso dos innovaciones importantes: primero, el uso de los nombres únicos y binomiales, donde el primer epíteto aludía al género y el segundo a la especie particular. Esta estrategia pretendía además evitar la plétora de sinónimos para enunciar a la misma entidad, lo que generaba confusiones entre los botánicos de la época. Esta implementación tuvo dos efectos secundarios: que se afianzara el criterio tipológico para asignar especies discretas y que se sistematizara la noción de que existían entidades más cercanamente relacionadas entre sí que podían ser agrupadas en un mismo género (intentando reflejar relaciones naturales de similitud entre organismos). La segunda aportación fundamental fue el establecer como uno de los criterios normativos de clasificación el estudio de las estructuras reproductivas de las plantas, utilizando de manera sistemática los órganos sexuales, particularmente el número y posición de los estambres, así como el número de pistilos (Smyth, 2005) como los órganos sexuales masculinos y femeninos (Stevens, 1997).

Pocos años después, Antoine-Laurent de Jussieu refinó el esquema de clasificación para las plantas (incluidos hongos y algas), que ahora estaba basado en reflejar las relaciones "naturales" (de parentesco) entre ellas, proponiendo la naturalidad de los rangos superiores utilizados en los esquemas clasificatorios (desde género y familia hasta clase). Este trabajo, publicado en 1774 es el *Genera Plantarum,* cuya lógica sigue normando muchos de los esfuerzos clasificatorios actuales (Stevens, 1997).

Los avances descritos en los esquemas clasificatorios implicaron un cambio sustancial entorno a qué características de las plantas eran relevantes para el proceso de clasificación. El énfasis en la flor y sus órganos, junto con los avances en los instrumentos para hacer observaciones a nivel celular (fundamentalmente las lupas y microscopios) favorecieron el

estudio minucioso de los diferentes tejidos que conformaban a las plantas, dando paso a lo que serían la morfología y embriología comparadas (Kaplan, 2001). Estas innovaciones tecnológicas permitieron el descubrimiento de la reproducción sexual en las plantas por parte de Rudolph Jacob Camerarius (1665–1721), ya que hasta entonces el proceso no era entendido y el carpelo era visto como antecedente (quizá vegetativo) del fruto. A partir de este momento y con la sistematización de observaciones morfológicas, comenzarían a articularse diferentes hipótesis entorno a los mecanismos de desarrollo que unificarían a las diferentes especies (Kaplan, 2001). En el caso de la flor, otro acontecimiento importante fue la documentación del proceso de fertilización de la célula madre por polen dentro del saco embrionario y la observación de que este fenómeno daba paso al desarrollo del fruto, fenómeno observado por Robert Brown en 1833 y que sentaría las bases para la morfología floral comparada (ver Smyth, 2005 y referencias ahí contenidas).

Otro antecedente conceptual que resultó ser muy importante en el estudio del desarrollo de las plantas fue la propuesta articulada por Goethe, quien con base en estudios de morfología comparada y fisiología, propondría la teoría "foliar" (el órgano primigenio fue llamado por Goethe *Blatt*) cuya tesis considera que los diferentes órganos tanto estériles como fértiles de las plantas terrestres se derivan de modificaciones de un órgano laminar (*Blatt*) parecido pero no reducido a la hoja, que sería la estructura básica de construcción de una planta (ver prefacio de Miller [2009] a la obra de Goethe [1790]). Esta estructura foliar –como estructura platónica ideal- daría paso mediante diferentes fusiones y transformaciones a todos los órganos de las plantas (Goethe, 1790; traducción al inglés de Miller, G., 2009). La noción de un origen derivado a partir de una estructura básica de todos los órganos presentes en las plantas, acoplado con las nociones sobre evolución que serían propuestas por Darwin a partir de 1859, tendrían un impacto directo en las teorías subsecuentes en torno a la evolución de las plantas terrestres y en particular, en la hipótesis de origen de la flor. Más recientemente, las teorías de Goethe se han confirmado con estudios de genética del desarrollo que han demostrado que el desarrollo de los órganos florales es regulado por una red que subyace el desarrollo foliar, que se revela cuando se desactivan algunos de los genes específicos de la flor (Pelaz et al., 2001).

1.2. Origen y evolución de la flor

El grupo de las Espermatofitas o plantas con semilla (originadas hace 380-325 Millones de Años Antes del Presente; MAAP; Bell y Hemsley, 2000) está dividido en gimnospermas, angiospermas y varios linajes de plantas fósiles como las Bennetitales, Pteridospermas, Caytoniales, y Glossopteriales, por nombrar a algunas (Bell y Hemsley, 2000).

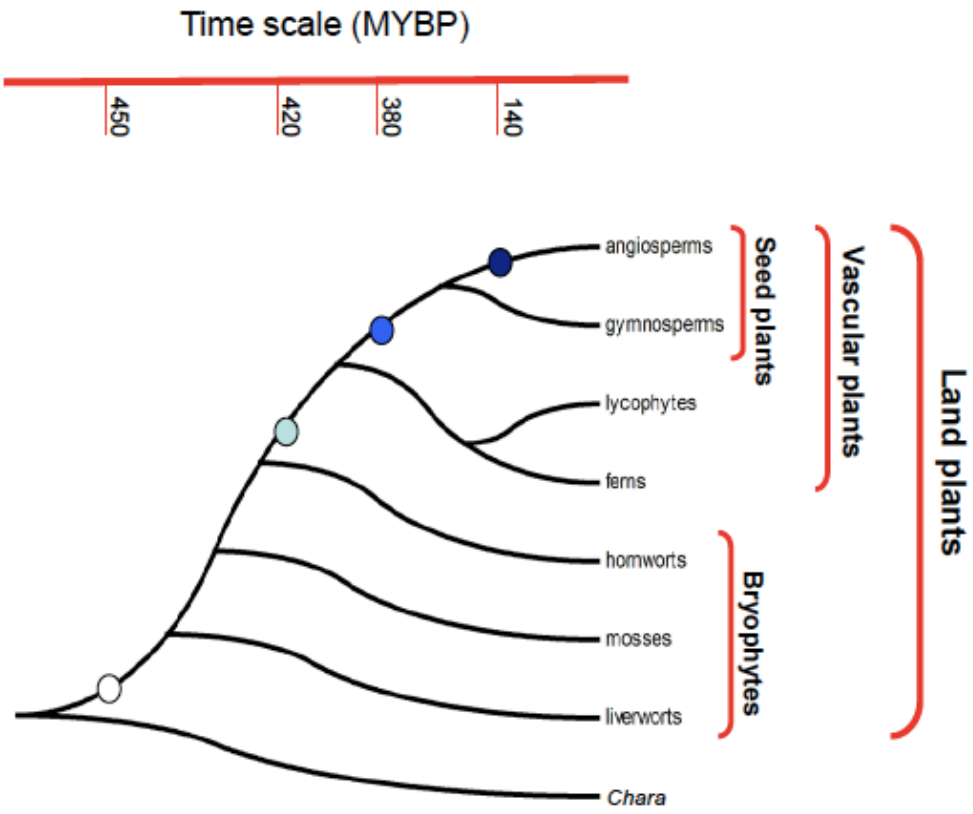

Figura 1. Árbol filogenético que resume puntos clave en la historia evolutiva de las plantas terrestres
Árbol filogenético de la evolución de las plantas terrestres sobre el cual los nodos coloreados marcan algunos eventos de especiación. Nodo blanco: origen de las plantas terrestres; nodo azul claro: origen de las plantas vasculares; nodo azul, origen de las espermatofitas; nodo azul oscuro: origen de las angiospermas. Aquí, *Chara spp*. del orden Charales es usada como grupo externo, ya que esta especie ha sido utilizada para enraizar diferentes filogenias moleculares recientes. La topología del árbol está basada en los estudios de Soltis y colegas (1999) y de Nickrent y colegas (2000). La escalas de tiempo están en Millones de Años Antes del Presente (en inglés: MYBP) y fueron tomadas de Judd y colaboradores (2002). (Modificado de Álvarez-Buylla et al., 2010; ver artículo anexo en capítulo 2 de esta tesis).

En el caso de las angiospermas, las primeras estructuras florales encontradas hasta el momento en el registro fósil son de hace 160-147 MAAP (Frohlich, 2006), dejando un periodo prolongado en el que no se detectan formas intermedias que ayuden a explicar la evolución floral temprana de este grupo, lo que aunado al rápido proceso de especiación observado en especies contemporáneas que, dando lugar a una gran diversidad de formas y síndromes florales, están ausentes en el registro fósil conocido, constituye el famoso "misterio abominable" de Charles Darwin (para una reflexión, ver Friedman, 2009).

Las angiospermas comparten con otras plantas terrestres la heterosporia, o la existencia de dos tipos de esporofitos diferenciados en megagametofito (donde se desarrollan los gametos femeninos) y un microgametofito (donde se forman los gametos masculinos), así como una reducción progresiva del gametofito (es decir, las estructuras reproductivas) y la presencia de un embrión diploide. Si bien estas características son compartidas entre los diferentes linajes de espermatofitas, tanto actuales como extintas, las características definitorias de una flor son: (1) un carpelo cerrado (sea por fusión congénita, posgenital o por secreciones/tricomas) que contiene uno o más óvulos comúnmente bitégmicos; (2) una nucela que contiene el saco embrionario dentro del cual, después de la doble fertilización, se desarrolla un embrión diploide rodeado de un endospermo triploide. Por último, las estructuras presentes en (2) conforman los estratos presentes en la semilla (Judd et al., 2002). Otra característica relevante es la existencia de hermafroditismo verdadero (Judd et al., 2002; Frohlich, 2006). Si bien hay linajes de angiospermas que varían en alguno de estos criterios (como puede ser óvulos unitégmicos, gametofito femenino compuesto por 9 en lugar de 8 núcleos [*Amborella trichopoda*; Friedman 2006] o ploidías superiores al 2n o 3n, como ocurre en numerosas poales y especies dentro de las familias Malphigiaceae, Myricaceae, Berberidopsidaceae, entre otras; Hanson et al., 2001; Judd et al., 2002), la posición relativa de los órganos florales (sépalos, pétalos, estambres y carpelos) y la estructura general del carpelo están ampliamente conservadas, excepto en casos excepcionales de homesis floral, tal como ocurre en la especie de estudio de la presente tesis, *Lacandonia schismatica* (ver sección 1.4.). Dada la importancia de la flor como estructura de interés taxonómico así como la acumulación de estudios de morfología y embriología comparada en diferentes linajes de espermatofitas, tanto actuales como extintas, aunado al impulso provisto por la caracterización de genes homeóticos involucrados en el establecimiento de los órganos sexuales en las antofitas, varias teorías

morfogenéticas sobre el origen de la flor han sido desarrolladas en los últimos veinte años (Frohlich, 2006). A continuación hacemos una breve síntesis de dichas teorías, con especial énfasis en aquellas que incorporan información proveniente de la genética molecular del desarrollo.

Entre 1980 y 1990, varios autores plantearon de manera paralela y complementaria, que las Gnetales y Angiospermas eran linajes hermanos que conformaban (comúnmente junto con el linaje fósil Benettitales) a las "antofitas" o plantas con estructuras parecidas a la flor (Loconte y Stevenson, 1990). Dicho planteamiento se derivaba de estudios de morfología y embriología comparadas de diferentes linajes de espermatofitas (incluyendo linajes extintos que poseen un registro fósil), cuyos datos fueron integrados en un análisis cladista (Loconte y Stevenson, 1990). En la teoría antofítica, la topología presente en los cladogramas recuperados sugería que las angiospermas muy probablemente se habrían derivado de un ancestro con características similares a las observadas en las Gnetales contemporáneas (para un resumen, ver: Loconte y Stevenson [1990] y Donoghue y Doyle [2000]). Esta propuesta tenía dos implicaciones importantes: que la flor como estructura era producto de un proceso de evolución gradual y que las flores ancestrales eran muy probablemente hermafroditas (a juzgar por las estructuras reproductivas existentes en especies contemporáneas de Gnetales (géneros *Gnetum*, *Ephedra* y *Welwitschia*) mientras que las gimnospermas eran parafiléticas (Loconte y Stevenson, 1990).

Poco tiempo después de esta propuesta, se publicaron los primeros estudios de filogenias moleculares de las espermatofitas. Si bien estos estudios necesariamente excluían a los linajes fósiles, disminuyendo el universo muestreado, estas investigaciones arrojaron un resultado inesperado: las gimnospermas ahora incluían a las Gnetales, las cuales estaban asociadas con Pinaceae, mientras que las angiospermas formaban un clado aparte (ver resumen en Donoghue y Doyle, 2000).

A la par que se publicaban estas propuestas filogenéticas, también se avanzaba en el estudio de las bases genéticas del desarrollo floral, las cuales de manera independiente cuestionaban la teoría antofítica (ver Winter et al., 1999). Dentro de los genes aislados y caracterizados como fundamentales en la ontogenia floral, se encontró a LEAFY (LFY; Wiegel et al., 1992), un factor de transcripción exclusivo de las plantas verdes, que es de

copia única en las angiospermas pero que tiene dos copias en gimnospermas con NEEDLY como parálogo (NLY; Mouradov et al., 1998). En las angiospermas, LFY se expresa primordialmente en el meristemo de inflorescencia y después en el meristemo de flor, donde interactúa con otros factores de transcripción para establecer el "pre-patrón" floral donde se desarrollarán los diferentes órganos florales (típicamente: sépalos, pétalos, estambres, carpelos). En contraste, en gimnospermas el estudio de los patrones de expresión *in situ* del los RNAm del homólogo de LFY en los conos unisexuales de *Pinus radiata* mostraron que se expresa en primordios laterales, brácteas estériles y microgametofitos de conos masculinos, pero no en brácteas y megagamefitos de conos femeninos (Mellerowicz et al., 1998). Estas observaciones, junto con la monofilia de las gimnospermas, llevaron a Frohlich y Parker (2000) a plantear una nueva teoría del origen de la flor (bisexual) en angiospermas: la teoría del *Origen Mayoritariamente Masculino* (Mostly Male Theory). Dado que NLY no se encuentra en angiospermas y que el LFY de gimnospermas se expresa en conos masculinos, los autores plantean que la flor bisexual de las angiospermas se pudo haber originado a partir de un proceso de homeosis en un cono masculino en desarrollo. En este escenario, Frohlich y Parker (2000) plantean que la contracción de la región del meristemo de inflorescencia donde se expresan los genes implicados en el establecimiento de los estambres (gen B más gen C; ver capítulo 2) pudo ocasionar que en las brácteas en desarrollo en la región más apical sólo se expresaran LFY y el gen implicado en el desarrollo de óvulos (gen C), lo que habría posibilitado la formación de óvulos ectópicos sobre las brácteas más internas, las cuales, al cerrarse sobre sí mismas darían origen a los carpelos (cerrados) característicos de las angiospermas (Frohlich y Parker, 2000), y por lo tanto, a flores hermafroditas con carpelos centrales.

Teorías alternativas donde se minimiza la importancia de LFY, y en su lugar se da prioridad a los genes B y C son aquellas que a lo largo de varios años (2003-2010) ha elaborado Theissen junto con sus colegas. La primera propuesta elaborada por Becker y Theissen en 2003 propone, a partir de un análisis de los patrones de duplicación de genes B y C(D) en angiospermas, junto con la observación de estos genes en gimnospermas, una variante de la teoría de Frohlich y Parker (2000): la *Salida de Hembra o Salida de Macho* (Out of Male or out of Female). En esta teoría, Becker y Theissen (2003) argumentan que el mecanismo involucrado en la formación de una flor bisexual estaría más relacionado con la expresión ectópica de genes B en los bordes de una estructura otrora femenina (Salida

de Hembra) o con la heterotopía en la expresión del gen C al centro de una estructura originalmente masculina (Salida de Macho) más que con un gen "genérico" como LFY.

Unos años después, Theissen y Melzer refinan esta hipótesis (2007), focalizando la atención en los genes B como los genes homeóticos que estarían fundamentalmente involucrados en favorecer la aparición de una estructura reproductiva bisexual, donde dado un cambio en su dominio de expresión que favorecería un cambio de los interactores proteicos disponibles para el gen C en la zona más apical de un cono originalmente masculino, (en línea con la noción de que los genes MADS-box ejercen su función regulatoria mediante tetrámeros proteicos llamados "cuartetos florales", noción articulada por el propio Theissen en 2002) se daría paso al desarrollo de una estructura reproductiva bisexual (Theissen y Melzer, 2007). Además, la formación del carpelo estaría relacionada directamente con la duplicación del gen C que se ha observado en la base de las angiospermas; una copia retendría la función ancestral relacionada con la formación de óvulos (dando paso al linaje de genes óvulo-específicos llamados genes D), mientras que la otra copia (el gen C propiamente dicho) tendría funciones más genéricas en cuanto a la especificación de los órganos sexuales, interactuando con genes B (para formar estambres) o sólo con LFY y otros genes para formar los carpelos (Theissen y Melzer, 2007). En un artículo posterior (2010) Melzer y colegas sugieren que otro factor determinante que favoreció la aparición de la "especificidad" de los tetrámeros proteicos involucrados en definir la identidad de los cuatro verticilos florales encontrados comúnmente en angiospermas, es la diversificación del super-clado que contiene a los genes MADS-box involucrados con la determinación de la identidad y agotamiento del meristemo floral: los genes del linaje SQUAMOSA (AP1), SEPALLATA1 (SEP1) y AGL6 (SEPALLATA3; SEP3), que según los autores habrían estado presentes en el ancestro común de gimnospermas y angiospermas, mientras que AP1 y SEP1 se habrían perdido en el linaje que dio pie a las gimnospermas (Melzer et al., 2010). Con esta base, asumen que los genes del super clado SQUA/SEP1/AGL6 son fundamentales para dotar de regulación fina, especificidad y posibilitar el desarrollo de una estructura bisexual mediante la acción de unos genes B y C que en angiospermas (a diferencia de lo observado en gimnospermas), ya no pueden formar tetrámeros proteicos en ausencia de algún gen SQUA/SEP1/AGL6. Esta hipótesis sigue siendo insuficiente para explicar el porqué de la emergencia de estructuras bisexuales comprimidas (flores), donde está fijada la posición de los órganos masculinos

con respecto de los órganos femeninos en la gran mayoría de las angiospermas. La respuesta de los autores es proponer que alguno de los genes de este super-clado equivale a un "morfógeno" (probablemente AGL6, por su acción en *P. abies*), que se expresa en un gradiente apico-basal en el eje reproductivo, al cual son receptivos de manera diferencial los genes B y C (Melzer et al., 2010). De esta manera, los autores regresan a la noción de "morfógeno" asignada a LFY por Frohlich y Parker (2000), añadiendo a este argumento, que la evolución y especialización de los tetrámeros proteicos en angiospermas (que contrastan con la situación en gimnospermas, donde hay menos genes MADS-box II y los SEP1 no son necesarios para la formación de tetrámeros) es lo que ha generado una canalización del fenotipo floral en las angiospermas. Por último plantean que esta especialización de categorías de genes MADS-box y su red de interacciones constituyen un "grano" (*kernel*, en el sentido bioinformático que se le da a este término), en una lógica modular del desarrollo.

Es interesante añadir que la noción de módulos genéticos involucrados en el desarrollo de eucariontes y en particular, en el desarrollo de la flor, ya había sido explorada y formalizada por otros estudiosos (Mendoza et al., 1999; Espinosa-Soto et al., 2004). Bajo esta óptica, se analiza la acción concertada de diferentes genes involucrados en una red de regulación genética (RRG), donde configuraciones génicas particulares y estables (atractores) subyacen a los diferentes tipos celulares encontrados en los cuatro verticilos flores de las angiospermas, así como en los meristemos de inflorescencia y flor (Espinosa-Soto et al., 2004). La delimitación de los genes necesarios y suficientes para recuperar las configuraciones genéticas características de las células primordiales de los órganos florales, que se incluyeron en la RRG, así como las interacciones entre ellos fueron asignados con base en datos experimentales de genes individuales caracterizados funcionalmente en *A. thaliana*, no de simulaciones o interpretación de datos *ad hoc* (Espinosa-Soto et al., 2004). Aún así, la lógica de biología de sistemas, que favorece el entendimiento de patrones de desarrollo que emergen a partir de interacciones génicas específicas entre un grupo de genes homeóticos y/o sus reguladores y/o el ambiente celular, que actúan como módulos durante el desarrollo, en lugar de la estrategia de buscar el "gen maestro" que controle una función particular aún no ha terminado de incidir cabalmente en la comunidad de biólogos experimentales, o en las teorías sobre origen y evolución de la flor. En la comunidad es común la presencia de esquemas de

"cascadas génicas" donde se esquematizan las interacciones positivas o negativas de un grupo de genes que han sido caracterizados como importantes en alguna vía de desarrollo o metabólica, hasta las aproximaciones de secuenciación masiva de genomas o ensayos de expresión génica diferencial (microarreglos, secuenciación masiva de ARNm, etc), pero aún falta articular los resultados obtenidos en modelos dinámicos explicativos del desarrollo, más que en la suma de partes o interacciones entre partes (genes) en esquemas estáticos.

Otra aproximación reciente al estudio de los mecanismos moleculares involucrados en el desarrollo de la flor y sus diferencias con respecto a las estructuras reproductivas en gimnospermas, ha sido el uso de datos producto de esfuerzos de secuenciación masiva. En este sentido, en un estudio reciente de Chanderbali y colegas se investigan las "cascadas transcripcionales" asociadas con el desarrollo de los órganos florales en varias especies ahora consideradas basales dentro de las angiospermas como *Amborella trichopoda* (Amborellales), *Nuphar advena* (Nymphaeales) y *Persea americana* (Laurales [Chanderbali et al., 2010]). Dichos perfiles transcripcionales fueron comparados con la eudicotiledónea *Arabidopsis thaliana* y la gimnosperma *Zamia vazquezii*. De esta última se secuenciaron los transcriptomas de conos masculinos y femeninos (Chanderbali et al., 2010). Este estudio encontró que los perfiles transcripcionales asociados con el desarrollo tanto de perianto como de estambres eran más variables entre gimnospermas y angiospermas basales que las cascadas asociadas a desarrollo de carpelos/óvulos. En el primer caso, las cascadas transcripcionales se parecían más a las gimnospermas a nivel de transcriptoma, ya que se documentó un gradiente continuo de expresión de los factores de transcripción involucrados en los programas de los órganos del perianto y de los estambres, misma que se refleja en el desarrollo de estructuras donde hay una intergradación de pétalos estaminoides, estambres, etc. En contraste, la cascada transcripcional asociada con el desarrollo de los órganos sexuales femeninos –en particular, los óvulos- estaba mucho más conservado entre gimnospermas y todas las angiospermas (incluyendo *A. thaliana*; Chanderbali et al., 2010). Estos resultados a decir de Chanderbali y colaboradores (2010) apoyaban la hipótesis del Origen Mayoritariamente Masculino propuesta por Frohlich y Parker (2000).

Al margen de discernir el proceso evolutivo y morfogenético que dio lugar a la flor primigenia, las angiospermas contemporáneas conforman un linaje muy exitoso evolutiva y ecológicamente, estando presentes en casi todos los ambientes terrestres y en gran parte de los acuáticos (Judd et al., 2002). A su vez, el proceso de especiación dentro de este linaje parece haber sido bastante rápido y haber generado muy diversas formas florales, con variantes importantes en tamaño, color, fenología, síndrome de polinización, entre muchas otras características. A pesar de ello, el 73% de las 250,000 especies conocidas hasta ahora (Drinnan et al., 1994), presentan flores hermafroditas con una arquitectura floral conformada de verticilos concéntricos que de afuera hacia adentro incluyen: sépalos, pétalos, estambres y carpelos (Judd et al., 2002). Este patrón floral o tema morfogenético floral conservado se ha alterado en varias especies; pero el caso mas sorprendente es el que se estudio en esta tesis y se refiere a la homeosis de órganos reproductivos que se encuentra en *Lacandonia schismatica,* cuya flor tiene estambres centrales rodeados de carpelos. En la siguiente sección se abordarán instancias de homeosis floral donde esta configuración estructural se ve modificada.

1.3. Instancias de homeosis floral en angiospermas

La definición amplia de homeosis (término acuñado por Bateson en 1894) se refiere a "la asunción de una parte [de un organismo] a la semejanza de otra parte" (Holmes, 1979, citado en Sattler, 1988). Los cambios homeóticos se refieren a cuando un órgano adquiere la identidad de otro; este fenómeno ha sido observado en todas las estructuras de las plantas, desde las raíces, tallo, hojas, flores, etc. (Sattler, 1988). Las instancias de homeosis parecen ser importantes en moldear la diversidad de antofitas contemporáneas, ampliando el universo de formas existentes en las plantas, que en muchos casos son producto de la respuesta de una planta a su ambiente (Sattler, 1988). En este sentido, varios procesos homeóticos, en particular aquellos que involucran la aparición de órganos quiméricos (como puede ser variaciones en el número de pinas que conforman una hoja compuesta así como hojas individuales que son sustituidas por zarcillos, estípulas, etc. fenómeno documentado en leguminosas; Sattler, 1988) parecen estar estrechamente relacionados con la respuesta de la planta al ambiente físico y suelen englobarse en lo que se ha llamado "plasticidad fenotípica". Estos procesos no suelen basarse en modificaciones

genéticas sino epigenéticas que no siempre son heredables y que pueden ocurrir en un individuo, no en una población.

En la discusión en torno a los procesos ontogenéticos que pueden llevar a fenotipos homeóticos que han sido analizados desde la morfología, se ha considerado la presencia de órganos quiméricos que se desarrollan a partir de la modificación de un primordio floral que toma la identidad del verticilo contiguo (por ejemplo, un primordio de estambre que se diferencia en un órgano petaloide), como evidencia de instancias donde el traslape de programas genéticos que subyacen al desarrollo de cada tipo de órgano floral dan como resultado órganos quiméricos que generan un gradiente morfológico en las poblaciones de una especie que a su vez puede impactar en la evolución gradual de nuevas especies (Ronse De Craene, 2003). En contraposición, existen fenómenos homeóticos donde un órgano es totalmente sustituido por otro, dando pie a un proceso de evolución saltacional donde hay sustitución entre estructuras no homólogas (desde el punto de vista de categorías morfológicas; Ronse De Craene, 2003; Sattler, 1988). *Lacandonia schismatica* constituye un ejemplo extremo de este fenómeno.

En esta sección nos restringimos a revisar los casos de homeosis floral en donde una parte o la totalidad de uno o más verticilos florales adquieren la identidad de otra parte y dicho cambio puede ser trazado al menos parcialmente, a cambios genéticos o epigenéticos que son heredables. Este fenómeno, al afectar a una parte de la población, puede generar impactos evolutivos importantes al incrementar la diversidad de morfos florales existentes en una o varias poblaciones o llevando a un proceso de especiación (Ronse De Craene, 2003).

Dentro de las variantes homeóticas que han sido observadas en la naturaleza, algunas de ellas han sido investigadas para determinar la base genética involucrada (Ronse De Craene, 2003). Si bien en la mayoría de especies modelo se ha observado que la pérdida de función de uno o más genes involucrados en procesos del desarrollo que tienen mutaciones que anulan la transcripción de ARNm o producen proteínas truncas y por lo tanto no funcionales y que subyacen al fenotipo homéotico observado (Bowman et al., 1989; Coen y Meyerowitz, 1991), en otros casos los fenotipos homeóticos han sido relacionados con modificaciones epigenéticas que modulan (habitualmente mediante

silenciamiento transcripcional) la expresión espacio-temporal de genes del desarrollo particulares cuyo estado silenciado puede ser transmitido de una generación a otra (Cubas et al., 1999; Jaligot et al., 2011).

Dentro de las especies cultivadas, existen numerosos ejemplos de mutantes homeóticas que han sido seleccionadas por los agricultores u horticultores por sus cualidades agronómicas o estéticas, y que explican lo abrupto de algunos cambios morfológicos durante la domesticación de plantas cultivadas. Un caso de silenciamiento de genes activos durante el desarrollo floral que han dado paso a fenotipos homéoticos heredables en especies cultivadas ha sido documentado en la palma de aceite *Elaeis guineensis* (Arecaceae; Adam et al., 2007). Esta especie posee individuos monóicos que son funcionalmente dióicos al tener ciclos alternados de producción de inflorescencias con flores hembra o macho. En la variante homeótica bautizada como "mantled", los estambres de las flores macho degeneran a estaminodios estériles y se forma un carpelo generalmente estéril, mientras que en las flores hembra los estaminodios que se encuentran presentes son transformados a estructuras carpeloides estériles (Adam et al., 2007 y Jaligot et al., 2011). Esta homeosis ha sido correlacionada con cambios epigenéticos que afectan negativamente la expresión de los genes relacionados con la formación de estambres ("genes B"; ver capítulo 2) a consecuencia de alteraciones hormonales producto de la propagación somato-clonal de *E. guineensis* para satisfacer las demandas de material forestal genéticamente homogéneo (Jaligot et al., 2011). El fenotipo "mantled" puede revertir de manera espontánea en generaciones subsecuentes (Adam et al., 2007).

Con respecto a mutantes florales que han sido activamente seleccionadas por horticultores por sus fenotipos vistosos están las llamadas "flores dobles", comunes en variedades de rosas ornamentales donde varios verticilos de pétalos/órganos petaloides se desarrollan a expensas de verticilos de estambres (constituyendo mutantes débiles del gen homeótico AGAMOUS que se retrae hacia el centro del meristemo floral; Dubois et al., 2010 y capítulo 2 de esta tesis). Este fenómeno también ha sido documentado en muchas otras especies de ornato, como algunas papaveráceas, liliáceas, etc. (Ronse de Craene, 2003, Pavord, 2005).

A su vez, existen linajes de diferentes plantas silvestres donde se han fijado fenotipos considerados homeóticos con respecto al ancestro común. Un ejemplo de este fenómeno se encuentra en el orden Zingiberales, donde existe una amplia variación morfológica floral, tanto en la simetría como en el número y estructura de órganos del perianto (Kirchoff, 1991). En cuanto al perianto, algunas familias han desarrollado órganos petaloides adicionales –como el labelo-, a costa de transformaciones homeóticas de los primordios de estambres (Kirchoff, 1991). Este proceso llega a tal extremo en algunos linajes de Zingiberales que en la familia Costaceae sólo hay la mitad de un estambre funcional (una teca dentro de una antera originalmente bitecada), mientras que los otros cinco primordios de estambres forman el labelo y dos pétalos laterales (Kirchoff, 1991). Las bases morfogenéticas de este fenómeno han sido recientemente investigadas y se ha sugerido que la sustitución de estambres por órganos petaloides está directamente relacionada con la expansión del dominio de expresión de uno de los genes B (GLO/PI) hacia el centro del meristemo floral (Bartlett y Specht, 2010). En cuanto a la variación en simetría (radial a bilateral), ésta parece estar relacionada con cambios en el dominio de expresión de un gen homeótico de la misma familia génica que CYC: TEOSINTE BRANCHED-1 like (originalmente descrito en maíz; Bartlett y Specht, 2011).

Este fenómeno de pérdida de simetría bilateral también ha sido documentado en algunas especies de orquídeas, donde hay mutantes pelóricos o pseudopelóricos (donde un verticilo del perianto tiene simetría radial pero otros verticilos mantienen la zigomorfía; Mondragón-Palomino y Theissen, 2009). En este linaje, las bases genéticas de los fenotipos observados han sido trazadas a la expresión diferencial de algunos parálogos de genes B que se encuentran extensamente duplicados en algunos linajes de orquídeas, por lo que diferentes combinaciones de genes B dan origen a cada verticilo del perianto. En algunos casos, esta homeosis modifica no sólo los órganos del perianto, si no también los verticilos de los estambres y hasta los carpelos, dando pie a flores con múltiples tépalos pero que son macho-estériles o totalmente estériles (ver ejemplos en Mondragón-Palomino y Theissen, 2009).

En términos generales, las instancias de homeosis que han sido documentadas en estructuras reproductivas pueden ser catalogadas en aquellas donde se pierde la simetría de la flor, comúnmente pasando de una simetría bilateral a una radial (aunque puede ser

en sentido inverso, como el mutante *Radialis* en *A. majus*; Gufstavsson 1979); aquella que atañe al desarrollo de órganos supernumerarios consecuencia de la modificación de uno o más verticilos de órganos florales que adoptan la identidad de los órganos de un verticilo contiguo, favoreciendo, por ejemplo, la transformación de órganos como los estambres en pétalos (como sucede en diversas variedades de rosas cultivadas; Dubois et al., 2010). Un caso extremo de esta transformación puede llevar al desarrollo de flores unisexuales en especies con flores habitualmente hermafroditas, dada la generación de órganos del perianto a expensas de los estambres (como en las "rosas dobles"; Dubois et al., 2010 o en algunas orquídeas; Mondragón-Palomino y Theissen, 2009). También se han documentado casos de flores unisexuales donde se da un cambio en la identidad sexual (por ejemplo, las flores "mantled" de *E. guineensis*; Jaligot et al., 2011).

Si bien muchos de los fenotipos florales descritos corresponden a especies domesticadas o a linajes donde las variaciones homeóticas han sido fijadas durante la evolución y diversificación históricas (Zingiberales, orquídeas), existen especies silvestres donde el proceso de fijación de este fenotipo en subpoblaciones naturales ha podido documentarse dentro de una escala de tiempo humana. Una ejemplo notable se presenta en la especie *Capsella bursa-pastoris* (Brassicaceae) en donde una variante homeótica desarrolla un verticilo adicional de estambres a costa de los pétalos (Hameister et al., 2009). Plantas con este fenotipo han comenzado a formar poblaciones fenológicamente aisladas de las poblaciones tipo silvestre, a pesar de crecer en simpatría (formando subpoblaciones estables; Hameister et al., 2009).

La variación floral más extrema documentada hasta el momento y que ha dado pie a una homeosis *sui generis*, se refiere a la especie objeto de esta tesis: *Lacandonia schismatica*, que representa a una de las dos especies conocidas hasta el momento en donde se ha documentado la presencia de flores bisexuales heterotópicas que poseen estambres centrales y carpelos laterales (Martínez y Ramos, 1989). La otra especie recientemente descubierta pertenece al mismo género y fue encontrada en un fracción de la Mata Atlántica del noreste de Brasil: *L. brasiliana* (Melo y Alves, 2012). Como parte de la investigación doctoral que se resume en esta tesis, la base molecular que subyace al desarrollo de estambres centrales en *L. schismatica* ha sido investigada y se ha podido determinar que el desplazamiento de uno de los genes de la función B al centro del

meristemo floral (*LsAP3*) aunado al traslape con la función C, es suficiente para explicar la heterotopía observada (Álvarez-Buylla et al., 2010b), dado que además los genes B de *L. schismatica* son capaces de determinar estambres en *Arabidopsis thaliana*. Las bases regulatorias que favorecen la expresión central de *LsAP3* son abordadas en el capítulo 2 de esta tesis; en la siguiente sección de este capítulo se presentan las características generales de *L. schismatica*.

1.4. La flor homeótica de *Lacandonia schismatica*

L. schismatica es una monocotiledónea micoheterótrofa situada originalmente en la familia Lacandoniaceae (Martínez y Ramos, 1989), que actualmente es considerada un género dentro de Triuridaceae (Davis et al 2004, Vergara-Silva et al., 2003; Rudall y Bateman, 2006 y capítulo 3 de esta tesis). Esta especie es endémica a la selva Lacandona (Chiapas, México) y habita zonas restringidas de alta humedad, creciendo en montículos de materia orgánica que sobresalen por encima de zonas semi-inundables como turberas (localidad Frontera Corozal), riachuelos someros semi-permanentes (localidad San Javier) o en estrechos de selva mediana perennifolia (localidad Benito Juárez; observaciones personales).

L. schismatica fue descubierta en 1985 por el botánico Esteban Martínez Salas, siendo descrita en 1989 (Martínez y Ramos, 1989). *L. schismatica* marcó un hito en la botánica y ha suscitado gran interés en la comunidad científica, en donde se generó un debate en torno a la identidad floral de las unidades reproductivas de *L. schismatica*, ya que mientras los descubridores de esta planta y otros autores la han considerado una especie con flores verdaderas o euantios (Martínez y Ramos, 1989; Márquez-Guzmán et al., 1989 y 1993; Vergara-Silva et al., 2003; Ambrose et al., 2006), otros han propuesto que podría tener inflorescencias comprimidas o pseudantios (Stevens, 1991; Rudall 2003). En la publicación anexa a este capítulo: ***Development and Evolution of the Unique Floral Organ Arrangement of Lacandonia schismatica*** (Piñeyro-Nelson et al., 2010), se presenta una síntesis conceptual y reseña de los de los avances en el conocimiento de esta especie así como propuestas novedosas acerca de aproximaciones al debate en torno la identidad de sus estructuras reproductivas, e hipótesis originales para explicar la base molecular del peculiar patrón morfogético de la flor de *L. schismatica* que son abordadas en el capítulo 2 de esta tesis.

2. BASES GENÉTICO-MOLECULARES DE LA MORFOGÉNESIS FLORAL ÚNICA DE LACANDONIA SCHISMATICA.

2.1. Modelo ABC de determinación de los órganos florales

La sistematización de los trabajos de genética del desarrollo realizados en mutantes homeóticas de las especies modelo de eudicotiledóneas, *Arabidopsis thaliana thaliana* (Brassicaceae) y *Anthirrinum majus* (Scrophulariaceae) en la década de los ochenta del siglo XX (para un resumen ver: Jack, 2004; Kaufmann et al., 2005; Krizek and Fletcher, 2005; Causier et al., 2010; Bowman et al., 1989; Coen y Meyerowitz, 1991), caracterizaron a un grupo de genes necesarios para la formación de los órganos florales, así como la determinación del meristemo floral (Bowman et al., 1989; Coen y Meyerowitz, 1991). Nos referimos al "Modelo ABC de determinación floral" que agrupa las funciones combinatorias de tres grupos de genes (Bowman et al., 1989; Coen y Meyerowitz, 1991).

El modelo ABC integra la acción combinatoria de cinco genes homeóticos que son necesarios para la determinación de los cuatro verticilos florales comúnmente encontrados en angiospermas: sépalos, pétalos, estambres y carpelos (Bowman et al., 1989). En este esquema, los genes de la categoría A se expresan en los dos verticilos más externos y son necesarios para la determinación de sépalos; en combinación con los genes B, son necesarios para la determinaciónn de pétalos; los genes de la categoría B se expresan en los verticilos de los pétalos y estambres y el gen C se expresa en los verticilos de estambres y carpelos. La combinación de los genes B y C son necesarios para la determinación de estambres y los genes C solos para la determinación de carpelos. Adicionalmente, los genes A y C son mutuamente excluyentes y la expresión del gen C lleva a la diferenciación de todas las células del meristemo floral en los carpelos, y cuando se muta la flor se vuelve una estructura indeterminada (Bowman et al., 1989; Coen y Meyerowitz, 1991).

Aquí se sigue la nomenclatura usada para nombrar a dichos genes homeóticos acorde a los nombres usados en *A. thaliana*. En este sentido, los genes A son *APETALA1* [*AP1*] y *APETALA2* [*AP2*]; los genes B son *APETALA3* [*AP3*] y *PISTILLATA* [*PI*] y el gen C es *AGAMOUS* [*AG*]. Todos los genes mencionados con excepción de *APETALA2*, pertenecen

a la familia de genes que codifican para factores de transcripción con dominio MADS tipo II, que comparten un alto grado de similitud en su dominio MADS con los genes MEF de animales (Álvarez-Buylla et al., 2000). *APETALA2* pertenece a la familia de factores de transcripción AP2/EREBP. En la figura 2.1. se presentan los fenotipos de cada mutante de los genes ABC (modificada de Álvarez-Buylla et al., 2010[a]).

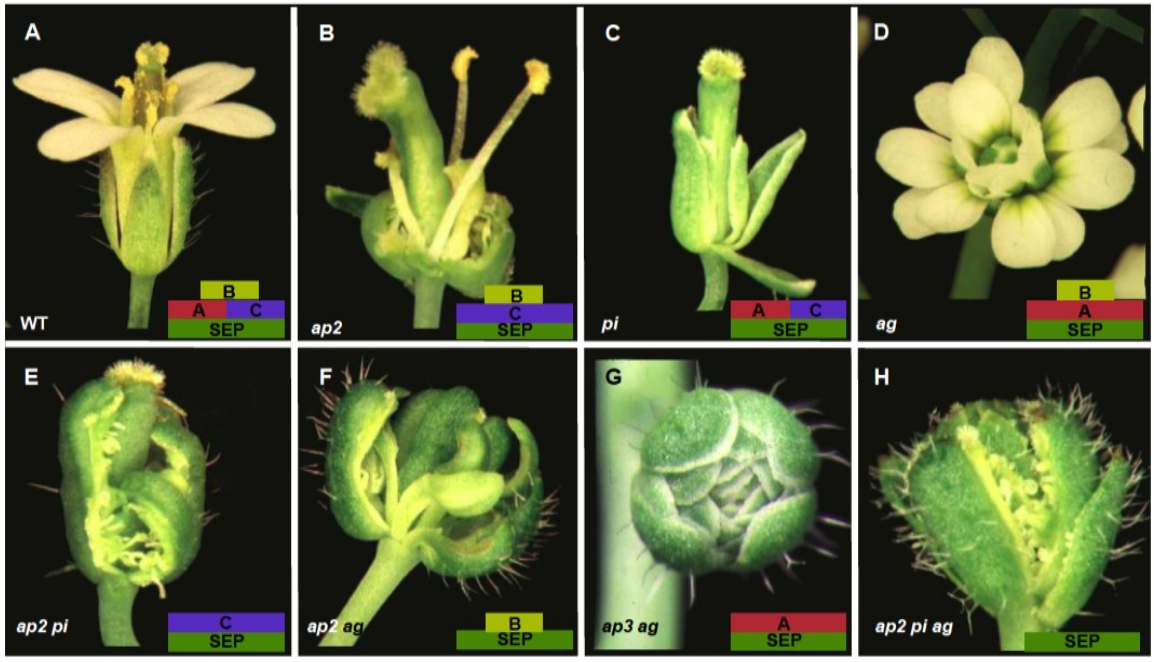

Figura 2.1. Mutantes florales de genes ABC en *A. thaliana*. Se presentan fotografías del fenotipo floral de mutantes ABC sencillos, dobles y triples. Cada foto está acompañada de un esquema donde los rectángulos representan la combinatoria de las funciones transcripcionales de los genes A (*AP1* y *AP2*), B (*AP3* y *PI*) y C (*AG*), así como los genes SEP (llamados genes de función E; *SEP1-4*). A continuación se enlistan los órganos florales presentes en cada mutante así como la identidad de los verticilos florales descritos de afuera hacia adentro, a menos que se especifique lo contrario.
(A) Flor tipo silvestre (WT). Verticilos compuestos por sépalos, pétalos, estambres y carpelos.
(B) Mutante *ap2* compuesta de sépalos carpeloides, sépalos, estambres, estambres y carpelos.
(C) Mutante *pi* compuesta de sépalos, sépalos, carpelos y carpelos.
(D) Mutante *ag* tiene sépalos, pétalos, pétalos y en lugar de carpelo se forma otra flor que repite el mismo patrón de desarrollo.
(E) La mutante doble *ap2 pi* presenta flores compuestas de carpelos sepaloides.
(F) La mutante doble *ap2 ag* tiene flores con órganos parecidos a hojas en el primer y cuarto verticilo y órganos quiméricos con características de pétalos/estambres en los verticilos dos y tres.
(G) El mutante doble *ap3 ag* produce flores compuestas de verticilos de sépalos.

(H) El triple mutante *ap2 pi ag* tiene verticilos compuestos de órganos parecidos a hojas con algo de tejido carpeloide. (Figura modificada a partir de la figura 11 de Álvarez-Buylla et al., 2010[a]).

Investigaciones posteriores realizadas en otras angiospermas además de *A. thaliana* han demostrado que el modelo ABC está bastante bien conservado en cuanto a: la acción combinatoria y mutuamente excluyente de los genes A y C, así como en el papel de los genes B y C en la determinación de los verticilos de pétalos, estambres y carpelos. La función del gen C también está conservada en lo tocante al agotamiento (determinación) del meristemo floral (ver trabajos de Xiao et al., 2003; Irish and Litt, 2005; Kim et al., 2005; Soltis et al., 2007; Theissen y Melzer, 2007; Whipple et al., 2007; Mondragón-Palomino y Theissen, 2009, entre otros, así como Figura 2.2.). En el caso de los genes A, su papel en la determinación de primordios de sépalos no está conservado en otras angiospermas además de *A. thaliana*, sin embargo, la expresión de los genes A es fundamental en todas las angiospermas analizadas para la delimitación y diferenciación del meristemo de flor a partir del meristemo de inflorescencia (Soltis et al., 2007).

2.2. La excepción a la regla: modificación del modelo ABC en *Lacandonia schismatica*

La existencia de estambres centrales en la flor bisexual de *Lacandonia schismatica* (Martínez y Ramos 1989), junto con la evidencia de que dicho fenotipo está fijado genéticamente en las poblaciones de esta especie analizadas hasta ahora (Vergara-Silva et al., 2003), sugirió una modificación sustancial al esquema ABC de determinación floral en esta especie. Se generó una hipótesis que pudiese probarse experimentalmente para entender las bases genético-moleculares que subyacen al fenotipo homeótico de la flor de *L. schismatica*. Los genes homeóticos caracterizados como necesarios para determinar la identidad y desarrollo espacio-temporal de los cuatro órganos canónicos de las flores (genes ABC) se encuentran muy conservados, tanto a nivel de secuencia como a nivel de función en todas las angiospermas analizadas, y con base en ello, se propuso que el factor determinante para explicar la heterotopía de los estambres en *L. schismatica* podría estar relacionado con el desplazamiento al centro del meristemo floral de la función B (genes *AP3* y *PI*; Vergara-Silva et al., 2000), donde en conjunción con la función C, que, al igual que en otras angiospermas estaría presente en los dos verticilos más internos de la flor de *L. schismatica*, podría ser el factor causal de la homeosis observada (Vergara-Silva et al., 2003).

El desplazamiento al centro del meristemo floral de *L. schismatica* podría incluir una expresión disjunta de los genes B, expresándose tanto en los primordios de los tépalos como en el verticilo central, dando lugar a un perianto de identidad petaloide y a estambres centrales, respectivamente, o, si el perianto fuese de origen sepaloide y/o no necesitara de función B para desarrollarse, la función B sólo se encontraría desplazada al centro del meristemo floral.

Recientemente, el estudio de Álvarez-Buylla y colaboradores (2010b) analiza la conservación a nivel de secuencia así como el dominio de expresión y la conservación funcional de los ortólogos de los genes B y C de *L. schismatica* (*LsAP3*, *LsPI* y *LsAG*). Los experimentos de hibridación *in situ* del ARNm de estos tres genes demuestran que el patrón de expresión espacio-temporal de *LsPI* y *LsAG* está conservado con respecto a otras angiospermas, ya que ambos genes se expresan en los últimos dos verticilos florales. En contraste, *LsAP3* se expresa únicamente y desde etapas tempranas del desarrollo en el centro del meristemo floral (Álvarez-Buylla et al., 2010b; ver Figura 2.2. para un resumen del patrón de expresión). Dado que sólo en el centro del meristemo floral se traslapan los sitios de expresión de *LsPI* y *LsAP3*, sólo en esta región de la flor en desarrollo existe función B, que junto con la expresión de *LsAG*, determina la formación de estambres centrales (ver Figura 2.2.).

Con el fin de evaluar si los cambios a nivel de secuencia detectados en los genes B de *L. schismatica* eran suficientes para desplazar el dominio de expresión de ambos al centro del meristemo floral, y si además mostraban conservación funcional con respecto a sus ortólogos en otras angiospermas, se llevaron a cabo experimentos de genética funcional donde *LsPI* y *LsAP3* fueron expresados en mutantes florales y también usados para generar líneas de sobreexpresión en *A. thaliana*, especie que se usó como sistema heterólogo puesto que *L. schismatica* hasta el momento no ha podido cultivarse en el laboratorio. Dichos experimentos demostraron que ambos genes estaban funcionalmente conservados con respecto a otras angiospermas ya que eran capaces de recuperar la formación de estambres en mutantes de genes B, sin embargo, *LsPI* recuperaba pétalos mientras que las líneas de rescate de *LsAP3* no (Álvarez-Buylla et al., 2010b). En congruencia con estos resultados, en las líneas transgénicas de sobreexpresión de *LsAP3* y

LsPI, donde estos genes fueron fusionados al promotor 35S del Virus del Mosaico de la Coliflor y transformados en líneas tipo silvestre de *A. thaliana*, *LsPI* producía un fenotipo similar al observado en las líneas de sobreexpresión usando *PI* de *A. thaliana*, mientras que *LsAP3* no recuperaba dicho fenotipo (Álvarez-Buylla et al., 2010b). Con el fin de evaluar si las diferencias en la habilidad de *LsPI* y *LsAP3* de recuperar un fenotipo silvestre se debían a diferencias en la afinidad para formar heterodímeros proteicos entre sí o con las proteínas endógenas de *A. thaliana*, se realizaron experimentos de doble y triple híbrido en levadura, que corroboraron la sospecha de que LsPI se encuentra más conservado funcionalmente que LsAP3 ya que LsPI lograba heterodimerizar con AP3 mientras que LsAP3 no lo hacía con PI. Adicionalmente, se encontró que LsPI y LsAP3 no heterodimerizaban entre sí excepto cuando SEP3 era incluido (Álvarez-Buylla et al., 2010b).

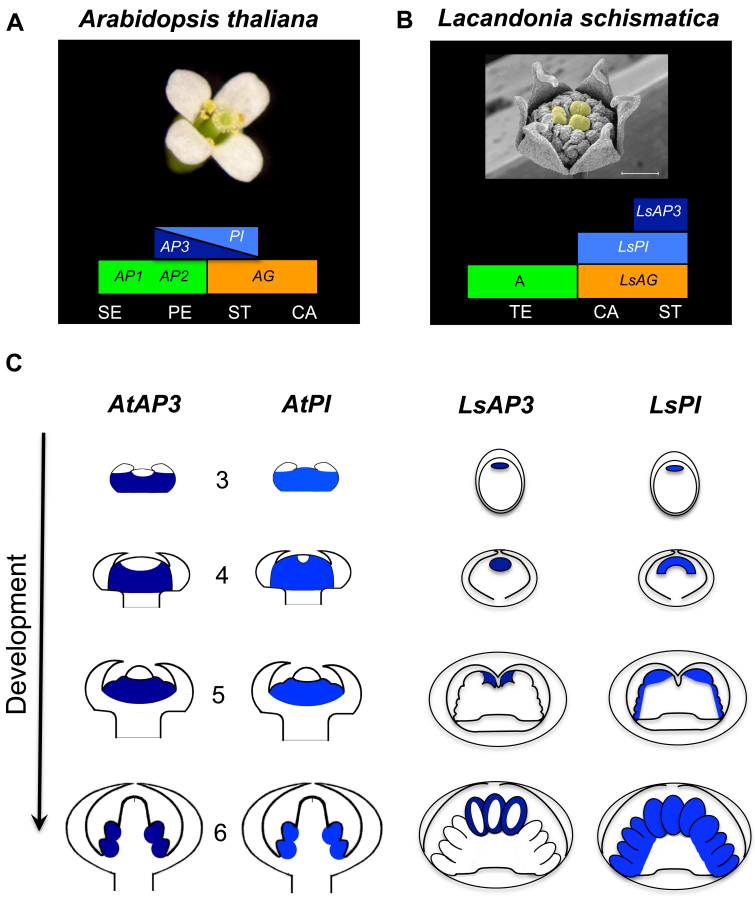

Figura 2.2. Modelo ABC en *A. thaliana* y *L. schismatica* con representación de patrones de expresión de genes B en ambas especies modificado de Álvarez-Buylla et al., 2010b).

(A) EL modelo combinatorio ABC de identidad de los órganos florales que está conservado entre eudicots, donde la función B está restringida a los primordios de pétalos y estambres (Coen and Meyerowitz, 1991).
(B) Hipótesis de genética molecular que explica el fenotipo de *L. schismatica*: la función B ejercida por el heterodímero *LsAP3-LsPI* está restringida al centro del meristemo floral, donde se desarrollarán los estambres.
Función A (verde), B (AP3 en azul oscuro y PI en azul claro) y C (naranja). CA, carpelos; PE, pétalos; SE, sépalos; ST, estambres; TE, tépalos. Barra = 500 mm.
(C) Cambios en el patrón de expresión in situ del ARNm de genes B en diferentes momentos del desarrollo de *A. thaliana* (Jack et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996) y *L. schismatica* (Álvarez-Buylla et al., 2010b). Las diferencias en el patrón de expresión entre estas dos especies representan dos instancias de cambios evolutivos durante la evolución

de las angiospermas. Representación esquemática de diferentes estadíos de desarrollo floral de *A. thaliana* y *L. schismatica*, empezando en la etapa 3 de desarrollo en *A. thaliana*, cuando los primordios de sépalos se vuelven visibles. Los primordios de sépalos continúan creciendo hasta que cubren el meristemo floral en la etapa 6. Mientras tanto, en la etapa 5 los primordios de pétalos y estambres aparecen y el carpelo se comienza a formar en la etapa 6. Estadíos similares fueron escogidos para *L. schismatica* basados en características morfológicas (Ambrose et al., 2006).

Los resultados de Álvarez-Buylla y colaboradores (2010) confirman la hipótesis del desplazamiento de la función B (por desplazamiento desde etapas tempranas del desarrollo floral de *LsAP3*) al centro del meristemo floral como la base causal del desarrollo de estambres centrales en *L. schismatica* (ver Figura 2.2.) a su vez que demuestran que tanto *LsAP3* como *LsPI* están globalmente conservados funcionalmente, pero que el ortólogo de *AP3* muestra mayor cantidad de cambios. Estos pueden afectar su función proteica y la formación de dímeros con otras proteínas MADS.

Como señalan los propios autores, para continuar en la comprensión de las bases morfogenéticas del desarrollo floral de *L. schismatica* aún queda por evaluar cuáles son los mecanismos regulatorios que subyacen al desplazamiento de la expresión de *LsAP3* al verticilo central en esta especie, fenómeno que podría estar relacionado con cambios en los módulos regulatorios del promotor de *LsAP3*, así como cambios en la afinidad proteica o dominio de expresión de sus reguladores transcripcionales (Álvarez-Buylla et al., 2010b; Piñeyro-Nelson et al., 2010; Garay-Arroyo et al., 2012).

Otro aspecto que queda pendiente investigar es qué procesos dentro del linaje de las Pandanales en general, y las Triuridáceas en particular, pudieron favorecer la emergencia del fenotipo homeótico de *L. schismatica* (ver figura 2.3). En este capítulo se abordan los experimentos realizados en torno a la regulación de *LsAP3* (ver siguiente sección), mientras que en el capítulo 3 se presenta un análisis filogenético del orden Pandanales que analiza las afinidades entre familias al interior de este clado, explorando a su vez las transformaciones morfológicas que pudieron dar origen a *L. schismatica* (ver capítulo 3 y manuscrito Piñeyro-Nelson et al., en preparación, anexo al mismo).

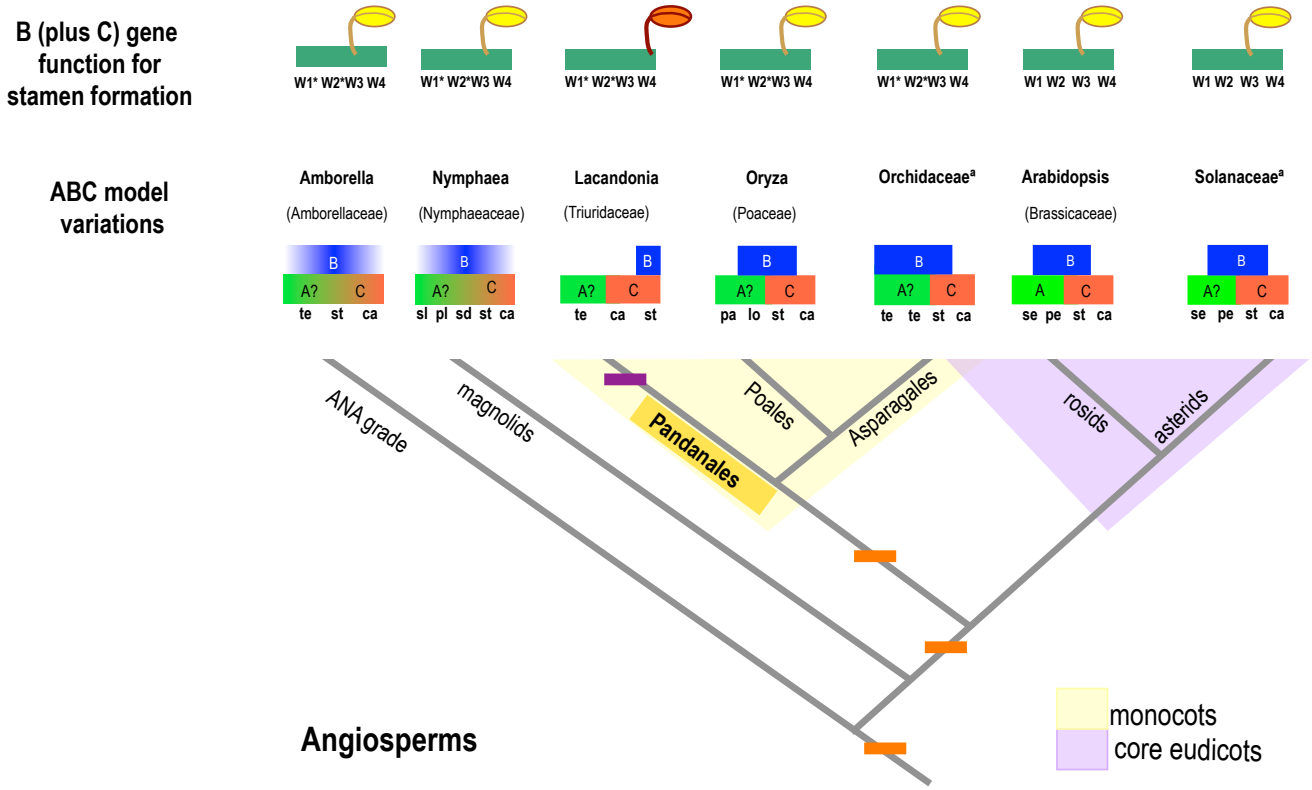

Figura 2.3. Evolución de las interacciones entre genes ABC en las angiospermas. Filogenia simplificada (basada en APG III, 2009) a la que se ha añadido diferentes variaciones al modelo ABC documentadas en taxones selectos, así como el sitio donde la función BC define el desarrollo de los estambres. Taxones representativos de varias angiospermas son mostrados en las puntas del árbol con su familia en paréntesis, excepto por Orchidaceae y Solanaceae (a), familias para las cuales el modelo ABC correspondiente representa a varias especies estudiadas experimentalmente (para Orchidaceae; *Phalaenopsis spp*, Tsai et al., 2004 y *Dendrobium crumenatum*, Xu et al., 2006); para Solanaceae, *Petunia* Vandenbussche et al., 2004 y tomate; de Martino et al., 2006). Las función A fue mantenida para todos los linajes representados con el fin de facilitar la comparación con *A. thaliana*, aunque un signo de interrogación fue agregado para enfatizar el dudoso papel que tienen los genes A en la formación de los órganos del perianto en otras angiospermas además de *A. thaliana*. Los genes A son considerados importantes para la diferenciación del meristemo de flor (Soltis et al., 2007). En las diferentes ramas se indican los diferentes momentos donde las variantes del modelo ABC pudieron haber surgido. Las sinapomorfías están en rectángulos naranjas y las autapomorfías que pudieron favorecer la emergencia del fenotipo homeótico en Triuridaceae. *L. schismatica* están representadas con rectángulos morados. En contraste con otras angiospermas, la expresión de *LsAP3* está restringida al centro del meristemo floral, mientras que en otras angiospermas bisexuales AP3 tiene una expresión temprana que abarca varios verticilos, aunque la función BC esté restringida al tercer verticilo en especies aparte de *L. schismatica*. Tépalos (te); tépalos sepaloides (sl); tépalos petaloides (pl); estaminodios (sd); estambres (st); carpelos (ca); sépalos petaloides (te); pétalos (pe); palea/lema (pa); lodículos (lo); sépalos (se); pétalos sepaloides (sp); los verticilos están numerados desde fuera hacia adentro (W1-W4); *; mientras que en numerosas angiospermas W1 correspondería al verticilo de los sépalos, W2 a los pétalos, W3 a estambres y W4 a carpelos, varios linajes de angiospermas difieren de este acomodo ya sea por tener filotaxia espiral (*Amborella* y *Nimphaea*); por tener órganos del perianto muy modificados (*Oryza*) o por carecer de una diferenciación de sépalos y pétalos (llamados tépalos; *Amborella*, *Lacandonia*, orquídeas). Figura modificada de Figura 6 de Álvarez-Buylla et al., 2010b (datos resumidos de publicaciones por: Xiao et al., 2003; Irish and Litt, 2005; Kim et al., 2005; Soltis et al., 2007; Theissen y Melzer, 2007; Whipple et al., 2007; Mondragón-Palomino y Theissen, 2009). Figura modificada de Álvarez-Buylla et al., 2010b.

2.3. Mecanismos regulatorios implicados en el patrón de expresión de *LsAP3*: factores *cis vs trans*

En la sección anterior se han descrito los experimentos que han corroborado que la base molecular del desarrollo que subyace la formación de estambres centrales en *L. schismatica* está directamente relacionada con el desplazamiento al centro del meristemo floral de la expresión del gen B, *LsAP3* (Álvarez-Buylla et al., 2010b). Ahora bien, los mecanismos moleculares que sesgan el patrón de expresión de *LsAP3* hacia el centro de la flor aún no han sido caracterizados y no sabemos si es debido a cambios en *cis* en las secuencias regulatorias de *LsAP3*, mientras que el resto de la red de regulación floral se encuentra conservada, o si existen alteraciones en otros reguladores en *trans* que afectan tanto el dominio de expresión de *LsAP3*, como la red de regulación genética en la cual participa este gen B.

Los posibles cambios en *cis* pueden involucrar cambios en la región promotora y/o en los intrones. Estos cambios pueden afectar las afinidades de unión de factores de transcripción que regulen positiva o negativamente la expresión de una secuencia dada, en este caso, *LsAP3*.

En plantas, estudios comparativos utilizando la técnica de *phylogenetic footprinting* (por ejemplo; De Bodt et al., 2006), en donde se detectan motivos conservados y divergentes entre secuencias promotoras aisladas de especies cercanamente emparentadas, han demostrado que cambios en *cis* pueden impactar la expresión espacio-temporal de genes involucrados en procesos de desarrollo (Hong et al., 2003; Lee et al., 2005). Ejemplo de lo anterior es la comparación entre las brasicales *Lepidium, A. thaliana* y *Brassica* de la región 5' del factor de transcripción CRABS CLAW (CRC), donde se detectaron motivos conservados involucrados tanto en la regulación positiva como negativa de este gen (Lee et al., 2005). Estos motivos parecen funcionar de manera modular, ya que construcciones reporteras que incluían estos motivos demostraron que cada módulo podía guiar la expresión del reportero a sitios específicos del meristemo floral, como si la suma de todos los motivos (módulos) pudiese recuperar el perfil de expresión tipo silvestre de CRC (Lee et al., 2005). En otro artículo relacionado, la comparación del segundo intrón del gen

AGAMOUS (AG) aislado de 29 especies del género *Brassica* permitió delimitar dos sitios conservados para la unión de WUSCHEL y LEAFY; dichos sitios parecen ser específicos a las Brasicales (Hong et al., 2003).

A su vez, la posibilidad de cambios en *trans* que afectan la capacidad de unión a ADN y/o interacciones proteicas entre factores de transactivación, también podrían estar involucrados en la expresión atípica de *LsAP3* (Garay-Arroyo et al., 2012). Bajo este escenario, composiciones alternativas de los tetrámeros de proteínas MADS-box pudieran tener diferentes afinidades con los diferentes motivos regulatorios presentes en las regiones promotoras e intrónicas de *LsAP3*.

Adicionalmente, las proteínas que forman estos complejos pudieron a su vez sufrir cambios en sus patrones de expresión espaciales o temporales. Uno de los factores de transcripción que podría estar afectado por un cambio en el patrón de expresión de LFY es la proteína F-box UNUSUAL FLORAL ORGANS (UFO), proteína que en *A. thaliana*, es un co-factor necesario para que LFY pueda activar a AP3 en los primordios de los verticilos 2 y 3 en etapas muy tempranas del desarrollo (Lee et al., 1997). Lo anterior se debe a la interacción física entre LFY y UFO en un complejo que se une directamente al promotor de AP3 aunque aún no es claro si dicho complejo marca a un represor para degradación, o si aumenta directamente la tasa de transcripción de AP3 (Lee et al., 1997; Chae et al., 2008).

En el caso de *L. schismatica*, se ha propuesto (Piñeyro-Nelson et al., 2010; Garay-Arroyo et al., 2012), que el desplazamiento de *LsAP3* al centro del meristemo floral podría estar relacionado directamente con que LsUFO permanezca en el verticilo central durante un periodo más extendido que lo documentado en *A. thaliana*, presentando un patrón "neoténico" similar a lo observado en etapas tempranas del desarrollo de *A. thaliana*, lo que llevaría a un sesgo en el sitio donde *LsAP3* puede expresarse. Esta hipótesis implicaría que el fenotipo homeótico de *L. schismatica* podría deberse al patrón de expresión de UFO, un gen que tiene patrones de expresión variables en diferentes angiospermas (Souer et al., 2008).

2.3.1 Investigación experimental: clonación del promotor *LsAP3* y evaluación de cambios en *cis*.

En esta tesis se puso a prueba la hipótesis de si cambios en motivos regulatorios en *cis* podrían ser los principales factores causales del desplazamiento del patrón de expresión de *LsAP3* al centro del meristemo floral en *L. schismatica*. Para poner a prueba esta hipótesis, se clonó un fragmento del promotor de *LsAP3* que fue analizado mediante herramientas bioinformáticas para determinar posibles motivos conservados y divergentes con respecto a promotores del mismo gen secuenciados de otras especies. Posteriormente, se generaron líneas reporteras pro*LsAP3*::GUS en *A. thaliana* con el fin de investigar si los cambios en el promotor de *LsAP3* eran suficientes para desplazar la expresión de un gen reportero al centro del meristemo floral.

MATERIALES Y MÉTODOS

Amplificación del promotor de *LsAP3*

Para clonar el promotor de *LsAP3* se utilizó el sistema GenomeWalker™ (Clontech Laboratories, Inc.) avanzando hacia el extremo 5' a partir de la región conocida de *LsAP3*, que consistía en el cDNA y un fragmento de los UTRs (Álvarez-Buylla et al., 2010b). Se prepararon 3 alícuotas de ADN genómico de *L. schismatica* previamente purificado por el método CTAB detallado de CIMMYT (CIMMYT, 2006) que fueron cortadas con las enzimas de restricción DraI, PvuII y EcorV, respectivamente, siguiendo las instrucciones provistas en el manual del sistema GenomeWalker™. Se usó el protocolo de ciclaje para la amplificación por PCR sugerido en el manual del GenomeWalker™ y el kit para amplificación Advantage® 2 PCR Kit (Clontech Laboratories Inc.), ambos de acuerdo a lo sugerido por el fabricante. Los oligonucleótidos utilizados se presentan en la Tabla 1.

Tabla 1. Oligonucleótidos utilizados para amplificar el promotor de *LsAP3*

Nombre	Secuencia	Dirección	Tamaño (nt)
Adaptor Primer 1 (AP1)*	5′ GTAATACGACTCACTATAGGGC 3′	Sentido	22
Nested Adaptor Primer 2 (AP2)*	5′ ACTATAGGGCACGCGTGGT 3′	Sentido	19
***LsAP3*(long)-GSP1.R**	5′TCTCGATCTTTCCCCTGCCCAT GGCCG GAG 3′	Antisentido	30
***LsAP3*(long) -GSP2.R**	5′AGAGGGAGAGCGGGCGGTGG AGGAAGT TAG 3′	Antisentido	30

*** Estos oligos fueron provistos en el kit de GenomeWalker™. Los oligos específicos para *LsAP3* fueron diseñados por Eduardo Flores y corroborados por la autora como útiles para amplificar el promotor *LsAP3*. nt: nucleótidos.**

Con este método, se amplificaron por PCR y clonaron en el vector pGEM®-T Easy (Promega Corporation, USA) varios fragmentos que podrían corresponder al promotor de *LsAP3*. Un fragmento de 1096pb contenía la secuencia del juego de oligonucleótidos utilizados en sus extremos (AP2 + *LsAP3*(long)-GSP2) y traslapaba el extremo 3′ con la región 5′UTR conocida de *LsAP3* (ver Figura 2.4.), por lo que esta secuencia fue considerada un fragmento del promotor *LsAP3*.

Análisis bioinformáticos sobre la secuencia putativa del promotor *LsAP3*

Para corroborar la identidad de la secuencia clonada como parte del promotor *LsAP3*, se verificó la continuidad del segmento clonado con la región conocida del 5′ UTR mediante alineación manual en los programas Se-AL v2.0a11 (Rambaut, 1996-2002) y Jalview (Clamp et al., 2004). La secuencia clonada empezaba en el -9. Se corroboró que en el promotor putativo de *LsAP3* había 50 nucleótidos más del extremo 5´UTR que eran exactamente iguales a la región previamente clonada de *LsAP3*. Una vez establecida la continuidad de la secuencia, se llevaron a cabo análisis comparativos para determinar las

regiones más conservadas y divergentes con respecto a las secuencias promotoras de otros ortólogos de AP3 disponibles en la base de datos del National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). En este mismo portal se utilizó el algoritmo de BLAST (http://blast.ncbi.nlm.nih.gov/) con el fin de analizar motivos de más de 12pb que estuvieran conservados en el promotor de *LsAP3* y el promotor de *AP3* de *A. thaliana* (de aquí en adelante llamado *AtAP3*), usado como referencia (ver Figura 2.5.). Para detectar posibles motivos regulatorios se analizó la secuencia del promotor de *LsAP3*, así como los promotores de *AtAP3* y *DEF* (=*AP3*) de la monocotiledónea *Lilium regale* (*LrDEF*) en la base de datos PLACE (Plant Cis-acting Regulatory DNA Elements: http://www.dna.affrc.go.jp/PLACE/).

Generación de líneas reporteras pro*LsAP3*::GUS en *A. thaliana*

Con el fin de analizar la función del fragmento del promotor *LsAP3* clonado, se extrajo el promotor del plásmido vector pGEM®-T Easy (clona T15) mediante digestión con EcoRI y se introdujo a un vector binario con el gen reportero de la beta-galactosidasa (GUS); pD1849 (amablemente facilitado por Dr. Thomas Jack). Se corroboró la dirección del inserto mediante secuenciación y la clona elegida fue transformada a células electrocompetentes de la cepa C58 de *Agrobacterium tumefaciens*. Las colonias transformantes fueron seleccionadas mediante plateo en placas LB+Rifamicina+Ampicilina según métodos estándar. Una vez corroborada la presencia del vector binario en *A. tumefasciens*, se transformaron plantas tipo silvestre del ecotipo Col-0 de *A. thaliana* por el método de "floral dip" (Clough y Bent, 1998). Se recuperaron varias líneas transformantes y 14 de ellas fueron propagadas hasta la T2. Plantas de la T2 fueron genotipificadas mediante la amplificación de GUS::pro*LsAP3* por PCR y propagadas hasta la T3.

Ensayos de tinción de *GUS*

Plantas de la T2 y T3 fueron sometidas a ensayos de tinción de GUS (Beeckman y Engler, 1994). Como control positivo en cada ensayo de tinción de GUS se utilizó una línea reportera con el gen de la ciclina CYCD3.1 fusionado a GUS (construcción proCYCD3.1::GUS, facilitada por la Biól. Karla Verónica García Cruz); como control

positivo del patrón de expresión esperado para AP3 de *A. thaliana*, se utilizó la línea reportera 890-7 (construcción: proAP3:cDNA-AP3-3'AP3::GUS en *A. thaliana* Wt ecotipo Col-0, provista por el Dr. Thomas Jack) y como control negativo plantas de *A. thaliana* de tipo silvestre (ecotipo Col-0).

RESULTADOS Y DISCUSIÓN

Motivos conservados en la secuencia clonada del promotor *LsAP3* con respecto a promotores AP3 de otras angiospermas.

Acorde con lo esperado, al someter el promotor de *LsAP3* a análisis BLASTX con el algoritmo megablast, se observó que el fragmento de promotor de *LsAP3* tiene regiones de mayor similitud con monocotiledóneas que con el promotor de *A. thaliana* (ver Figura 2.5.). A su vez, las secuencias disponibles de promotores de AP3 para diferentes especies de angiospermas fueron bajadas de la base de datos del NCBI para llevar a cabo análisis comparativos.

Al llevar a cabo un análisis de alineamiento global de los promotores de AP3 disponibles, fue interesante observar que al excluir la secuencia de *LsAP3*, éstos compartían un mayor número de nucleótidos en la región donde se encuentran las cajas CArG descritas para *A. thaliana* (Hill et al., 1998), que cuando se incluía la secuencia de *L. schismatica* (ver Figura 2.6.). Sin embargo, todos los promotores analizados presentaban las tres cajas CArG esperadas (ver Figura 2.6.). Con el fin de detectar otros motivos regulatorios, se analizaron los promotores de *L. schismatica*, *L. regale* y *A. thaliana* en la base de datos PLACE (Plant Cis-acting Regulatory DNA Elements: http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999; Prestridge, 1991). De manera relevante, el promotor de *LsAP3* presentaba muchos más motivos relacionados con regulación de estrés osmótico que los otros dos promotores analizados, mientras que los tres promotores presentaban cajas TATA, GATA, MYB y diversas cajas relacionadas con regulación hormonal. El desglose de los motivos regulatorios predichos se encuentra en el Apéndice I del Anexo A de esta tesis. En conclusión, se logró clonar un fragmento importante del promotor del ortólogo de *AP3* de *L. schismatica*. El fragmento clonado tendría que ser suficiente para observar la generación de patrones de expresión similares a los descritos para *A. thaliana*, en caso de que el

promotor clonado guardara suficiente conservación con el de esta última especie. Para probar esta hipótesis se generaron líneas transgénicas de *A. thaliana* con las líneas reporteras del promotor clonado de *L. schismatica.*

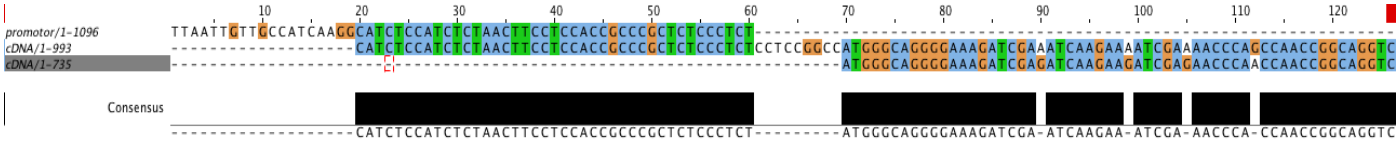

Figura 2.4. Comprobación de sitio de unión entre región del promotor de *LsAP3* clonada mediante experimentos de *GenomeWalker*™ y secuencia conocida (cDNA) de *LsAP3*. El promotor clonado y la secuencia de cDNA comparten 40 nucleótidos correspondientes al 5′ UTR que están 9 nucleótidos río arriba del sitio de inicio de la transcripción (marcado por el triplete "ATG", correspondiente a la metionina inicial). Imagen obtenida en el editor de secuencias *Jalview* (Clamp *et al.,* 2004).

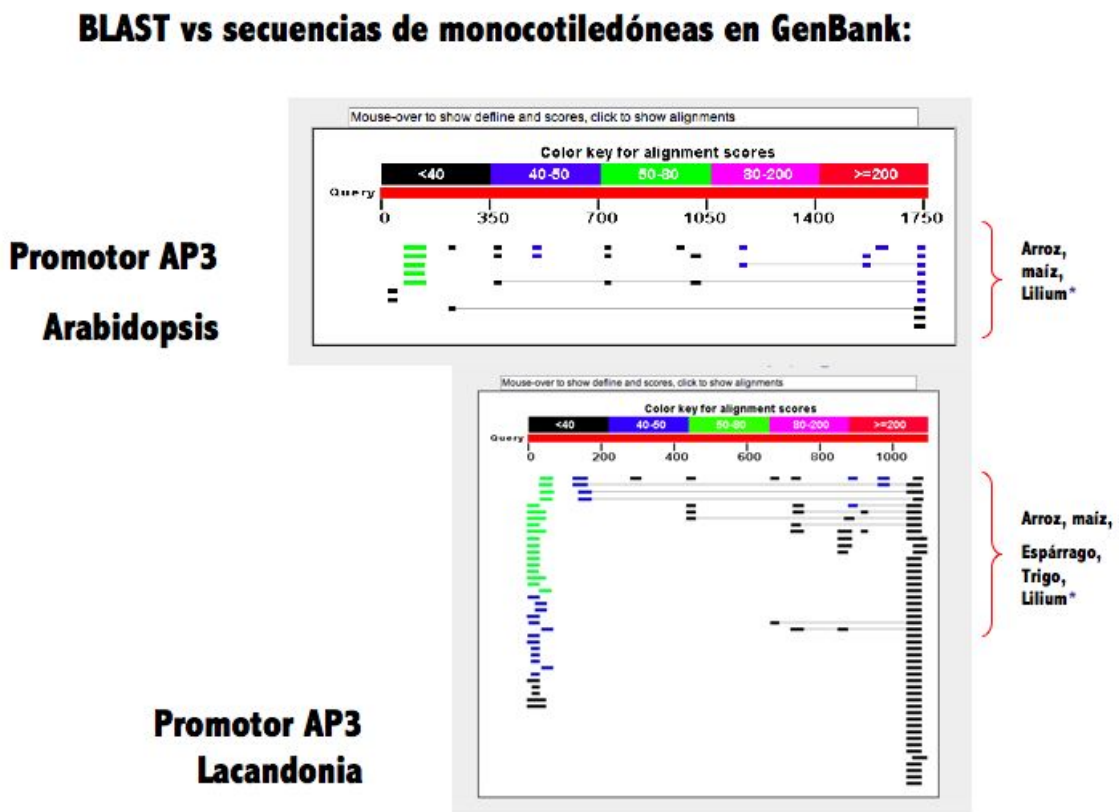

Figura 2.5. Análisis tipo BLASTX realizado contra la base de datos del NCBI (algoritmo megablast) utilizando los promotores de AP3 de *A. thaliana* (panel superior) y *L. schismatica* (panel inferior) con el fin de detectar regiones con identidad en ambas secuencias con respecto a los genes disponibles en la base de datos del NCBI. La búsqueda fue limitada a las

monocotiledóneas. Existen varias secciones que muestran homología con otros promotores AP3, (rectángulos de diferentes colores en cada una de las hileras debajo de la secuencia de referencia (rectángulo rojo), sin embargo el promotor de *L. schismatica* tiene más registros que el de *A. thaliana*, como era de esperarse dado que *L. schismatica* es una monocotiledónea.

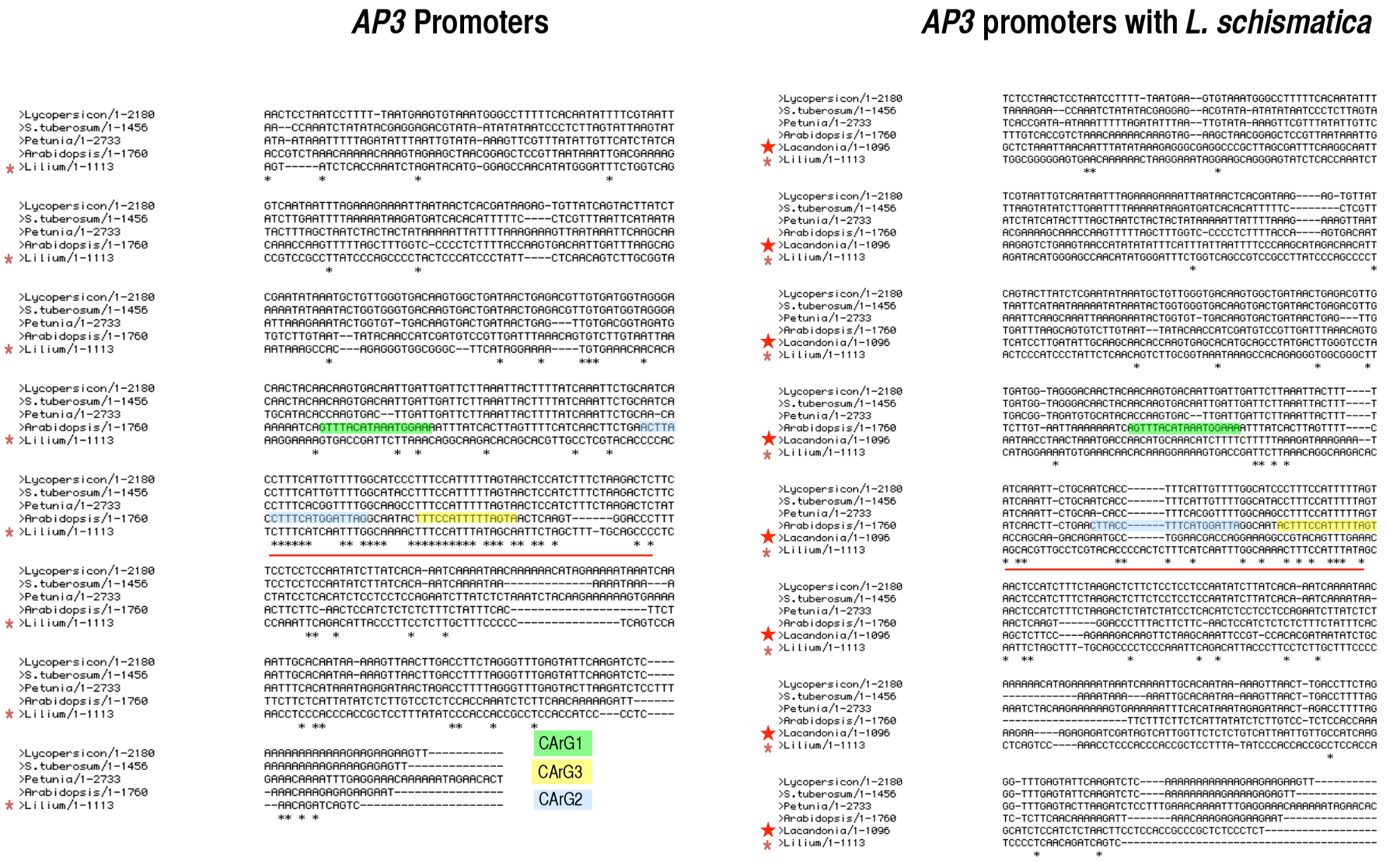

Figura 2.6. Primeras 350pb de la alineación global de secuencias ortólogas de los promotores de AP3 de *Lycopersicon esculentum* (tomate); *S. tuberosum* (papa); *Petunia hybrida* (petunia); *A. thaliana thaliana* (A. thaliana); *Lilium regale* (Lilium) y *Lacandonia schismatica* (Lacandonia) a partir del nucleótido -9. El alineamiento a la izquierda no incluye la secuencia promotora de *L. schismatica*, mientras que el alineamiento de la derecha sí. En verde, azul y amarillo se encuentran señalizadas las secuencias consenso de las cajas CArG1, 2 y 3, respectivamente. Estas cajas CArG han sido experimentalmente caracterizadas en *A. thaliana* como necesarias y suficientes para que diversos factores de transcripción MADS-box II se unan al promotor (Tilly *et al.*, 1998). Alineación global realizada con ClustalW.

Patrones de tinción de GUS en líneas reporteras pro*LsAP3*::GUS

El análisis de los patrones de expresión de GUS en las líneas reporteras con la construcción pro*LsAP3*::GUS muestran que los cambios en *cis* presentes en dicha secuencia no son suficientes por si solos para desplazar el patrón de expresión del gen que dirige al centro del meristemo floral (ver Figura 2.7.), ya que GUS es expresado en los estambres de las líneas reporteras de *A. thaliana*. A pesar de no ser suficiente para desplazar el dominio de expresión al centro del meristemo floral, el fragmento analizado del promotor de *LsAP3* presenta varias diferencias notables con respecto al patrón de expresión observado en el control positivo de *AP3* (línea 890-7): i) el pro*LsAP3* dirige la expresión de GUS únicamente a los estambres de *A. thaliana* y a la base del receptáculo de la flor una vez que se comienza a elongar el fruto (silicua). ii) En contraste con lo reportado para *A. thaliana*, pro*LsAP3*::GUS no se expresa en pétalos en ninguna etapa del desarrollo floral, además; iii) la expresión de GUS en estambres en líneas reporteras pro*LsAP3*::GUS sólo se detectó en etapas tardías del desarrollo floral (flores pre-antéticas y antéticas) y únicamente en las anteras (ver Figura 2.7.).

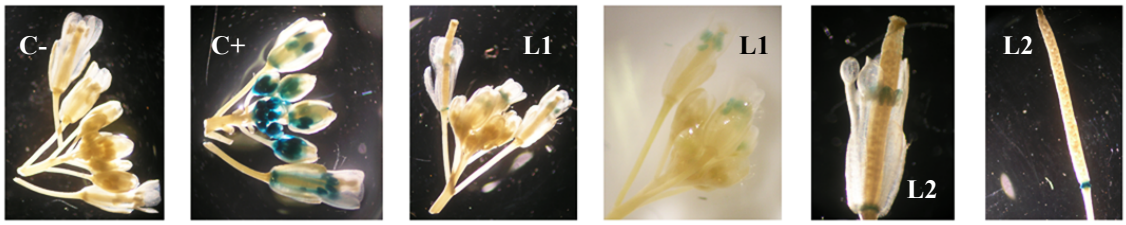

Figura 2.7. Patrón de expresión del gen reportero *GUS* después de manipulación bioquímica. Las plantas presentadas son líneas reporteras de *A. thaliana* que contienen diferentes construcciones. C-: control negativo consistente en una planta no transgénica tipo silvestre; C+: línea 890-7 (construcción proAP3::cDNA-AP3::3'AP3::GUS; línea provista por el Dr. Thomas Jack); L1 y L2: dos líneas reporteras independientes que contienen el fragmento de 1kb del promotor de *LsAP3* (pro*LsAP3*::GUS). Nótese que los patrones de expresión en el C- y C+ son consistentes con lo esperado, mientras que en las Líneas L1 y L2 el gen reportero sólo se expresa en la base del receptáculo y en las anteras, en etapas tardías del desarrollo floral (flores pre-antéticas u antéticas).

El patrón de expresión de pro*LsAP3*::GUS sugiere que 1.1kb del promotor de *LsAP3* no es suficiente para recuperar la expresión espacio-temporal de *LsAP3* previamente documentada *in situ* o que las diferencias entre ambos y/o en la función de factores en *trans* explican los distintos patrones observados (Álvarez-Buylla et al., 2010b). En claro

contraste con el caso de *A. thaliana*, donde un fragmento de 1kb del promotor de *AtAP3* fue caracterizado experimentalmente como suficiente para recuperar los patrones espacio-temporales más importantes observados en experimentos con *AtAP3* (Tilly et al., 1998), el fragmento equivalente del promotor del ortólogo de *L. schismatica* solo recuperó algunos aspectos del patrón de *AP3*.

El trabajo de Tilly y colaboradores junto con el de Hill y colegas (1998) demostraron que las primeras 300pb del promotor *AtAP3* contenían motivos regulatorios que eran fundamentales para recuperar la expresión temprana de *AtAP3* en primordios de estambres y pétalos. En el caso del promotor de *LsAP3*, el fragmento clonado diverge más con respecto a otros promotores AP3 en esta región (-1 a -300pb aproximadamente; ver Figura 2.6.), lugar donde se encuentran las tres cajas CArG que son fundamentales para la unión de factores de transcripción tipo MADS-box II y otros que regulan la expresión de AP3 (Tilly et al., 1998).

Los experimentos aquí presentados sugieren que con el fin de determinar la contribución de los cambios en el promotor de *LsAP3* al patrón de expresión *in situ* observado para este gen en *L. schismatic,a* es necesario, además de clonar una región más extensa del promotor *LsAP3* para investigar si río arriba se encuentran regiones regulatorias importantes, abordar de manera simultánea análisis de los posibles cambios en *trans* que pueden afectar la regulación endógena de *LsAP3*. En este sentido, será importante: i) caracterizar molecularmente y a nivel de expresión *in situ*, a los genes *LsLFY* y *LsUFO*, con el fin de determinar si éstos están sesgando de inicio el patrón de expresión posible de *LsAP3*, en línea con lo observado en *A. thaliana* (Chae et al., 2008); ii) realizar experimentos de hibridización promotor-proteína con aquellos factores de transcripción propios de *L. schismatica* cuyos ortólogos han sido previamente caracterizados en *A. thaliana* y otras angiospermas como necesarios para la transcripción temprana y sostenida de genes AP3: LFY, UFO, PI, entre otros (Piñeyro-Nelson et al., 2010; Garay-Arroyo et al., 2012); iii) clonar los genes ortólogos de *AP3* y sus promotores en el taxón hermano de *L. schismatica* -*Triuris brevistylis*- con el fin de llevar a cabo análisis de *phylogenetic footprinting* que permitan determinar candidatos de motivos regulatorios en *cis* importantes para la expresión de *AP3* en cada una de estas especies. En este sentido, es importante resaltar que, dado que en *T. brevistylis* se han encontrado dos genes *AP3*, uno mucho más parecido

a nivel de secuencia a *LsAP3* y otro más divergente (datos de análisis de transcriptoma [Cappello et al., en preparación] y tesis de licenciatura de Alejandra Dolores Fuentes) y dado que el linaje del que se deriva *T. brevistylis* (tribu Triurideae; ver capítulo 3 de esta tesis) parece ser más antiguo que el linaje del género *Lacandonia*, podría existir la posibilidad de que en *L. schismatica* lo que observamos en el caso de *LsAP3* es producto de una subfuncionalización ancestral a este linaje. Bajo este escenario, el gen *LsAP3* sería la copia involucrada con la formación de estambres que, adicionalmente con cambios en sus factores reguladores en *trans*, haya favorecido la expresión central de *LsAP3*, mientras que la otra copia presente en *T. brevistylis* pudiera estar involucrada en regular otros fenómenos de desarrollo o quizá estar en proceso de volverse un pseudogen.

La propuesta anterior se deberá probar experimentalmente y mediante el análisis de las tasas de sustitución y regiones o residuos sujetos a diferentes procesos selectivos de las secuencias *AP3* que se comparen. Pero además la caracterización de la expresión *in situ* de los dos genes *AP3* de *T. brevistylis* y el análisis de sus regiones promotoras serán útiles. Por otro lado, es importante tener en mente que esta especie, si bien fue descrita originalmente como dioica (flores unisexuales en plantas separadas), *T. brevistylis* presenta en baja frecuencia morfos florales con flores bisexuales que en algunos casos tienen estaminodios fértiles embebidos en lo que sería un tercer verticilo floral (y en ocasiones los estambres están al centro con respecto a los carpelos), o estambres distribuidos de manera azarosa en el receptáculo de flores otrora macho o hembra (Vergara-Silva et al., 2003; Espinosa-Matías et al., 2012). Es posible que esta ontogenia compleja esté relacionada con la función ejercida por las dos copias de *AP3* detectadas.

3. CONTEXTO FILOGENÉTICO DE LACANDONIA SCHISMATICA: EVOLUCIÓN DE PATRONES MORFOGENÉTICOS EN LAS PANDANALES

Los estudios de *evo-devo* implican aproximaciones evolutivas o de las causas últimas del fenotipo de interés y con este enfoque se responde a preguntas del por qué de un patrón morfológico particular en el taxón bajo estudio. Este enfoque comparativo permite poner en un contexto histórico el origen de una novedad evolutiva, como lo es el patrón homeótico de *Lacandonia schismatica.* Pero la evolución del desarrollo también implica estudios mecanísticos o proximales de los procesos de desarrollo de las estructuras bajo estudio. Este componente de los estudios de *evo-devo* implican cuestionamientos acerca del cómo es que los mecanismos de desarrollo a nivel molecular, celular, estructural, organogénico, entre otros, dan lugar a las estructuras adultas bajo estudio.

L. schismatica puede considerarse un "monstruo esperanzado" dado que implica que en este taxón se fijó una macromutación a nivel morfológico en poblaciones naturales, a pesar de los "costos" adaptativos que este tipo de saltos morfológicos pueden tener (Goldschmidt, 1940). Por ello, es muy importante indagar aspectos evolutivos del origen del fenotipo homeótico único de las flores de *L. schismatica*. Por qué es que este fenotipo apareció solo en el linaje de las Triuridaceae, qué fuerzas evolutivas pudieron haber jugado un papel fundamental en la fijación de este fenotipo en las poblaciones naturales en donde se originó, pero también cual es el contexto histórico más amplio en el cual se originó aún no están resueltas. Como parte de mi tesis, me enfoqué justamente en este último aspecto de los enfoques comparativos y evolutivos de las investigaciones de *evo-devo* que se realizan en torno al origen y desarrollo de la flor de *L. schismatica* en el laboratorio de Genética Molecular, Desarrollo y Evolución de Plantas del Instituto de Ecología. Como parte de mi formación doctoral completé un estudio de sistemática molecular y morfológica, que es útil para plantear una hipótesis mas robusta acerca de las relaciones filogenéticas de las Triuridaceae dentro del orden de las Pandanales, así como para plantear análisis comparativos formales y explorar distintas hipótesis acerca de la naturaleza y evolución de las estructuras reproductivas de las Triuridaceae.

5.1. Posición filogenética de *L. schismatica*: afinidades contrastantes afectan las inferencias de identidad de las unidades reproductivas.

L. schismatica originalmente fue puesta dentro de su propia familia (Lacandoniaceae; Martínez y Ramos, 1989) por la singularidad de sus flores homeóticas. Sin embargo, análisis recientes de morfología comparada y sistemática molecular sugieren que debe ser considerada un género dentro de Triuridaceae (Rudall, 2003; Vergara-Silva et al., 2003 a y b; Davis et al., 2004; Ambrose et al., 2006; Rudall y Bateman, 2006). En concordancia con estos últimos estudios, en este capítulo se considera que el género *Lacandonia* es parte de Triuridaceae. La familia Triuridaceae (Gardner, 1843) se encuentra actualmente situada dentro del orden Pandanales (Chase et al., 2000), clado que adicionalmente incluye a Cyclanthaceae (Poit. Ex A. Rich 1824); Pandanaceae (R.Br. 1810); Stemonaceae (Caruel 1878, nom. cons.) y Velloziaceae (Endler 1841).

Este orden botánico es de creación reciente, siendo propuesto por Chase y colaboradores en 1993, a partir de un análisis filogenético de las monocotiledóneas basado en datos moleculares (secuencias del gen 18S ribosomal y el gen codificante para la enzima Rubisco [Rbcl] del genoma de cloroplasto). En este análisis Chase y colaboradores (1993) recuperaban de manera sorpresiva pero consistente a través de diferentes reconstrucciones filogenéticas un clado monofilético que agrupaba a las especies representantes de las familias Velloziaceae, Pandanaceae y Cyclanthaceae. Las afinidades entre estas tres familias no habían sido propuestas previamente por otros estudiosos, dada la divergencia morfológica entre estos tres taxones, donde Cyclanthaceae y Pandanaceae poseen especies preponderantemente arborescentes que son superficialmente similares a las palmas (Arecaceae), con inflorescencias modificadas que incluyen flores muy reducidas. Por ejemplo, en *Cyclanthus bipartitus* [Cyclanthaceae] las inflorescencias comprenden fascículos alternados de flores macho y flores hembra que están reducidas a estambres y carpelos desnudos, respectivamente (Harling, 1958). En contraste, Velloziaceae posee especies preponderantemente herbáceas (aunque algunas llegan a ser arborescentes), con flores agrupadas en inflorescencias sencillas como racimos o flores verdaderas solitarias, que además tienen el merismo trímero típico de monocotiledóneas (De Menezes, 1980; Mello-Silva et al., 2011).

En un análisis filogenético posterior basado únicamente en la secuencia del gen 18S ribosomal pero que incorporaba muchas más especies de monocotiledóneas (Chase et al., 2000), las familias Stemonaceae y Triuridaceae fueron agrupadas dentro del orden Pandanales. Adicionalmente, Triuridaceae y Pandanaceae aparecían como familias hermanas (usando una sola especie de Triuridaceae: *Sciaphila albescens*; Chase et al., 2000). Esta posición filogenética en conjunto con análisis morfológicos de las flores maduras de Triuridaceae y de las Pandanaceae, llevó a Rudall (2003) a concluir que las unidades reproductivas de las primeras no eran flores verdaderas (euantios), sino inflorescencias reducidas (pseudantios), como las de las Pandanaceae.

Ahora bien, la posición filogenética de Triuridaceae no ha sido resuelta, ya que diferentes reconstrucciones filogenéticas basadas en diferentes marcadores han dado lugar a hipótesis de relación contrastantes. Por ejemplo, utilizando marcadores moleculares, Triuridaceae ha sido puesto como grupo hermano a Pandanaceae (Chase et al., 2000); como grupo hermano al resto de Pandanales (Davies et al., 2004; Chase et al., 2004); como grupo hermano de Velloziaceae (Chase et al., 2004; Davis et al., 2004); o como grupo hermano de Stemonaceae (Vergara-Silva et al., 2003b). De manera sugerente, estas reconstrucciones no sólo son contradictorias entre sí, si no que tienen un muy bajo soporte estadístico (Rudall y Bateman, 2006; Piñeyro-Nelson et al., en preparación). Adicionalmente, en un análisis filogenético utilizando caracteres morfológicos, Triuridaceae fue reconstruido como un grupo parafilético con Stemonaceae (Rudall y Bateman, 2006).

La dificultad para determinar la posición filogenética de Triuridaceae dentro de Pandanales parece radicar al menos parcialmente, en la imposibilidad para utilizar marcadores moleculares provenientes del genoma del cloroplasto en las Triuridáceas. Estos marcadores han sido muy útiles para esclarecer las relaciones filogenéticas entre grupos de plantas cercanamente emparentados (Givnish et al., 2010). A su vez, el uso de caracteres morfológicos para discernir las relaciones evolutivas entre miembros de las Pandanales ha sido complejo ya que este orden comprende a especies con morfologías muy contrastantes entre sí, donde varias familias presentan modificaciones estructurales *sui generis* que han hecho difícil la asignación de homologías, aún en los estudios más meticulosos (Rudall y Bateman, 2006).

Adicionalmente, se mantiene el debate abierto en torno a la interpretación de los ejes reproductivos en Triuridaceae, donde algunos autores han interpretado estos ejes como estructuras monoaxiales (o flores verdaderas/euantios; Maas y Rübsamen 1986; Martínez y Ramos, 1989; Márquez-Guzmán et al., 1993; Maas-van de Kamer and Weustenfeld, 1998; Vergara-Silva et al., 2003; Ambrose et al., 2006; Álvarez-Buylla et al., 2010) o como estructuras poliaxiales (o inflorescencias comprimidas/pseudantios de algún tipo; Stevens, 1991; Rudall, 2003; Rudall 2008), ya sea de manera directa o como derivadas de un eje originalmente monoaxial que ha perdido la delimitación genética y morfológica entre una flor verdadera y una inflorescencia (Rudall y Bateman, 2006; Rudall, 2008; 2010).

En el centro de este debate está la discusión en torno a si *L. schismatica* posee una flor homeótica verdadera con un acomodo único de los órganos sexuales o una inflorescencia comprimida con flores macho (reducidas a estambres) apicales y flores hembra (reducidas a carpelos) distales (para un resumen de esta discusión, ver: Piñeyro-Nelson et al., 2010 y Álvarez-Buylla et al., 2010).

Si bien ahora se cuenta con evidencia morfológica y morfogenética que sugiere fuertemente que bajo todos los parámetros botánicos normativos, *L. schismatica* posee flores verdaderas (Vergara-Silva et al., 2003; Ambrose et al., 2006; Álvarez-Buylla et al., 2010), la resolución de las afinidades de Triuridaceae dentro del orden Pandanales será fundamental para poder elaborar reconstrucciones florales fundamentadas en la filogenia del orden.

En el trabajo anexo a este capítulo (Piñeyro-Nelson et al., en preparación), se reporta el análisis filogenético que incorpora marcadores moleculares (un fragmento del 18S ribosomal y un fragmento del gen nuclear atpA), así como una versión revisada y expandida de la matriz morfológica previamente publicada por Rudall y Bateman (2006), que ahora incluye un mayor número de caracteres, así como más especies de los grupos externos más cercanos: Dioscorales y Petrosaviales, con el fin de llevar a cabo una reconstrucción filogenética que permita determinar de manera robusta las relaciones filogenéticas al interior de las Pandanales.

Métodos

Los métodos de inferencia filogenética utilizados fueron Máxima Parsimonia (en PAUP*; Swofford 2001) y métodos Bayesianos (Mr.Bayes; Huelsenbeck and Ronquist 2001), con el fin de contrastar las topologías reconstruídas a partir tanto de supuestos epistémicos como estadísticos distintos. Con base en las filogenias inferidas por métodos Bayesianos, se llevó a cabo una reconstrucción de los estados de carácter floral más probables en el ancestro común a las Pandanales y a las Triuridaceae. Para estos análisis se utilizó las librería *ace* implementada en la plataforma estadística R (Paradis, 2006).

Resultados y Discusión

Los resultados de esta investigación corroboran la monofilia de las Pandanales, mientras que al interior del orden se recuperaron topologías contrastantes dependiendo del método de inferencia filogenética utilizado: bajo Máxima Parsimonia, Triuridaceae es reconstruído como el grupo hermano al resto de las Pandanales (tanto en filogenias elaboradas a partir de marcadores moleculares solos o con marcadores moleculares y morfológicos combinados), mientras que con Métodos Bayesianos utilizando sólo los marcadores moleculares se recupera una politomía basal en Pandanales, que incluye tres grupos claramente definidos: Velloziaceae, Triuridaceae y un grupo que sitúa a Stemonaceae como grupo hermano de Cyclanthaceae y Pandanaceae.

En las reconstrucciones basadas en la combinación de marcadores moleculares y morfológicos, se recuperan dos grupos claramente definidos: Velloziaceae y Triuridaceae como familias hermanas y otro grupo que de nuevo recupera a Stemonaceae como grupo hermano a Cyclanthaceae y Pandanaceae.

En las reconstrucciones morfológicas basadas en las topologías recuperadas bajo métodos Bayesianos, los estados de carácter más probables para el ancestro común a las Pandanales corresponden a una flor verdadera, situación que se repite en el ancestro de Velloziaceae y Triuridaceae, sin importar si la interpretación de las estructuras florales en Triuridaceae es codificada como de flor verdadera o inflorescencia.

Dados los resultados presentados en este trabajo (Piñeyro-Nelson et al., en preparación), las reconstrucciones filogenéticas tanto bajo MP como Métodos Bayesianos sugieren que Triuridaceae posee flores verdaderas. Esta conclusión refuerza el interés del proyecto de

evo-devo de *L. schismatica* empeñado en integrar enfoques evolutivos y de biología del desarrollo para desentrañar las causas últimas y proximales del origen de una novedad evolutiva única en la historia de las angiospermas: flores con los verticilos reproductivos invertidos en su posición.

4. DISCUSIÓN Y CONCLUSIONES

Los aportes de esta tesis se circunscriben dentro de una línea de investigación más amplia que ha sido liderada por Elena Álvarez-Buylla y qué ha tenido como motivación central entender los mecanismos genético-moleculares y procesos evolutivos del origen y fijación del fenotipo floral único presente en las poblaciones naturales de *Lacandonia schismatica*, una planta que ha sido considerada un "monstruo esperanzado" desde su descubrimiento. El término monstruo esperanzado fue acuñado por Goldschmidt (1940) para referirse a una entidad biológica que sufre un cambio morfogenético discreto (también llamado macromutación) que no se puede explicar por la acumulación paulatina y selección de cambios pequeños y continuos. *L. schismatica* posee flores bisexuales que desarrollan estambres centrales rodeados de carpelas, que en el resto de las angiospermas son los órganos centrales y por lo tanto presenta una heterotopía floral única.

En los capítulos anteriores he presentado las contribuciones que he hecho durante mi doctorado a este proyecto, en colaboración con otros miembros del laboratorio de Genética Molecular, Desarrollo y Evolución de Plantas. Se han resumido las investigaciones para abordar aspectos proximales y últimos sobre el origen de la flor *de Lacandonia schismatica,*(Piñeyro-Nelson et al., 2010; Garay-Arroyo et al., 2012; Capítulo 1). Estos estudios han incluído los últimos avances en torno al estudio de las bases genético-moleculares involucradas en la formación de estambres centrales en *L. schismatica*, que son consecuencia del desplazamiento al centro del meristemo floral de uno de los dos genes implicados en la función B: *LsAP3* (Álvarez-Buylla et al., 2010). Como continuación de estos descubrimientos, se analizó la contribución que cambios en los motivos regulatorios en *cis* presentes en el promotor de *LsAP3* pudieran tener en dirigir la expresión de *LsAP3* al verticilo central, encontrándose que dichos cambios si bien modifican el patrón de expresión de este promotor con respecto a otros promotores *AP3,* no son suficientes para explicar el desplazamiento de la expresión del gen reportero GUS al verticilo central (Capítulo 2, sección 2.3.1).

Estos datos sugieren que el patrón de expresión *in situ* de *LsAP3* debe estar gobernado por cambios tanto en las secuencias promotoras como en los factores de transactivación de

LsAP3, sugiriendo un escenario complejo desde el punto de vista genético-molecular donde cambios en la región regulatoria están acoplados con cambios en las propiedades proteicas, la afinidad para heterodimerizar y/o el patrón de expresión de los factores de transcripción que regulan a *LsAP3* (Piñeyro-Nelson et al., 2010, Garay-Arroyo et al., 2012); lo que potencialmente puede implicar cambios en la red de regulación genética que subyace a la formación de los diferentes verticilos florales y que ha sido previamente descrita en la especie modelo *A. thaliana* (Espinosa-Soto et al., 2004). Los datos recabados en esta tesis abren, por lo tanto, nuevas hipótesis acerca de los mecanismos proximales que subyacen tras el desarrollo de la flor única de *Lacandonia schismatica.*

En esta tesis también se hicieron contribuciones importantes para abordar la pregunta de: ¿en qué momento de la evolución del linaje que llevó al origen de *Lacandonia schismatica* se fueron generando los cambios ontogenéticos que posibilitarían la emergencia del fenotipo homeótico de este género? Se ha hecho una contribución para esclarecer las afinidades al interior del orden de las Pandanales en donde se encuentran las Triuridáceas. Esta contribución es una aproximación útil para sugerir patrones evolutivos presentes en el ancestro de este grupo, así como para abordar desde el punto de vista filogenético si las estructuras reproductivas de esta familia de micoheterótrofas son flores verdaderas o inflorescencias reducidas. La evidencia y análisis realizados hasta el momento sugieren fuertemente que las Triuridáceas son más afines con otra familia con flores verdaderas; Velloziaceae y que el ancestro común a las Triuridáceas muy probablemente tenía flores verdaderas (ver Capítulo 3 y Piñeyro-Nelson et al., en preparación). Por lo tanto, la hipótesis más parsimoniosa de acuerdo a la evidencia presentada en esta tesis en torno a la identidad de las estructuras reproductivas de las Triuridaceae es que sean flores verdaderas.

En las siguientes secciones se reseñan algunas de las líneas de investigación que quedan abiertas y que complementarían de manera importante la información presentada previamente en torno a *Lacandonia* y en esta tesis y los artículos científicos anexos, con respecto a la comprensión de los procesos evolutivos que hicieron posible la aparición del género *Lacandonia* en el continente americano y el establecimiento de *L. schismatica* en la

selva Lacandona de México. También se plantean algunas líneas de investigación adicionales en las cuales *L. schismatica* y su taxón hermano podrían resultar muy útiles.

PERSPECTIVAS

4.1. Estudio comparativo de los patrones de expresión de los genes BC en Triuridáceas

El enfoque comparativo de procesos de desarrollo en especies cercanamente emparentadas es también muy útil para entender la evolución de mecanismos de desarrollo y características morfológicas de las plantas. Este enfoque ya ha sido utilizado por otros autores en el estudio de la ontogenia de *L. schismatica*, donde se ha investigado la variación morfológica de individuos del taxón hermano *Triuris brevistylis*, otra especie de Triuridácea que es dioica. Los análisis realizados en estas dos especies se han enfocado en documentar si los fenotipos florales de *L. schismatica* y *T. brevistylis* están fijados en las poblaciones conocidas de estas especies, además de observar la frecuencia de flores atípicas (Vergara-Silva et al., 2003). También se ha estudiado el desarrollo floral en ambas especies a través de series de desarrollo comparativas (Ambrose et al., 2006). Recientemente se ha publicado otro estudio donde se documentan con mayor detalle las variaciones florales presentes en *T. brevistylis*, así como la existencia de flores "hermafroditas crípticas" en esta especie, las cuales al igual que *L. schismatica*, presentan fertilización cleistogámica (Espinosa-Matías et al., 2012).

La información recabada en estos estudios comparativos, donde se observa que en *T. brevistylis* ya existen patrones de desarrollo floral reminiscentes de lo observado en *Lacandonia*, queda por ser complementada con estudios de la expresión *in situ* de los genes de función B (*TbPI, TbAP3v1* y *TbAP3v2*) y C (*TbAG*) en este taxón, los cuales pueden generar hipótesis adicionales acerca de los mecanismos y procesos morfogenéticos que subyacen tras el desarrollo floral en estas especies y explorar distintos escenarios evolutivos y sus estructuras reproductivas en estos dos taxones Adicionalmente, será interesante documentar los patrones de expresión *in situ* y la evolución molecular de los genes que regulan a los genes ABC, como son los genes de la familia *SEPALLATA* (Honma y Goto, 2001; Kauffman et al.,

2009), así como aquellos factores de transcripción que regulan la expresión temprana de *AP3* (*LFY* y *UFO*) y otros que delimitan la frontera en el domino de expresión de *AP3* en etapas posteriores de desarrollo (por ejemplo, *SUPERMAN*; Bowman et al., 1992), tanto en *L. schismatica* como en *T. brevistylis*.

Otra aproximación comparativa que permitirá definir de manera más exhaustiva los posibles motivos regulatorios en *cis* de los genes *AP3* en estas dos especies, será mediante un análisis tipo "phylogenetic footprinting" (por ejemplo: De Bodt et al., 2006) donde se comparen los motivos compartidos y divergentes presentes en el promotor de *LsAP3* con los promotores de *TbAP3v1* y *TbAP3v2*. La clonación de los promotores de *T. brevistylis* se está realizando en el laboratorio de Elena Álvarez-Buylla.

4.2. Estudio de las Triuridáceas americanas: desentrañando el origen evolutivo de *Lacandonia*

El estudio presentado en el capítulo 3 en torno a las relaciones filogenéticas al interior de las Pandanales y el mapeo de diversos caracteres florales sobre la filogenia de este orden sugieren que la evolución floral de este clado ha sido bastante compleja (Piñeyro-Nelson et al, en preparación). Este análisis, junto con la información sobre los diferentes tipos de sistemas sexuales (plantas monoicas, dioicas, plantas con flores bisexuales) al interior de las Triuridáceas (Maas-van de Kramer y Weustenfeld, 1998; Rudall y Bateman, 2006) sugieren que con el fin de obtener una mejor comprensión de la evolución floral en *L. schismatica* y *T. brevistylis*, sería importante incorporar en un análisis tanto morfológico como filogenético a más miembros de las Triuridáceas, con especial énfasis en especies que poseen flores bisexuales no homeóticas y especies que pertenecen a la misma tribu de estas dos especies. En este sentido, sería interesante poder realizar series de desarrollo floral en especies como *Sciaphila picta* o *S. rubra,* que poseen flores hermafroditas típicas (Maas y Rubsamen, 1986), con el fin de averiguar si la ontogenia floral en estas especies bisexuales se parece más a la presente en otras angiospermas o si existen indicios de una "predisposición" a la homeosis floral.

L. schismatica y *T. brevistylis* pertenecen a la tribu Triurideae que adicionalmente incluye a los géneros *Peltophyllum* y *Triuridopsis* (Maas-van de Kramer y Weustenfeld, 1998). Esta tribu presenta varias características que la hacen particularmente útil para entender los procesos históricos de especiación y posibles modificaciones en la ontogenia floral que dieron origen al género *Lacandonia*: 1) a diferencia de las otras dos tribus de Triuridáceas, las Triurideae son exclusivas del continente americano y, a excepción de *T. brevistylis* y *L. schismatica*, todas las especies conocidas hasta ahora se encuentran en Sudamérica; 2) todas las especies que componen esta tribu han sido descritas como dioicas, con la excepción de las dos especies descritas para el género *Lacandonia*: *L. schismatica* y *L. brasiliana* (Melo y Alves, 2012). En este sentido, el reciente descubrimiento de *L. brasiliana* en una región de la Mata Atlántica brasileña sugiere fuertemente que en contraste con lo previamente propuesto (Vergara-Silva et al., 2003); 3), el género *Lacandonia* es más viejo de lo que se pensaba, pudiéndose haber originado en Sudamérica para después migrar al norte del continente y dar origen a *L. schismatica* y, 4) dada la reciente descripción de flores hermafroditas en *T. brevistylis* presentes en baja frecuencia dentro de esta especie (Martínez y Gómez, 1994; Espinosa-Matías et al., 2012), será interesante averiguar si esta predisposición a la homeosis floral es privativa del linaje de *Triuris* y *Lacandonia* o es extensiva a otros géneros dentro de la tribu.

Por último, el estudio de los procesos microevolutivos del género *Lacandonia* será fundamental para generar inferencias a nivel de genética de poblaciones y filogeografía que permitan establecer: i) si estamos ante dos especies distintas o una sola especie con una amplia distribución geográfica, así como sugerir ii) la historia evolutiva reciente de este género, elaborando hipótesis de las vías de dispersión más probables con base en datos de diversidad genética y estructuración de las poblaciones de cada especie o, en su defecto, de una sola especie: *L. schismatica*. Este tipo de investigaciones también podrán aportar evidencia acerca de la historia demográfica de estas especies, del papel de las fuerzas evolutivas en la fijación de sus morfologías florales, y de la estructura de sus poblaciones.

4.3. Otras investigaciones

De manera adicional a lo presentado en los tres capítulos anteriores, en el contexto de esta tesis de doctorado he estado colaborando en otras líneas adicionales y complementarias a las exploradas de manera central en mi tesis. Colaboré en la colecta y extracción de ARNm para llevar a cabo esfuerzos de secuenciación masiva del transcriptoma de los ejes reproductivos de *L. schismatica* y los dos morfos (inflorescencias macho y hembra) de *T. brevistylis*, así como otra especie mycoheterótrofa no relacionada: *Gymnosiphon divaricatus* (Burmanniaceae).

Las secuencias recuperadas a partir de la secuenciación, ensamble y curaduría de transcriptomas y otros "omas" (proteoma, genoma, etc.), han demostrado ser útiles para inferir procesos de evolución genómica, como son las duplicaciones genómicas ancestrales que han ocurrido en diferentes linajes de seres vivos (Lynch y Conery, 2000), permitiendo en algunos casos reconstruir las historias evolutivas de un linaje particular tomando en cuenta los posibles efectos macroevolutivos de cambios drásticos en el genoma (Cui et al., 2007). A su vez, la secuenciación masiva de transcriptomas y proteomas ha posibilitado la realización de un inventario fino de los transcritos y proteínas que se expresan en un organismo o tejido en un momento particular del desarrollo y/o los cambios regulatorios que ocurren ante un cambio en las condiciones bióticas o abióticas (por ejemplo, Meyers et al., 2004). Este tipo de aproximaciones pueden ayudar a obtener un repertorio más exhaustivo de las moléculas que se expresan en diferentes etapas de vida de un organismo, pero también a elaborar hipótesis en torno a los módulos genéticos involucrados en las vías de respuesta a ciertos procesos de desarrollo o estímulos externos que pueden después ser probadas experimentalmente.

En el caso de proyectos de investigación enfocados a especies que no han podido reproducirse *ex situ* y que por lo tanto existe un acceso restringido a su material biológico, como es el caso de *L. schismatica* y *T. brevistylis*, estas estrategias son particularmente pertinentes. Por ejemplo, permiten aislar las secuencias de los homólogos de genes que se han documentado en otras especies como importantes para el desarrollo, metabolismo, homeostasis, etc., sin recurrir a métodos de clonación experimentales que generalmente

requieren de bastante material para la extraccion de ácidos nucleicos (ADN o ARN). En este mismo sentido, diversos genes se están aislando y caracterizando bioinformáticamente, y se están llevando a cabo análisis de evolución molecular.

Otra línea que se ha iniciado es la de recuperar los genes involucrados en la red de regulación genética implicada en el establecimiento de las configuraciones genéticas estables que subyacen a la especificación de los cuatro verticilos florales en otras angiospermas (Espinosa-Soto et al., 2004). En una primera aproximación, se puede investigar si los cambios en los patrones de evolución molecular de ciertas secuencias o residuos dentro de genes particulares de esta red pudieran ocasionar cambios en la dinámica de la misma, mientras que se puede recurrir a aproximaciones experimentales para corroborar las inferencias hechas.

Actualmente se está terminando la curaduría y descripción de las secuencias obtenidas para los transcriptomas de estas dos Triuridáceas, mientras que se están aislando y analizando aquellos genes considerados importantes en la red de regulación genética floral en ambas especies.

Otros fenómeno que no es privativo de las Triuridáceas pero que es poco común en angiospermas y que sería interesante abordar con los datos derivados del transcriptoma, es la evolución del hábito micoheterotrófico (Bidartondo, 2005). Realizando un esfuerzo de minería de datos se podrían aislar aquellos genes relacionados con las vías de síntesis, traslocación y polimerización de azúcares que estuviesen presentes en *L. schismatica* y *T. brevistylis*, lo que potencialmente podría permitir inferir qué partes de este metabolismo de azúcares se encuentran silenciadas en estas dos especies que no poseen cloroplastos funcionales (se mantienen como amiloplastos durante todo el ciclo de vida de, al menos, *L. schismatica*; Jiménez-García et al., 1998). Realizando el mismo análisis en especies tales como *G. divaricatus* y otros micoheterótrofos se podría investigar si hay patrones de evolución convergente entre angiospermas que han adquirido este hábito de manera independiente (Bidartondo, 2005). En este mismo sentido, estas especies podrían servir para refinar la caracterización de los aportes del genoma de cloroplasto en el metabolismo de las plantas más allá de la síntesis de azúcares simples, puesto que en estos

micoheterótrofos, los genes relacionados con producción de clorofila no están expresados. sin embargo, en análisis preliminares de los genes obtenidos en el transcriptoma de *L. schismatica* se han encontrado transcritos de genes relacionados con el cloroplasto. De los datos obtenidos no se podrá definir si éstos transcritos provenían de los amiloplastos o de genes de cloroplasto que han migrado al genoma nuclear, fenómeno que ha sido documentado en varias plantas, sin embargo será importante analizar de manera sistemática qué genes de cloroplasto se encuentran representados en los datos del transcriptoma, así como si éstos presentan indicios de estar conservados mediante procesos de selección o si, por el contrario, están evolucionando hacia convertirse en pseudogenes. Las inferencias que puedan surgir en torno a la evolución de la micoheterotrofía en estas especies podrán contrastarse mediante análisis comparativos de los transcriptomas generados en linajes que poseen desde especies aclorofílicas obligadas hasta aquellas que lo son únicamente durante una parte de su ciclo de vida, como es el caso del género *Cuscuta* (Convolvulaceae). Este tipo de estudios servirían para entender cuáles son los caminos evolutivos para perder la funcionalidad de un organelo que define a las viridofitas.

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6. ANEXOS

Anexo A: Material suplementario

Apéndice I: motivos de regulación en *cis* de los promotores de AP3 de *L. schismatica, L. regale* y *A. thaliana*.

Web Signal Scan Program

Database searched: PLACE

This is the sequence you submitted
>Arabidopsis/thaliana AP3 promoter from -12, 1748 bases, 7026D9F4 checksum.
AAGCTTCTTAAGAATTATAGTAGCACTTGTTGATATCGGGGTTCTTTTTT
CTTAACATAGGTTTTGGTGGGTAGATCAACAGAAGACCTCGCTGTGGACA
TTGATTTGGGAAGAGAAAAGCGGGGTAGCAAAATATCTCGACGACAGGTC
GGTCAATAGTAGATACTTCTATTTGTATTTTAGGTCTTTAAGTTGTATGA
GAAGCAGCAGCCCAGGATCTGTAATGGTTGTTGTGTATGTTTTTTTCCTT
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... .

RESULTS OF YOUR SIGNAL SCAN SEARCH REQUEST

../../tmp/sigscan//signalseqdone.14570: 1748 base pairs
Signal database file: user.dat

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE #
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ARR1AT	site 756 (+)	NGATT	S000454 </sigscan/disp.cgi?S000454>
CAATBOX1	site 758 (-)	CAAT	S000028 </sigscan/disp.cgi?S000028>
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DOFCOREZM site 1594 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
ARR1AT site 1601 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
CAATBOX1 site 1609 (+) CAAT S000028 </sigscan/disp.cgi?S000028>
CACTFTPPCA1 site 1612 (+) YACT S000449 </sigscan/disp.cgi?S000449>
AGL2ATCONSENSUS site 1614 (+) NNWNCCAWWWWTRGWWAN S000339 </sigscan/disp.cgi?S000339>
CARG3ATAP3 site 1614 (+) CTTTCCATTTTTAGTAAC S000349 </sigscan/disp.cgi?S000349>
DOFCOREZM site 1614 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
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SEF4MOTIFGM7S site 1620 (+) RTTTTTR S000103 </sigscan/disp.cgi?S000103>
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EBOXBNNAPA site 1633 (+) CANNTG S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT site 1633 (+) CANNTG S000407 </sigscan/disp.cgi?S000407>
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DOFCOREZM site 1643 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
TAAAGSTKST1 site 1643 (-) TAAAG S000387 </sigscan/disp.cgi?S000387>
CACTFTPPCA1 site 1646 (+) YACT S000449 </sigscan/disp.cgi?S000449>
CAREOSREP1 site 1654 (+) CAACTC S000421 </sigscan/disp.cgi?S000421>
CIACADIANLELHC site 1654 (+) CAANNNNATC S000252 </sigscan/disp.cgi?S000252>
CTRMCAMV35S site 1662 (+) TCTCTCTCT S000460 </sigscan/disp.cgi?S000460>
NODCON2GM site 1667 (+) CTCTT S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE site 1667 (+) CTCTT S000468 </sigscan/disp.cgi?S000468>
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POLLEN1LELAT52 site 1670 (-) AGAAA S000245 </sigscan/disp.cgi?S000245>
BOXIINTPATPB site 1671 (-) ATAGAA S000296 </sigscan/disp.cgi?S000296>
INRNTPSADB site 1677 (+) YTCANTYY S000395 </sigscan/disp.cgi?S000395>
GTGANTG10 site 1678 (-) GTGA S000378 </sigscan/disp.cgi?S000378>
CACTFTPPCA1 site 1679 (+) YACT S000449 </sigscan/disp.cgi?S000449>
DOFCOREZM site 1684 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
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GATABOX site 1698 (-) GATA S000039 </sigscan/disp.cgi?S000039>
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DOFCOREZM site 1733 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
NODCON1GM site 1733 (+) AAAGAT S000461 </sigscan/disp.cgi?S000461>
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ARR1AT site 1735 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
ANAERO1CONSENSUS site 1740 (+) AAACAAA S000477 </sigscan/disp.cgi?S000477>
DOFCOREZM site 1744 (+) AAAG S000265 </sigscan/disp.cgi?S000265>

Web Signal Scan Program

Database searched: PLACE

This is the sequence you submitted
>Lacandonia schismatica proLsAP3 since -1, 1107 bases, 1AD9F67F checksum.
--CGACCTGCAGGCGGCCGCGAATTCACTAGTGATTACTATAGGGCACGC
GTGGTCGACGGCCCGGGCTGGTAAACAAAATGTACTAGGAGAACCGACCG
TAGTCTTTTGGTTCTCAAGTATGAAGGCAAACCAACAAAAGCATATTATA
ATATTTGCATAAACATAAATGCATCTCATTTAACTGGAAACATAAAGCTG
CCCTTCCCATCTCTCTGAAAGGATCCCAGTTTTCAAAGTGTTTCCTCACA
CCTCTCAGTTGTTCAATCTAATTCCGATCTCCTGGAATCATGCATTTTAT
CCCTTAGGATTTAACAACAAGCAACTGCTTACTTAACAACTGATTTGCAT
TTGTTAACCTCTTTTAACAAACCTTAATCCCAACTGGCTTAGCTAAAACT
GAGTCTAACTGACTCTAACAAATCTCCTCTCTAGAGTCCCTGCTGACTCC
TCCCCTTTGAATCCAAGATTAGCCAACCGCGGCTACTTAATTATTATTGC
TAACAACGAAGTAGGCCACATGCAGACTAGGCCCTGGTTCTAACTTAGCT
CCATAACCTCAATCAAGTGATGAATCGATCTCTTTATCTGAAGGAACCTT
TATTTGGTTCCTAGACTAATAATCCCTCGTCTAATCTGGCACCACGGGCT
CTGCGCTCTAAATTAACAATTTATATAAAGAGGGCGAGGCCCGCTTAGCG
ATTTCAAGGCAATTAAGAGTCTGAAGTAACCATATATATTTCATTTATTA
ATTTTCCCAAGCATAGACAACATTTCATCCTTGATATTGCAAGCAACACC
AAGTGAGCACATGCAGCCTATGACTTGGGTCCTAAATAACCTAACTAAAT
GACCAACATGCAAACATCTTTTCTTTTTAAAGATAAAGAAATACCAGCAA
GACAGAATGCCTGGAACGACCAGGAAAGGCCGTACAGTTTGAAACAGCTC
TTCCAGAAAGACAAGTTCTAAGCAAATTCCGTCCACACGATAATATCTGC
AAGAAAGAGAGATCGATAGTCATTGGTTCTCTCTGTCATTAATTGTTGCC
ATCAAGGCATCTCCATCTCTAACTTCCTCCACCGCCCGCTCTCCCTCTCC
TCCGGCC

... .

RESULTS OF YOUR SIGNAL SCAN SEARCH REQUEST

../../tmp/sigscan//signalseqdone.14617: 1107 base pairs
Signal database file: user.dat

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE #
INTRONLOWER	site 6 (-)	TGCAGG	S000086 </sigscan/disp.cgi?S000086>
INTRONLOWER	site 8 (+)	TGCAGG	S000086 </sigscan/disp.cgi?S000086>
GCCCORE	site 12 (-)	GCCGCC	S000430 </sigscan/disp.cgi?S000430>
E2FCONSENSUS	site 17 (-)	WTTSSCSS	S000476 </sigscan/disp.cgi?S000476>
GTGANTG10	site 25 (-)	GTGA	S000378 </sigscan/disp.cgi?S000378>
CACTFTPPCA1	site 26 (+)	YACT	S000449 </sigscan/disp.cgi?S000449>
CACTFTPPCA1	site 30 (-)	YACT	S000449 </sigscan/disp.cgi?S000449>
GTGANTG10	site 31 (+)	GTGA	S000378 </sigscan/disp.cgi?S000378>
ARR1AT	site 32 (+)	NGATT	S000454 </sigscan/disp.cgi?S000454>
CACTFTPPCA1	site 36 (+)	YACT	S000449 </sigscan/disp.cgi?S000449>
ABRERATCAL	site 46 (+)	MACGYGB	S000507 </sigscan/disp.cgi?S000507>
CGCGBOXAT	site 47 (+)	VCGCGB	S000501 </sigscan/disp.cgi?S000501>
ABRERATCAL	site 47 (-)	MACGYGB	S000507 </sigscan/disp.cgi?S000507>
CGCGBOXAT	site 47 (-)	VCGCGB	S000501 </sigscan/disp.cgi?S000501>
CBFHV	site 54 (+)	RYCGAC	S000497 </sigscan/disp.cgi?S000497>
CRTDREHVCBF2	site 54 (+)	GTCGAC	S000411 </sigscan/disp.cgi?S000411>
CBFHV	site 54 (-)	RYCGAC	S000497 </sigscan/disp.cgi?S000497>
CRTDREHVCBF2	site 54 (-)	GTCGAC	S000411 </sigscan/disp.cgi?S000411>
CGACGOSAMY3	site 56 (+)	CGACG	S000205 </sigscan/disp.cgi?S000205>
HEXAMERATH4	site 56 (-)	CCGTCG	S000146 </sigscan/disp.cgi?S000146>
SORLIP2AT	site 60 (-)	GGGCC	S000483 </sigscan/disp.cgi?S000483>
GT1CONSENSUS	site 70 (+)	GRWAAW	S000198 </sigscan/disp.cgi?S000198>
ANAERO1CONSENSUS	site 73 (+)	AAACAAA	S000477 </sigscan/disp.cgi?S000477>
CURECORECR	site 82 (+)	GTAC	S000493 </sigscan/disp.cgi?S000493>
CURECORECR	site 82 (-)	GTAC	S000493 </sigscan/disp.cgi?S000493>

CACTFTPPCA1 site 83 (+) YACT S000449 </sigscan/disp.cgi?S000449>
CBFHV site 93 (+) RYCGAC S000497 </sigscan/disp.cgi?S000497>
DRE2COREZMRAB17 site 93 (+) ACCGAC S000402 </sigscan/disp.cgi?S000402>
DRECRTCOREAT site 93 (+) RCCGAC S000418 </sigscan/disp.cgi?S000418>
LTRECOREATCOR15 site 94 (+) CCGAC S000153 </sigscan/disp.cgi?S000153>
PRECONSCRHSP70A site 94 (+) SCGAYNRNNNNNNNNNNNNNNHD S000506 </sigscan/disp.cgi?S000506>
DOFCOREZM site 105 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
REALPHALGLHCB21 site 108 (-) AACCAA S000362 </sigscan/disp.cgi?S000362>
CACTFTPPCA1 site 118 (-) YACT S000449 </sigscan/disp.cgi?S000449>
MYB1AT site 129 (+) WAACCA S000408 </sigscan/disp.cgi?S000408>
REALPHALGLHCB21 site 130 (+) AACCAA S000362 </sigscan/disp.cgi?S000362>
RAV1AAT site 133 (+) CAACA S000314 </sigscan/disp.cgi?S000314>
DOFCOREZM site 138 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
ROOTMOTIFTAPOX1 site 143 (+) ATATT S000098 </sigscan/disp.cgi?S000098>
ROOTMOTIFTAPOX1 site 150 (-) ATATT S000098 </sigscan/disp.cgi?S000098>
ROOTMOTIFTAPOX1 site 151 (+) ATATT S000098 </sigscan/disp.cgi?S000098>
MARARS site 160 (-) WTTTATRTTTW S000064 </sigscan/disp.cgi?S000064>
L1BOXATPDF1 site 166 (+) TAAATGYA S000386 </sigscan/disp.cgi?S000386>
MYB2AT site 181 (+) TAACTG S000177 </sigscan/disp.cgi?S000177>
MYB2CONSENSUSAT site 181 (+) YAACKG S000409 </sigscan/disp.cgi?S000409>
MYBCORE site 181 (-) CNGTTR S000176 </sigscan/disp.cgi?S000176>
TAAAGSTKST1 site 193 (+) TAAAG S000387 </sigscan/disp.cgi?S000387>
DOFCOREZM site 194 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
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CEREGLUBOX2PSLEGA site 228 (-) TGAAAACT S000033 </sigscan/disp.cgi?S000033>
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ARR1AT site 265 (-) NGATT S000454 </sigscan/disp.cgi?S000454>
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RYREPEATLEGUMINBOX site 289 (+) CATGCAY S000100 </sigscan/disp.cgi?S000100>
GT1CONSENSUS site 296 (-) GRWAAW S000198 </sigscan/disp.cgi?S000198>
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WBOXNTERF3 site 410 (+) TGACY S000457 </sigscan/disp.cgi?S000457>
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CACTFTPPCA1 site 566 (-) YACT S000449 </sigscan/disp.cgi?S000449>
GTGANTG10 site 567 (+) GTGA S000378 </sigscan/disp.cgi?S000378>
ANAERO3CONSENSUS site 567 (-) TCATCAC S000479 </sigscan/disp.cgi?S000479>

ARR1AT	site 573 (-) NGATT	S000454 </sigscan/disp.cgi?S000454>
NODCON2GM	site 580 (+) CTCTT	S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE	site 580 (+) CTCTT	S000468 </sigscan/disp.cgi?S000468>
DOFCOREZM	site 582 (-) AAAG	S000265 </sigscan/disp.cgi?S000265>
TAAAGSTKST1	site 582 (-) TAAAG	S000387 </sigscan/disp.cgi?S000387>
GT1CONSENSUS	site 583 (-) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
IBOXCORE	site 584 (-) GATAA	S000199 </sigscan/disp.cgi?S000199>
GATABOX	site 585 (-) GATA	S000039 </sigscan/disp.cgi?S000039>
DOFCOREZM	site 598 (-) AAAG	S000265 </sigscan/disp.cgi?S000265>
TAAAGSTKST1	site 598 (-) TAAAG	S000387 </sigscan/disp.cgi?S000387>
POLASIG1	site 599 (-) AATAAA	S000080 </sigscan/disp.cgi?S000080>
TATABOX5	site 600 (+) TTATTT	S000203 </sigscan/disp.cgi?S000203>
REALPHALGLHCB21	site 604 (-) AACCAA	S000362 </sigscan/disp.cgi?S000362>
CPBCSPOR	site 616 (-) TATTAG	S000491 </sigscan/disp.cgi?S000491>
POLASIG3	site 618 (+) AATAAT	S000088 </sigscan/disp.cgi?S000088>
ARR1AT	site 621 (-) NGATT	S000454 </sigscan/disp.cgi?S000454>
ARR1AT	site 633 (-) NGATT	S000454 </sigscan/disp.cgi?S000454>
CAATBOX1	site 667 (+) CAAT	S000028 </sigscan/disp.cgi?S000028>
TATABOX2	site 669 (-) TATAAAT	S000109 </sigscan/disp.cgi?S000109>
TATAPVTRNALEU	site 670 (+) TTTATATA	S000340 </sigscan/disp.cgi?S000340>
TATABOX4	site 671 (-) TATATAA	S000111 </sigscan/disp.cgi?S000111>
TATABOX4	site 672 (+) TATATAA	S000111 </sigscan/disp.cgi?S000111>
TATAPVTRNALEU	site 672 (-) TTTATATA	S000340 </sigscan/disp.cgi?S000340>
TAAAGSTKST1	site 676 (+) TAAAG	S000387 </sigscan/disp.cgi?S000387>
DOFCOREZM	site 677 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
NODCON2GM	site 678 (-) CTCTT	S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE	site 678 (-) CTCTT	S000468 </sigscan/disp.cgi?S000468>
SORLIP2AT	site 688 (-) GGGCC	S000483 </sigscan/disp.cgi?S000483>
BS1EGCCR	site 690 (-) AGCGGG	S000352 </sigscan/disp.cgi?S000352>
ARR1AT	site 699 (+) NGATT	S000454 </sigscan/disp.cgi?S000454>
CAATBOX1	site 710 (+) CAAT	S000028 </sigscan/disp.cgi?S000028>
NODCON2GM	site 715 (-) CTCTT	S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE	site 715 (-) CTCTT	S000468 </sigscan/disp.cgi?S000468>
CACTFTPPCA1	site 725 (-) YACT	S000449 </sigscan/disp.cgi?S000449>
MYB1AT	site 727 (+) WAACCA	S000408 </sigscan/disp.cgi?S000408>
ROOTMOTIFTAPOX1	site 736 (+) ATATT	S000098 </sigscan/disp.cgi?S000098>
POLASIG1	site 744 (-) AATAAA	S000080 </sigscan/disp.cgi?S000080>
TATABOX3	site 746 (+) TATTAAT	S000110 </sigscan/disp.cgi?S000110>
GT1CONSENSUS	site 751 (-) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
GT1CONSENSUS	site 752 (-) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
RAV1AAT	site 768 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>
GATABOX	site 783 (+) GATA	S000039 </sigscan/disp.cgi?S000039>
ROOTMOTIFTAPOX1	site 784 (+) ATATT	S000098 </sigscan/disp.cgi?S000098>
CAATBOX1	site 786 (-) CAAT	S000028 </sigscan/disp.cgi?S000028>
RAV1AAT	site 794 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>
EBOXBNNAPA	site 800 (+) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT	site 800 (+) CANNTG	S000407 </sigscan/disp.cgi?S000407>
EBOXBNNAPA	site 800 (-) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT	site 800 (-) CANNTG	S000407 </sigscan/disp.cgi?S000407>
CACTFTPPCA1	site 802 (-) YACT	S000449 </sigscan/disp.cgi?S000449>
GTGANTG10	site 803 (+) GTGA	S000378 </sigscan/disp.cgi?S000378>
EBOXBNNAPA	site 808 (+) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCATRD22	site 808 (+) CACATG	S000174 </sigscan/disp.cgi?S000174>
MYCCONSENSUSAT	site 808 (+) CANNTG	S000407 </sigscan/disp.cgi?S000407>
EBOXBNNAPA	site 808 (-) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCATERD1	site 808 (-) CATGTG	S000413 </sigscan/disp.cgi?S000413>
MYCCONSENSUSAT	site 808 (-) CANNTG	S000407 </sigscan/disp.cgi?S000407>
RYREPEATBNNAPA	site 810 (+) CATGCA	S000264 </sigscan/disp.cgi?S000264>
WBOXHVISO1	site 821 (+) TGACT	S000442 </sigscan/disp.cgi?S000442>
WBOXNTERF3	site 821 (+) TGACY	S000457 </sigscan/disp.cgi?S000457>
WRKY71OS	site 821 (+) TGAC	S000447 </sigscan/disp.cgi?S000447>
TATABOX5	site 834 (-) TTATTT	S000203 </sigscan/disp.cgi?S000203>
MYBPLANT	site 838 (+) MACCWAMC	S000167 </sigscan/disp.cgi?S000167>
MYB26PS	site 838 (-) GTTAGGTT	S000182 </sigscan/disp.cgi?S000182>
WBOXNTERF3	site 850 (+) TGACY	S000457 </sigscan/disp.cgi?S000457>
WRKY71OS	site 850 (+) TGAC	S000447 </sigscan/disp.cgi?S000447>
RAV1AAT	site 854 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>

RYREPEATBNNAPA	site	857 (+) CATGCA	S000264 </sigscan/disp.cgi?S000264>
NODCON1GM	site	866 (-) AAAGAT	S000461 </sigscan/disp.cgi?S000461>
OSE1ROOTNODULE	site	866 (-) AAAGAT	S000467 </sigscan/disp.cgi?S000467>
DOFCOREZM	site	868 (-) AAAG	S000265 </sigscan/disp.cgi?S000265>
POLLEN1LELAT52	site	870 (-) AGAAA	S000245 </sigscan/disp.cgi?S000245>
CARGCW8GAT	site	873 (+) CWWWWWWWWG	S000431 </sigscan/disp.cgi?S000431>
CARGCW8GAT	site	873 (-) CWWWWWWWWG	S000431 </sigscan/disp.cgi?S000431>
DOFCOREZM	site	873 (-) AAAG	S000265 </sigscan/disp.cgi?S000265>
TAAAGSTKST1	site	878 (+) TAAAG	S000387 </sigscan/disp.cgi?S000387>
DOFCOREZM	site	879 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
NODCON1GM	site	879 (+) AAAGAT	S000461 </sigscan/disp.cgi?S000461>
OSE1ROOTNODULE	site	879 (+) AAAGAT	S000467 </sigscan/disp.cgi?S000467>
GATABOX	site	882 (+) GATA	S000039 </sigscan/disp.cgi?S000039>
GT1CONSENSUS	site	882 (+) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
IBOXCORE	site	882 (+) GATAA	S000199 </sigscan/disp.cgi?S000199>
TAAAGSTKST1	site	884 (+) TAAAG	S000387 </sigscan/disp.cgi?S000387>
DOFCOREZM	site	885 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
POLLEN1LELAT52	site	887 (+) AGAAA	S000245 </sigscan/disp.cgi?S000245>
DOFCOREZM	site	925 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
CURECORECR	site	932 (+) GTAC	S000493 </sigscan/disp.cgi?S000493>
CURECORECR	site	932 (-) GTAC	S000493 </sigscan/disp.cgi?S000493>
NODCON2GM	site	948 (+) CTCTT	S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE	site	948 (+) CTCTT	S000468 </sigscan/disp.cgi?S000468>
POLLEN1LELAT52	site	955 (+) AGAAA	S000245 </sigscan/disp.cgi?S000245>
DOFCOREZM	site	957 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
PALBOXAPC	site	979 (+) CCGTCC	S000137 </sigscan/disp.cgi?S000137>
CMSRE1IBSPOA	site	979 (-) TGGACGG	S000511 </sigscan/disp.cgi?S000511>
GATABOX	site	989 (+) GATA	S000039 </sigscan/disp.cgi?S000039>
GT1CONSENSUS	site	989 (+) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
IBOXCORE	site	989 (+) GATAA	S000199 </sigscan/disp.cgi?S000199>
ROOTMOTIFTAPOX1	site	992 (-) ATATT	S000098 </sigscan/disp.cgi?S000098>
GATABOX	site	994 (-) GATA	S000039 </sigscan/disp.cgi?S000039>
POLLEN1LELAT52	site	1002 (+) AGAAA	S000245 </sigscan/disp.cgi?S000245>
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NODCON2GM	site	1005 (-) CTCTT	S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE	site	1005 (-) CTCTT	S000468 </sigscan/disp.cgi?S000468>
GATABOX	site	1015 (+) GATA	S000039 </sigscan/disp.cgi?S000039>
WBOXHVISO1	site	1018 (-) TGACT	S000442 </sigscan/disp.cgi?S000442>
WBOXNTERF3	site	1018 (-) TGACY	S000457 </sigscan/disp.cgi?S000457>
WRKY71OS	site	1019 (-) TGAC	S000447 </sigscan/disp.cgi?S000447>
CAATBOX1	site	1022 (-) CAAT	S000028 </sigscan/disp.cgi?S000028>
CCAATBOX1	site	1022 (-) CCAAT	S000030 </sigscan/disp.cgi?S000030>
REALPHALGLHCB21	site	1023 (-) AACCAA	S000362 </sigscan/disp.cgi?S000362>
BIHD1OS	site	1034 (+) TGTCA	S000498 </sigscan/disp.cgi?S000498>
WRKY71OS	site	1035 (-) TGAC	S000447 </sigscan/disp.cgi?S000447>
CAATBOX1	site	1042 (-) CAAT	S000028 </sigscan/disp.cgi?S000028>
RAV1AAT	site	1044 (-) CAACA	S000314 </sigscan/disp.cgi?S000314>
BS1EGCCR	site	1085 (-) AGCGGG	S000352 </sigscan/disp.cgi?S000352>

Web Signal Scan Program

Database searched: PLACE

This is the sequence you submitted
>Lilium regale proLRDEF 1-1096 , 1096 bases, 639CA571 checksum.
TTTAGTCGGTTACACGATTGCTTGATCACACTTCACAAGTCAAATTACTT
TGTATCACATTTTTAACCAGTTTGATTATAACTTCTTACCTGTATGATAC
TCAGCCTTAATTATCTGTACTTTGATTCATAAATAAAGAACCCTTGATCT
GTACCGTTAGACTAAATTTGGATGATAATTTTATTGCATTTTCAAAGAGG
AGCTTGGCTTGTGGGGTGAGTTTGCATATGTATCCAAGAGTTGGAGAGGA
TGGGGATTTTGGGAGGGAATGGAGGGAATTGTGGTTAGTGGGAGATATTG
TGTTTTCAGTGAAATAAGTAGTGAGGTATGATGGGTGAGGCTTGTAGTTG
GATGTTAGGGTATGTGAATGGGAGGCCATGATGAAAGTGTGTTGGCAGAA
GAACGATAAGGAGGAGGGTGATTGGTGGATAATAGTGGAACGTATAATTG
GGGGAATATGATTAGGGCCACCACAATTTTGGAGAGTTCGGATTCAGAAG
GGTAGTTTTTTTTTTAACATCCGATCTTTTTATTTTTCTGAATAGTAGAA
AATGAAAAAGAATGGAGGGTGTGATATGATGAGAGAGAAGAGTGATAGAG
ACAATTATGGATATTGAAGGAATGGTGAGGTGTCGATGATAAATGGTGTA
GGATGATTGTTAATGGCGGGGGAGTGAACAAAAAACTAAGGAAATAGGAA
GCAGGGAGTATCTCACCAAATCTAGATACATGGGAGCCAACATATGGGAT
TTCTGGTCAGCCGTCCGCCTTATCCCAGCCCCTACTCCCATCCCTATTCT
CAACAGTCTTGCGGTAAATAAAGCCACAGAGGGTGGCGGGCTTCATAGGA
AAATGTGAAACAACACAAAGGAAAAGTGACCGATTCTTAAACAGGCAAGA
CACAGCACGTTGCCTCGTACACCCCACTCTTTCATCAATTTGGCAAAACT
TTCCATTTATAGCAATTCTAGCTTTTGCAGCCCCTCCCAAATTCAGACAT
TACCCTTCCTCTTGCTTTCCCCCTCAGTCCAAACCTCCCACCCACCGCTC
CTTTATATCCCACCACCGCCTCCACCATCCCCTCAACAGATCAGTC

... .

RESULTS OF YOUR SIGNAL SCAN SEARCH REQUEST

../../tmp/sigscan//signalseqdone.14790: 1096 base pairs
Signal database file: user.dat

Factor or Site Name	Loc.(Str.)	Signal Sequence	SITE #
CBFHV	site 5 (-)	RYCGAC	S000497 </sigscan/disp.cgi?S000497>
DRE2COREZMRAB17	site 5 (-)	ACCGAC	S000402 </sigscan/disp.cgi?S000402>
DRECRTCOREAT	site 5 (-)	RCCGAC	S000418 </sigscan/disp.cgi?S000418>
LTRECOREATCOR15	site 5 (-)	CCGAC	S000153 </sigscan/disp.cgi?S000153>
MYBCORE	site 7 (+)	CNGTTR	S000176 </sigscan/disp.cgi?S000176>
ARR1AT	site 15 (+)	NGATT	S000454 </sigscan/disp.cgi?S000454>
CAATBOX1	site 17 (-)	CAAT	S000028 </sigscan/disp.cgi?S000028>
GTGANTG10	site 26 (-)	GTGA	S000378 </sigscan/disp.cgi?S000378>
CACTFTPPCA1	site 29 (+)	YACT	S000449 </sigscan/disp.cgi?S000449>
GTGANTG10	site 33 (-)	GTGA	S000378 </sigscan/disp.cgi?S000378>
WBBOXPCWRKY1	site 38 (-)	TTTGACY	S000310 </sigscan/disp.cgi?S000310>
WBOXHVISO1	site 38 (-)	TGACT	S000442 </sigscan/disp.cgi?S000442>
WBOXNTERF3	site 38 (-)	TGACY	S000457 </sigscan/disp.cgi?S000457>
WBOXATNPR1	site 39 (-)	TTGAC	S000390 </sigscan/disp.cgi?S000390>
WRKY71OS	site 39 (-)	TGAC	S000447 </sigscan/disp.cgi?S000447>
CACTFTPPCA1	site 46 (+)	YACT	S000449 </sigscan/disp.cgi?S000449>
TBOXATGAPB	site 47 (+)	ACTTTG	S000383 </sigscan/disp.cgi?S000383>
DOFCOREZM	site 48 (-)	AAAG	S000265 </sigscan/disp.cgi?S000265>
GATABOX	site 53 (-)	GATA	S000039 </sigscan/disp.cgi?S000039>
GTGANTG10	site 55 (-)	GTGA	S000378 </sigscan/disp.cgi?S000378>
SEF4MOTIFGM7S	site 59 (+)	RTTTTTR	S000103 </sigscan/disp.cgi?S000103>
GT1CORE	site 63 (-)	GGTTAA	S000125 </sigscan/disp.cgi?S000125>
MYB1AT	site 64 (+)	WAACCA	S000408 </sigscan/disp.cgi?S000408>
ARR1AT	site 73 (+)	NGATT	S000454 </sigscan/disp.cgi?S000454>
GATABOX	site 96 (+)	GATA	S000039 </sigscan/disp.cgi?S000039>
CACTFTPPCA1	site 98 (+)	YACT	S000449 </sigscan/disp.cgi?S000449>

GT1CONSENSUS site 110 (-) GRWAAW S000198 </sigscan/disp.cgi?S000198>
IBOXCORE site 111 (-) GATAA S000199 </sigscan/disp.cgi?S000199>
GATABOX site 112 (-) GATA S000039 </sigscan/disp.cgi?S000039>
CURECORECR site 117 (+) GTAC S000493 </sigscan/disp.cgi?S000493>
CURECORECR site 117 (-) GTAC S000493 </sigscan/disp.cgi?S000493>
CACTFTPPCA1 site 118 (+) YACT S000449 </sigscan/disp.cgi?S000449>
TBOXATGAPB site 119 (+) ACTTTG S000383 </sigscan/disp.cgi?S000383>
DOFCOREZM site 120 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
ARR1AT site 123 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
TATABOX5 site 131 (-) TTATTT S000203 </sigscan/disp.cgi?S000203>
POLASIG1 site 132 (+) AATAAA S000080 </sigscan/disp.cgi?S000080>
TAAAGSTKST1 site 134 (+) TAAAG S000387 </sigscan/disp.cgi?S000387>
DOFCOREZM site 135 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
CURECORECR site 151 (+) GTAC S000493 </sigscan/disp.cgi?S000493>
CURECORECR site 151 (-) GTAC S000493 </sigscan/disp.cgi?S000493>
MYBCORE site 154 (+) CNGTTR S000176 </sigscan/disp.cgi?S000176>
MYB2CONSENSUSAT site 154 (-) YAACKG S000409 </sigscan/disp.cgi?S000409>
MYBCOREATCYCB1 site 154 (-) AACGG S000502 </sigscan/disp.cgi?S000502>
GATABOX site 174 (+) GATA S000039 </sigscan/disp.cgi?S000039>
GT1CONSENSUS site 174 (+) GRWAAW S000198 </sigscan/disp.cgi?S000198>
IBOXCORE site 174 (+) GATAA S000199 </sigscan/disp.cgi?S000199>
POLASIG1 site 180 (-) AATAAA S000080 </sigscan/disp.cgi?S000080>
CAATBOX1 site 183 (-) CAAT S000028 </sigscan/disp.cgi?S000028>
GT1CONSENSUS site 188 (-) GRWAAW S000198 </sigscan/disp.cgi?S000198>
DOFCOREZM site 194 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
NODCON2GM site 195 (-) CTCTT S000462 </sigscan/disp.cgi?S000462>
OSE2ROOTNODULE site 195 (-) CTCTT S000468 </sigscan/disp.cgi?S000468>
GTGANTG10 site 216 (+) GTGA S000378 </sigscan/disp.cgi?S000378>
CATATGGMSAUR site 225 (+) CATATG S000370 </sigscan/disp.cgi?S000370>
EBOXBNNAPA site 225 (+) CANNTG S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT site 225 (+) CANNTG S000407 </sigscan/disp.cgi?S000407>
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TATCCAOSAMY site 231 (+) TATCCA S000403 </sigscan/disp.cgi?S000403>
GATABOX site 231 (-) GATA S000039 </sigscan/disp.cgi?S000039>
MYBST1 site 231 (-) GGATA S000180 </sigscan/disp.cgi?S000180>
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OSE2ROOTNODULE site 236 (-) CTCTT S000468 </sigscan/disp.cgi?S000468>
CAREOSREP1 site 238 (-) CAACTC S000421 </sigscan/disp.cgi?S000421>
ARR1AT site 254 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
CAATBOX1 site 278 (-) CAAT S000028 </sigscan/disp.cgi?S000028>
SV40COREENHAN site 281 (+) GTGGWWHG S000123 </sigscan/disp.cgi?S000123>
MYB1AT site 282 (-) WAACCA S000408 </sigscan/disp.cgi?S000408>
MYBATRD22 site 282 (-) CTAACCA S000175 </sigscan/disp.cgi?S000175>
CACTFTPPCA1 site 287 (-) YACT S000449 </sigscan/disp.cgi?S000449>
BOXCPSAS1 site 288 (-) CTCCCAC S000226 </sigscan/disp.cgi?S000226>
GATABOX site 294 (+) GATA S000039 </sigscan/disp.cgi?S000039>
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DOFCOREZM site 384 (+) AAAG S000265 </sigscan/disp.cgi?S000265>
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CANBNNAPA site 389 (-) CNAACAC S000148 </sigscan/disp.cgi?S000148>
RAV1AAT site 390 (-) CAACA S000314 </sigscan/disp.cgi?S000314>
GATABOX site 405 (+) GATA S000039 </sigscan/disp.cgi?S000039>
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IBOXCORENT site 405 (+) GATAAGR S000424 </sigscan/disp.cgi?S000424>

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TATCCAYMOTIFOSRAMY3D site 425 (-) TATCCAY S000256 </sigscan/disp.cgi?S000256>
TATCCAOSAMY site 426 (-) TATCCA S000403 </sigscan/disp.cgi?S000403>
MYBST1 site 427 (+) GGATA S000180 </sigscan/disp.cgi?S000180>
SREATMSD site 427 (-) TTATCC S000470 </sigscan/disp.cgi?S000470>
GATABOX site 428 (+) GATA S000039 </sigscan/disp.cgi?S000039>
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SV40COREENHAN site 435 (+) GTGGWWHG S000123 </sigscan/disp.cgi?S000123>
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E2FCONSENSUS site 450 (-) WTTSSCSS S000476 </sigscan/disp.cgi?S000476>
ROOTMOTIFTAPOX1 site 455 (-) ATATT S000098 </sigscan/disp.cgi?S000098>
ARR1AT site 459 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
SORLIP2AT site 465 (+) GGGCC S000483 </sigscan/disp.cgi?S000483>
SORLIP1AT site 467 (+) GCCAC S000482 </sigscan/disp.cgi?S000482>
CAATBOX1 site 474 (+) CAAT S000028 </sigscan/disp.cgi?S000028>
ARR1AT site 490 (+) NGATT S000454 </sigscan/disp.cgi?S000454>
MARTBOX site 506 (+) TTWTWTTWTT S000067 </sigscan/disp.cgi?S000067>
NODCON1GM site 524 (-) AAAGAT S000461 </sigscan/disp.cgi?S000461>
OSE1ROOTNODULE site 524 (-) AAAGAT S000467 </sigscan/disp.cgi?S000467>
DOFCOREZM site 526 (-) AAAG S000265 </sigscan/disp.cgi?S000265>
MARTBOX site 528 (+) TTWTWTTWTT S000067 </sigscan/disp.cgi?S000067>
POLASIG1 site 529 (-) AATAAA S000080 </sigscan/disp.cgi?S000080>
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GT1CONSENSUS site 533 (-) GRWAAW S000198 </sigscan/disp.cgi?S000198>
GT1GMSCAM4 site 533 (-) GAAAAA S000453 </sigscan/disp.cgi?S000453>
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GATABOX site 573 (+) GATA S000039 </sigscan/disp.cgi?S000039>
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OSE2ROOTNODULE site 588 (-) CTCTT S000468 </sigscan/disp.cgi?S000468>
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GTGANTG10 site 592 (+) GTGA S000378 </sigscan/disp.cgi?S000378>
GATABOX site 594 (+) GATA S000039 </sigscan/disp.cgi?S000039>
SURECOREATSULTR11 site 598 (+) GAGAC S000499 </sigscan/disp.cgi?S000499>
ARFAT site 598 (-) TGTCTC S000270 </sigscan/disp.cgi?S000270>
SEBFCONSSTPR10A site 598 (-) YTGTCWC S000391 </sigscan/disp.cgi?S000391>
CAATBOX1 site 602 (+) CAAT S000028 </sigscan/disp.cgi?S000028>
AMYBOX2 site 607 (-) TATCCAT S000021 </sigscan/disp.cgi?S000021>
TATCCAYMOTIFOSRAMY3D site 607 (-) TATCCAY S000256 </sigscan/disp.cgi?S000256>
TATCCAOSAMY site 608 (-) TATCCA S000403 </sigscan/disp.cgi?S000403>
MYBST1 site 609 (+) GGATA S000180 </sigscan/disp.cgi?S000180>
GATABOX site 610 (+) GATA S000039 </sigscan/disp.cgi?S000039>
ROOTMOTIFTAPOX1 site 611 (+) ATATT S000098 </sigscan/disp.cgi?S000098>
CAATBOX1 site 613 (-) CAAT S000028 </sigscan/disp.cgi?S000028>
GTGANTG10 site 625 (+) GTGA S000378 </sigscan/disp.cgi?S000378>
CBFHV site 632 (-) RYCGAC S000497 </sigscan/disp.cgi?S000497>
GATABOX site 638 (+) GATA S000039 </sigscan/disp.cgi?S000039>
GT1CONSENSUS site 638 (+) GRWAAW S000198 </sigscan/disp.cgi?S000198>

IBOXCORE	site 638 (+) GATAA	S000199 </sigscan/disp.cgi?S000199>
ARR1AT	site 654 (+) NGATT	S000454 </sigscan/disp.cgi?S000454>
CAATBOX1	site 656 (-) CAAT	S000028 </sigscan/disp.cgi?S000028>
WUSATAg	site 660 (+) TTAATGG	S000433 </sigscan/disp.cgi?S000433>
INRNTPSADB	site 671 (-) YTCANTYY	S000395 </sigscan/disp.cgi?S000395>
CACTFTPPCA1	site 673 (-) YACT	S000449 </sigscan/disp.cgi?S000449>
GTGANTG10	site 674 (+) GTGA	S000378 </sigscan/disp.cgi?S000378>
GT1CONSENSUS	site 690 (+) GRWAAW	S000198 </sigscan/disp.cgi?S000198>
CACTFTPPCA1	site 707 (-) YACT	S000449 </sigscan/disp.cgi?S000449>
GATABOX	site 709 (-) GATA	S000039 </sigscan/disp.cgi?S000039>
GTGANTG10	site 713 (-) GTGA	S000378 </sigscan/disp.cgi?S000378>
ARR1AT	site 719 (-) NGATT	S000454 </sigscan/disp.cgi?S000454>
GATABOX	site 725 (+) GATA	S000039 </sigscan/disp.cgi?S000039>
RAV1AAT	site 738 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>
CATATGGMSAUR	site 741 (+) CATATG	S000370 </sigscan/disp.cgi?S000370>
EBOXBNNAPA	site 741 (+) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT	site 741 (+) CANNTG	S000407 </sigscan/disp.cgi?S000407>
CATATGGMSAUR	site 741 (-) CATATG	S000370 </sigscan/disp.cgi?S000370>
EBOXBNNAPA	site 741 (-) CANNTG	S000144 </sigscan/disp.cgi?S000144>
MYCCONSENSUSAT	site 741 (-) CANNTG	S000407 </sigscan/disp.cgi?S000407>
ARR1AT	site 747 (+) NGATT	S000454 </sigscan/disp.cgi?S000454>
POLLEN1LELAT52	site 750 (-) AGAAA	S000245 </sigscan/disp.cgi?S000245>
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WBOXNTERF3	site 755 (-) TGACY	S000457 </sigscan/disp.cgi?S000457>
WRKY71OS	site 756 (-) TGAC	S000447 </sigscan/disp.cgi?S000447>
PALBOXAPC	site 761 (+) CCGTCC	S000137 </sigscan/disp.cgi?S000137>
IBOXCORENT	site 768 (-) GATAAGR	S000424 </sigscan/disp.cgi?S000424>
IBOX	site 769 (-) GATAAG	S000124 </sigscan/disp.cgi?S000124>
SREATMSD	site 770 (+) TTATCC	S000470 </sigscan/disp.cgi?S000470>
IBOXCORE	site 770 (-) GATAA	S000199 </sigscan/disp.cgi?S000199>
GATABOX	site 771 (-) GATA	S000039 </sigscan/disp.cgi?S000039>
MYBST1	site 771 (-) GGATA	S000180 </sigscan/disp.cgi?S000180>
CACTFTPPCA1	site 783 (+) YACT	S000449 </sigscan/disp.cgi?S000449>
-10PEHVPSBD	site 795 (+) TATTCT	S000392 </sigscan/disp.cgi?S000392>
RAV1AAT	site 801 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>
MYBCORE	site 801 (-) CNGTTR	S000176 </sigscan/disp.cgi?S000176>
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DOFCOREZM	site 820 (+) AAAG	S000265 </sigscan/disp.cgi?S000265>
SORLIP1AT	site 823 (+) GCCAC	S000482 </sigscan/disp.cgi?S000482>
SORLIP1AT	site 833 (-) GCCAC	S000482 </sigscan/disp.cgi?S000482>
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GTGANTG10	site 876 (+) GTGA	S000378 </sigscan/disp.cgi?S000378>
WBOXNTERF3	site 877 (+) TGACY	S000457 </sigscan/disp.cgi?S000457>
WRKY71OS	site 877 (+) TGAC	S000447 </sigscan/disp.cgi?S000447>
ARR1AT	site 881 (+) NGATT	S000454 </sigscan/disp.cgi?S000454>
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ABRELATERD1	site 906 (-) ACGTG	S000414 </sigscan/disp.cgi?S000414>
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OSE2ROOTNODULE	site 927 (+) CTCTT	S000468 </sigscan/disp.cgi?S000468>

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DOFCOREZM	site 1051 (-) AAAG	S000265 </sigscan/disp.cgi?S000265>
TAAAGSTKST1	site 1051 (-) TAAAG	S000387 </sigscan/disp.cgi?S000387>
GATABOX	site 1056 (-) GATA	S000039 </sigscan/disp.cgi?S000039>
MYBST1	site 1056 (-) GGATA	S000180 </sigscan/disp.cgi?S000180>
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RAV1AAT	site 1084 (+) CAACA	S000314 </sigscan/disp.cgi?S000314>
MYBCORE	site 1084 (-) CNGTTR	S000176 </sigscan/disp.cgi?S000176>

Anexo B: Artículos arbitrados relevantes a los capítulos de la tesis en los que participé como co-autora.

Apéndice II: Piñeyro-Nelson et al., 2010. *International Journal of Plant Developmental Sciences.*

Apéndice III: Álvarez-Buylla et al., 2010[a]. *The Arabidopsis Book*.

Apéndice IV: Álvarez-Buylla et al., 2010[b]. *The Plant Cell*.

Apéndice V: Garay-Arroyo et al., 2012. *Journal of Experimental Botany.*

Apéndice VI: Piñeyro-Nelson et al., en preparación para *Systematic Botany*.

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Development and Evolution of the Unique Floral Organ Arrangement of *Lacandonia schismatica*

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ABSTRACT

Lacandonia schismatica (Triuridaceae) is the only known angiosperm species with flowers composed of central stamens surrounded by carpels. If the reproductive axes of this species are interpreted as heterotopic flowers, crucial questions on the evolution of morphological novelties arise, such as: a) is this phenotype fixed or whether intermediate floral variants within *L. schismatica* populations exist, and b) what is the nature and number of molecular alterations involved in such a morphological saltation. Furthermore, the temporal progression of floral organ formation in this taxon is unaltered with respect to the great majority of angiosperms (perianth, then stamens and finally carpels). This suggests that the regulatory mechanisms underlying the spatial and temporal morphogenetic patterns of flower development can be altered independently of each other. Through developmental genetic studies, the underlying molecular components involved in the unique position of sexual organs in *L. schismatica* have started to be unravelled. However, studies on floral meristem identity genes, including B-function genes and their regulators (LFY, UFO and SEP) will be important to address the molecular basis of any regulatory alterations. In this contribution we summarize the developmental, systematic and structural data that nurture the on going debate concerning the nature of the Triurid reproductive structures, considered either true flowers (euanthia) or compressed inflorescences (pseudanthia). Finally, we discuss the theoretical approaches that are helping us to understand developmental constraints of the ABC gene regulatory network, and how such theoretical analyses could help explain the arrangement of *L. schismatica* flowers.

Keywords: ABC model, developmental genetics, homeosis, reproductive structures, Triuridaceae

CONTENTS

INTRODUCTION

The field of Evolutionary Developmental Biology or *Evo-Devo* has profited from the data and conceptual inputs from a diverse set of scholars and fields of study. The morphology-centered school of the 18th and 19th centuries was fundamental in describing plant sexual organs, which would prove fundamental for taxonomic and comparative morphology studies (Smyth 2005). In the 20th century, biologists such as C.H. Waddington and D'Arcy Thompson

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Invited Review

among others, made important propositions regarding the role of interactions among genetic and non genetic elements during development, as well as the need to explicitly acknowledge the role of physical and mechanical constraints affecting organismal form. More recently, *Evo-Devo* has benefited from the growing field of developmental genetics, which has utilized empirical data attained through the study of homeotic transformations in model systems, to propose developmental models such as the ABC model of plant floral organ formation (Bowman *et al.* 1989, 1991; Coen and Meyerowitz 1991; Smyth 2005). Such models have been instrumental to assess the role of homeotic genes in the development of the flower structure in both model and non-model plant species (Soltis *et al.* 2007).

Experimental data attained from *Arabidopsis thaliana* has also enabled the development of computational models that address the role of Gene Regulatory Networks (GRN) in the emergence of evolutionarily stable genetic expression patterns associated with organ development (Espinosa-Soto *et al.* 2004; Álvarez-Buylla *et al.* 2008). These contemporary experimental and theoretical approaches have benefited particularly from ideas put forward by Waddington (1957) who introduced the notion of epigenetic landscapes as a metaphor to explain the dynamic nature of development. In such a metaphor, the landscape topology is a dynamic system defined by complex interactions among genetic and non-genetic factors, and basins correspond to stable developmental pathways. In the epigenetic landscape, once a basin has been reached, the developmental process will be buffered against external or internal interventions, thus being *canalized* (Slack 2002). This and other concepts put forward by embryologists and developmental biologists, have been important in shaping the way we think about development (see review by Slack 2002). However, the *Modern Synthesis* did not consider developmental processes explicitly (Huxley 1942) thus failed to provide a useful theoretical framework for understanding phenotypic evolution from an ontogenetic perspective. Developmental constraints, epigenesis, and the dynamics and structure of gene regulatory networks should be explicitly addressed and ideally, comprehended, in order to contribute to the understanding of the evolution of organismal form and function and to map genetic variation into phenotypic variation.

In contrast to the *Modern Synthesis*, *Evo-Devo* has incorporated data and insights from comparative embryology, developmental biology, developmental genetics and theoretical biology, to understand the evolution of organismal form. *Evo-Devo* has also attempted to fulfill what has been called the most ambitious promise of the discipline: "[a] full conceptual continuity between evolutionary and developmental mechanisms and explanations" or, "[a] conceptual continuity from ontogeny through phylogeny" (Bolker 2008).

Some novel approximations put forward to address the non-linear nature of the phenotype-genotype mapping, have addressed the possible microevolutionary processes underlying morphological innovations in certain plant lineages, for example, discerning whether such innovations can be explained by continuous variation or by a discrete phenotypic change (Vergara-Silva *et al.* 2003). Other approaches have advocated the inclusion of genetic algorithms into evolutionary models. Such algorithms describe the mechanisms that determine how genetic information is mapped onto phenotypic traits (Espinosa-Soto *et al.* 2004). The molecular basis of developmental processes is being understood with greater detail and comparative genomics has revealed conservation in developmental gene families outside model systems. It is therefore becoming possible to address hypotheses concerning candidate genes involved in the origin of major morphological transitions. In fact, there are already some examples of relatively small changes to key regulators that are associated with major evolutionary innovations or morphological saltations (Shapiro *et al.* 2004; Brakefield 2006; Davidson and Erwin 2006; Jeong *et al.* 2008). Among such genetic events are homeotic alterations involving major changes in body plans in the case of flowers, or limb number in arthropods (Bowman *et al.* 1991; Ford and Gottlieb 1992; Ronshaugen *et al.* 2002). Similar homeotic alterations seem to be involved in the case of the remarkable inside-out flower of the Mexican monocot herb, *Lacandonia schismatica*.

Fig. 1 ***Lacandonia schismatica*** **flower.** Note semi-transparent tepals and three central stamens (dark yellow) surrounded by numerous carpels (photo by Esteban Martínez).

As a contribution to this special issue of the IJPDB, we synthesize here the state of knowledge about the evolution and development of *L. schismatica*, a living example of a natural heterotopic species (see **Fig. 1**).

This review provides an overview of the general boiology of *L. schismatica*; its taxonomy as well as the phylogenetic position of the Triuridaceae within the order Pandanales. We also summarize morphology-based studies of reproductive axes of Triuridaceae (from adult specimens of different Pandanales representatives, to developmental series of *L. schismatica*) (Rudall 2003; Ambrose *et al.* 2006; Rudall and Bateman 2006) and review data generated from developmental genetic studies based on the ABC model for floral organ determination (Bowman *et al.* 1991; Coen and Meyerowitz 1991).

Developmental genetics has generated a body of data that informs the debate regarding the nature of the reproductive axes of *L. schismatica*, a controversy that started immediately following formal description of this taxon (Martínez and Ramos 1989). Furthermore, we also address other questions that have been raised since the introduction of *L. schismatica* into the botanical literature, such as: Which changes in the underlying gene regulatory networks controlling flower morphogenesis enabled the emergence of this plant's unique floral phenotype? What is the identity of the reproductive axes of this species? What can we learn in terms of the mechanisms underlying floral morphogenesis from studying *L. schismatica* flower development? Can the understanding of the particular modifications present in *L. schismatica* at the functional/genetic levels help expand our view of the mechanisms and processes implicated in plant *Evo-Devo*?

We end this review with future perspectives on the research that remains ahead, which we continue to pursue with this challenging and fascinating taxon.

TAXONOMY OF *LACANDONIA SCHISMATICA* AND RELATED TRIURIDACEAE SPECIES

L. schismatica was assigned to a monotypic genus and a new family: Lacandoniaceae, although its affinity with Triuridaceae was also acknowledged (Martínez and Ramos 1989). Currently, morphological and phylogenetic analyses further support that this species should be considered a member of Triuridaceae (Maas van der Kamer 1995; Vergara-Silva *et al.* 2003; Davis *et al.* 2004; Ambrose *et al.* 2006; Rudall and Bateman 2006; Rudall 2008).

The family Triuridaceae comprises approximately 48

extant species and 11 genera (Maas-van de Kamer and Weustenfeld 1998; Cheek 2003) of mycoheterotrophic, achlorophyllous (white, yellow or crimson/purple) monocot herbs distributed in tropical rain forests of the Paleo and Neotropics (Maas-van de Kamer and Weustenfeld 1998). Members of this family are thought to be perennials (although further biological and ecological observations are needed to corroborate this observation for all species within this group). These species bear rhizomes from where simple stems with reduced leaves arise and give place to a raceme bearing minute flowers (2-10 mm in diameter) during the reproductive season. *L. schismatica* is a hyaline, racemose, bisexual species with central stamens (3 on average) surrounded by numerous (40-80) free carpels (polyapocarpy) and an actinomorphic perianth (Martínez and Ramos 1989).

Triuridaceae is subdivided in three tribes based on stamen morphology, embryo development, and the sexual reproduction system (Maas-van de Kamer and Weustenfeld 1998; Rudall and Bateman 2006; Rudall 2008). The basal tribe Kupeaeae, with monoecious unisexual or dioecious species includes the genera *Kupea* and *Kihansia* and is only distributed in Africa (Cheek 2003; Cheek *et al.* 2003). The tribe Sciaphileae comprises monoecious, dioecious and bisexual species and includes the genera *Sciaphila*, *Seychellaria*, *Soridium*, *Andruris* and *Hyalisma*. Finally, the tribe Triurideae, comprises dioecious individuals – with the exception of *L. schismatica*, which is hermaphroditic – and includes the genera *Peltophyllum*, *Triuridopsis*, *Triuris* and *Lacandonia.* The most species-rich tribe is Sciaphileae (with approximately 30 taxa; Maas-van de Kramer and Weustenfeld 1998).

While Triuridaceae was first established as a botanical family in 1843 (Gardner), it is still far from being thoroughly studied. One of the reasons for such situation is that the habitats where species from this group grow consist of: "dense and humid forests hidden under leaf mould at the base of large trees or along the bank of streams" (cited from Maas-van de Kamer and Weustenfeld p. 456, 1998). Another factor is their minute overall size and coloring. These two characteristics make them difficult to spot while conducting a botanical survey. Thus, new species are still being discovered and described as late as 2003 (Cheek 2003; Cheek *et al.* 2003). Hardly anything is known about these taxa patterns of pollination, dispersal and population renewal. In the next section of this review we will summarize the available cytological information for *L. schismatica* and address how findings at this level may affect other aspects of this plant's biology.

PECULIARITIES OF *LACANDONIA SCHISMATICA*

Reproductive and cell biology of *Lacandonia schismatica*

The flowers of this species have a cleistogamous mode of fertilization (Márquez-Guzmán *et al.* 1993) where pollen grains germinate in the closed flower bud and fertilize adjacent carpels through a lateral micropyle opening (Márquez-Guzmán *et al.* 1989, 1993). Anthesis is then thought to be important as a means for seed dispersal, rather than for pollinator interactions. Due to this pre-anthesis fertilization system, fixation of mutations within populations of this species could take place at a relatively higher rate than under random mating; this inference has been initially supported through a study based on 15 isoenzymatic loci undertaken on individuals from the best well-known population of *L. schismatica.* This study indeed showed that *L. schismatica* shows high homozygosity among the assayed loci (Coello *et al.* 1993); and the same pattern is expected for its most recently discovered populations.

Another interesting feature of this plant, is the unique development of the embryo-sac, which presents a new type of megagametophyte development called the *Lacandonia* type (Vázquez-Santana *et al.* 1998). The latter differs from other types described for other Triuridaceae species (Vázquez-Santana *et al.* 1998); in *L. schismatica* the micropylar megaspore, rather than the chalazal one, is the functional cell that gives rise to a monosporic female gametophyte that lacks cytokinesis during the first division of meiosis (Vázquez-Santana *et al.* 1998).

Peculiar nuclear architecture

At the subcellular level, *L. schismatica* has a standard plant cell structure but chloroplast development is arrested (Jiménez-García *et al.* 1998), an expected feature given its mycoheterotrophic habit. However, the nucleus presents abundant extranucleolar ribonucleoprotein particles that have been called "Lacandonia granules" (Jiménez-García *et al.* 1992; Agredano-Moreno *et al.* 1994). Interestingly, these particles vary in their quantity during flower development and life stage: they are abundant in flower buds (pre-anthesis) in relation to open flowers (post-anthesis), (Agredano-Moreno and Jiménez-García 2000) and are also more abundant during fertilization (Márquez-Guzmán *et al.* 1993; Vázquez-Santana *et al.* 1998). These granules are not *L. schismatica*-specific as was formerly believed; they have been found in many other monocot plant species but never in eudicot plants (Jiménez-García pers. comm. to AGA). Based on cytochemical and immunocytochemical features, Agredano-Moreno and Jímenez-García (2000) proposed that *L. schismatica* granules, perichromatin granules and Balbiani ring granules might be functionally similar.

The importance of the perichromatin region as a functional nuclear domain where DNA replication, transcription and probably most steps of pre-mRNA processing take place, have been acknowledged during the last 30 years (Fakan 2004) and might be correlated with the stages when these granules are more abundant. Also, ring-shaped bodies, which are found in both plants and animals, which contain a central core of RNA surrounded by an electron-dense external ring containing snRNP (small nuclear ribo-nucleoproteins) were found in the nucleus of *L. schismatica.* These have also been documented in animals and could be involved in pre-mRNA metabolism (Agredano-Moreno *et al.* 1994; Zavala and Vázquez-Nin 1997; Agredano-Moreno and Jiménez-García 2000; Agredano-Moreno *et al.* 2001).

It would be useful to investigate if these structures carry out the same function in *L. schismatica* as stated for other organisms, in particular, if the mycoheterotrophic habit of this species is correlated with these peculiar nucleic acid bodies. Furthermore, it would be of particular interest to compare these structures among achlorophyllous and chlorophyllous monocot species in order to study if there is any type of correlation between habit and presence or function of these structures.

PHYLOGENETIC POSITION OF *LACANDONIA SCHISMATICA* WITHIN PANDANALES

The original taxonomic placement of *L. schismatica* (Martínez and Ramos 1989) was contended by Maas-van der Kamer (1995), and to date Lacandoniaceae is considered synonymous to Triuridaceae, where it has been placed as a monospecific genus (Maas-van der Kamer 1995; Maas-van de Kramer and Weustenfeld 1998; Angiosperm Phylogeny Group II 2003). Independently, a study based on comparative developmental series of *L. schismatica* and a Mexican triurid representative; *Triuris brevistylis*, concluded that *L. schismatica* should be placed as a monotypic genus within the tribe Triurideae but not as a separate family (Ambrose *et al.* 2006). This classification has been adopted in recent publications (Rudall and Bateman 2006; Rudall 2008).

From a phylogenetic perspective, the position of Triuridaceae with respect to all other monocot taxa has changed markedly in the last 20 years. This family had been placed as sister to Petrosaviaceae on the basis of their morphology and mycoheterotrophic habits (Cameron *et al.* 2003). Cladistic analyses based on a fragment of the *rbcL* sequence grouped a series of families (Pandanaceae, Velloziaceae and

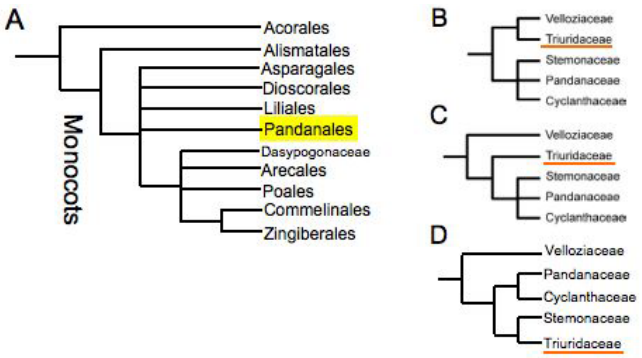

Fig. 2 Position of Pandanales in the Monocot clade and alternative phylogenetic relationships of families comprised within this order. (**A**) Diagramatic representation of the Monocot clade and position of Pandanales (shaded in yellow), based on the cladogram published by The APG II (2003). (**B**), (**C**) Alternative topologies for families within Pandanales (Velloziaceae, Stemonaceae, Cyclanthaceae, Pandanaceae and Triuridaceae) attained using a two-sequence gene analysis based on *atpA* and *Rbcl* sequences; for Triuridaceae only the *atpA* sequence was used (Davis *et al.* 2004; diagram taken from Rudall and Bateman 2006). (**D**) Position of Triuridaceae based on a morphology-based phylogenetic analysis of Pandanales. Simplified diagram drawn after cladogram published by Rudall and Bateman (2006). In (**B**), (**C**) and (**D**) Triuridaceae are underlined in orange. Diagram modified from **Rudall PJ, Bateman RM** (2006) Morphological phylogenetic analysis of Pandanales: Testing contrasting hypotheses of floral evolution. *Systematic Botany* **31**, 223-238, ©2009, with kind permission from the authors and the American Society of Plant Taxonomists, ASPT Publications, Wyoming, USA.

Cyclanthaceae) into a new order: Pandanales (Chase *et al.* 1993). Further analyses based on the 18S rDNA sequence included Stemonaceae and Triuridaceae into this order and placed Pandanaceae as sister group to Triuridaceae (Chase *et al.* 2000). By 2003, The Angiosperm Phylogeny Group corroborated this placement and integrated Pandanales into its lists of recognized orders. While the families comprising this order have remained consistent since its proposal, the relationships among families within this order, as well as of this group with respect to other orders, have remained unresolved. This lack of resolution within the Pandanales is an important issue: while Stemonaceae and Velloziaceae are groups with true hermaphroditic flowers, Cyclanthaceae and Pandanaceae comprise species with reduced or compressed inflorescences (pseudanthia) in which arrested floral organs are clearly visible in scanning electron micrographs of developing inflorescences (See for example *Freycinetia arborea* (Pandanaceae); Cox 1990). The ontogenetic differences between these two kinds of reproductive units are far from being trivial from the *Evo-Devo* perspective and can give further support to the two competing hypotheses regarding the identity of Triuridaceae reproductive structures.

Several phylogenetic analyses for Pandanales have been conducted based on morphological and/or molecular data and have been summarized by Rudall and Bateman (2006; see **Fig. 2**). Most of the relationships among these groups are not well resolved due to the low number of taxa sampled and the lack of available chloroplast markers for Triuridaceae due to their mycoheterotrophic habit, as well as to additional molecular markers. For instance, in some analyses Triuridaceae is placed as sister to Pandanaceae (Chase *et al.* 2000; Rudall 2003), while in others, it is sometimes placed as sister to Velloziaceae (Davis *et al*. 2004), or as a sister group to all other Pandanales (Davies *et al*. 2004; Davis *et al*. 2004).

The latest phylogenetic study of Pandanales based on 39 morphological characters, comparing 23 genera representing the five families of the order shows an unexpected result with Velloziaceae as sister to all Pandanales, and Cyclanthaceae and Pandanaceae forming a separate clade that is in turn sister to a paraphyletic group in which Triuridaceae nests within Stemonaceae. In this reconstruction, the recently discovered genus *Kupea* was placed as a basal member of Triuridaceae (Rudall and Bateman 2006). Confirmation of this result would likely require a comparison with a phylogenetic analysis based on plastid (*atpA*) and nuclear (18S rDNA) markers using a similar or broader number of taxa.

Results of phylogenetic analyses summarized here have complemented morphological analyses in the discussion concerning the identity of the reproductive axes of *Lacandonia schismatica* and other triurids.

CONTROVERSIES ON THE IDENTITY OF THE REPRODUCTIVE STRUCTURES OF TRIURIDACEAE

Euanthial vs Pseudanthial interpretation of the reproductive axes in Triuridaceae

Two main hypotheses have been advanced regarding the nature of *L. schismatica* reproductive axes. Stevens (1991) and Rudall (2003) proposed that the entire family of Triuridaceae comprised species that bore pseudanthia (reduced inflorescences with aborted floral organs of single flowers), rather than euanthia (true flowers). Rudall based her hypothesis in the position previously assigned to *Schiaphila* in a molecular phylogenetic analysis (Chase *et al.* 2000), where it was sister to Pandanaceae, and supported this placement through comparative morphological analyses of mature reproductive structures of both groups (Rudall 2003). An alternative hypothesis regarded *L. schismatica* reproductive units as true flowers (euanthia) with a homeotic inversion of sexual organs where stamens are interior to carpels (Márquez-Guzmán *et al.* 1989; Martínez and Ramos 1989; Vergara-Silva *et al.* 2003; Ambrose *et al.* 2006).

The pseudanthial hypothesis has been contended based on the observation that no aborted reproductive organs or perianth organs were observed in developmental series of *L. schismatica* and *T. brevistylis* flower development using both histological sections and scanning electron micrographs (Márquez-Guzmán *et al.* 1989; Vergara-Silva *et al.* 2003; Ambrose *et al.* 2006). The most recent of these studies, has provided new structural evidence in support of the euanthial hypothesis. In addition to documenting the absence of aborted floral organs, extra evidence includes the presence of a bract subtending and encapsulating each floral primordium, found in opposition to the perianth organ (tepal) that first arises during flower development; this trait is characteristic of most flowers. Also, the temporal progression of floral organ development is reminiscent of flowers and not inflorescences with perianth organs arising first in the most external whorl, then stamens (irrespective of their position) and finally, carpels.

Another interesting feature documented through SEM was the presence of common primordia for stamens and carpels that developed centrifugally (from the center of the floral meristem to the periphery of the flower). In *L. schismatica*, such common primordia bear stamens in the distal part, while carpels differentiate in the proximal (closest to the perianth) section (**Fig. 3**). Furthermore, carpels differentiate from these common primordia not in successive whorls, as could be expected if *L. schismatica*'s reproductive unit was an inflorescence, but in ridges that radiate from the distal part of each stamen primordia (Ambrose *et al.* 2006; Rudall 2008; see **Fig. 3**, panels C, D, E). This type of floral organ development from a common primordium is relatively rare among angiosperms, but had been documented previously in legume species from the subfamily Papilionoideae, which bear true flowers (Tucker 2003). Interestingly, in the Papilionoid species in which this type of ontogenesis has been reported, the common primordium gives rise to stamens and petals (Tucker 2003); while *L. schismatica* unique compound primordium gives rise to stamens in the

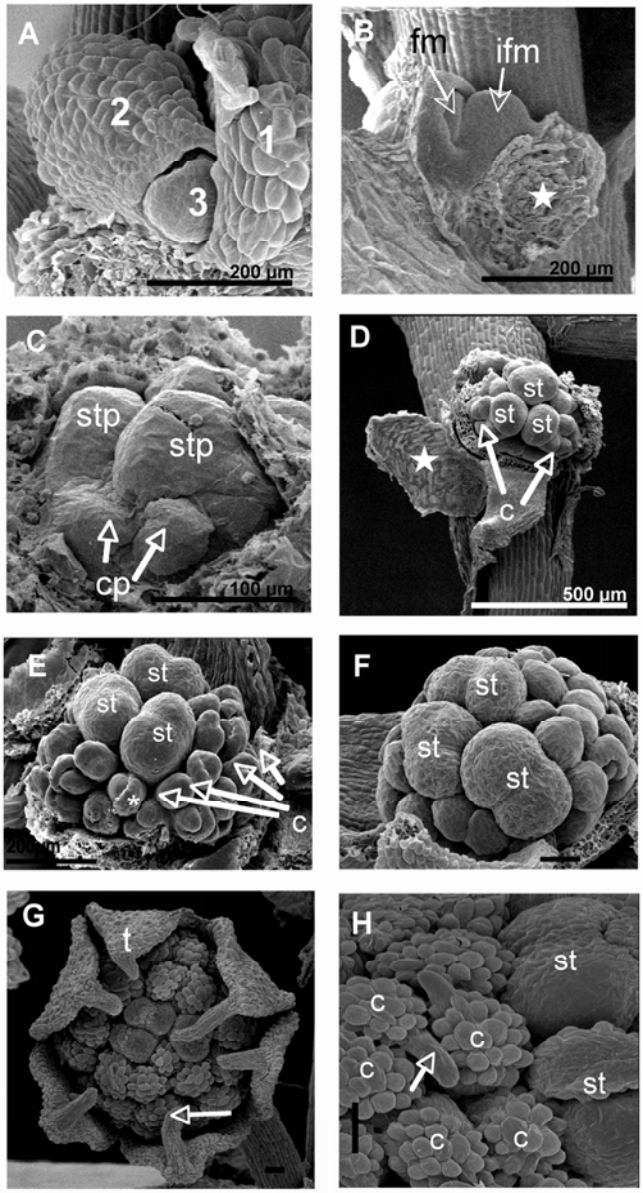

Fig. 3 SEM photographs of *Lacandonia schismatica* flower development. (**A**) Three floral buds before pedicel elongation. Flowers numbered from oldest (1) to youngest (3). (**B**) Inflorescence apex. The inflorescence meristem (ifm), is marked by an arrow. One floral bud has been removed (star) and a flower meristem (fm), covered with a bract is also indicated by an arrow. (**C**) Close-up of a flower bud. Bract and developing tepals have been removed. Common primordia with stamen primordia in the apical part and carpel primordia developing in the distal part are shown. (**D**) Older flower bud. Tepals have been removed and a section of the subtending bract is still apparent (star). Stamen and carpels are shown. (**E**) Close-up of another flower bud where multiple developing carpels are apparent. An asterisk shows a developing carpel wall enclosing an individual nucellus. (**F**) Another flower bud close-up. Bilobulation of developing stamens is apparent. (**G**) Mature flower at anthesis. Note tepals and filamentous tepal tips (arrow). (**H**) Detail of carpels from a mature flower. Note papillous carpel cells and stigmatic extensions (arrow). Figure abbreviations: ifm= inflorescence meristem; stp=stamen primordium; cp= carpel primordia; st= stamen; c=carpel; t= tepal.

most apical position, but instead of producing petals in their periphery, carpels develop. Also, the three stamen primordia develop in the central part of the receptacle since the first stages of organogenesis, thus, refuting the hypothesis of a secondary physical inversion, as already contended in other studies (Márquez-Guzmán *et al.* 1993). Additionally, all floral whorls have a trimerous arrangement that is reminiscent of true flower meristic arrangement proper of other monocot families: three stamen primordia from where three carpel primordia start to differentiate and fasciate, while the perianth is usually composed of six basally fused tepals (Vergara-Silva *et al.* 2003; Ambrose *et al.* 2006).

Although some of the characteristics described above can also be present within species bearing inflorescences (such as a trimerous arrangement of floral organs as well as the temporal progression of floral organ development), while other species with true flowers lack some of these traits, their overall presence in a single species is very suggestive of an euanthial interpretation.

Is there an unclear floral/inflorescence meristem distinction in Triuridaceae? Morphology-based hypotheses

In addition to the summarized developmental biology evidence, the pseudanthial hypothesis was further challenged by a comprehensive cladistic analysis based on morphological characters, which showed that Stemonaceae formed a paraphyletic group with Triuridaceae (Rudall and Bateman 2006). Based on the polyapocarpic flowers of Triuridaceae and given that Stemonaceae has true flowers with single free carpels (except for the case of *Pentastemona*), Rudall and Bateman (2006) speculate a scenario involving carpel multiplication in the origin of Triuridaceae. They argue that these events could have been coupled with a partial loss of determinacy in the flower meristem, that created chimeric axes where the boundary of flower – inflorescence is not clear (Rudall and Bateman 2006).

Morphological evidence of a seemingly blurry boundary between an inflorescence and floral meristem has been put forward for different plant species (see for example, Prenner and Rudall 2007 [Euphorbia]; Rudall *et al.* 2007 [Hydatellaceae]; Rudall 2008 [Triuridaceae]; Rudall 2009 [review of different plant families]; Rudall *et al.* 2009 [Hydatellaceae]) as well as for particular floral architectures, such as racemes, were it has been proposed that what could be interpreted as a terminal flower in these kind of undetermined inflorescences could actually be a very simplified pseudanthium (Sokoloff *et al.* 2006). Overall, a lack of a definitive boundary among meristems has been related to a possible spatio-temporal overlap among domains of expression of inflorescence determination genes and floral meristem genes. (Sokoloff *et al.* 2006; Prenner and Rudall 2007; Rudall *et al.* 2007; Rudall 2008, 2009).

Concerning the blurring of the distinction between the flower and inflorescence meristem, the data by Ambrose and collaborators (2006) showed that in *L. schismatica* these two types of meristems are clearly distinguishable, and while the inflorescence meristem is naked, the flower meristem is covered by a bract.

In the Triuridaceae, further morphological analyses performed by Rudall (2008) address the identity of the reproductive axes of members of this family by comparing the development of these strange polyapocarpic flowers in different species. This study notes several morphological novelties present in flowers of Triuridaceae, including filamentous structures in tepal tips or in central positions of male flowers of *Seychellaria* or *Triuridopsis*, fasciated carpels with stigmatic extensions, and a transition of centripetal to centrifugal carpel development (centrifugal carpel inception), in species within the tribe Triurideae. Based on the presence of these structures, Rudall (2008) considers two alternative scenarios for the origin of the inside-out flowers of *L. schismatica*: the first consists on a homeotic substitution (as previously proposed in Vergara-Silva *et al.* 2003) of the three central carpel primordia from female flowers by stamen primordia (somehow 'predisposed' by centrifugal carpel inception); or alternatively, a heterochronic scenario in which male flowers underwent secondary carpel development between the stamen and perianth whorls. In either case, an inversion in the position of stamens and carpels within hermaphroditic flowers arouse in *L. schismatica*.

Recently, a new interpretation of the identity of peculiar reproductive axes such as those in Triuridaceae, has been put forward by Rudall and collaborators (2009). In this

study, based on the morphological analysis of the reproductive structures of Hydatellaceae, which posses reduced bisexual axes with central stamens, the authors propose the term "nonflower" to describe either "an inflorescence-like structure that is derived from a secondarily modified flower" or alternatively, a pre-floral structure (Rudall *et al.* 2009; p. 79). Importantly, the authors suggest that the first definition of nonflower could possibly apply to Triuridaceae (see also Rudall 2008). Hydatellaceae has been recently recircunscribed as part of the basal angiosperm grade (Saarela *et al.* 2007), which is evolutionarily distant from derived groups such as Triuridaceae. The proposal of a convergent nonflower structure would imply multiple origins of this peculiar morphogenetic path during angiosperm evolution and as such is difficult to reconcile with current angiosperm systematics. Also, the SEM photographs of atypical reproductive structures of specimens of *Trithuria submersa* presented by Rudall and colleagues in their 2009 paper, suggest a closer relationship of this taxon with an inflorescence structure, as phillomes – which could be either subtending bracts of perianth organs – are present in between reproductive organs, while carpels are some times found at the center of the reproductive unit. In the case of *L. schismatica*, these two arrangements have not been documented either when assessing the natural variation of this taxon (Vergara-Silva *et al.* 2003), or through developmental series (Ambrose *et al.* 2006). A comprehensive set of developmental series of Hydatellaceae representatives would be convenient to further address this issue, although morphological evidence available to date (see Rudall *et al.* 2007, 2009), shows that this family has a floral morphology far more divergent than Triuridaceae with regards to the stereotypical floral *bauplan* of other angiosperms (for instance, no clear subtending bracts can be assigned, no clear perianth is visible, emergence of sexual organs is seemingly spiral, etc. see Rudall *et al.* 2007, 2009).

For the case of singular reproductive axes such as the ones found in *L. schismatica* and Hydatellaceae, controversial interpretations on the homology of structures and the understanding of the origin of new characters during flower evolution, require knowledge provided by developmental genetics. Thus, gene expression and functional experiments assessing the role of genes known to be fundamental for the inflorescence to flower meristem transition should be performed in these taxa.

Developmental genetics hypotheses

Much insight can be gained from the data attained in the model species *Arabidopsis thaliana*, where several transcription factors including *LEAFY* (*LFY*), *APETALA1* (*AP1*) (a member of the *SQUAMOSA* lineage) and more recently, *SEPALLATA3* (*SEP3*), have been suggested as crucial for the establishment and spatio-temporal patterning of the flower organ identity (ABC) genes (Bowman *et al.* 1993; Parcy *et al.* 1998; Lohmann *et al.* 2001; Liu *et al.* 2009). In this species, the domains of expression of these genes coincide with the places where flower meristems are being specified. Furthermore, homologous copies of these genes with similar functions have been found in other monocots. In rice, when the *LEAFY* homologue *RFL* is silenced, mutant plants lack flowers and gain-of-function lines promote early flowering (Rao *et al.* 2008), although not all characterized LFY orthologs within diverse angiosperm model species have the same function (see review by Moyroud *et al.* 2009). In the case of wheat, *Leafy Hull Sterile*, a homologue of SEPALLATA proteins, seems to be also involved in the determination of the spikelet meristem (Malcomber and Kellogg 2004). *SQUAMOSA*-like homologues are present in monocots but their role is yet unknown outside eudicots, although AP1 seems to be a more specific flower meristem marker in *A. thaliana* (Bowman *et al.* 1993).

Alternatively, floral regulators such as *AGAMOUS* (*AG*) have been shown to be involved in the determination of the floral meristem by suppressing its activator, *WUSCHEL* (*WUS*), in a negative feedback loop (Lohmann *et al.* 2001). *WUS* is a gene involved in stem cell population maintenance that activates *AG* by interacting with LFY (Lohmann *et al.* 2001). The expression profiles and functional characterization of *AG* orthologs of Triuridaceae could point at whether there are divergences in the determination of the floral meristems in this lineage. Recent studies with the proteins REBOLOTE (RBL), SQUINT (SQN) and ULTRAPETALA1 (ULT1) in *A. thaliana* have shown that they are also involved in floral meristem determination (Prunet *et al.* 2008). Multiple mutants of these genes result in indeterminate flowers with repetitions of whorls of carpels and stamens in their flower center, as well as flowers with extra whorls of stamens such as in *superman* mutants (*sup*; Bowman *et al.* 1992), due to a decrease in *AG* expression in the 4th whorl of the flower. Experiments aimed to resolve the spatio-temporal expression profiles of combinations of these genes in key species spanning the five families of the order Pandanales, with emphasis on the Triuridaceae, would be very informative.

This approach would also be useful for species of Hydatellaceae, as already noted by Rudall and colleagues (2009), who analyzed the gene expression pattern of LFY in developing floral units of *Trithuria submersa* (Rudall *et al.* 2009). While the data presented is informative, other organ specification genes such as the ABC class genes should be assayed; the patterns of expression of the former would yield important data that could aid in clarifying the identity of the reproductive units of this family of plants.

For the remaining of this paper, we will proceed under the assumption that the reproductive axes of the Triuridaceae correspond to true flowers and hence aim at analyzing the case of the inside-out flower of *L. schismatica*, as a case of morphological saltation in the context of flower angiosperm evolution.

THE HOMEOTIC FLOWERS OF *LACANDONIA SCHISMATICA* CONSTITUTE A MORPHOLOGICAL SALTATION: STUDIES AT THE POPULATION LEVEL

Some of the fundamental questions regarding the origin of *L. schismatica* heterotopic flower are related to the nature of the underlying genetic variation implicated in this homeosis. One of the possibilities is that such heterotopy is the outcome of microevolutionary forces affecting the genetic variation of homeotic genes, a phenomenon that has been reported (Barrier *et al.* 2001; Olsen *et al.* 2002), while another alternative is that this homeosis is of a "saltational" nature and as such, it is related to discrete changes in particular homeotic genes (Vergara-Silva *et al.* 2003). A means to approach these alternatives is to assess the natural variation within contemporary populations of *L. schismatica* in order to document if a continuum of forms is present in the flower morphs of this taxon. Furthermore, while the heterotopic flower of *L. schismatica* is regarded as an autapomorphy, this approach can help address another related question: was this homeosis present in the most recent common ancestor of *L. schismatica* and its putative sister taxon, *Triuris brevistylis*?

A study by Vergara-Silva and collaborators (2003), addressed the former questions through the study of the natural variation among inflorescences from contemporary populations of *L. schismatica* and *T. brevistylis*, both of which dwell in the Lacandon rainforest and are sympatric to each other (**Fig. 4**). In this study, Vergara-Silva and colleagues (2003) analyzed over 1000 inflorescences of *L. schismatica*. This study revealed an overall fixation of the inverted floral phenotype, with a few variants in this species. Approximately 2% of the analyzed flowers were unisexual (both male and female); a few others had variation in sexual organ number (stamens and carpels); while an additional few individuals bore fertile and sterile stamens. However, flowers with central carpels in bisexual flowers were not observed. A complementary morphological analysis was

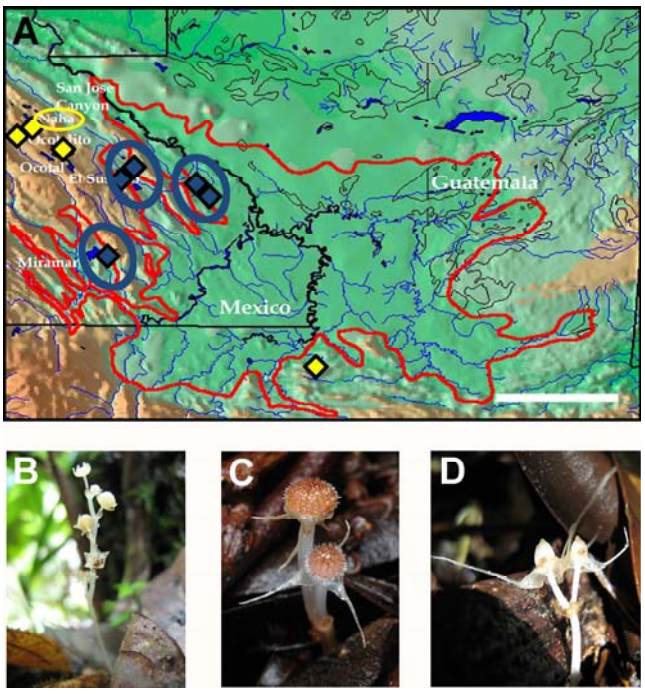

Fig. 4 Lacandon rainforest map with extant *Lacandonia schismatica* and *Triuris brevistylis* populations. (**A**) Former margins of glacial lake *ca*. 7000 years ago (red line), known *L. schismatica* populations (in blue diamonds) and *T. brevistylis* populations (in yellow diamonds). Note how *L. schismatica* populations are settled in close proximity of former lake borders. (**B**) *L. schismatica* inflorescence growing in the Lacandon rainforest. (**C**) *T. brevistylis* female inflorescence. (**D**) *T. brevistylis* male inflorescence growing in the Lacandon rainforest. (map in panel (**A**) modified from Vergara-Silva *et al.* 2003; photos in panels (**B**), (**C**) and (**D**) by Eduardo Flores, Joel McNeal and Esteban Martínez, respectively).

conducted on around 1000 inflorescences of *T. brevistylis* by the same authors (Vergara-Silva *et al.* 2003). In this species, morphological variation was also found: 1% of observed individuals bore male flowers and 0.5% of these were bisexual. Bisexual flowers had varying arrangements of sexual organs, with cases where stamens were present on one side of the androphore while carpels were present in the opposing side; other specimens had stamens surrounded by carpels and this was observed in flowers that seemed both, overall female or male.

The results regarding morphological variation in flowers from *L. schismatica* and *T. brevistylis* do not form a continuum of intermediate forms between extreme phenotypes and as such, strongly suggest that *L. schismatica* heterotrophic flower is the product of a "saltational" event regarding the change in a genetic module or set of modules (Vergara-Silva *et al.* 2003). Furthermore, given the results of *T. brevistylis*, it seems that the most recent common ancestor of these taxa bared unisexual flowers with a "pre-disposition" for homeosis.

Regarding the speciation event that could have originated these triurid species, a biogeographical hypothesis has been put forward based on current knowledge of the distribution of contemporary populations of these two taxa. *L. schismatica* extant populations rest in the Lacandon forest low-lands (around 200 m above sea level; see **Fig. 4**, panels A and B), encircling the perimeter of a glacial lake. *T. brevistylis* populations are localized in the high lands of this forest as well as in the Guatemalan forests, but at higher altitudes (600-800 m above sea level), in more temperate (6 to 8ºC lower than sites for *L. schismatica*) zones. The distribution of *T. brevistylis* also loosely encircles the perimeter of the ancient lake, but at higher elevations (**Fig. 4**, panels A, C and D). This setting, the morphological similitude, the over 40 plant species shared among habitats of *L. schismatica* and *T. brevistylis* contemporary populations, as well as the projected limits of the ancient margins and contemporary margins of the glacial lake, suggest that this two species probably derive from a common Triuris-like ancestor whose populations were isolated after the retraction of the glacial lake approximately five million years ago (Vergara-Silva *et al.* 2003).

The next interesting *Evo-Devo* question that needs to be addressed concerning the morphological saltation of *L. schismatica* floral organ arrangement, concerns the nature of the genetic changes involved, under the hypothesis that relatively small genetic alterations could be at the basis of such a relatively large morphological change.

MOLECULAR GENETIC MECHANISMS UNDERLYING THE SINGULAR SPATIAL ORGAN ARRANGEMENT OF *LACANDONIA SCHISMATICA* FLOWERS

The conserved floral organ arrangement pattern in angiosperms, suggests a restricted and robust developmental mechanism that was broken in the evolution of the Mexican triurids, with a saltation in such floral arrangement having been fixed in *L. schismatica* populations. Indeed, such unique floral morphology has not been found either among natural or experimentally induced mutant phenotypes, thus *L. schismatica* is particularly interesting for studies of the gene regulatory networks underlying flower development, and the constraints that these networks imply, as well as which of their aspects were altered along this species lineage.

We have been studying the molecular genetic basis of *L. schismatica* flower development, based on the knowledge accumulated for model organisms. In particular, we derived our first hypothesis from the ABC model of floral organ specification. The classical ABC model was inferred using *Arabidopsis thaliana* and *Antirrhinum majus* flower homeotic mutants (Bowman *et al.* 1989; Coen and Meyerowitz 1991). This model proposed that the distinct floral organs of a flower are the product of the concerted action or mutual repression of a set of type II MADS-box genes expressed in a precise spatio-temporal order in the floral meristem during flower development. In this model, A class genes alone induce the formation of sepals; A + B class genes specify the formation of petals; B + C class genes induce the development of stamens and C genes alone specify carpel formation. Additionally, A and C genes mutually repress each other (Coen and Meyerowitz 1991).

ABC model-based hypotheses to explain the inside-out flower of *Lacandonia schismatica*

Based on the ABC-model of floral development, the simplest hypothesis to explain the inversion of stamens and carpels in *L. schismatica* flowers, would involve a displacement of B-function gene expression to the center of the floral meristem, while C class gene function would remain in the third and fourth whorls of the developing flower primordium (Vergara-Silva *et al.* 2000; Vergara-Silva *et al.* 2003) (**Fig. 5**).

Such hypothesis is justified because ABC gene orthologs expression patterns and overall function during sexual organ flower development are conserved among angiosperms, particularly in eudicots and grasses (Ambrose *et al.* 2000; Nagasawa *et al.* 2003; Whipple *et al.* 2004, 2007). Overall, B genes have been always associated to stamen specification (Ambrose *et al.* 2000; Nagasawa *et al.* 2003; Rijpkema *et al.* 2006).

Not withstanding the former evidence, a number of publications using different basal angiosperms, non-model eudicot and monocot species as experimental systems have appeared in recent years, with important implications on the applicability and variation of the ABC model in members of this latter group and in *L. schismatica* (Kanno *et al.* 2007; Mondragón-Palomino and Theißen 2009). Some of the most relevant findings on ABC gene evolution and expression

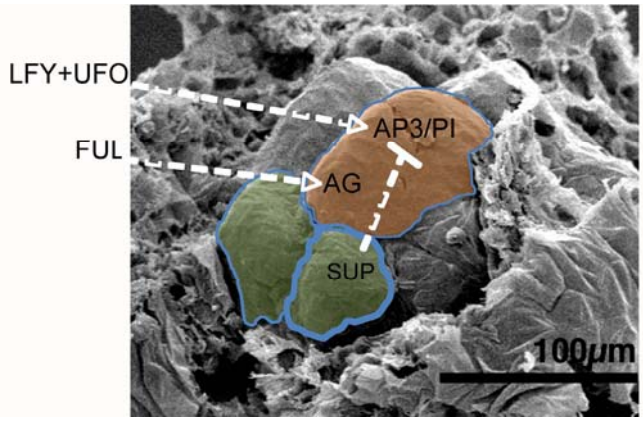

Fig. 5 SEM photograph of a *Lacandonia schismatica* flower bud artificially colorized to show hypothetic domains of expression of different transcription factors. One stamen primordium is colorized in orange; two carpel primordia colorized in yellow. Limits among organs depicted with blue lines. Dotted line with arrowhead implies activation of *AP3/PI* by LFY+ UFO and *AG* by FUL in stamen primordia. Dotted line with bar implies repression of *AP3/PI* by SUP. It should be noted that *AG* expression is present throughout stamen and carpel primordia (Álvarez-Buylla *et al.* unpublished). Figure abbreviations: LFY – LEAFY; FUL – FRUITFULL; UFO – UNUSUAL FLORAL ORGANS; AP3 – APETALA3; PI – PISTILLATA; AG - AGAMOUS; SUP – SUPERMAN.

patterns in different angiosperm lineages are: the existence of pervasive duplication events of MADS-box genes, particularly of the *PI* and *AP3* lineages; these duplications have translated into anomalous expression patterns of B class genes (Stellari *et al.* 2004) or C class genes (Kramer *et al.* 2004), with respect to the ABC model predictions (in basal angiosperms; see Soltis *et al.* 2007; for monocots, see, among others: Kanno *et al.* 2003; Hama *et al.* 2004; Tsai *et al.* 2004; Kanno *et al.* 2007; Xu and Kong 2007; for an eudicot, *Petunia hybrida*, see Vandenbussche *et al.* 2004; Rijpkema *et al.* 2006). Furthermore, such duplication events have led in several cases, to the subfunctionalization and/or neofunctionalization of such gene paralogs and to particular interactions between *PI*-lineage and *AP3*-lineage duplicates in different angiosperm groups (Kramer *et al.* 1998; Irish and Litt 2005; Rijpkema *et al.* 2006; Mondragón-Palomino and Theißen 2009).

It is important to note, however, that the divergences documented to date have never been associated with a homeotic inversion on the position of stamens and carpels caused by a displacement of the B function to the flower center (Kramer and Irish 1999; Kanno *et al.* 2003; Vandenbussche *et al.* 2004; Rijpkema *et al.* 2006; Xu *et al.* 2006, 2007).

Molecular genetics data on *Lacandonia schismatica* floral development

In our laboratory, the B and C gene orthologues of *L. schismatica LsAPETALA3* [LsAP3] and *LsPISTILLATA* [LsPI] and *LsAGAMOUS* [LsAG] have been cloned and characterized (Álvarez-Buylla *et al*. unpublished). Bayesian phylogenetic analyses supported the orthology of these genes as they group with the rest of the B genes and posses characteristic motifs characterized from other angiosperms (Kramer *et al.* 1998, 2003; Álvarez-Buylla *et al*. unpublished).

Based on gene expression analyses, we have observed that the expression domain of the B+C functions is clearly restricted to the most apical or central areas of the compound primordia (Ambrose *et al.* 2006), where stamens develop. Furthermore, B genes of *L. schismatica* are able to rescue or specify stamen development in transgenic complementation and overexpression *A. thaliana* lines which showed very similar phenotypes to corresponding transgenic plants harbouring *A. thaliana* B function genes (Jack *et al*. 1994; Krizek and Meyerowitz 1996; Álvarez-Buylla *et al*. unpublished). However, petals are not completely recovered in these lines (Álvarez-Buylla *et al*. unpublished), which suggests the need for protein complexes involving additional *L. schismatica* native proteins in order to form the multimers which would properly recover both stamens and petals (Honma and Goto 2001).

Alterations of *cis* versus *trans* factors in the regulation of B genes underlying the inside-out flower of *Lacandonia schismatica*

The genetic-molecular data obtained up to date (Álvarez-Buylla *et al.* unpublished) provide a sufficient molecular genetic explanation for the unique spatial inversion of stamens and carpels in *L. schismatica* flowers. However, we still do not know to what extent are other aspects of the GRN in which ABC genes are embedded altered in this species with respect to model organisms. For example, is the atypical expression pattern of *LsAP3* due to changes in the *cis*- regulatory sequences of this gene, while the rest of the GRN is conserved, or are alterations in other *trans*-regulatory components also involved? We provide here some specific hypotheses concerning the possible molecular mechanisms and the involvement of other GRN components in the origin of the *L. schismatica* flower.

Changes in *cis* involve particular mutations in promoter and other regulatory sequences like introns, which could affect the binding affinities of transcription factors that regulate the expression of *LsAP3*. In plants, comparative studies using phylogenetic footprinting of the type to be undertaken in *LsAP3-like* promoters, have shown that the particular inputs of *cis*-motifs can be dissected by comparing promoters or introns of developmental genes of closely related species (Hong *et al.* 2003; Lee *et al.* 2005). In the case of the transcription factor *CRABS CLAW* (*CRC*), comparisons of 5′ upstream regulatory regions between *Arabidopsis*, *Lepidium* and *Brassica* showed conserved modules that have both positive and negative regulatory elements. Noteworthy, each particular motif has the ability to drive reporter gene expression in specific sites as if the overall summation of inputs gave rise to the wild type expression profile (Lee *et al.* 2005). In a related paper, the comparison of the second intron of *AG* between 29 species of *Brassica* allowed dissection of two conserved binding sites for the binding of WUS and LFY; these sites seem to be specific to the Brassicales (Hong *et al.* 2003).

In a conceptually connected but still alternative explanation, divergences in the types, interactions or binding capacities of *trans* activating factors could also render the atypical expression pattern of *LsAP3*. In this scenario, alternative compositions of MADS-domain protein tetramers – the putative functional transcriptional units in *A. thaliana* flower development – could have different affinities for specific regulatory sequences in the *LsAP3* transcriptional regulatory regions. Additionally, proteins forming those complexes could have also experienced spatial or temporal modifications in their expression domains. One such floral regulatory gene that could be concomitantly affected with a change in LFY domain of expression could be *UFO* (UNUSUAL FLORAL ORGANS), an F-box protein that in *A. thaliana*, is a necessary cofactor of LFY required to activate *AP3* expression in the primordia of floral whorls 2 and 3 of this plant. This is achieved by a physical interaction between LFY and UFO in a complex that binds directly to the *AP3* promoter, seemingly tagging a yet unknown *AP3* repressor for degradation (Lee *et al.* 1997; Chae *et al.* 2008). In this paper, we propose that the displacement of *LsAP3* towards the center of the flower meristem of *L. schismatica* could be related to *UFO* being only expressed in the central whorl of this flower, resembling the pattern of *UFO* observed during early *A. thaliana* flower development. Furthermore, the fact that both LFY and *UFO* orthologs from other angiosperms show divergent expression patterns, suggest that alterations in the domain of expression of these

genes could be involved in the inversion of stamens and carpels in *L. schismatica* (Souer *et al.* 2008; Moyroud *et al.* 2009).

It is important to notice that with respect to type II MADS-box genes, the *SQUAMOSA*-like *FRUITFUL* (*FUL*) and *AP3* seem to have the greatest degree of conservation in their promoter sequences (Koch *et al.* 2001). Whether this responds to a particular developmental or historical constraint is yet to be tested. Overall, the structure of the promoters of *AP3* in angiosperms should be related to the following factors: initial recognition by UFO and LFY and later-developmental state recognition by the AP3-PI heterodimer (coupled with AG and possibly SEP3) which keeps the *AP3* active in a positive feed back loop. Molecular techniques such as Yeast One Hybrid analysis that allow identification of proteins interacting with a specific DNA sequence, or Chip on Chip, that allow identification of DNA sequences to which transcription factors bind to, should be used once the *LsAP3* promoter and genetic sequences are characterized in order to dissect the genetic-regulatory basis of the inside-out floral phenotype of *L. schismatica*.

L. schismatica SEP3 orthologs may also be involved in mechanisms related to the inversion and partial indeterminacy of the carpel whorl. In *A. thaliana*, SEP3, as stated above, is a regulator of floral meristem patterning and may induce ectopic B and C gene expression when ectopically expressed (Castillejo *et al.* 2005). It is a key factor for the co-localization of AP3 and PI proteins to the cell nucleus, forming ternary complexes with these proteins *in planta* (Immink *et al.* 2009). *SEP3* seems to be also important during the transition from inflorescence to flower meristem (Liu *et al.* 2009), and it could be a useful marker to establish the boundary and the distinction between these two types of meristems in *L. schismatica* and other triurids. Furthermore, SEP3 has been shown to bind to the *AP3* promoter and enhance its expression, although loss of function mutants of *SEP3* do not seem to affect stamen formation (but lack petals, Kaufmann *et al.* 2009), maybe due to redundancy with *SEP1,2-4* genes (Kaufmann *et al.* 2009). Another gene that can be implicated in the molecular mechanism related to the homeosis of *L. schismatica*, is *SUP*. This gene encodes a transcription factor with a C2H2-type zinc finger protein important for delimiting the stamen and carpel whorls and controlling cellular proliferation of both sexual organ types, but specially stamens (Bowman *et al.* 1992; Sakai *et al.* 2000). This gene could be particularly relevant during the specification of the stamen-carpel boundary within the common primordia: stamens in the most apical portion and carpels in the most basal one. Interestingly, it is the second whorl from the inside towards the periphery of the flower the one that has a proliferative stage in *L. schismatica.* In *A. thaliana*, *sup* mutants show supernumerary organs, but in this species, stamens rather than carpels are differentiated in this position (see **Fig. 5** for a representation of proposed genetic interactions).

DEVELOPMENTAL CONSTRAINTS NOT BROKEN DURING FLOWER EVOLUTION IN *L. SCHISMATICA:* THE TEMPORAL PATTERN OF FLORAL ORGAN FORMATION AND THE PRESENCE OF COMPOUND PRIMORDIA

It is interesting to note that despite the fact that *L. schismatica* flowers present a unique spatial sexual organ arrangement, the temporal sequence in which floral organs arise during flower development is not altered with respect to the stereotypical temporal pattern found in the large majority of angiosperms. Namely, perianth organs are specified and appear first, and then stamens and carpels are formed next (see Section **CONTROVERSIES ON THE IDENTITY OF THE REPRODUCTIVE STRUCTURES OF TRIURIDACEAE**). The fact that the temporal progression of floral organ formation is conserved, while the spatial pattern is altered in *L. schismatica*, suggests that the genetic mechanisms that underlie such developmental restrictions can be altered in the course of evolution independently of each other.

Recent efforts in GRN modeling grounded on experimental data of model organisms (Espinosa-Soto *et al.* 2004; Álvarez-Buylla *et al.* 2008) have revealed that a robust network leads to stable gene configurations that characterize each of the four types of primordial cell lineages found at early stages of flower development in *A. thaliana*, which later yield the mature floral organs. Interestingly, the conserved temporal progression of floral organ formation seems to emerge also from the dynamics of such gene regulatory module (Alvarez-Buylla *et al.* 2008). Present modeling efforts are geared towards addressing if alterations of the same GRN may independently change the temporal and spatial patterns of floral organ arrangement. Such approaches provide insights of the specific components of the GRN under study that could have been altered in *L. schismatica* floral evolution. Remarkably, models based on *A. thaliana* genetic data have been able to recuperate stable gene activation profiles that give way to the wild type for this plant and also, infer the genetic configuration of known mutants. An interesting outcome of the study by Espinosa-Soto and collaborators (2004) was that a regulatory module or submodule can be altered independently of others and generate mutant phenotypes. This observation further supports the suggestion made by Vergara-Silva and collaborators (2003) after studying inflorescences of *L. schismatica* and *T. brevistylis*, where they concluded that, given the discontinuous nature of the morphological variations documented in different extant populations of these taxa, the heterotopic flower of *L. schismatica* was most probably a consequence, at the genetic level, of a discrete change in one or several modules of the GRN of this plant.

In order to fully assess the former proposal, it will be instrumental to document the ontogeny of a hermaphrodite *Sciaphila* species, through developmental series. If the development of sexual organs in hermaphrodite triurids follows the common ontogenetic path of angiosperms, this could hint to the possibility that only taxa that posses secondary carpel development (or centrifugal carpel inception; all within tribe Triurideae; Rudall 2008), are the ones where a separation between the spatial and temporal patterns of development could have been favored during the course of evolution. While we were working on a revised version of this paper, an article by Paula Rudall (2009) was published that gives additional examples of angiosperm taxa where a spatio-temporal decouplement of flower development has taken place. This paper analyses this issue from the perspective of the centripetal vs centrifugal floral organ progression.

CHANGES IN FLOWER MERISTEM IDENTITY GENES UNDERLYING COMMON PRIMORDIA AS AN ADDITIONAL EXPLANATION OF *LACANDONIA SCHISMATICA* ONTOGENY

An additional explanation of the peculiar development of *L. schismatica* sexual organs could be related to the underlying developmental genetics network of a common primordium. Common primordia have been documented in different plant species within monocots, in members of the Zingiberaceae and Costaceae families (i.e. *Alpinia ceorulea* and *Costus igneus*; Endress 1995), as well as eudicots, such as some legume species (Tucker 2003). In *L. schismatica* the common primordium from where stamens and carpels develop, can be interpreted as being apically determinate and distally –somewhat-indeterminate (proliferative).

The underlying genetic mechanisms that could be involved in this singular primordium could imply not only a shift to the flower center of the B class genes but also, a similar displacement of the domain of expression of floral pre-patterning genes also involved in meristem determinacy such as *LFY* (or UFO, depending on which of these two proteins is displaced). We advance this hypothesis based on findings in *Pisum sativum* and *Lotus japonicus*, two legume

species that bare a common primordium from where petal and stamen whorls develop (Taylor *et al.* 2001; Tucker 2003; Dong *et al.* 2005).

In *L. japonicus*, a plant species with a clear flower-like morphology, the ABCE genes plus *LFY* and *UFO* orthologs have been functionally characterized through loss of function mutants. Dong and collaborators (2005) show that in *L. japonicus* the LFY ortholog is not necessary for A and C-function gene initial expression, given that both *LFY* and *UFO* loss of function mutants in this species have flowers with carpel and sepal whorls, but have no distinctive second and third whorls. Rather, a single whorl is present in the place normally occupied by petals and stamens where repetitive floral meristems arise. This data suggest that the functions of LFY and UFO can diverge significantly in plant species with a common primordium (maybe due to extra *LFY* copies that have undergone subfunctionalization) and also, could imply that in *L. schismatica* the domain of expression of its *LFY* (and/or *UFO*) orthologs could be displaced to the flower center (where determinate stamens arise) and only weakly expressed or completely repressed in the area surrounding the stamens, that is partially indeterminate, and where multiple carpels develop. This hypothesis could also help explain the partial indeterminacy of the carpel whorl, while being congruent with the pattern of expression of the B and C genes observed through *in situ* hybridizations in *L. schismatica* (Alvarez-Buylla *et al.* unpublished). An additional characteristic that further supports this scenario is the fact that *L. schismatica* flowers only have three floral whorls comprised of stamens, carpels and a sepal-like perianth (tepal), which is also the number of whorls present in the *L. japonicus lfy* mutant. The underlying mechanism in *L. schismatica* could imply additional genes, but there are relevant structural similarities concerning the common primordia and the *lfy* mutant of the legume with respect to *L. schismatica.* Furthermore, the LFY ortholog present in triurids could have a divergent function from that characterized in *A. thaliana*; in several angiosperm species this has proven to be the case, where LFY orthologs have been documented to be involved in compound leaf development, as well as plant and inflorescence architecture (Moyroud *et al.* 2009).

It could be possible that changes in the spatial and/or temporal expression of certain flower meristem determinacy genes can be a unifying theme of common primordia in distantly related species that independently evolved this type of organ meristem. This proposal is partially supported by an interesting suggestion made by Moyroud and collaborators (2009) who hint to the idea that *trans* regulation of LFY orthologs within different flowering plants can be fundamental in determining the function of this evolutionary conserved transcription factor, as diverse LFY orthologs used to rescue *lfy* mutants in *A. thaliana* fulfill this task, regardless of having different roles in their species of origin.

Alternatively, a de-coupling of the spatial and temporal pattern of organ progression could be a common trait in compound primordia, as in *L. japonicus* the temporal progression of organs is: sepals, carpels, petals and stamens (Dong *et al.* 2005). Further studies en *L. schismatica* and related species, as well as members of the Zingiberaceae and Costaceae will be instrumental to shed light on this subject.

CONCLUSIONS

In this review we have addressed different lines of research that have complemented or given alternative explanations of the heterotopic flower of *L. schismatica*. A common theme to such research lines is that they have used various strategies at hand to investigate the ontogeny, genetics, development and overall evolutionary history of this taxon, with special emphasis on and *Evo-Devo* perspective committed to an integrative approach to biological reality.

As such, evidence on the morphological variation present in contemporary populations of *L. schismatica* (Vergara-Silva *et al.* 2003), developmental data documenting the spatial and temporal pattern of floral organ development (Ambrose *et al.* 2006; Rudall 2008), and developmental genetics data (Álvarez-Buylla *et al.* unpublished), as well as phylogenetic analyses based in morphology and/or molecular markers (Chase *et al.* 2000; Davis *et al.* 2004; Rudall and Bateman 2006), have generated a body of data that substantiate the importance of studying homeosis and heterotopy as phenomena that can underlie "saltational" events during evolution, such as the one that probably occurred in the *L. schismatica* flower.

The studies undertaken in this unique flowering plant contribute to further our understanding of the nature and extent of developmental constraints during morphological evolution and as such, will help address the possible changes in the overall topology and basins of the epigenetic landscape of *L. schismatica*. In line with this conceptual approach, the data at hand suggest that the regulatory mechanisms underlying the spatial and temporal morphogenetic patterns of floral organ formation seem to be decoupled and can be altered independently from each other during the course of evolution. This is based on the fact that while *L. schismatica* spatial floral arrangement is altered and unique, its temporal pattern of floral organ emergence is conserved with respect to most other angiospems. Previous studies suggest that both processes may be controlled by the same GRN (Alvarez-Buylla *et al.* 2008) and thus suggest that the same network may be altered in such a way that independently alters the spatial and the temporal patterns of floral organs.

New and exciting questions are now ready to be addressed with a broader comparative approach and additional developmental genetics molecular tools. On the evolutionary front, one of the most exciting questions concerns how discrete developmental changes arise and become fixed in a subset of individuals in a population. Addressing this interesting issue will require population genetics data of highly variable molecular loci analyzed in a geographical context, paleogeographic reconstructions and natural history research. The ongoing debate regarding the structural nature of Triuridaceae flowers and inflorescences, as well as the mechanisms beyond the ABC model, that underlie the heterotopic *LsAP3* expression, continue to pose intriguing questions. These will contribute to unravel the developmental mechanisms of the phenotypes of *L. schismatica* and other Triuridaceae, but may also contribute to our general understanding of the gene regulatory networks at play during flower development. In the context of the 150th anniversary of the publication of The Origin of Species, *L. schismatica* is keeping yet another old debate in evolutionary biology alive; the gradualistic versus saltational nature of major morphological transitions during evolution and the genetic mechanisms underlying such transitions, as well as the origin of morphological novelties.

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Flower Development

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Flowers are the most complex structures of plants. Studies of *Arabidopsis thaliana*, which has typical eudicot flowers, have been fundamental in advancing the structural and molecular understanding of flower development. The main processes and stages of Arabidopsis flower development are summarized to provide a framework in which to interpret the detailed molecular genetic studies of genes assigned functions during flower development and is extended to recent genomics studies uncovering the key regulatory modules involved. Computational models have been used to study the concerted action and dynamics of the gene regulatory module that underlies patterning of the Arabidopsis inflorescence meristem and specification of the primordial cell types during early stages of flower development. This includes the gene combinations that specify sepal, petal, stamen and carpel identity, and genes that interact with them. As a dynamic gene regulatory network this module has been shown to converge to stable multigenic profiles that depend upon the overall network topology and are thus robust, which can explain the canalization of flower organ determination and the overall conservation of the basic flower plan among eudicots. Comparative and evolutionary approaches derived from Arabidopsis studies pave the way to studying the molecular basis of diverse floral morphologies.

1. INTRODUCTION: WHEN DID THE FLOWER EVOLVE?

The flower is the most complex structure of plants. Flowers distinguish the most recently diverged plant lineage, the angiosperms or flowering plants, from the other land plants (Figure 1). Embryophytes originated approximately 450 million years before present (MYBP) and have as distinctive features a thick cuticle resistant to desiccation, sporopollenin, pores or true stomata that aid in gas exchange, a glycolate oxidase system that improves carbon fixation at high oxygen tensions, and importantly, distinctive multicellular diploid (sporophytic) and haploid (gametophytic) stages within their life cycles (Judd et al., 2002). The major extant land plant lineages are Bryophytes (Liverworts, Hornworts and Mosses), which do not have a vascular system, and Tracheophytes, vascular plants. Within the large latter group, Lycophytes, ferns, and seed bearing plants (Spermatophytes) can be distinguished. The Spermatophyte group has been further divided into Gymnosperms (originating 380-325 MYBP) and Angiosperms. According to the fossil record, flower-like structures originated 160-147 MYBP (Frohlich, 2006). A general trend within land plant evolution is the appearance of heterospory: the existence of a megagametophyte, including the female gametes, and a microgametophyte, including the male gametes, a progressive reduction in gametophyte size (sexual reproductive structures), and within the seed plants, the presence of a diploid embryo. While these characteristics are shared among both extant and extinct seed plant lineages, the defining features of the angiosperm flower are: (1) a closed carpel bearing the ovules, which are each generally comprised of two integuments and (2) a nucellus that contains the embryo sac within which, after double fertilization, a diploid embryo and a triploid endosperm (nutritional tissue for the embryo) will develop to form a seed (Judd et al., 2002). Another characteristic of angiosperms is true hermaphroditism (Judd et al., 2002; Frohlich, 2006).

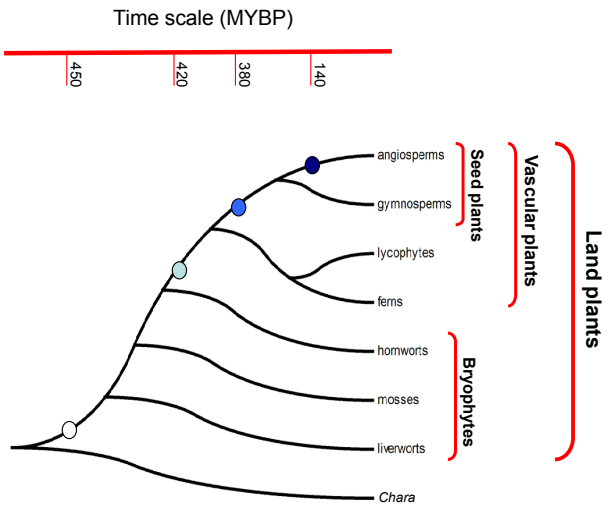


Figure 1. Phylogenetic context of *Arabidopsis thaliana*: Evolutionary history of land plants.

Phylogenetic tree of land plant evolution with some speciation events shown as colored nodes. White node, origin of land plants; light blue node, origin of vascular plants; blue node, origin of seed plants; dark blue node, origin of flowering plants. Here, *Chara* spp. from the green algae order Charales is the outgroup, since it has been used to root several recent molecular land plant phylogenies. The topology of this tree is based on studies by Soltis et al. (1999) and Nickrent et al. (2000). Time references in million years before present (MYBP) were taken from Judd et al. (2002).

Flower structure has been studied in a variety of ways. Studies of the natural history and evolutionary biology of flowers have emphasized understanding the ultimate (evolutionary) causes of the wide range of variants such as color, symmetry, meristic arrangements (e.g. flower organ number), size, pollination syndrome, etc. Other studies have addressed the cellular, tissue type, morphological and physical factors that can account for both the phenotypic plasticity and developmental constraints in flower form (for a review of the developmental framework of angiosperm morphology, see Endress, 2006). A different approach flourished in the late 1980s and early 1990s, the molecular genetics of flower development in two model eudicot species: *Arabidopsis thaliana* and *Antirrhinum majus* (see reviews in: Jack, 2004; Kaufmann et al., 2005; Krizek and Fletcher, 2005; Theissen and Melzer, 2007).

Genetic studies of floral homeotic mutants in both plant species yielded the now classic combinatorial ABC developmental model for floral organ determination (Bowman et al., 1989; Coen and Meyerowitz, 1991). While much work has been and continues to be done in *Antirrhinum* and other eudicot species, including *Petunia hybrida*, the genomic and life-cycle characteristics of Arabidopsis make it the preferred experimental system for in-depth studies on the molecular components underlying cell differentiation and morphogenesis during flower development.

The basic floral architecture is mostly conserved among the so-called core eudicots, that make up over 73% of extant flowering plants (Drinnan et al., 1994) including Arabidopsis. Flowers within this group generally have four concentric whorls of organs that are specified, from the outside to the center of the flower, in the sequence: sepals, petals, stamens, and carpels. Arabidopsis has this typical floral architecture. An interesting exception to the conserved floral ground plan of eudicots is found in a Mexican rainforest monocotyledon, *Lacandonia schismatica* (Triuridaceae), which bears central stamens surrounded by carpels (Martínez and Ramos, 1989; Vergara-Silva et al., 2003; Ambrose et al., 2006).

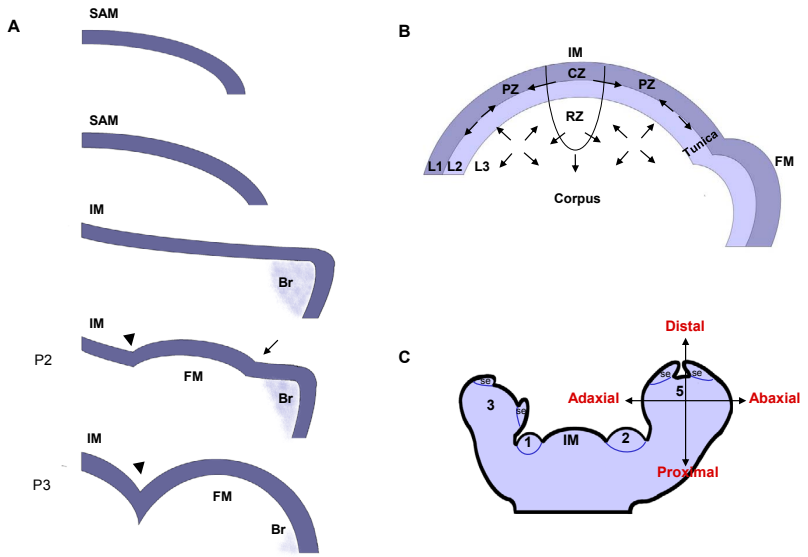


Figure 2. Schematic representation of the shoot apical meristem (SAM): the inflorescence shoot apical meristem and floral meristem.

(A) Diagram outlining the geometry of the inflorescence shoot apical meristem (IM) and flower meristem (FM) during the first stages of development of the latter. On the flank of the IM a first bulge that corresponds to the rudimentary bract (Br) appears. In its axil, a second bulge forms and this continues to grow engulfing the first one and forming the FM proper. Theses stages of FM development correspond to P2 and P3 according to Reddy et al. (2004). The arrow and arrowhead indicate the first and second visible grooves respectively (see section 2.3 for further detail).
(B) Three distinctive zones make up the IM: the central zone (CZ) which contains the stem cells; the peripheral zone (PZ) on the flanks of the CZ that gives rise to the bract and floral primordia; and the rib zone (RZ) underneath the CZ that yields stem tissue. Three cell layers are distinguished: L1 and L2 layers constitute the tunica and include portions of both the CZ and the PZ. The rest of the cells form the L3 layer or corpus. In L1 and L2, cell divisions are anticlinal, while in L3 they occur in all directions (arrows, direction of cell division). The structure is maintained in the FM.
(C) Schematic representation of the boundary zones (blue lines) and axes of polarity during floral development with the differentiation of sepals (se) from the floral primordium illustrated.

Even though the basic floral architecture is overall conserved among core eudicots, variation in the symmetry and size of flowers, the number of whorls of each organ type, the number of organs per whorl, and their arrangement, size, shape and color is common (e.g., Judd et al., 2002).

The overall conservation of the flower plan suggests that robust gene regulatory network (GRN) modules controlling the basic features of flower development were established early in the evolution of angiosperms and have persisted in the great majority of lineages throughout 140 million years of flower evolution. Recent integrated approaches to study the concerted action of the molecular components in flower development (Mendoza and Alvarez-Buylla, 1998; Espinosa-Soto et al., 2004), have led to a hypothesis that helps explain such robustness and conservation at the level of the GRN underlying floral organ specification. However structural (e.g., mechanical) constraints could also be involved in conserving floral architecture (see section 4). Approaches that integrate genetic and structural aspects of flowers should be pursued further to understand flower development in Arabidopsis and other angiosperms.

2. STRUCTURAL ASPECTS OF ARABIDOPSIS FLOWER DEVELOPMENT

In this section, we provide a summary of structural features of Arabidopsis flower development. This is essential background to the molecular genetics reviewed in section 3.

2.1 Structural Organization of the Inflorescence Meristem and Origin of the Flower Meristem

During the vegetative phase of the Arabidopsis life cycle, the shoot apical meristem (SAM) produces leaves on its flanks and on transition to flowering, the shoot bolts and the SAM becomes the inflorescence shoot apical meristem (IM). On bolting, some of the pre-existing leaf primordia become cauline leaves subtending lateral inflorescence shoots (paraclades) and the shoot apex starts to produce flowers (Hempel and Feldman, 1995). A primary IM produces lateral meristems that may go on to produce flowers or secondary inflorescences. Arabidopsis inflorescences are subtended by fully developed bracts, but flowers only by rudimentary ones. It is generally said that the IM generates the floral meristems (FM) on its flanks, but to be more precise, Arabidopsis FM are formed in the axils of the rudimentary bracts (Figure 2A; Long and Barton 2000; Hepworth et al., 2006; Kwiatkowska, 2006; reviewed in Kwiatkowska, 2008).

The SAM of the Arabidopsis inflorescence consists of a small dome of cells organized into different regions (Figure 2B) with different gene expression profiles (see section 4.1), cellular behaviors and structures. The tunica at the SAM surface and corpus are distinguished on the basis of cell division planes. In Arabidopsis, the tunica consists of two clonally distinct cell layers called L1 and L2 (Vaughan, 1952; Steeves and Sussex, 1989). Cell divisions within these meristem layers are exclusively anticlinal and the new cell walls are formed perpendicular to the surface of the meristem. The progeny of cells in the L1 will therefore remain in this same layer within the meristem similar to the underlying L2 progeny. Since outside the meristem the L1 derived cells continue to divide only anticlinally the L1 eventually gives rise to epidermal cells. The cells originating from L2 also divide periclinally (outside the SAM) and contribute for example to the leaf mesophyll or stem ground tissue formation during organogenesis. This is also the germ line in the angiosperm SAM (Ruth et al., 1985, Klekowski, 1988; Kwiatkowska, 2008). Below the tunica, cell divisions are both anticlinal and periclinal. This region of the SAM is the corpus or L3 from which the innermost tissues, like vascular tissues, are formed (Figure 2B; Brand et al., 2001).

The SAM is also organized into three different cytohistological zones each with characteristic cytoplasmic densities and cell division rates: the central zone (CZ), the peripheral zone (PZ) surrounding the CZ and the rib zone (RZ) underneath the CZ (Figure 2B; Bowman, 1994; Bowman and Eshed, 2000).

Flower primordia are derived from the PZ of the IM and are initiated from a block of four so-called founder cells (Bossinger and Smyth, 1996; Reddy et al., 2004). This estimate was based on sector boundary analysis. However, using a non-invasive replica method and a 3-D reconstruction algorithm, Kwiatkowska (2006; 2008) argues that more cells are assigned to the flower primordium, and this is consistent with the observations by Grandjean et al. (2004). The difference could be due to the fact that not all of the cells estimated to be involved in the latter approaches are incorporated into the flower meristem proper. Some of them may form a part of the subtending rudimentary bract (Figure 2A; see next section for further discussion).

The first cells produced by the RZ following the transition to flowering are rectangular with their long axis perpendicular to the major axis of the stem, but the subsequent elongation of these cells reverses this situation (Vaughan, 1955). The RZ gives rise to stem tissue. The CZ encompasses the reservoir of stem cells that divide less frequently than cells at the periphery (Grandjean et al., 2004; Reddy et al., 2004). The CZ maintains itself and yields daughter cells that form both the PZ and RZ (Bowman and Eshed, 2000). Fifteen stages of Arabidopsis flower development have been distinguished (Smyth et al., 1990). The first stages of flower meristem development are: stage 1, when a flower buttress arises, stage 2 when the flower meristem is formed and stage 3 when sepal primordia appear. Recently researchers have been able to study early flower meristem development in greater detail (Reddy et al., 2004; Kwiatkowska, 2006; reviewed by Kwiatkowska, 2008) and have proposed subdividing stage 1 (see section 2.3).

2.2 Floral Organ Primordia

Once a flower primordium is initiated, the geometry changes and a rapid and coordinated burst of cell expansion and division occurs in three dimensions generating a concentric group of cells as an almost spherical flower primordium, from which all floral tissues are derived (Bossinger and Smyth, 1996; Reddy et al., 2004; Kwiatkowska, 2006). Jenik and Irish (2000) found that the regulation of cell divisions during early and late stages of flower development seems to depend upon different mechanisms. Early in flower development, when the floral meristem of Arabidopsis is divided into four concentric rings (each with a characteristic multigenic expression profile; see section 3.3), cell division patterns depend upon the cell's radial position in the floral meristem, and not on the future identity of the floral organ to be formed in each ring. After stage 6, during organogenesis, the ABC homeotic genes (see section 3.3) seem to control the rate and orientation of cell divisions. As a result, the continuity of the concentric rings is broken giving distinct floral organ primordia within each whorl, then cells subdifferentiate into distinct types within each organ. The initiation and identity of floral organs are also regulated by different and largely independent molecular modules. This is suggested, for example, by the fact that conversion of petals into sepal-like organs in mutant plants does not alter the number of cells involved in their initiation (Crone and Lord, 1994; Bossinger and Smyth, 1996).

Tissues of floral organs are organized according to coordinated patterns and rates of cell division in the different cell layers of the meristem that dynamically acquire distinct fates. Clonal analysis shows that L1 contributes to the epidermis, the stigma, part of the transmitting tract and the integument of the ovules, while L2 and L3 contribute to the mesophyll and other internal tissues (Jenik and Irish, 2000).

Sector boundary analysis of surface cells has shown that sepals and carpels are initiated from eight cells, stamens from four

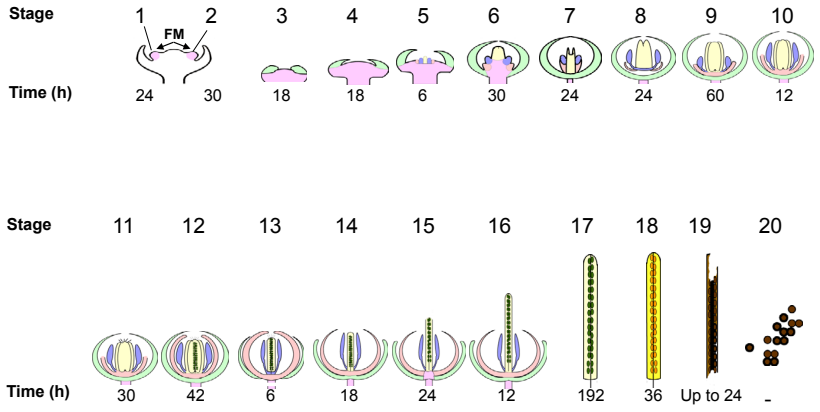


Figure 3. Summary of the 20 stages of flower development.

Schematic representation of developmental stages of Arabidopsis flowers. Briefly, the flower primordium is formed at stages 1 and 2. At stage 3, sepal primordia are already visible and continue growing until they enclose the flower meristem (from stage 4 to 6). Meanwhile, at stage 5, petal and stamen primordia are beginning to be visible, and the gynoecium starts to form (stage 6). Organ development continues and by stage 9, stigmatic papillae arise at the top of the gynoecium. At stage 12, petals are similar in length to stamens. Anthesis occurs at stage 13, fertilization occurs, and the flower opens at stage 14. Siliques reach their maximum size and are green by stage 17, then they loose water and turn yellow (stage 18) until valves separate from dry siliques (stage 19) and seeds fall (stage 20). Floral meristems (FM), pink; sepals, green; petals, bright pink; stamens, blue; gynoecia, yellow; ovules, dark green; seeds orange and brown. Duration of each stage in hours (h) is given under the figures (from Smyth et al., 1990).

cells, and petals from two cells (Bossinger and Smyth, 1996). Each organ primordium arises as a set of cells separated by boundary regions of slow-dividing cells (Figure 2C and section 3.4.2; Breuil-Broyer et al., 2004). Flower development ends when mature organs are formed and all the flower meristem cells are used up (Takeda et al., 2004; Krizek and Fletcher, 2005).

2.3 Stages of Flower Development

We provide an illustrated description of 20 states of floral development and fruit formation (Figures 2-7), mostly based on Bowman (1994), Smyth et al. (1990), Ferrándiz et al. (1999) and Roeder and Yanofsky (2006), with updates and substages as proposed by Long and Barton (2000), Reddy et al., 2004; Hepworth et al. (2006), Kwiatkowska (2006) and Kwiatkowska (2008).

STAGE 1: The first sign of flower primordium formation is the bulging of the peripheral surface of the IM in a lateral direction. This stage was referred to as P1 by Reddy et al. (2004). It is hypothesized that a lateral protrusion formed during bulging is a rudimentary bract (Figure 2A; Kwiatkowska, 2006). At this early stage, growth is fast and strongly anisotropic, with maximal growth in a meridional (i.e. radial when viewed from the top of the meristem) direction (Kwiatkowska, 2006) eventually leading to formation of a shallow crease, which corresponds to the first visible groove and to the P2 stage (according to Reddy et al., 2004) of flower development (Figure 2A). This shallow crease corresponds to the axil of the putative rudimentary bract (Kwiatkowska, 2006, 2008). Soon after the bract is formed, another bulge occurs in its axil in an upward direction. This second bulging corresponds to the formation of a flower primordium proper and to stage P3 according to Reddy et al. (2004). This stage corresponds to stage 2 according to Smyth et al. (1990). Hence, during early stages of flower development in Arabidopsis, two types of primordia (bract and flower primordium proper) and organ boundaries are observed. The first boundary is the adaxial boundary of the rudimentary bract, while the second is the boundary between the IM and the flower primordium proper (Figure 2A). The expression patterns of several genes confirm the developmental stages distinguished here (see more data on gene expression in the next section).

A significant increase in mitotic activity is observed upon formation of the primordium. The mitotic activity can be estimated as the increase in the number of cells per 24 h or the accompanying area growth rates on the condition that the mean cell size does

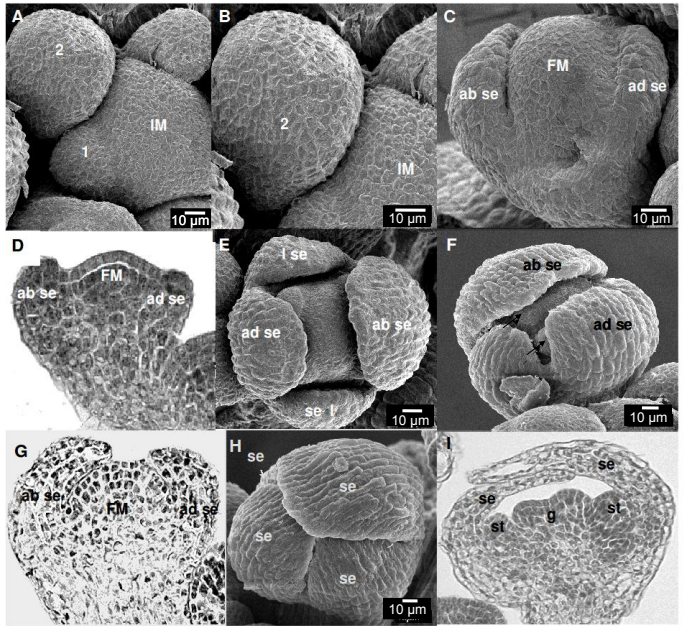


Figure 4. Stages 1 to 6 of Arabidopsis flower development.

(A) and **(B)** Inflorescence shoot apical meristem (IM) and floral meristem (FM) at stage 1 and 2 as indicated.
(C) and **(D)** Stage 3 FM showing abaxial (ab) and adaxial (ad) sepals (se).
(E) At stage 4, lateral sepals **(I)** shown growing perpendicularly to the abaxial and adaxial ones.
(F) and **(G)** At stage 5, stamen primordia are visible (arrows) and sepals almost cover the rest of the meristem.
(H) Flower bud where sepals are covering the stamens and the gynoecium primordium.
(I) Section through a stage-6 flower primordium where the gynoecium (g), stamens (st), and sepals (se) are apparent.
Pictures are scanning electron micrographs (SEM), except **(D)**, **(G)** and **(I)** which are optical images of histological sections. All pictures are of Columbia-0 wild-type plants.

not increase (Grandjean et al., 2004; Kwiatkowska, 2006; Reddy et al., 2004). During these early stages of flower development, periclinal cell divisions occur in the corpus while L1 and L2 cells only divide anticlinally (Vaughan, 1955). Hence, the two-layered tunica organization is maintained in the flower meristem, but all of its cells are mitotically active.

STAGE 2: During this stage, the hemispherical primordium continues to grow forming almost a right angle with the surface of the SAM, which itself lengthens and widens rebuilding the portion of the periphery that has been used for primordium formation (Figures 3 and 4A-B). At this stage the flower primordium becomes clearly delimited from the IM, and starts to grow larger very quickly in all directions (Figures 3 and 4A-B; Reddy et al., 2004; Kwiatkowska, 2006).

STAGE 3: This stage begins when sepal primordia become visible. By now the flower primordium is 30-35 µm in diameter and is becoming stalked with an incipient pedicel. It has also started to grow vertically. The two lateral (l) sepal primordia appear first, but are soon outgrown by the abaxial (ab) then the adaxial (ad) sepal primordia. Sepal primordia arise initially as ridges that lengthen and curve inwards until they begin to overtop the remaining dome-shaped portion of the flower primordium (Figures 3 and 4C-D).

STAGE 4: During this stage, the elongation of the pedicel continues concurrently with an increase in the diameter of the developing flower primordium to 65-70 µm. The medial sepal primordia have already partly overtopped the remaining floral meristem (Figure 4E).

STAGE 5: This stage is when the petal and stamen primordia become visible. Primordia of the four medial (long) stamens are first seen as wide outgrowths on the flanks of the central dome of the FM. The four petal primordia that arise between the sepals close to their base are just visible during this stage. The two lateral (short) stamens develop from primordia that appear later during this stage (Figures 3 and 4F-G).

STAGE 6: The sepals grow to completely cover the floral bud and the primordia of the four long stamens bulge out and become distinct from the central dome of cells that comprise the FM. The two lateral stamen primordia arise slightly lower on the dome and develop later. The petal primordia grow somewhat but are still relatively small. A rim around the central dome of the flower primordium now begins to grow upward to produce an oval tube that will become the gynoecium (Figures 3 and 4H-I).

STAGE 7: This stage begins when the growing primordia of the long stamens become stalked at their base. The stalks give rise to the filaments, and the wider upper region to the anthers. By this stage, petal primordia have become hemispherical although they are still relatively small (ca. 25 µm in diameter; Figures 3 and 5A-B).

STAGE 8: The beginning of stage 8 is defined by another landmark in stamen development: anther locules are visible as convex protrusions on the inner (adaxial) surface of the long stamens. At this stage stamens are 55-60 µm long most of which is the developing anther. Locules also appear soon after in the short stamens. Petal growth now accelerates and petal primordia become apparent (Figures 3 and 5C-E).

STAGE 9: This stage begins when the petal primordia elongate. There is a rapid lengthening of all organs especially of petals that acquire a tongue-like shape and increase in length from about 45 µm to up to 200 µm. Nectary glands appear and the stamens grow rapidly. By the end of stage 9, the medial stamens are around 300 µm long. Most of this growth occurs in the anther region, which still accounts for over 80% of their total length. At this stage the floral bud remains completely closed (Figures 3 and 5F-G).

STAGE 10: The rapidly growing petals reach the top of the lateral stamens. The cap of papillae that will constitute the stigma starts to form at the top of the gynoecium (Figures 3 and 5H-I).

STAGE 11: This stage begins when the upper surface of the gynoecium develops stigmatic papillae (Figures 3 and 6A-C) although their outward growth is limited at first to regions not in contact with the overlapping sepals. By the end of this stage petal primordia reach the top of the medial stamens.

STAGE 12: Petals continue to lengthen relatively rapidly. Lateral sepals continue to grow while the stamens and gynoecium lengthen coordinately. The anthers have almost reached their mature length of 350-400 µm and the filaments now lengthen rapidly. The upper part of the gynoecium differentiates into the style (Figure 6D) and a sharp boundary separates it from the cap of stigmatic papillae. Stage 12 ends when the sepals open (Figures 3 and 6D-F).

STAGE 13: Petals become visible between the sepals and continue to elongate rapidly. The stigma is receptive at this stage (Figures 3 and 6G-H). Stamen filaments extend even faster so the stamens outstrip the gynoecium in length and self pollination takes place. The gynoecium is now mature and its three distinct regions can be distinguished: an apical stigma, a style, and a basal ovary. After pollination, pollen tubes grow to fertilize the ovules, the stamens extend above the stigma, and furrows at both valve/replum boundaries appear.

STAGE 14: This is also defined as the stage zero hours after flowering (0 HAF), and it marks the beginning of silique (the fertilized pistil or fruit) and seed development. Cells in the exocarp continue to divide anticlinally and expand longitudinally in the replum and the valve, where there is also some expansion in other directions. There is also division and expansion in the mesocarp and many chloroplasts develop (Figures 3 and 6I).

STAGE 15: The stigma extends above the long anthers. In the carpel walls, cell division and expansion continue. The medial vascular bundles continue to grow and xylem lignifies, while the lateral bundles branch out through the mesocarp (Figures 3 and 6J-K).

STAGE 16: At this stage the silique is twice as long as a stage-13 pistil. Petals and sepals wither and tissues in the silique continue expanding (Figures 3 and 6L).

STAGE 17: This stage is defined by the abscission of the senescent floral organs from the silique, ~2 days after fertilization. The green silique grows to reach its final length and matures, a phase lasting about 8 days making this the longest stage. The dehiscence zone also differentiates (Figures 3, 7A and 7E; Substages 17A and 17B, see Roeder and Yanofsky, 2006).

STAGE 18: The silique begins to yellow from the tip to the base. One of the endocarp cell layers (the second from the inside) lignifies further, and the inner endocarp cell layer disintegrates, while the mesocarp begins to dry out. It has been suggested that lignification may contribute to the silique shattering process, acting in a springlike manner to create mechanical tensions (Figures 3 and 7B).

STAGE 19: The valves begin to separate from the dry silique, apparently owing to the lack of cell cohesion at the separation layer.(Figures 3 and 7C).

STAGE 20: At this stage the valves become separated from the dry silique and the mature seeds are ready to be dispersed (Figures 3, 7D and 7F).

2.4 Morphology, Histology and Development of Floral Organs

Sepals: In sepals L1-derived cells form the epidermis, the mesophyll originates from the L2, and the L3 contributes to the vasculature in the basal part (Jenik and Irish, 2000). Sepals and petals together form the perianth. Both organ types have a simple laminar structure, consisting of an epidermis, mesophyll and rather delicate vascular bundles (veins). The four sepal primordia (the abaxial, adaxial, and two lateral sepal primordia) are the first floral organ primordia to appear. They arise at stage 3 of flower development in a cruciform pattern (Figures 4C-D; Smyth et al., 1990; Bowman, 1994). Whether all four sepals occupy one whorl or the two lateral sepals occupy a separate outer whorl, has been the subject of discussion (Figure 4E; Smyth et al., 1990; Bowman, 1994; Choob and Penin, 2004), but all sepal primordia are formed at around the same time, shortly after they are specified (Figure 4E; Bowman, 1994).

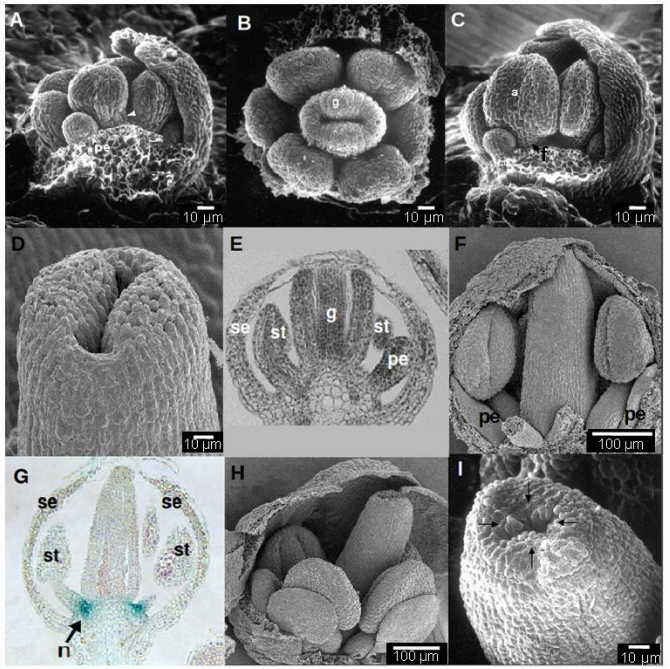


Figure 5. Stages 7 to 10 of Arabidopsis flower development.

(A) Stage 7 in which petal (pe) and stamen (arrowhead) primordia are indicated.
(B) Vertical view of the gynoecium (g) in a stage 7 floral primordium.
(C) to **(E)** Carpels and stamens at stage 8 of floral development are shown. Filament (f) and anther (a) regions of the stamen are differentiated **(C)** and a slot is formed at the tip of the style in the gynoecium **(D)**. Section through the floral bud with sepals (se), petals (pe), stamens (st) and gynoecium (g) indicated **(E)**.
(F) and **(G)** Floral bud at stage 9 in which petal primordia (pe) are indicated **(F)**. Section through flower primordium **(G)** in which *XAL1:GUS* is shown staining nectaries (n).
(H) and **(I)** Stage10 flowers. Flower bud showing the enlarged sepals which cover other floral organs, stalked petals and stamens, and developing carpels in the center **(H)**. Stigma starts to be formed at the top of the gynoecium (**I**, arrows)

Bars = 10 µm except in **(F)** and **(H)**. Images **(A)**, **(B)**, **(C)**, **(G)** and **(I)** are of Lansberg *erecta* ecotype, from Smyth et al. (1990) provided by Dr J. Bowman. Some sepals were removed from flower buds shown in **(A)**, **(B)**, **(C)**, **(D)**, (F), **(H)** and **(I)**. All images except **(E)** and **(G)** are SEM. **(D)**, **(E)**, **(F)**, **(H)** are of Columbia-0 ecotype.

The adaxial and abaxial surfaces of the sepal epidermis are different (Figures 8B and 8D-E). On the abaxial surface, cells have irregular shapes and sizes with some quite long cells (with nuclei of various sizes) and fringes of smaller cells. Unlike the adaxial surface, the abaxial surface has stomata and may have unbranched trichomes (Figure 8E; Smyth et al., 1990; Bowman, 1994; Hase et al., 2000; Krizek et al., 2000).

Petals: In the petal primordium the meristematic layer L1 contributes to the epidermis and L2 to the mesophyll; as yet cells originating from L3 have not been found to form part of the petal

(Figure 2B; Jenik and Irish, 2000). These primordia become apparent almost at the same time as stamen primordia at stage 5 of flower development. Visible signs of petal differentiation are seen by stage 9 (Figure 5F; Smyth et al., 1990; Bowman, 1994). The four petals of Arabidopsis are white and flat and approximately the same size and shape. They are narrower and greenish toward the base (Figure 8C; Takeda et al., 2004).

Cells on the adaxial surface are conical with epicuticular thickenings running from the cell base to the apex, whereas those on the abaxial surface are flatter and more cobblestone-like with cuticular thickening (Figures 8F-G). Stomata are absent from both petal surfaces (Bowman, 1994; Krizek et al., 2000). Cells toward the base of petals resemble those of stamen filaments (Bowman, 1994).

Stamens: Primordia appear at stage 5 of flower development (Figure 4F) due to periclinal divisions in the subprotodermal cell layer (L2) and sometimes in L3 (Crone and Lord, 1994; Jenik and Irish, 2000). Stamen primordia are visible at stage 6. By stage 7, differentiation can be observed and long stamen primordia appear stalked at their bases (Figures 4I and 5A-B; Bowman, 1994; Smyth et al., 1990). At this stage stamen primordia are composed of an L1-derived epidermis, one layer of L2-derived subepidermis, and an L3-derived core (Figure 2B; Jenik and Irish, 2000). Locules appear in the anthers by stage 8 (Figure 5C). Growth of the internal anther tissue at this stage is due to divisions of L2-derived cells (Jenik and Irish, 2000). At stage 14, anthers extend above the stigma (Figure 5I; Bowman, 1994). In the mature anther, the L3 cells contribute only to the vasculature (Jenik and Irish, 2000). Stamens of the Arabidopsis flower are not formed simultaneously: four long medial stamens arise a little earlier than the two short lateral ones (Smyth et al., 1990).

Each stamen consists of two distinct parts, the filament and the anther. At the tip of the filaments, the anther develops both reproductive and non-reproductive tissues that produce, harbor, and release pollen grains upon maturity (Goldberg et al., 1993). The anther is a bilocular structure with longitudinal dehiscence (Figure 6G; Bowman, 1994). Each locule develops from successive divisions of subprotodermal archesporial cells formed in the anther primordium that gives rise to three morphologically distinct layers: the endothecium, the middle layer, and the tapetum which surrounds the pollen mother cells (PMCs). The PMCs undergo meiosis and form the haploid microspores. The tapetum is a source of nutrients and is indispensable for microspore maturation (Xing and Zachgo, 2008). Anther development and microspore formation in Arabidopsis is a complex process that has been divided into 14 stages (See also section 3.4.5; Sanders et al., 1999).

Once formed, PMCs are surrounded by a layer of callose. After meiosis, the anther contains most of its specialized cells and tissues, and tetrads of microspores are present within the pollen sacs; with microspores in each tetrad surrounded by a callose wall. Callose dissolves and microspores are released. As pollen grains develop, the anther enlarges and is pushed upward in the flower by the elongating filament (Scott et al., 2004).

Carpels: The fourth and innermost whorl is occupied by the gynoecium that is composed of two fused carpels. Carpel primordia start to form at stage 6 of flower development (Figure 4I) due to periclinal cell divisions in the L3 layer (Jenik and Irish, 2000). Carpels enclose and protect the developing ovules, mediate pollination, and after fertilization develop into a fruit within which fertilized ovules develop into seeds (Bowman et al., 1999). The gynoecium consists of two valves separated by a false septum with ovules arising from parental placental tissue on each side of the septum (Bowman, 1994). The valves grow upward from the flower meristem to form a closed cylinder. At early stage 8, the walls of the cylinder are composed of an L1-derived epidermis, one L2-derived subepidermal layer and a two-cell thick, L3-derived core. At this stage the distal L2 cells start to divide periclinally (with respect to the top surface of the cylinder), contributing to the longitudinal growth of the carpel (Figure 2B; Jenik and Irish, 2000). Later the inner surfaces of septal outgrowths within this cylinder will fuse, the tip will close and ovules will develop along the margins of the fused walls (placenta) of the bilocular chamber (Bowman, 1994; Sessions and Zambryski, 1995). The gynoecium is oriented in the flower so that the septum coincides with the medial plane (Figures 4D-E and 4G; Sessions and Zambryski, 1995).

At the distal end of the gynoecium, the stigma, an epidermal structure composed of stigmatic papillae (bulbous elongated cells), functions in pollen binding and recognition and participates in the induction of pollen germination (Figures 6A-B). After germination, the pollen tubes will grow between the papillar cells into the transmitting tract at the center of the style and the septum of the ovary (Bowman, 1994; Sessions and Zambryski, 1995).

At about stage 11, the inner and outer integuments of the ovule are formed. By stage 12, the integuments of the developing ovule grow to cover the nucellus and megagametogenesis occurs (Figures 6E-F; Bowman, 1994).

Nectaries: These organs produce and secrete nectar. Nectar is a protein- and carbohydrate-rich solution, which varies in composition among different plant species (Davis et al., 1998). Nectar may be a reward for pollinators or for insects that protect the plant against herbivores, or even a lure for animal prey in carnivorous plants (Davis et al., 1998; Baum et al., 2001; Lee et al., 2005a).

In Arabidopsis, the nectarium (multiple nectary) found in individual flowers (Davis et al., 1998) is composed of two parts: nectary glands that form below the stamen filament, and the connective tissue linking the glands in a continuum around the androecium (Bowman, 1994; Baum et al., 2001). The nectarium is always situated in the third whorl of the flower and its location is independent of the identity of the other organs occupying this whorl. These glands are formed from stage 9 to 17 of flower development (Figure 5G; Bowman, 1994; Bowman and Smyth 1999; Baum et al., 2001; Tapia-López et al., 2008).

3. MOLECULAR GENETICS OF ARABIDOPSIS FLOWER DEVELOPMENT

Plant organogenesis, including flower formation, occurs from actively proliferating meristems over the entire life cycle. In the next section we provide a very brief summary of the molecular mechanisms that maintain an active SAM. In section 3.2, we explain how the flower meristem is specified and becomes determinate after the flower organs are formed.

3.1 Shoot Apical Meristem Proliferation and Maintenance

The balance between cell proliferation and cell recruitment to differentiated tissues in the SAM is dependent on mechanisms

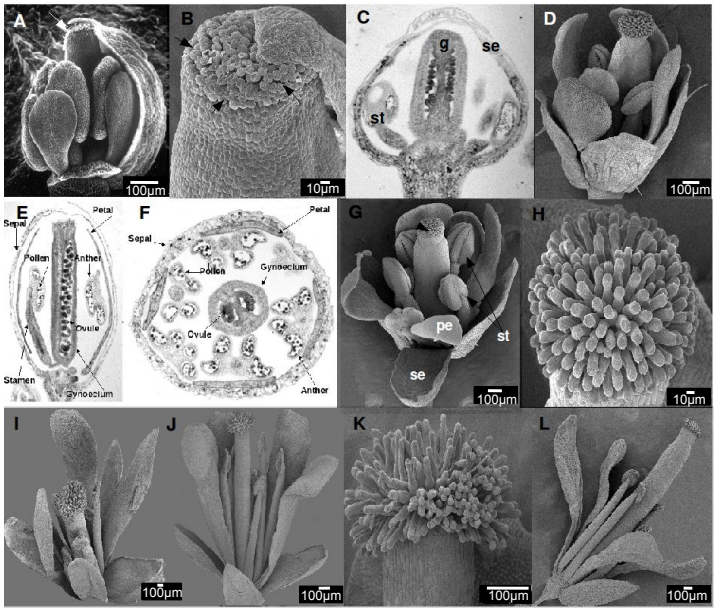


Figure 6. Stages 11 to 16 of Arabidopsis flower development.

(A) to **(C)** Stage 11 of flower development where the gynoecium develops stigmatic papillae (arrows) **(A)** and **(B)**. Longitudinal section where sepals (se), stamen (st), and gynoecium (g) are indicated **(C)**.
(D) to **(F)** Flower primordium at stage 12. Longitudinal **(E)** and transverse **(F)** sections showing all the organs as well as ovules and pollen grains.
(G) and **(H)** Flower anthesis at early stage 13 when the stigma (arrowhead) is already receptive **(G)**; a close-up view of the stigma **(H)**.
(I) to **(L)** Flower primordium at stages 14 **(I)** and 15 where the gynoecium has begun to enlarge to form the silique **(J)**. Close-up of a stage-15 stigma **(K)** and stage-16 flowers where sepals and petals are beginning to wither **(L)**.
Bars = 100 µm. All images except **(C)**, **(E)** and **(F)** are SEM. Images are of Columbia-0 ecotype, except **(A)** that is of Landsberg *erecta* (from Smyth et al.,1990, provided by Dr. J. Bowman).

regulated by *WUSCHEL* (*WUS*; Laux et al., 1996; Sablowski, 2007). The homeodomain-containing WUS transcription factor has the role of the maintaining the identity of stem cells in the organizing center of the CZ; *wus* mutants lack stem cells in the SAM (Mayer et al., 1998). *WUS* expression is limited to the cells immediately below the stem cells, an expression domain regulated by the receptor-kinase signaling system that includes the *CLAVATA1*, *2* and *3* (*CLV1, 2, 3*) gene products (Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000). *CLV1* is expressed in most L3 stem cells while *CLV3* is expressed in all three stem cell layers but mostly in L1 and L2 stem cells (see Figure 2B; Clark et al., 1997; Fletcher et al., 1999). In *clv* mutants, there is an imbalance between cells retained within meristems versus those recruited to form lateral organs. *clv* mutations cause an expansion of the *WUS* expression domain resulting in an enlarged stem cell niche. *CLV3* expression is, in turn, positively regulated by *WUS*, suggesting that meristem size depends greatly on a *WUS-CLV* regulatory loop (Clark et al., 1993, 1995; Kayes and Clark, 1998; Brand et al., 2000). Overexpression of *CLV3* represses *WUS* expression and decreases meristem activity, suggesting that CLV3, a secreted CLE-domain peptide, is the signal that regulates *WUS* expression via the CLV1/CLV2 LRR protein-kinase transduction complex (Fletcher et al., 1999; Jeong et al., 1999; Trotochaud et al., 1999; Clark, 2001a

and 2001b; Ni and Clark, 2006). It has been shown that other LRR-protein kinases closely related to CLV1 like BARELY ANY MERISTEM1 and 2 (BAM1, 2) are also involved in meristem maintenance possibly by sequestering CLV3 on the flanks of the meristem where they are expressed (DeYoung et al., 2006; DeYoung and Clark, 2008).

SHOOT MERISTEMLESS (*STM*) is a *KNOTTED1-like homeobox* (*KNOX*) gene that encodes a protein expressed in the SAM's CZ, RZ and regions of the PZ that have not been assigned to a primordium, i.e. it is expressed throughout the meristem except for anlagen, the sites of primordium formation (Figure 2B). STM promotes the profileration of stem cell derivatives until a critical cellular mass is attained sufficient to form either leaves or floral primordia. It also inhibits the expression of *ASYMMETRIC LEAVES1* and *2* (*AS1, 2*) genes in the SAM, preventing these cells from undergoing premature differentiation (Byrne et al., 2000; Byrne et al., 2002). Thus, the *STM* gene is considered to play a pivotal role in meristem maintenance (Long et al., 1996; Carles et al., 2004). *ULTRAPETALA1* (*ULT1*) encodes a cysteine-rich protein with a B-box like domain that restricts the size of shoot and floral meristems. It functions antagonistically to the proliferative roles of WUS and STM during most of the Arabidopsis life cycle but it in an independent genetic pathway (Carles et al., 2004).

3.2 Floral Meristem Specification and Determination

The changes in cellular characteristics, growth and geometry observed in the transition of the SAM to an IM (Kwiatkowska, 2006) are correlated with dynamic changes in the spatial and temporal expression of certain genes. The Arabidopsis IM produces rudimentary bracts in whose axils flower meristems emerge. *STM* and *AINTEGUMENTA* (*ANT)* expression patterns correlate with the development of this rudimentary bract primordium (Long and Barton, 2000).

The expression of *LEAFY* (*LFY*), a transcription factor found only in plants (Schultz and Haughn, 1991; Weigel et al., 1992; Maizel et al., 2005; Weigel, 2005) and *ANT* has been used in order to trace the cells that form the flower primordium (Grandjean et al., 2004). First, tens of cells are rapidly recruited to those already committed to become part of the flower meristem. This stage may correspond to the upward bulging at the shallow crease formed between the rudimentary bract and the IM described by Kwiatkowska (2006). These cells which express *LFY* then continue to proliferate. Interpretating this, the first cells that express *LFY* would correspond to the rudimentary bract (but not its axil or shallow crease), and later the domain of *LFY* expression would expand to include the cells committed to the flower primordium proper (Kwiatkowska, 2006). This interpretation can explain the discrepancy in the number of founder cells estimated using sector boundary analysis (Bossinger and Smyth, 1996) and using *in vivo LFY* expression patterns (Grandjean et al., 2004). Bossinger and Smyth (1996) concluded that a FM derives from four founder cells directly on the surface of the IM (or SAM). In support of this, evidence from confocal laser scanning microscopy indicates that flower primordia are formed from two rows of cells in a radial arc (Reddy et al., 2004). In contrast, the number of cells expressing *LFY* at these early stages (Reddy *et al.,* 2004) suggest that

Figure 7. Stages 17 to 20 of Arabidopsis flower development.

(A) to **(D)** Photographs of developing and mature siliques at stages 17 **(A)**, 18 **(B)**, 19 **(C)**, and 20 **(D)** of flower development.
(E) SEM of seeds from a silique at stage 17.
(F) Close-up view of a seed from a stage-20 dehiscent silique.
All photographs are of Columbia-0 ecotype.

a flower meristem has more founder cells. An explanation that resolves the discrepancy is that the *LFY*-expressing cells could include those that eventually form the rudimentary bract, as well as those which form the flower primordium (Kwiatkowska, 2006; reviewed in Kwiatkowska, 2008)

The gene *CUP-SHAPED COTYLEDON2* (*CUC2*) is expressed in the slow-dividing cells that expand in a latitudinal direction (Reddy et al., 2004) to define the second boundary between the floral primordium proper and the IM (Breuil-Broyer et al., 2004). Several regulators of *CUC* including a miRNA have been described as important components of the GRN involved in this developmental process (Laufs et al., 2004; Aida and Tasaka, 2006a).

Flower versus inflorescence meristem identity is controlled by a complex GRN that integrates environmental and internal

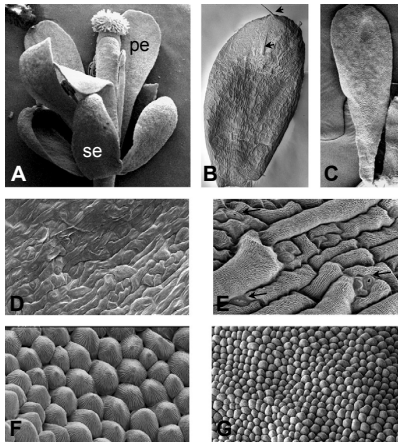


Figure 8. Sepal and petal cell types.

Scanning electron micrographs (SEM) of wild-type flowers and flower organs.
(A) A mature flower with sepals (se) and petals (pe) fully expanded and the stigma extending above the long stamens.
(B) Sepal blade showing simple unbranched trichomes (arrowheads) characteristic of the abaxial surface.
(C) Mature petal blade consisting of a basal claw and a distal blade.
(D) Adaxial sepal surface with irregular sizes and shapes of cells, some elongated (800x).
(E) Abaxial sepal surface bearing stomata (arrows) and characteristic elongated cells (500x).
(F) Adaxial surface of a mature petal blade showing conical cells with epicuticular thickenings running from the base to the apex (800x).
(G) Abaxial petal surface showing flatter, cobblestone-shaped cells with cuticular thickenings. Both petal surfaces lack stomata.

cues (Figure 9). On induction of flowering, the IM genes, such as *TERMINAL FLOWER 1* (*TFL1*; Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991 and 1993, Ohshima et al., 1997) and *EMBRYONIC FLOWER 1 and 2* (*EMF1, 2*; Chen et al., 1997; Aubert et al., 2001), are repressed in the FM, while the floral meristem identity (FMI) genes, mainly *LFY*, *APETALA1* (*AP1*), *APETALA2* (*AP2*), and *CAULIFLOWER* (*CAL*), are upregulated (Figure 10; Blazquez et al., 1997; for review Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Kempin et al., 1995; Mandel and Yanofsky, 1995a; Blazquez et al., 2006).

Mutual repression of the IM and FMI genes seem to underlie the co-existence, identity and boundaries of both types of meristem in the SAM in the transition to flowering (Chen et al., 1997; Liljegren et al., 1999; Ratcliffe et al., 1999). For example, if genes such as *TFL1* or *EMF1* or *2* are mutated, *LFY* and/or *AP1* are ectopically expressed in the IM that is then transformed into a FM (Shannon and Meeks-Wagner, 1991; 1993; Weigel et al., 1992, Bowman et al., 1993; Gustafson-Brown et al., 1994; Bradley et al., 1997; Chen et al., 1997; Moon et al., 2003). On the contrary, if *AP1, CAL* and *LFY* are repressed, the FM attains IM identity (Ratcliffe et al., 1998; Ratcliffe et al., 1999). *TFL1* is an important regulator of inflorescence development (Alvarez et al., 1992; Ratcliffe et al., 1998; Parcy et al., 2002). It encodes a phosphatidyl ethanolamine-binding protein (PEBP) that is transcribed in the center of the IM but the protein moves to other cells where *AP1* and *LFY* are downregulated (Bradley et al., 1997; Conti and Bradley, 2007). *EMF* genes are required for vegetative growth, but they seem to regulate flowering time and inflorescence development too (Sung et al., 1992; Aubert et al., 2001; Yoshida et al., 2001). Loss-of-function mutants in these genes produce flowers immediately after germination skipping the vegetative phase (Yang et al., 1995; Chen et al., 1997). *EMF1* encodes a transcription factor that represses *AP1* but not *LFY*, and *EMF2* encodes a novel zinc finger protein related to the polycomb group (Aubert et al., 2001; Yoshida et al., 2001).

LFY is necessary and sufficient to specify FMI (Weigel et al., 1992; Weigel and Nilsson, 1995). In *lfy* mutants, leaves and secondary shoots are produced instead of flowers (Schultz and Haughn, 1991; Weigel et al., 1992) and *LFY* overexpression causes the conversion of leaves and axillary meristems to flowers (Weigel and Nilsson, 1995). *LFY* is expressed in the leaf primordia during vegetative growth, but when induced by external (vernalization and light) and/or internal (gibberellins) signals, it is strongly expressed and relocates to the SAM flanks where floral meristems are formed (Figure 9; Blazquez et al., 1997; Hempel et al., 1997; for *LFY* regulation see: Nilsson et al., 1998; Blazquez and Weigel, 2000; Liu et al., 2008). *LFY* expression persists at high levels in the FM until stage 3 of development and then diminishes in the center of the flower (Figure 10; Blazquez et al., 1997; Wagner et al., 2004). LFY protein abundance, however, is homogenous in the FM, probably because it moves between cells (Parcy et al., 1998; Sessions et al., 2000; Wu et al., 2003).

LFY and *AP1* have overlapping functions in establishing the FM; while the *ap1* mutant has shoots with inflorescence characteristics, the *lfy ap1* double mutant has an almost complete conversion of flowers into shoots (Huala and Sussex, 1992; Bowman et al., 1993). Both genes when overexpressed cause a terminal flower phenotype suggesting that each one is sufficient to determine the IM (Mandel and Yanofsky, 1995a; Weigel and Nilsson, 1995). *CAL*, the closest paralogue of *AP1*, and *FRUITFULL* (*FUL*) from the same gene clade within the MADS-box phylogenetic tree (Alvarez-Buylla et al., 2000; Martínez-Castilla and Alvarez-Buylla, 2003; Parenicová et al., 2003), may also act redundantly to *AP1* in FM specification. Single *cal* and *ful* mutants do not show any FMI disorders, but in combination with *ap1* in double or triple mutants, the *ap1* phenotype is greatly intensified (Bowman et al., 1993; Kempin et al., 1995; Ferrándiz et al., 2000a). *FUL* is expressed at the same time as *LFY* during the establishment of the FMI (Mandel and Yanofsky, 1995b; Hempel et al., 1997), but is mostly localized in the IM (Figure 10). Later during flower development, *FUL* is expressed again during carpel and silique development where it plays an important role (Gu et al., 1998). Despite its close similarity to *AP1*, overexpression of *CAL* is not able to determine the IM as does overexpression of *AP1*, indicating that CAL does not interact with the same partners as AP1. The unique functions

of AP1 rely on residues within the K and COOH domains that are not found in CAL (Alvarez-Buylla et al., 2006).

LFY directly regulates *AP1* and *CAL* transcription by binding to the consensus sequence CCANTG (CArG-box; Parcy et al., 1998; Wagner et al., 1999; Wagner et al., 2004; William et al., 2004). However, expression reminiscent of *AP1* is seen in the *lfy* mutant, while it is completely abolished in the double mutant *lfy ft* (*flowering locus t*; Ruiz-Garcia et al., 1997; Schmid et al., 2003; Wigge et al., 2005). Thus FT, a homolog of TFL1 (Koornneef et al., 1991; Kardailsky et al., 1999), together with FD, a bZip transcription factor (Abe et al., 2005), redundantly regulate *AP1* with LFY. AP1 and CAL in turn regulate *LFY* by positive feedback, allowing it to exert its transcriptional regulation during flower development (Bowman et al., 1993; Liljegren et al., 1999). Recently, additional LFY targets have been found (William et al., 2004), among them *LATE MERISTEM IDENTITY1* (*LMI1*), which encodes a homeodomain leucine-zipper transcription factor and functions as a FMI gene. Interestingly, LMI1 acts together with LFY to activate *CAL* expression (Figure 9; Saddic et al., 2006).

AP2 encodes a putative transcription factor of a plant-specific gene family (*AP2/EREBP*) with diverse functions (Riechmann and Meyerowitz, 1998). Mutations in *AP2* enhance both *ap1* and *lfy* mutant phenotypes, indicating that *AP2* also plays a role in specifying FMI (Huala and Sussex, 1992; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993; Simpson et al., 1999).

MADS-box genes are key components of the regulatory module that integrates flowering transition signaling pathways (for review see Jack, 2004; Parcy, 2005, Blazquez et al., 2006), IM and FM identities (Mandel et al., 1992; Bowman et al., 1993; Mandel and Yanofsky, 1995a, 1995b), and floral organ specification (see section 3.3; Coen and Meyerowitz, 1991). To specify the FM, LFY and/or AP1 are also required to downregulate flowering induction genes such as *AGAMOUS-LIKE 24* (*AGL24*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *SHORT VEGETATIVE PHASE* (*SVP*), and *FUL* (Figures 9 and 10). Overexpression of any of these genes causes FM to revert to IM-like structures as when LFY and/or AP1 are mutated (Mandel and Yanofsky 1995b; Yu et al., 2004a; Liu et al., 2007).

Floral reversion is often found in plants heterozygous for *lfy-6* (*LFY/lfy*) and homozygous for *agamous-1* (*ag-1*), suggesting a key role for *LFY* and *AG* in the maintenance of determinate floral meristems (Okamuro et al., 1996). The reason for this is that late in floral organogenesis *AG*, induced by *WUS*, *LFY* and *PERIANTHIA* (*PAN*) among others, positively regulates *KNUCKLES* (*KNU*) which in turn represses *WUS* expression to terminate the stem cell niche after a limited number of organs have been formed (Parcy et al., 1998; Busch et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001; Das et al., 2009; Maier et al., 2009; Sun et al., 2009). In fact, while *WUS* expression declines after stage 6 in wild-type flowers, it persists in *pan* or *ag* flowers (Lenhard et al., 2001; Lohmann et al., 2001; Das et al., 2009; Maier et al., 2009). *ULT* also participates in meristem determinancy together with *AG* downregulating *WUS* (Carles et al., 2004).

Although it is very rare to observe spontaneous or induced reversion from FM to IM, a set of genes that actively maintain FM identity could conform to a "flower developmental module" that prevents reversion. The genetic mechanisms involved in maintaining FMI are closely linked to hormone balance and environmental factors (Tooke et al., 2005). For example, we now know that *STM* is a positive regulator of local cytokinin (CK) biosynthesis and accumulation (Jasinski et al., 2005; Yanai et al., 2005), and a repressor of gibberellin (GA) production (Jasinski et al., 2005). On the other hand, *WUS* enhances CK activity by repressing ARABIDOPSIS *TYPE A RESPONSE REGULATORS* (*ARRs*) (Leibfried et al., 2005). The resulting high CK:auxin ratio and low GA levels promote indeterminate growth (Shani et al., 2006). While a high auxin concentration restricts *STM* and *CUC* expression (see section 3.4.2), it also downregulates CK biosynthesis and activity, thus yielding a high auxin:CK ratio and high levels of GA, which induce floral meristem formation. Raising GA levels or response, for example by crossing with the *spindly* (*spy*) mutant, is sufficient to suppress FM reversion to IM in *lfy*, *ap1*, *ap2* and *ag* mutants. This demonstrates the importance of GA in the maintenance of FM identity (Okamuro et al., 1996; Okamuro et al., 1997).

Light signal transduction pathways are also involved in FM maintenance. Spontaneous floral reversion in wild-type Arabidopsis has only been observed at low frequencies in the first flowers of Landsberg *erecta* grown in short days. However, *long hypocotyl* (*hy1-1*), a mutant in which phytochrome activity is blocked, suppresses floral reversion of both *lfy and ag* single mutants in short days (Okamuro et al., 1996). Floral reversion seems to be a developmental abnormality with no apparent adaptative significance, unless plant resources are somehow saved under certain conditions if flowering is reversed. Further ecological and evolutionary developmental studies of Arabidopsis ecotypes will continue to elucidate the genetic, epigenetic, physiological, and environmental mechanisms involved in the maintenance of the FMI.

3.3 Specification of Floral Organs: The ABC Genes

Very soon after FM specification (11-13 days after germination in Landsberg *erecta* ecotype), the flower meristem is subdivided into four regions. Each one will give rise to the primordia of the different floral whorls, which from the outside to the inside are: sepals, petals, stamens, and carpels. The genes responsible for floral whorl specification attain their spatio-temporal pattern as a result of regulatory interactions among themselves, interactions with meristem identity genes and with some other genes, such as *WUS* and *UNUSUAL FLORAL ORGANS* (*UFO*; Levin and Meyerowitz, 1995). The complexity of the interactions involved is shown in the 'floral organ specification gene regulatory network' (FOS-GRN) model, analyzed in Section 4.1. This model includes a set of interacting genes sufficient to pattern the IM and FM during the first stages of flower development.

One of the key FM identity genes is *LFY*. The protein encoded by this gene requires co-factors to set the spatial limits of expression of the floral organ identity genes *AP3*, *PI*, and *AG*. For example, LFY participates with UFO in the regulation of *AP1* and *AP3* transcription (Lee et al., 1997; Chae et al., 2008), and with WUS co-regulates the expression of *AG* (Lenhard et al., 2001; Lohmann et al., 2001). LFY also regulates the expression of the *SEPALLATA* (*SEP*) genes *SEP1*, *SEP2* and *SEP3*, additional MADS-box genes required for organ identity specification (Krizek and Fletcher, 2005).

UFO is expressed in the second and third whorls during floral stage 3, probably restricting the B-gene expression domain to these whorls, together with LFY (Lee et al., 1997; Traas and

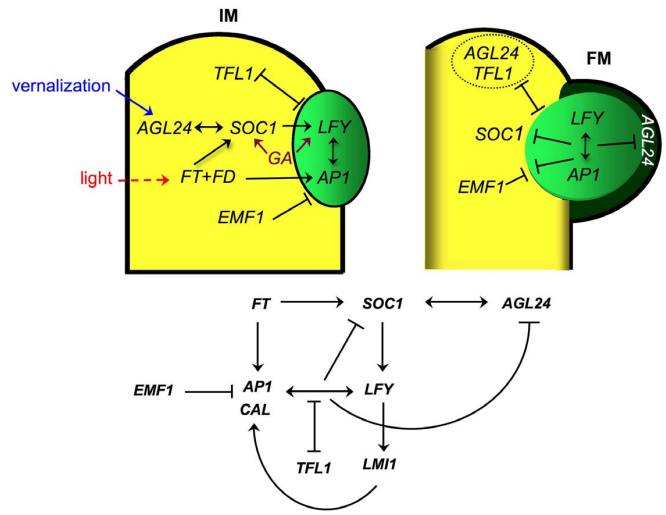


Figure 9. Inflorescence shoot apical meristem (IM) versus flower meristem (FM).

Simplified model of a gene regulatory network (GRN) that induces and maintains the FM. Flowering induction genes like *FT, SOC1* and *AGL24* are highly expressed in the IM in response to external (vernalization and light) and internal (gibberellins; GA) signals. These proteins in turn promote the expression of flower meristem identity (FMI) genes, *LFY* and *AP1*. Paradoxically, during the establishment of the FM, genes like *TFL1* and *EMF1* that help to maintain the IM identity are also expressed, keeping the expression of the FMI genes out of the IM. Later in development, LFY and AP1 repress the expression of *TFL1* and flowering genes *SOC1* and *AGL24*, among others, thus maintaining the FMI. Arrows and bars indicate positive and negative regulatory interactions respectively. (See references in main text).

Doonan, 2003). The *UFO* gene encodes a protein containing an F-box domain, which is a characteristic of E3 ubiquitin ligases that are components of SCF (Skp Cullin F-box containing) complexes and mark proteins for proteosome-dependent degradation (Deshaies, 1999). It was recently shown that LFY interacts with UFO in order to directly bind the *AP3* promoter. Furthermore, the proteosome activity mediated by UFO is required for the transcriptional activation of *AP3* by LFY (Chae et al., 2008).

Key components of the GRN that underlies the early patterning of the flower meristem are the so-called ABC homeotic genes, *AP1*, *AP2*, *AP3*, *PI*, and *AG*, which are all transcription factors belonging to the MADS-box gene family, except *AP2* (Coen and Meyerowitz, 1991; Wagner et al., 1999; Ng and Yanofsky, 2001; Lamb et al., 2002).

The classic ABC model was inferred using Arabidopsis and *Antirrhinum* homeotic flower mutants (Coen and Meyerowitz, 1991). In these mutants two floral organ types are replaced by two other floral organ types as follows: A- class mutant flowers have carpels-stamens-stamens-carpels (from the outermost to the innermost whorl), B-class mutant flowers have sepals-sepals-carpels-carpels, and C-class mutant flowers have sepals-petals-petals-sepals (Coen and Meyerowitz, 1991). It was shown that mutations in all three functions lead to the transformation of all floral organs into leaf-like organs, suggesting that flowers are modified leaves (reviewed in Robles and Pelaz, 2005). The Arabidopsis ABC mutants are shown in Figure 11.

Hence, three different classes of homeotic genes with overlapping activities were proposed to be necessary for floral organ specification. The A function specifies sepals, the A and B functions specify petals, the B and C functions specify stamens and the C function specifies carpels (Figure 12; Bowman et al., 1991). The A and C functions negatively regulate each other and the B function is restricted to the second and third whorls. The latter was originally thought to be independent of A and C functions (Bowman et al., 1991; Drews et al., 1991), but it was later shown that the A function gene *AP1* regulates the B genes. AP1 binds to the promoter of *AP3* (Hill et al., 1998; Tilly et al., 1998). AP1 can also specify petals by regulating the spatial domain of B genes together with UFO in the first flowers to arise, and independently of UFO in later flowers (Ng and Yanoksky, 2001).

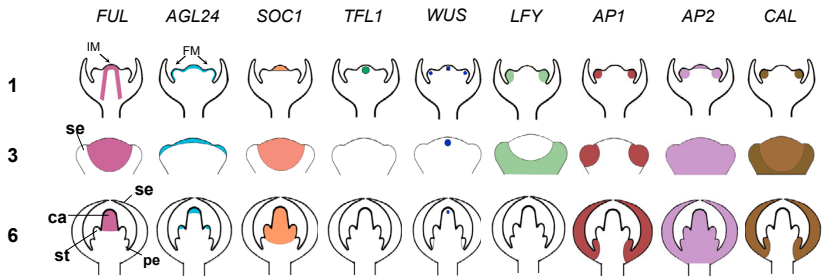


Figure 10. Schematic representation of some inflorescence shoot apical (IM) and flower (FM) meristem gene expression patterns at stages 1, 3 and 6.

Flowering (*FUL*, *AGL24* and *SOC*), indeterminate (*WUS* and *TFL1*), and FMI (*LFY, AP1*, *AP2* and *CAL*) gene expression patterns based on in situ hybridization data during floral primordium developmental stages 1, 3 and 6. At stage 1, expression patterns correspond to their functions in IM and FM identities. Sepal (se), petal (pe), stamen (st) and carpel (ca) primordia are indicated. At stages 3 to 6, all with the exception of *TFL1* are expressed in the FM, probably because their respective proteins also affect organ development. FUL will participate in fruit development, LFY will induce all the ABC genes and AP1 and AP2 are fundamental in sepal and petal formation (see references in main text).

Once identified at the molecular level, the mRNA expression patterns of the ABC genes were shown to overlap with the floral regions where the corresponding mutants had a phenotype (Yanofsky et al., 1990; Mandel et al., 1992; Goto and Meyerowitz, 1994; Jack et al., 1994). *AP1* and *AP2* are A-function genes. *AP1* is expressed in the two outer whorls of the floral meristem (Figures 10, 12, 13A; Mandel et al., 1992) and is important for the establishment of sepal and petal identity as well as the FM (section 3.2). *AP1* expression is first up-regulated by LFY and FT/FD (section 3.2), but later is maintained by the B class genes in a positive feedback loop (Sundström et al., 2006). Strong *ap1* alleles (*ap1-1*) often lack petals in the second whorl, while weaker mutant alleles of this gene do not have a full homeotic conversion of floral organs (see section 3.2; Irish and Sussex, 1990).

In contrast to the MADS-box ABCs, the expression pattern of *AP2* does not correlate with the site where it exerts its function in floral organ identity. *AP2* mRNA is found throughout the flower meristem (Figures 10 and 12; Jofuku et al., 1994). Recent data has shown that *AP2* is repressed at the translational level by a microRNA (miR172), which is active only in whorls 3 and 4 (Chen, 2004), thus explaining why the function of AP2 is restricted to the first two whorls of flower organs. In a recent experiment using double mutants of *ag* and an *ap2* allele, which is insensitive to repression by miR172, it was shown that both *AG* and miR172 independently downregulate *AP2,* but miR172 is more important than *AG* (Zhao et al., 2007). *ap2* mutants rarely develop petals and their sepals are transformed into carpelloid structures due to ectopic *AG* expression (Figure 11), which is negatively regulated by AP2 itself (Drews et al., 1991). AP2 is also implicated in the upregulation of the B genes, *AP3* and *PI* (Zhao *et al.*, 2007).

The B class genes (*AP3* and *PI*) are expressed in the second and third whorls and mutant flowers of any or both of these two genes lack petals and stamens, as predicted in the ABC model (Figure 11, 12 and 13; Coen and Meyerowitz 1991; Goto and Meyerowitz, 1994; Jack et al., 1994; Honma and Goto, 2000). The fact that both single mutants yield the same phenotype shows their interdependence. *AP3* and *PI* are regulated in two steps: they are first induced by LFY/UFO in response to flowering signals and they later maintain their expression in a self-regulatory loop (Honma and Goto, 2000). The proteins encoded by these two genes form heterodimers to exert their B function during petal and stamen development (Figure 14; Jack et al., 1992; Goto and Meyerowitz, 1994; Zik and Irish, 2003a) and this oligomerization is necessary for them to move into the nucleus (McGonigle et al., 1996).

Both genes are also regulated positively in a regulatory loop by *AP1* and negatively by *EARLY BOLTING IN SHORT DAYS* (*EBS*), a gene that encodes a nuclear protein that participates in petal and stamen development and regulates flowering time by repressing *FT* (Gómez-Mena et al., 2001; Piñeiro et al., 2003*). ANT*, a member of the *AP2* gene family, is another regulator of the B function, positively inducing *AP3* (Klucher et al., 1996; Nole-Wilson and Krizek, 2006; see section 3.4.2).

The only C-type gene discovered up to now is the MADS-box gene *AG* (Bowman et al., 1989). *ag* mutant flowers lack stamens and carpels, and also bear indeterminate flowers with reiterating sepals and petals (Figure 11), suggesting that *AG* is important for floral meristem determinancy (see section 3.2), besides its role in stamen and carpel identity (Yanofsky et al., 1990; Mizukami and Ma, 1997). The regulation of *AG* has been much studied; at least ten proteins repress and five activate it to maintain its expression in the appropriate whorl (Figures 12 and 13A).

AG is repressed by a transcriptional co-repressor complex formed by *LEUNIG* (*LUG*) and *SEUSS* (*SEU*) (Figure 15; Franks et al., 2002). *LUG* encodes a transcription protein similar to *TUP1* from yeast and interacts with *SEU,* which encodes a plant specific protein (see Table S1; Conner and Liu, 2000; Franks et al., 2002; Sridhar et al., 2004). Neither of these proteins are able to bind DNA sequences and AP1 and SEP3 recruit SEU/LUG to the second intron of *AG* to perform their inhibitory function and prevent the ectopic expression of *AG* (Sridhar et al., 2006). Recently, another transcriptional repressor of *AG* was identified, *LEUNIG_HOMOLOG* (*LUH*). This gene is the closest homolog of *LUG* and its inhibitory function on *AG* is completely dependent on SEU (Sitaraman et al., 2008).

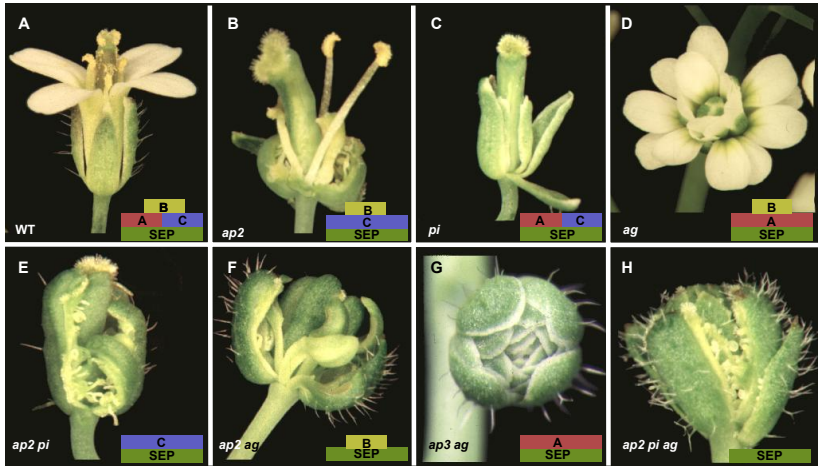


Figure 11. Arabidopsis ABC homeotic floral mutants.

Photos of single, double and triple ABC gene mutant flowers. Each photo is accompanied by a small diagram where rectangles represent the A (*AP1* and *AP2*), B (*AP3* and *PI*), and C (*AG*) combinatorial transcriptional regulatory functions and the *SEP* (*1, 2, 3, 4*) genes active in these mutants. Organs are listed below from the outer to the inner whorl unless stated otherwise.
(A) Wild-type (WT) flower.
(B) Single *ap2* mutant flower composed of carpelloid sepals, stamens, stamens and carpels.
(C) The *pi* mutant has flowers composed of sepals, sepals, carpels and carpels.
(D) The *ag* flower has the stamens transformed into petals and the carpels are replaced by another flower repeating the same pattern.
(E) The *ap2 pi* double mutant displays flowers composed only of sepalloid carpels.
(F) The *ap2 ag* flowers have leaf-like organs in the first and fourth whorls and mosaic petal/stamen organs in the second and third whorls.
(G) *ap3 ag* double mutants produce flowers composed of repeated whorls of sepals.
(H) The *ap2 pi ag* mutant has leaf-like organs with some residual carpel properties. (Photographs provided by Dr. J. Bowman).

Another repressor of *AG* is BELLRINGER (BLR), a homeodomain protein that binds to regions in the second intron of *AG* and prevents ectopic *AG* expression in the two outer whorls of the flower (see Table S1; Bao et al., 2004). *AG* is also negatively regulated epigenetically by a histone acetyltransferase *GCN5* (Bertrand et al., 2003). Other genes that participate in floral organogenesis are repressors of *AG*, namely *RABBIT EARS* (*RBE*, see section 3.4.4), *ANT* and *STERILE APETALA* (*SAP*) (see Table S1). *AG* is also positively regulated at the post-transcriptional level by several *ENHANCER OF AG-4* (*HUA*) and *HUA ENHANCER* (*HEN*) genes. All of these genes play a major role in pre-mRNA processing of *AG* (Cheng et al., 2003).

The ABC proteins exert their regulatory function as multimers. In *Antirrhinum majus*, a ternary complex between A and B function proteins was found to bind CArG DNA boxes more efficiently than single proteins (Egea-Cortines et al., 1999). More specifically, a higher-order complex consisting of SQUAMOSA *(SQUA*, the *AP1* ortholog*)*, DEFICIENS, and GLOBOSA *(DEF* and *GLO* are *A. majus AP3* and *PI* orthologues, respectively) bound DNA more efficiently than DEF/GLO or SQUA alone (Egea-Cortines et al., 1999). These results suggest that transcriptional complexes that combine A and B function proteins are more stable than those formed with proteins of any one function alone.

The fact that the ABC genes are necessary but not sufficient to determine floral organ identity was later confirmed in Arabidopsis. Honma and Goto (2001) used a yeast three-hybrid method to

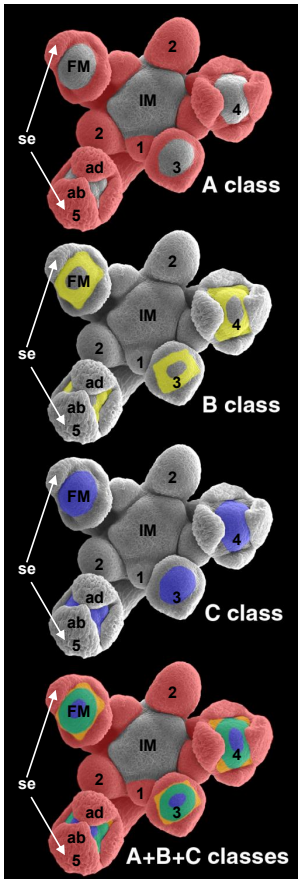


Figure 12. Expression patterns of the ABC genes during early stages of *Arabidopsis* flower development.

SEM of meristems have been colored to show expression patterns of **A class** (red, outer whorls), **B class** (yellow, petal and stamen primordia) and **C class** (blue, inner whorls) genes. Five flowers at early stages of development are marked 1 to 5 (5 being the oldest). Inflorescence shoot apical meristem (IM), floral meristem (FM) and sepals (se): adaxial (ad) and abaxial (ab) are indicated. (Photographs provided by Dr. J. Bowman).

show that SEP3 and AP1 are able to interact with the heterodimer AP3/PI but not with AP3 or PI alone. Moreover, they described this interaction as essential, since the heterodimer AP3/PI lacks the activation domain necessary for a transcription factor to function, a domain which both SEP3 and AP1 possess (Honma and Goto, 2001). These findings suggest that the inclusion of SEP3 or AP1 together with AP3/PI could result in an active tetrameric transcriptional complex (Figure 14). It was also demonstrated that the ABC proteins on their own or combined according to the ABC model (A, AB, BC, or C) were not sufficient to determine floral organs when expressed in leaves under the action of the 35S constitutive promoter (Pelaz et al., 2001). However, floral organs could indeed be recovered in leaves once appropriate combinations of genes were expressed (Honma and Goto, 2001; Pelaz et al., 2001).

The *SEP* genes received their names because the floral organs that develop in all four whorls in triple *sep* mutants resemble sepals (Pelaz et al., 2000). This *sep1 sep2 sep3* triple mutant phenotype is markedly similar to that of double mutants that lack both B and C class activity, such as *pi ag* and *ap3 ag* (Figure 11G; Bowman et al., 1989; Pelaz et al., 2000) in which the floral meristem becomes indeterminate as well. Single or double mutants for these *SEP* genes yield flowers indistinguishable from wild type, thus suggesting that the three *SEP* genes are functionally redundant and are important in determining three of the four floral organs: petals, stamens, and carpels (Honma and Goto, 2001; Pelaz et al., 2001; Robles and Pelaz, 2005).

Given that the triple *sep1 sep2 sep3* mutant does not show alterations in sepal identity, an additional gene is likely to be involved in sepal specification. Indeed, another *SEP*-like MADS-box gene, *SEP4* (previously *AGL3*), has now been characterized (Ditta et al., 2004), and the quadruple *sep1 sep2 sep3 sep4* mutants produce flowers with leaf-like organs in all whorls, thus confirming the *SEP* genes contribute to each floral organ identity (Figure 14). Coincidently, *SEP* genes are expressed in the whole floral meristem during flower development (Figure 13B; Flanagan and Ma, 1994), are important in regulating B and C gene expression (Liu et al., 2009), and encode proteins that apparently interact with the ABC proteins (Figure 14; Robles and Pelaz, 2005).

Based on data from *Antirrhinum* and yeast two-hybrid and three-hybrid protein interactions, and on the phenotypes of the ABC mutants, three models have been proposed to explain how the MADS domain proteins interact to constitute functional transcriptional complexes and bind DNA. None of the models completely explains the experimental data available, but the quartet model seems the most plausible (Jack, 2001; de Folter et al., 2005). This model proposes that MADS domain proteins form tetrameric complexes during floral organ determination (Figure 14; Theissen, 2001; Theissen and Saedler, 2001; Becker and Theissen, 2003; Jack, 2004). Within each transcriptional complex, there would be two MADS dimers, each one binding to a single CArG-binding site causing the DNA of the promoter region to bend, enabling the MADS dimers to act cooperatively in a tetrameric complex to regulate the gene. For example, binding of one dimer within the tetramer to DNA could increase the affinity of the second dimer for local DNA binding (Melzer et al., 2009). Besides, one of the dimers could function as the activation domain of the tetramer allowing for efficient transcriptional activation (Honma and Goto, 2001). Interestingly, several dimers

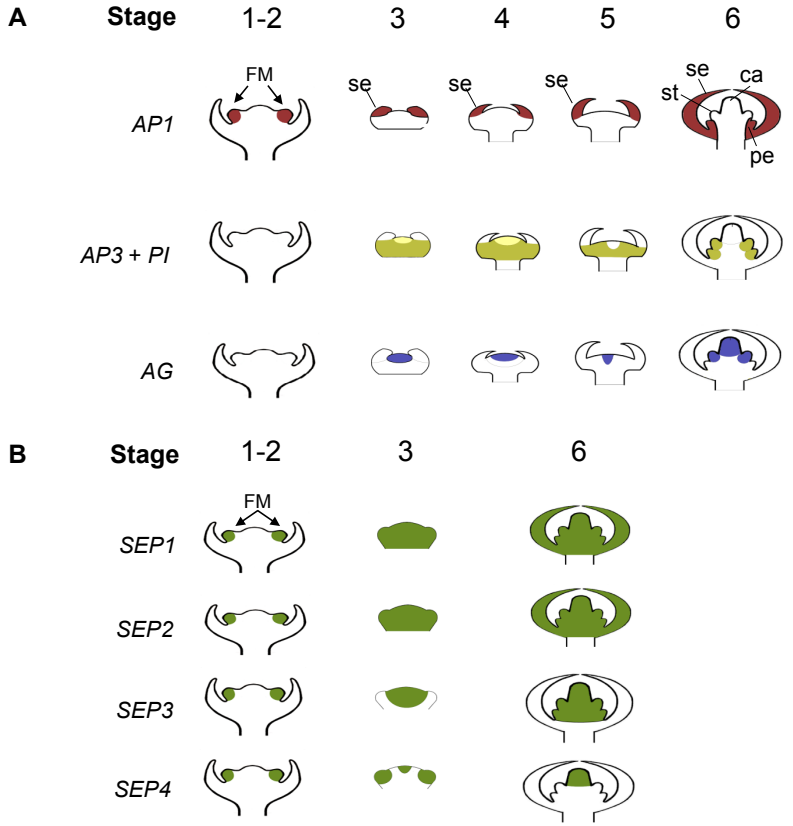


Figure 13. Diagram illustrating mRNA expression patterns of Arabidopsis ABC and SEP genes during different stages of flower development.

(A) ABC gene expression patterns illustrated from stage 1 to 6. The A function gene *AP1* is expressed (red) in the two outer floral primordia whorls that will later develop into sepals (se) and petals (pe) (Mandel et al., 1992; Gustafson-Brown et al, 1994; Parcy et al., 1998). The A function gene *AP2* is expressed in all four whorls of the flower (see figure 10; Jofuku et al., 1994). The B function genes (dark yellow) *AP3* and PI are expressed from stage 3 in the next two inner whorls of the flower (Weigel and Meyerowitz, 1993; Parcy et al., 1998). Interestingly *PI* is also expressed at stages 3 and 4 in cells that will generate the fourth whorl (light yellow). After stage 5, the pattern of PI expression largely coincides with that of *AP3*, only in petal and stamen (st) primordia (Goto and Meyerowitz, 1994). The C function gene AG is expressed (blue) in the two inner whorls that will become the stamens and carpels (ca) (Yanofsky et al., 1990; Gustafson-Brown et al., 1994; Parcy et al., 1998; Ito et al., 2004).

(B) SEP gene expression pattern during several stages (1 or 2, 3 and 6) of flower development. *SEP1* and *SEP2* are expressed in all whorls of the flower (Savidge et al., 1995). *SEP3* is first detected in late stage 2 flower primordia and afterwards in petal (pe), stamen (st), and carpel (ca) primordia. The expression pattern at stage 6 was deduced that from at stage 7 (Mandel and Yanofsky, 1998). *SEP4* is weakly expressed in sepal primordia at stage 3 and strongly expressed in carpel primordia from stage 3 to 6. (Ditta et al., 2004). Both figures have been modified and expanded from Krizek and Fletcher (2005).

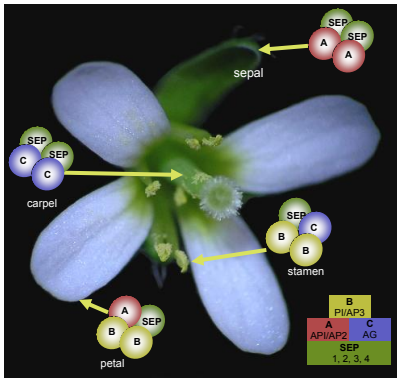


Figure 14. Schematic representation of the interaction of ABC and SEP proteins in the quartet model for *Arabidopsis* floral organ specification.

Possible MADS-domain protein complexes (circles) of the ABC model are sufficient for the specification of each of the four floral organs. In the ABC model, rectangles represent the A (*AP1* and *AP2*), B (*AP3* and *PI)*, and C (*AG*) combinatorial transcriptional regulatory functions necessary for sepal, petal, stamen and carpel primordia specification. The green rectangle below represents the SEP (1, 2, 3 and 4) proteins that interact with proteins encoded by the ABC genes (unknown for AP2 which has not been tested) to specify each floral organ (modified from Bowman et al., 1993; Robles and Pelaz, 2005).

and potential tetramers have been documented in a complete Arabidopsis MADS-domain family protein-protein interactome via yeast two-hybrid interactions (de Folter et al., 2005). This data base has been updated with a yeast three-hybrid screen for MADS-domain proteins (Immink et al., 2009). Future studies should test which of the complexes inferred from the MADS interactomes are functional and what their roles are during Arabidopsis development.

3.3.1 Target genes of the ABCs

Target genes of the ABC genes link the floral organ specification - gene regulatory network (FOS-GRN) with processes in organ primordia establishment and development (for review of MADS target genes, see de Folter and Angenent, 2006). Among the direct targets of the ABC genes, transcriptional regulators and hormone-related genes are prominent (Sablowski, 2009). But the sets of target genes change as organ development progresses; at later stages of floral organ development, several components of what could be common modules have been found that are involved in generic developmental processes (see below) during sepal, petal, stamen and carpel development. Finally, multiple genes having cell-specific roles are turned on especially during stamen and carpel development that is much more complex than perianth development (Sablowski, 2009).

The first examples of genes regulated by the ABC genes were two MADS-box genes, *AGL1* and *AGL5* (renamed the *SHATTERPROOF* genes (*SPH1* and *2*, respectively). There is virtually no expression of either gene in *ag* mutants (Savidge et al., 1995). *SHP2* is only expressed in carpels and *AG* can activate an *SPH2:GUS* reporter construct; furthermore, *AG* binds its promoter in vitro (Savidge et al., 1995).

The first non-ABC gene identified as a target of a MADS A, B or C protein was *NAC-LIKE ACTIVATED BY AP3/PI* (*NAP*), a target of the AP3/PI complex. It is important for the transition between cell division and cell expansion during petal and stamen development (see section 3.4.5 and Table S1; Sablowski and Meyerowitz, 1998).

A recent study identified two genes negatively regulated by AP3/PI, *GATA NITRATE INDUCIBLE, CARBON-METABOLISM-INVOLVED* (*GCN*) and *GCN-LIKE* (*GNL*), a GCN paralog (Mara and Irish, 2008). Both genes regulate chlorophyll biosynthesis in plant cells. Thus, their downregulation could be important in preventing chlorophyll accumulation in petals and anthers. The same study shows that both *GNC* and *GNL*, together with the B class genes, regulate the expression of a number of other GATA-motif-containing target genes like *HEXOKINASE1* (*HXK1*; Mara and Irish, 2008).

SUPERMAN (*SUP*; Bowman et al., 1992) is upregulated by AP3/PI and AG and by *LFY* (Riechmann et al., 1996; Sakai et al., 2000). *SUP* encodes a transcription factor with a C2H2-zinc finger motif and is involved in the maintenance of the stamen/carpel whorl boundary (Sakai et al., 2000; Dathan et al., 2002; see section 3.4.2). While the B genes and *LFY* seem to regulate early *SUP* expression, *AG* and the B genes are involved in maintaining its expression in flowers from stage 5 onward (Sakai et al., 2000).

Recent microarray experiments have proved useful in revealing new targets of the ABC MADS homeotic genes, as well as many putative components of the complex networks involved in floral organogenesis. For example, it was shown that the AP3/PI dimer regulates, directly or indirectly, 47 target genes. Only two of these are transcription factors, while most participate in basic cellular functions required for stamen and petal development (Zik and Irish, 2003a). By contrast, AG controls, directly or indirectly, the expression of 149 genes most of which are transcription factors, including other members of the MADS-box gene family. Ten of these were also shown to be direct targets of AG using chromatin immunoprecipitation (ChIP), including *AG* itself, *AP3*, *CRC* and *ATH1*, a gene that encodes a *BELL*-type homeodomain protein that participates in the development of the basal region of shoot organs (Gómez-Mena et al., 2005).

A more exhaustive experiment used four homeotic mutants (*ap1*/*ap2*, *ap3*, *pi* and *ag*) in two types of microarray assays: a whole genome microarray with approximately 26,090 gene-specific oligonucleotides and a flower specific-cDNA microarray with 5,000-6,000 genes. To summarize the assay results, transcription factors were neither over or underrepresented as being regulated by the ABC genes; on the contrary, genes involved in general cellular maintenance (DNA recombination and protein synthesis) were underrepresented. Genes specifically expressed in each of the four different whorls were identified: 13 genes for sepals, 18 for petals, 1162 for stamens, and 260 for carpels. As expected

from their structural and cellular complexity, the reproductive floral organs had many more specific target genes than the perianth organs (Wellmer et al., 2004; Sablowski, 2009).

In another genomic study of early floral stages it was found that many genes were downregulated in incipient floral primordia while many of them were activated during the differentiation of floral organs (Wellmer et al., 2006). However, some genes were overrepresented during all stages analyzed (i.e. transcription factors including the family of MADS-box genes, *PIN* dependent auxin transport genes, as well as auxin and GA metabolism genes). Even though the MADS box genes were overrepresented, the promoter regions of the genes expressed during these different stages are not enriched in CArG-box sequences compared to random samples from the whole genome. This result suggests that MADS-domain transcription factors may be able to bind sequences other than CArG motifs, or that they have few direct targets during the developmental stages analyzed (Wellmer et al., 2006).

In a different approach, an inducible post-translational version of *AG* was used in gene expression profiling to detect AG target genes. One of the genes identified that is upregulated by AG is *SPOROCYTELESS* (*SPL*). AG is able to bind *in vitro* to the 3' region (downstream of the stop codon) of the *SPL* gene (Ito et al., 2004). *SPL* has been described as a key regulator of sporogenesis later during stamen and carpel development (see sections 3.4.5, 3.4.6 and Table S1; Schiefthaler et al., 1999; Yang et al., 1999).

3.4 Floral Organogenesis

The challenge of inferring the topology of the gene regulatory network (GRN) underlying the establishment of floral organ primordia, and their development (cell differentiation, morphogenesis and growth) is still ahead. However, some key components and GRN functional modules characterized to date are summarized in Section 4. Such modules involve several functional feedback loops and underlie different generic developmental processes mainly: primordia type specification; delimitation; floral organ primordia positioning that depends on fundamentally on auxins; primordia number; inter-whorl and within-whorl boundaries; and primordia and organ adaxial-abaxial polarity (Figures 2C and 15; Irish, 2008). At later stages of floral organ development, subcellular differentiation and patterning, as well as overall organogenesis takes place and more specific regulatory modules are involved. The genes within such modules are treated separately for each organ type (Figures 16-17).

As a precursor to integrating GRN modules in the above categories, we now provide a synthesis of the molecular genetic studies of how such generic developmental processes are regulated. Several of these have also been identified as important regulators of leaf development, substantiating the proposal of Goethe that all plant organs are elaborations or modifications of a core leaf-like developmental program (for review of common pathways see Sablowski, 2009). ABC floral organ identity genes are also important in fine-tuning or coordinating the role of genes involved in some of the generic developmental modules during flower development (Figure 15; Sablowski, 2009). Some genes participate in more than one process or module and are important for making connections between different GRN modules. In such cases, they are considered in more than one category.

Regulatory modules controlling distinct components of floral organ development have been elucidated to different extents depending on available mutant phenotypes. In correlation with anatomical and morphological complexity, the size and complexity of the regulatory modules underlying stamen and carpel development are much greater than those that regulate sepal or petal development. Carpel development is covered in the "Fruit Development" chapter (Roeder and Yanofsky, 2006) in this book, and is only briefly considered here.

In the flower meristem, normal organogenesis depends upon a homeostatic equilibrium between stem cell specification and cellular differentiation (Green et al., 2005). Plant morphogenesis is influenced both by the orientation and rate of cell division, as well as by cell expansion and differentiation (see section 2 for a description of floral organ initiation and morphogenesis). How the molecular aspects of these processes are coordinated has been very difficult to elucidate. However, it is generally accepted that cells in meristematic regions respond to positional information important for inducing and controlling morphogenesis (Sussex, 1954; 1955; Meyerowitz 1997; Hauser et al., 1998). One of these positional signals is auxin (see Section 3.4.1; Reinhardt et al., 2000; Benková et al., 2003; Reinhardt et al., 2003; de Reuille et al., 2006). Several mutations that affect the number, size, and/or shape of one or several floral organs have also been characterized. Some of these phenotypes are pleiotropic consequences of mutations in genes acting from earlier steps of plant and flower development. Others are the result of alterations in organ specific genes (Figures 16-17). An extensive list of genes involved in flower organ morphogenesis with their inferred functions, mutant phenotypes and mRNA expression patterns is given in Table S1.

3.4.1. Floral meristem and organ primordia positioning: the role of auxin

The shoot apical meristem produces leaves and then flowers in a highly predictable and regular phyllotactic pattern (Tanaka et al., 2006). One of the key compounds that regulate this developmental process is the hormone auxin (Reinhardt et al., 2000). Increased auxin levels mark the initiation sites for organ primordia (including those of floral organs) and local application of auxin is sufficient to trigger leaf or flower formation in the shoot apex (Reinhardt et al., 2000; Tanaka et al., 2006). Once the primordium is established, there is a depletion of auxin around it and another peak of auxin is only able to form in cells at a specific distance from pre-existing primordia, generating a phyllotactic pattern (Reinhardt et al., 2000; Reinhardt et al., 2003; de Reuille et al., 2006; Tanaka et al., 2006; Berleth et al., 2007; Kuhlemeier, 2007). After initiation, the primordium grows by cell proliferation and cell expansion, and the organ differentiates along the apical-basal and dorsal-ventral axes (Heisler et al., 2005; Golz, 2006).

The overall distribution of auxin depends on its biosynthesis, metabolism, and directional transport. Most auxin is synthesized in young tissues of the shoot and distributed throughout the plant by two physiologically distinct pathways. One of them is passive and occurs only by diffusion through the mature phloem. The other one is an active polar auxin transport (called PAT) that mediates cell-to-cell movement of auxin through two different types of

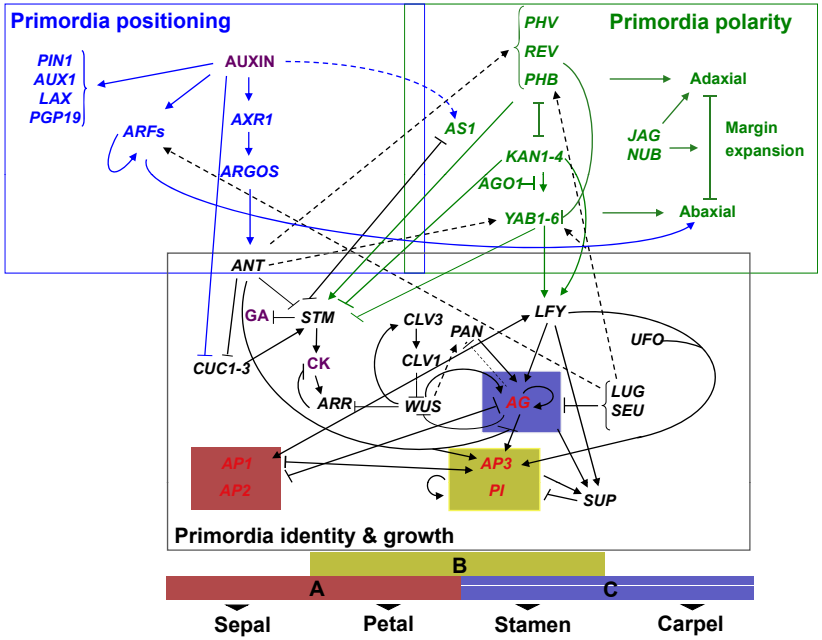


Figure 15. Functional gene regulatory modules during early flower development.

Common molecular modules act during early meristem morphogenesis from the SAM both before and after reproduction. During floral organogenesis, these modules interact among themselves and with the FOS-GRN that includes the floral homeotic genes. Anlagen positioning in the SAM flanks depends on auxin gradients. Transport and signal transduction proteins, as well as other factors regulated by auxins (letters in blue), participate in the establishment of such gradients and finally determining the position of primordia. The auxin pathway also downregulates some members of the NAC family (*CUC1* to *3* are important for organ boundary establishment), which also participate in the positive regulation of *STM* and *KNOX* genes. Since *WUS* maintains the apical meristem stem cells in a proliferating state with CLV proteins that in turn regulate its expression in a non cell-autonomous negative-positive feedback loop, and *STM* prevents meristem cell differentiation by inducing the production of cytokinins (CK) and the *ARR* transduction pathway (see text), floral primordia may emerge if cells in the anlagen are able to downregulate *STM*. This can be achieved by the action of AS1 and ANT. Upregulation of *LFY* by the flowering genes (Section 3.2; Figure 9) in conjunction with some KAN and YAB proteins, activate the expression of ABC homeotic genes (in red) for the establishment of the floral organ primordia identity and growth (gene acronyms in black, see text and Table S1 for full names). Lateral organ primordia acquire apical/basal, lateral/medial and adaxial/abaxial polarities by the action of protein families that include PHABs (PHB, PHV and REV), KANs (KANADI1-3, ATS/KAN4), YABs (FIL/YAB1, YAB2, YAB3, INO/YAB4, YAB5 and CRC/YAB6), JAG and NUB (letters in green). Some of these are organ-specific while others are shared by different floral organ primordia (see section 3.4). Not all the genes involved in each module are depicted, just some of the most representative ones, which help us to understand how they are interconnected. Arrows and bars indicate positive and negative regulatory interactions, respectively, and dashed lines a postulated interaction not yet proven. The text color used for the gene names in each module is the same as in Figures 16, 17, and 19 where specific organ developmental processes are summarized and the ABC genes are shown in boxes on the organ specified as in the model shown below. Hormones are in purple.

This figure was composed partially from information in Clark (2001b), Blazquez et al. (2006), Hord et al. (2006), Shani et al. (2006) and Feng and Dickinson (2007).

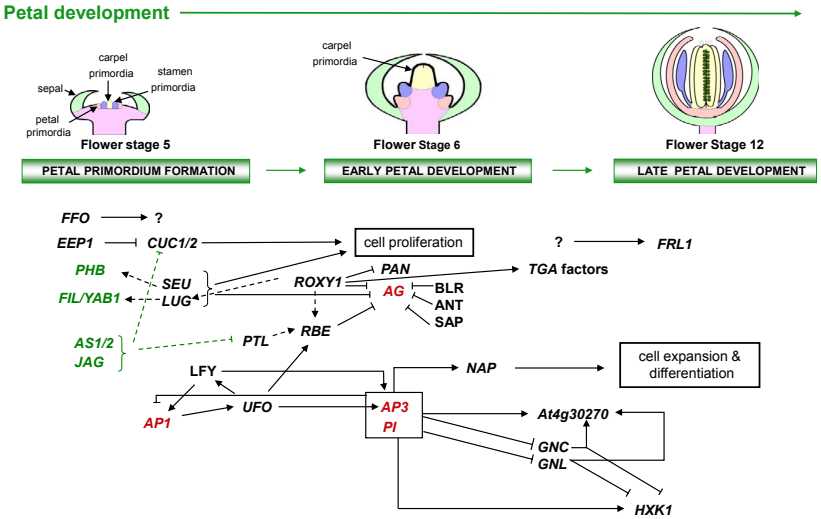


Figure 16. Main stages of petal development and some genes involved.

Schemes at the top illustrate three different stages of petal development (for details see section 2). Briefly, GRN modules (genes) in petal development include those involved in the establishment of the second whorl domain, the specification of petal identity and cell differentiation. *CUC* genes under the regulation of miR164c are involved in establishing whorl boundaries. Genes involved in polarity determination like *JAG*, *PHB* and *YAB1* are also necessary for petal development. A, B and *SEP* genes, and the absence of C genes, determine petal identity (AP2 and SEP genes are not shown here for clarity; see Figures 11 and 14). Petal blades are formed by active cell division at early developmental stages and by cell enlargement and differentiation at later stages. Some of the genes expressed early need to be continuously expressed throughout petal growth, including *ROXY1*, *SEU*, and *LUG*. Downregulation of the *GNC, GNL*, and *HXK1* genes inhibits chlorophyll accumulation and expression of photosynthetic genes. *At4g30270* might be necessary for correct cell wall dynamics during petal growth (see text section 3.4.5 and Table S1 for details; Franks et al., 2006; Irish, 2008;). Gene color code as in Figure 15; arrows and bars indicate positive and negative regulatory interactions, respectively.

proteins, efflux and influx carriers. Some of the genes that encode these transporters (or carriers) have been cloned: *PIN-FORMED* (*PIN*) and *P-GLYCOPROTEINS* (ABCB/PGP) for auxin efflux, and *AUXIN1* (*AUX1*) and its paralogs *LIKE-AUX1* (*LAX1-3*) for auxin uptake/influx (Figure 15; Bennett et al., 1996; Friml, 2003; Yang et al., 2006; Bandyopadhyay et al., 2007).

The *PIN* gene family encodes eight protein members in total; three of them (*PIN5*, *6*, and *8*) of unknown function. All of the PIN proteins characterized until now are asymmetrically distributed on the plasma membrane and some of them can be found in specific cell types with no pronounced polarity (Vieten et al., 2007). The direction of auxin flow is believed to be determined by the asymmetric cellular localization of PIN proteins (Friml, 2003). The first of these proteins to be characterized was *PIN1*, and its mutation (*pin1*) results in pin-shaped inflorescence meristems without flowers. *PIN1* expression is induced by auxin and it encodes a protein with 10-12 putative transmembrane domains and shares some similarity with bacterial transporters (Gälweiler et al., 1998). *pin1* mutant plants accumulate high amounts of auxin in vegetative meristems and a deficiency in the apical inflorescence meristem, which results in a defective organ initiation of leaves and flowers, a phenotype that can be imitated in wild type using auxin efflux inhibitors (Okada et al., 1991; Reinhardt et al., 2000). Of the other PIN proteins, only *pin3* and *pin7* loss-of-function mutants have flowers, and these bear fused petals, no stamens, and occasionally no sepals (Benková et al., 2003). *PIN3* is essentially involved in mediating differential shoot growth (Friml et al., 2002) and *PIN7* is important during early embryo development (Friml et al., 2003).

Auxin movement mediated by PIN carrier proteins determines the growth axis of the developing organ by establishing an auxin

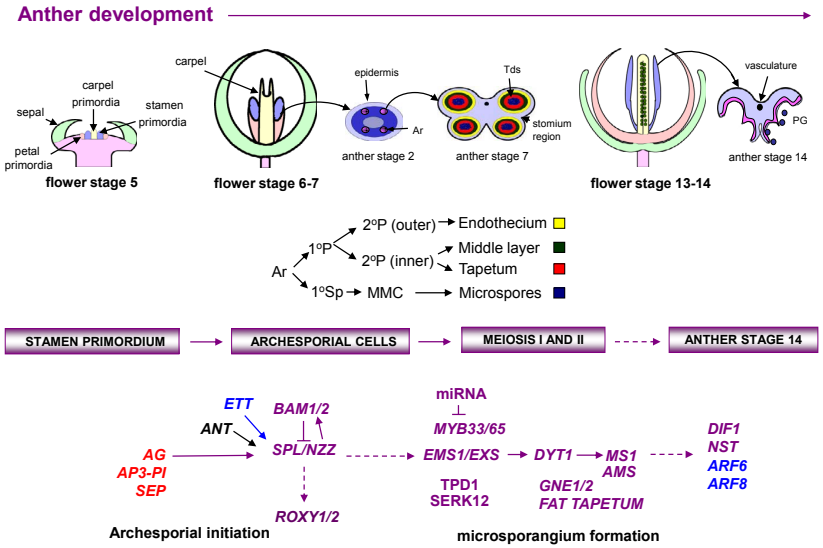


Figure 17. Stages of stamen development with emphasis on the genes implicated in anther formation.

Schemes of some stages of flower development showing representative stages of anther cell differentiation (Sanders et al., 1999) are shown at the top. At stage 1 of anther development and microspore formation, rounded stamen primordia emerge with three cell layers, L1, L2 and L3. During stage 2, the archesporial cells (Ar) arise in the four "corners" of the L2 layer and the epidermis in the L1. Before meiosis the Ar cells divide and generate the primary parietal layer (1°P) and the primary sporogenous layer (1°Sp). The 1°P then divides into two secondary parietal layers (outer and inner, 2°P). The outer layer gives rise to the endothecium, the inner cells to the middle layer and the tapetum. 1°Sp produces the microspore mother cell (MMC) that undergoes meiosis and gives rise to the microspores (Alves-Ferreira et al., 2007). At stage 7, meiosis is completed and the four locules carrying tetrads (Tds) of microspores are seen. At stage 14, cells shrink and the anther dehisces liberating the pollen grains (PG; Sanders et al., 1999). Some of the known genetic interactions important during anther development are shown in purple. AG (in red) induces the expression of *SPL* (the first gene known to be committed to anther development); later during microsporangium formation the action of the *EMS, DYT, MS1* and *AMS* genes is also indispensable (Feng and Dickinson, 2007). See section 3.4.6 for further explanation and Figure 15 for gene color code. Arrows and bars indicate positive and negative regulatory interactions, respectively, and dashed lines possible indirect interactions.

gradient with its maximum at the tip (Benková et al., 2003; Tanaka et al., 2006). As the primordium rapidly expands, auxin is depleted from the tip. Two hypotheses have been proposed to explain this observation: either auxin is transported through the primordium interior into the vascular network (Benková et al., 2003; Tanaka et al., 2006) or it is depleted from primordial regions as a result of specific reversals in PIN1 polarity (Heisler et al., 2005).

The ABCB/PGPs are also transmembrane proteins that belong to the ATP-binding cassette (ABC) transporter superfamily. In Arabidopsis, three of their members, ABCB1, ABCB4, and ABCB9, are able to transport auxin away from apical tissues and are important in maintaining long-distance auxin transport (Titapiwatanakun et al., 2009). One of the PGP proteins (PGP19) co-localizes and interacts with PIN1 and the ABCB protein is apparently important in stabilizing plasma membrane microdomains necessary for enhanced PIN1 activity (Bandyopadhyay et al., 2007; Titapiwatanakun et al., 2009).

Auxin enters the cell passively by simple diffusion and also by the import activity of AUX1 and related LAX proteins. The *AUX1* gene encodes a protein with 11 putative transmembrane domains (Hobbie, 2006) similar to plant amino acid permeases (Bennett et al., 1996). The mutant form (*aux1*) was identified in a screen

for auxin resistant and agravitropic mutants (Bennett et al., 1996; Vieten et al., 2007). The AUX1 protein also has polar subcellular localization in some cells and co-localizes with PIN1 in the shoot apical meristem. AUX1/LAX function could be essential for stabilizing the phyllotactic pattern. The proposed model for AUX1/LAX function is that these proteins concentrate auxin in the cytoplasm of cells of the L1 layer, preventing auxin diffusion in the apoplast (Bainbridge et al., 2008).

PINOID (*PID*) encodes a Ser/Thr protein kinase (Christensen et al., 2000) which has been implicated to function in redirecting subcellular PIN polarities, because the loss of its activity causes a shift in apical-basal PIN polarity (Friml et al., 2004; Berleth et al., 2007; Michniewicz et al., 2007). *pid* mutants have a defect in organ formation similar to that of *pin1*, but they do produce a few flowers (Reinhardt et al., 2003) with altered floral organ numbers (more petals but fewer stamens) (Bennett et al., 1995). Recently, Michniewicz et al., (2007) reported that *in vivo* PIN1 phosphorylation is directly dependent on the kinase PID and a phosphatase PP2A, which may act directly by dephosphorylating PIN1 or indirectly through PID. This phosphorylation status determines the intracellular apical-basal localization of PIN1 and therefore auxin transport-dependent development. PIN1 is targeted to the apex when it is phosphorylated and to the base when it is dephosphorylated (Michniewicz et al., 2007; Vieten et al., 2007).

Accumulation of auxin activates downstream processes through specific receptors and the combinatorial action of members of two large families of transcription factors, AUXIN RESPONSE FACTORS (ARF) and IAA/AUX (Kuhlemeier, 2007). The Aux/IAA proteins are degraded when the levels of free auxin rise, resulting in derepression of ARFs. *ETTIN* (*ETT*)/*ARF3* has a dynamic role in patterning by acting in specific cells within floral meristems and reproductive organs. At early stages, *ETT* functions in determining the number of organ primordia, whereas later it is involved in the outgrowth and patterning of tissues within organ primordia (Figure 15; Sessions et al., 1997). *ett* mutant plants show altered flower development; some flowers have missing petals and rudimentary radialized stamens, and others have normal fertile stamens, but radialized petals (Pekker et al., 2005). ETT is also involved in prepatterning apical and basal boundaries in the gynoecium primordium (see Table S1; Sessions and Zambryski, 1995; Sessions et al., 1997). MONOPTEROS (MP)/ARF5 mutants (*mp*) have inflorescences with smaller or absent flowers, similar to *pin1* mutants (Przemeck et al., 1996).

3.4.2. Floral organ primordia number, size, and boundaries

In Arabidopsis, which is a self-fertilizing (autogamous) and partially cleistogamous (before flower bud opens) plant, floral organ size might not be under strong evolutionary pressure compared to allogamous species. However, it has been an important model to study genes that control size and architectural traits of flowers (Weiss et al., 2005).

Several mutations that affect meristem size and maintenance lead to alterations in flower organ number or size. Mutations in the *CLV* genes (Clark et al., 1993 and 1995; Kayes and Clark, 1998) cause an increase in meristem size, thus yielding additional whorls and a change in floral organ number with altered phyllotaxis (Clark et al., 1993; Clark et al., 1997; Fletcher et al., 1999; Brand et al., 2000; Doerner, 2000). Mutations in genes that control cell proliferation in the SAM, such as the *CLV* genes, are similar to *ULT* in that they have larger SAM and primordia (Fletcher, 2001; Carles et al., 2004) and *WIGGUM* (*WIG*; Running et al., 1998).

When *WUS* is repressed and the number of cells for floral primordia formation is reduced, organ architecture is compromised suggesting that there is a threshold number of cells required to form a normal organ (Weiss et al., 2005). In fact, the loss of organs observed in A-function mutants, or any other AG repressor mutant could be explained as a result of premature repression of *WUS* by AG in these organs (Crone and Lord, 1994; Liu and Meyerowitz, 1995; Laux et al., 1996).

Other mutants that have altered floral organ numbers are *pan* (Running and Meyerowitz, 1996; Chuang et al., 1999), *ett* (Sessions et al., 1997) and *sup* (Jacobsen and Meyerowitz, 1997). Both *pan* and *ett* have more sepals and petals and fewer stamens, whereas *sup* produces more stamens at the expense of carpels (Weiss et al., 2005). Double *pan sup* mutants however have an attenuated *sup* phenotype in the fourth whorl, probably because in this mutant *AG* is downregulated and the domain of expression of *WUS* is expanded (Das et al., 2009).

The *PAN* gene mutation specifically alters floral organ number, yielding fertile plants with a pentamerous meristic pattern (Running and Meyerowitz, 1996). *PAN* encodes a member of the bZIP class of transcriptional regulators (Chuang et al., 1999) and is thought to act in the process by which cells assess their position within the developing floral meristem. This gene may affect the switch that commits floral organ primordia cells to enter an organ initiation program (Running and Meyerowitz, 1996). *PAN* and *WUS* expression overlaps and in *clv* mutants both genes are ectopically expressed (Chuang et al., 1999; Maier et al., 2009). *WUS* overexpression causes *PAN* overexpansion too suggesting that this gene is positively regulated by *WUS* (Maier et al., 2009).

Interestingly, pentameric symmetry is characteristic of flowers in early-diverging angiosperm lineages, thus suggesting that *PAN* may have been involved in changes to meristic patterns during angiosperm diversification; particularly the evolution from pentamerous to tetramerous flowers in the Brassicaceae lineage (Chuang et al., 1999).

Organ size is also regulated by the same components in all whorls. The *ANT* gene encodes a transcription factor of the AP2 family, which seems to be a general regulator of organ size during organogenesis (Elliott et al., 1996; Klucher et al., 1996; Krizek, 1999; Krizek et al., 2000; Mizukami and Fischer, 2000). The overexpression of *ANT* causes increased cell division in sepals and increased cell expansion in the inner three whorls, probably affecting both the rate and duration of cell divisions which are important determinants of the final size of lateral organs (Krizek, 1999; Mizukami and Fischer, 2000; Weiss et al., 2005). *ARGOS* participates in the same transduction pathway as *ANT* and acts downstream of *AUXIN RESISTANT 1* (*AXR1*). Interestingly, increased organ size observed in *ARGOS* overexpression lines is due to an extended period of cell division rather than to an increase in growth rate (Hu et al., 2003; Weiss et al., 2005). So, it is plausible to assume that these two genes (and probably others) affect organ size by transducing signals from plant growth regulators, such as auxin, which is a key player in establishing SAM

primordia and a general regulator of cell proliferation and expansion (Figure 15).

ANT also participates in defining abaxial-adaxial organ polarity in combination with *FILAMENTOUS FLOWER*/*YABBY1* (*FIL*/*YAB1*; Nole-Wilson and Krizek, 2006; see next section) and thus may be one of the links between the modules controlling primordia growth and the polarity establishment (Figure 15).

Ectopic expression of UFO (Levin and Meyerowitz, 1995) also causes increased floral organ size (Lee et al., 1997), due to increased cell division (Mizukami, 2001; Weiss et al., 2005). This pathway is regulated by UFO independently of its role in B gene expression, because ectopic expression of the B genes does not induce any increase in organ size, so missexpression of other unknown UFO-dependent factors may account for this phenotype (Ni et al., 2004). UFO and two gene enhancers of the *ufo* phenotype, *FUSED FLORAL ORGANS 1* and *3* (*FFO1* and *FFO3*), could also participate in establishing and maintaining organ boundaries probably by affecting cell proliferation (Levin et al., 1998).

Morphological boundaries are established in the early stages of the formation of a primordium separating it from surrounding tissues, and later from adjacent organ primordia (Figure 2C; Aida and Tasaka, 2006a). Cells in the boundary are distinctly narrow and elongated with low proliferation rates (Aida and Tasaka, 2006b). Genes expressed in the boundary may affect both meristem and organ development by upregulating cell differentiation genes and downregulating meristematic genes (Borghi et al., 2007). *CUC1, 2*, and *3* encode NAC-domain transcription factors that promote morphological separation of lateral organs through growth repression (Aida et al., 1997; Vroemen et al., 2003; Taoka et al., 2004). *cuc1 cuc2* double mutant seedlings have fused cotyledons with no shoots. However, when adventitious stems are induced in this genotype, flowers have fused sepals and stamens, fewer petals and stamens number, and reduced fertility (Aida et al., 1997). *CUC* genes are epigenetically regulated (Laufs et al., 2004; Kwon et al., 2006).

Other genes, such as *LATERAL ORGAN BOUNDARY* (*LOB*) and *JAGGED LATERAL ORGANS* (*JLO*), members of the *LATERAL ORGAN BOUNDARY DOMAIN* (*LBD*) gene family, encode putative transcription factors with a leucine-zipper motif that are also expressed in boundary cells (Shuai et al., 2002; Borghi et al., 2007). *JLO* along with the *CUC* genes probably coordinate auxin accumulation and loss of meristem-specific gene expression in organ anlagen (Takada et al., 2001; Borghi et al., 2007).

3.4.3. Floral organ polarity

Establishing organ polarity is an important aspect of morphogenesis and it is sometimes clearly associated with specific functions of plant organs. Both, adaxial-abaxial and proximal-distal polarities are regulated by genetic circuits that are similar for all lateral organs (Figure 2C; Feng and Dickinson, 2007), although each organ type has distinct cell types and morphogenesis in the abaxial versus adaxial surfaces, and in the proximal versus distal regions (Figures 2C and 8). Organ polarity is also linked to the establishment of hormone gradients.

Briefly, abaxial fate is conferred by members of the *YABBY* family (Sawa et al., 1999; Siegfried et al., 1999) and by some of the *KANADI* genes (Eshed et al., 2001; Kerstetter et al., 2001), whereas adaxial cell fate is determined by members of the *PHAB* family: *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), and *PHAVOLUTA* (*PHV*) (McConnell et al., 2001; Emery et al., 2003; reviewed in Bowman et al., 2002; Zik and Irish, 2003b; Golz, 2006) together with *JAGGED (JAG)* and *NUBBIN (NUB)* (Figure 15; Dinneny et al., 2004; Dinneny et al., 2006).

YABBY proteins (YAB) are transcription factors with a Zn-finger and a helix-loop-helix (YABBY) domain that are promoters of abaxial cell fate in all lateral organs, among other functions (Bowman 1999; Sawa et al., 1999; Siegfried et al., 1999). During flower development they participate in establishing the primordium domain and meristem patterns, and later in maintaining abaxial polarity (Siegfried et al., 1999; Goldshmidt et al., 2008). *FIL/YAB1*, *YAB2*, and *YAB3* are expressed in a polar manner in all lateral organs of the flower meristem, while *CRABS CLAW* (*CRC*/*YAB6*) is only expressed in carpels and nectaries, and *INNER NO OUTER* (*INO/YAB4*) is restricted to outer integuments (see section 3.4.6 and 3.4.7; Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Villanueva et al., 1999).

KANADI (KAN) genes encode transcription factors of the GARP family. *KAN1, KAN2*, and *KAN3* have been implicated in promoting abaxial cell fates (Eshed et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). The *kan1* mutant was selected as a genetic enhancer of *crc* gynoecium phenotype, producing a mirror-image of adaxial tissues in the *kan1 crc* double mutant, indicating that both genes participate in a redundant manner to promote abaxial identity (Eshed et al., 1999). In *kan1 kan2* double mutants, all floral organs are also extremely adaxialized (Eshed et al., 2001; Kerstetter et al 2001). Although these *KAN* genes are not necessary for the activation of *YAB* genes, they are important in controling their proper abaxial localization (Eshed et al., 2001). Even though *KAN* and *YAB* genes may have common targets, they also have different ones, since the phenotype of the *fil yab3* double mutant is not quite the same as the extreme phenotype of *kan1 kan2* (Bowman et al., 2002).

It has been hypothesized that the "default" state of cells is the abaxial fate (Sussex 1954, 1955). Genes that belong to the *PHAB* family (class III homeodomain-leucine zipper, HD-ZIP III; Sessa et al., 1998; McConnell et al., 2001; Golz, 2006) of transcription factors, like *PHB* and *PHV*, might be activated by a proximal signal coming from the apical meristem. These cells that are programmed to yield the adaxial portion of the lateral organ, are predicted to in turn have *YAB* and *KAN* genes repressed (Bowman et al., 2002). In this respect, semidominant gain-of-function mutants of *PHB* and *PHV* genes cause adaxialization of lateral organs (McConnell and Barton, 1998; McConnell et al., 2001). *PHB*, *PHV*, and *REV* have similar expression patterns. They are expressed in the SAM initiating lateral organs and later become adaxially restricted as the primordium emerges (McConnell et al., 2001; Otsuga et al., 2001; Prigge et al., 2005). Finally, phenotypes of the loss-of-function *rev* mutants could be interpreted as having a partial loss of adaxial identity (Talbert et al., 1995; Otsuga et al., 2001).

Besides the PHAB function in polarity, it is also interesting to note that a *phb phv cna* (*corona*, another member of the HD-ZIP III gene family) triple mutant has a very similar phenotype to those of *clv* mutants with a distinct increase in organ number in each whorl. This would suggest that HD-ZIP III genes and the *CLV* pathway regulate meristem function in a similar manner. The possible interrelation of these modules could contribute to homeostasis between stem cell maintenance and organ formation (Prigge et al., 2005).

NUB and *JAG* are similar genes which encode C2H2-zinc finger transcription factors that are proposed to play redundant functions in proliferation and differentiation of adaxial cells, particularly during anther and carpel development (Dinneny et al., 2004; Ohno et al., 2004; Dinneny et al., 2006; Xu et al., 2008). They specifically work together in determining the number of cell layers formed in floral organs, and like the *PHAB* family, they are not cell-fate genes. Hypothetically, *JAG* suppresses the premature differentiation of tissues by slowing down the cessation of cell division in distal regions of organs until it finally arrests after normal morphogenesis has occurred (Dinneny et al., 2004).

AS1 and *AS2* have redundant functions in the establishment of adaxial identity (Ori et al., 2000; Sun et al., 2000; Semiarti et al., 2001). *AS1* encodes a MYB-domain transcription factor, and *AS2* is a member of the LBD gene family (Serrano-Cartagena et al., 1999; Byrne et al., 2000; Semiarti et al., 2001; Sun et al., 2002). AS1 protein is expressed in organ initials and physically interacts with AS2 to inhibit KNOX gene expression, thus guiding primordia toward differentiation (Figure 15; Ori et al., 2000; Byrne et al., 2002; Xu et al., 2003; Guo et al., 2008).

Other reviews on polarity determination in embryos and in leaves are found in other chapters in this series: "Embryogenesis: pattern formation from a single cell" (Berleth and Chatfield, 2002) and "Leaf development" (Tsukaya, 2002).

3.4.4 Sepals and petals

Sepals and petals constitute the sterile perianth in the first and second flower whorls, respectively. The sepal whorl or calyx protects the developing floral bud and in some plants, but not in Arabidopsis, it may be involved in fruit development (He et al., 2004). The petal whorl or corolla is generally thought to be important for attracting pollinators (Krizek and Fletcher, 2005), but in an autogamous plant such as Arabidopsis, the corolla is generally not showy.

According to the ABC model, sepal identity specification depends on the activity of both A and *SEP* genes (see section 3.3; Coen and Meyerowitz, 1991; Pelaz et al., 2000), and petal identity specification depends on the overlapping activities of A, B and *SEP* genes (see section 3.3; Coen and Meyerowitz, 1991; Pelaz et al., 2000). Also, it has been shown that sepal and petal identity specification depends, at least in part, on the correct downregulation of *AG* expression in the second whorl (see below).

Several molecular components known to influence development of sepals, influence petals too. But knowledge is still limited especially of sepal developmental gene networks. However, a basic GRN for petal development can be constructed based on available data (Figure 16). As stated earlier, organ identity determination, boundary establishment, and expression of polarity determinants are common features needed for the correct development of all the flower organs (Figure 15). There are several pieces of evidence that suggest that genes involved in these processes might be acting at the same time (for example, expression profiles and *in situ* hybridization assays), at least momentarily during flower development. However, we still do not understand fully how such functional modules interact with each other.

As it was said before sepal and petal boundary and organ number establishment are controlled by the *CUC* and *FFO2* genes (see Figure 16 and section 3.4.2; Aida et al., 1997; Levin et al., 1998). *CUC* gene expression is regulated by the miR164c (encoded by *EEP1*) in an organ specific manner (Laufs et al., 2004; Baker et al., 2005).

Several genes are involved in establishing and maintaining the sepal and/or petal domain and, in a way, determining the boundaries between the organs. One of the main activities of these genes is to exclude *AG* expression from the first and second whorl. As stated in section 3.3, *AG* is repressed by *RBE*, *LUG*, *SEU, ROXY1*, *AP2*, *BLR*, *ANT* and *SAP* (for more information about each gene, see Table S1; Figures 15 and 16).

Briefly, *RBE* is mainly involved in boundary and organ number determination of both sepals (non-autonomously) and petals, and in AG exclusion from the second whorl at early stages of flower organ development. But it is also important during late petal development as mutants may form filamentous organs in the second whorl. *RBE* expression is controlled by both PTL and UFO (Takeda et al., 2004; Krizek et al., 2006). PTL is a trihelix transcription factor that is expressed at early stages in four zones between the initiating sepal primordia and in lateral regions of stamen primordial. Later on, *PTL* expression can be detected at the margins of expanding sepals, petals, and stamens (Brewer et al., 2004). Thus PTL may delimit the *AG* expression region indirectly by activating *RBE* expression (Irish, 2008), and it may also be controlling lateral outgrowth of mature sepals, petals and stamens defining their final shape and orientation (Griffith et al, 1999; Brewer et al., 2004).

UFO is also an important regulator of petal development. Its action toward *RBE* may be indirect, as it may be degrading (as part of an SCF E3 ubiquitin ligase complex) an unknown repressor of *RBE* (Irish, 2008). But *UFO* is an important network link between the *AG* inactivation pathway and the B gene identity determination pathway, because UFO interacts with LFY to activate *AP3* expression (See section 3.3; Lee et al., 1997; Samach et al., 1999; Chae et al., 2008). Importantly, *UFO* expression is also required for normal petal blade outgrowth after petal identity has been established (Laufs et al., 2003), as well as for determination of sepal shape and number in the first whorl (Levin and Meyerowitz, 1995; Samach et al., 1999).

SEU and *LUG* also repress *AG* expression in the first and second whorls by forming a protein complex with AP1 and SEP3 (see section 3.3; Sridhar et al., 2004; Sridhar et al., 2006). But these genes are also part of the adaxial/abaxial polarity establishment pathway in the petal GRN, as they are required for normal *PHB* and *FIL* expression (Figure 15). SEU and LUG participate in petal shape regulation by controlling blade cell number and petal vasculature development in an AG independent manner (Franks et al, 2006). Finally, SEU is also involved in auxin response pathways by directly interacting with ETT, and influencing the final shape, number and phyllotaxy of petals (Pfluger and Zambryski, 2004).

As part of the regulatory network that represses *AG* expression, AP2 is itself negatively regulated by miR172 when second whorl boundaries are determined (Chen, 2004; Zhao et al., 2007). Besides being a negative regulator of *AG*, ANT also affects organ number and morphology in the first three whorls (Elliott et al., 1996; Klucher et al., 1996). *SAP*, another regulator of the mor-

phology of all organs, but mostly of petals, is unexpectedly more important in later flowers (Byzova et al., 1999).

Another important indirect repressor of *AG* is ROXY1. As a glutaredoxin, ROXY1 seems to be a postranslational modifier of AP2, LUG, UFO and RBE giving them the specificity to repress AG in the second whorl (Xing et al., 2005; Irish, 2008). ROXY1 is also important for repressing *PAN* expression and for activating other *TGA* factors at different stages of petal development (Li et al., 2009).

Genes that usually work in the establishment of lateral organ polarity (see section 3.4.3) are also important in determining the polarity of sepals and petals, e.g. *PHB*, *JAG*, *FIL*, *YAB3*, *KAN*, *AS1* and *AS2* (Figure 16). Experimental data suggest that AS1, AS2 and JAG are negative regulators of *CUC1/2* and *PLT* (Xu et al., 2008). This links the expression of these genes with those important for boundary determination in the GRN of both sepals and petals. *PHB* and *FIL* expression are also part of the network and are regulated by SEU and LUG (Franks et al., 2006). Lateral-axis dependent development is determined by the *PRESSED FLOWER* (*PRS*) homeobox gene (Matsumoto and Okada, 2001). As with some other genes involved, its position in the GRN is unknown, but by analyzing the mutant phenotypes, it becomes clear that the same regulatory modules that underlie polarity determination are involved in organ shape regulation.

In Arabidopsis, as in other plants, several mutants featuring a foliose-sepal-syndrome (FSS) (leaf-like sepals) have been isolated. Ectopic expression of the MADS-box genes *AGL24, SVP*, and *ZMM19* (from *Zea mays),* belonging to the *STMADS11*-clade (according to Theissen et al., 2000), result in FSS (He et al., 2004). The main feature of these leaf-like sepals is that they are large and have leaf-like stellate trichomes on their outer surface. One of the characteristics of *ap1* mutant plants is that they also have large or foliose sepals. Thus, it has been proposed that, in addition to their roles in floral transition and/or organ determination, *AP1*, *SVP*, and *AGL24* may also have a role regulating sepal size (He et al., 2004). But how they interact among themselves or with other sepal specific genes is still unknown.

Final sepal and/or petal morphology is also determined by *FRL1* (Hase et al., 2000; Hase et al., 2005), *TSO1* (Hauser et al., 1998), the AP3/PI regulated genes *GNC*, *GNL*, *At4g30270, HXK1* (Mara and Irish, 2008), and *NAP* (Sablowski and Meyerowitz, 1998). Except for FRL1, which is involved in endoreduplication control, and TSO1, which is likely involved in chromatin remodelling, the position of these genes in the petal GRN has already been established (see Figure 16).

Using microarray approaches Wellmer et al. (2004), compared gene expression levels within different floral homeotic mutants (see section 3.3.1). Their first study of stage 2 flowers identified only 13 genes as being sepal-specific and only 18 genes expressed exclusively (or predominantly) in petals. However, a more recent study of flowers at stage 3, when sepal primordia have just formed, revealed that 199 genes are upregulated and 161 genes downregulated (Figures 3-4; Wellmer et al., 2006). One speculation is that sepals are relatively simple organs and not many specific genes are involved in their development. But more detailed studies are still required. Results also suggest that genes regulating sepal and petal development may have been recruited from leaf developmental pathways, and, hence, are not specific for the development of these organs.

Petals have been proposed as an excellent model system in which to study development because they have a simple organization and are not essential for survival or reproduction (Irish, 2008). Although much progress has been made, much work is still needed for an integrated and dynamical understanding of petal development.

3.4.5 Stamens

Six stamens occupy the third whorl in the Arabidopsis flower. Stamen specification depends on the overlapping activities of B, C and *SEP* MADS-box genes (Coen and Meyerowitz, 1991; Pelaz et al., 2000). A complex network of gene regulatory modules is simultaneously activated in young stamen primordia, and these are also important for organ morphogenesis (Figure 15). These modules include those that regulate adaxial-abaxial primordium polarity (also affecting other vegetative and reproductive lateral organs) including genes from the *PHAB* (*PHB, PHV*, and *REV*), *KANADI* (*KAN1-4*), and *YABBY* (*FIL/YAB1, YAB2, and YAB3*) families. At later stages of stamen development, genes involved in sporogenesis such as *SPL and BAM1/2*, and in anther development, such as *JAG* and *NUB*, are activated (see Figures 15 and 17 for regulatory modules and genes; Scott et al., 2004; Ma, 2005; Feng and Dickinson, 2007).

Among the most striking stamen development mutants is *fil* (also called *antherless* and *undeveloped anther*) which bears normal filaments with neither anthers nor pollen. The *FIL* gene is *YABBY*-like and the *fil* phenotype suggests that the developmental programs of the filament and anther are controlled by independent regulatory modules (Sanders et al., 1999).

As mentioned in section 3.3.1, *SPL/NZZ* is essential for male and female reproductive development and is probably the first reproductive gene to be activated in the anther or, at least, it is the only gene that remains active during most of early anther development. This transcription factor gene is expressed during micro- and megasporogenesis. *AG* directly induces *SPL* but *AG* is not necessary for maintaining its expression (Ito et al., 2004). *spl* mutants are not able to produce microsporogenous cells or tapetal tissue, and show several alterations in anther wall and nucellus development (Schiefthaler et al., 1999; Yang et al., 1999). Interestingly, BAM1 and BAM2, which participate in the first cell division of the archesporial cells and the subsequent periclinal divisions to produce the somatic cell layers, are proposed to form a regulatory loop with *SPL* (Figure 17; Hord et al., 2006; Feng and Dickinson, 2007). Since *SPL* maintains the sporogenous activity in the microsporogenous cells, and *BAM1/2* maintain somatic differentiation, *bam1 bam2* anthers have cells interior to the epidermis with characteristics of pollen mother cells (Hord et al., 2006).

Although *SPL* is one of the genes expressed the earliest in stamen development, it is not the only one. Ectopic expression of *SPL* in all the whorls of an *ag* mutant, results in the formation of microsporangia only in the lateral parts of the staminoid 'petals', suggesting that microsporangial localization is established independently of *AG*, and that there is at least one other *SPL* inducer that is expressed in the second whorl, and not in other whorls (Ito et al., 2004; Feng and Dickinson, 2007). Two other genes, *JAG* and *NUB* play a crucial role in the formation of the four-locular anther architecture, independent of *SPL* induction. *jag nub* double mutants do not have a proper microsporangium. Instead, they

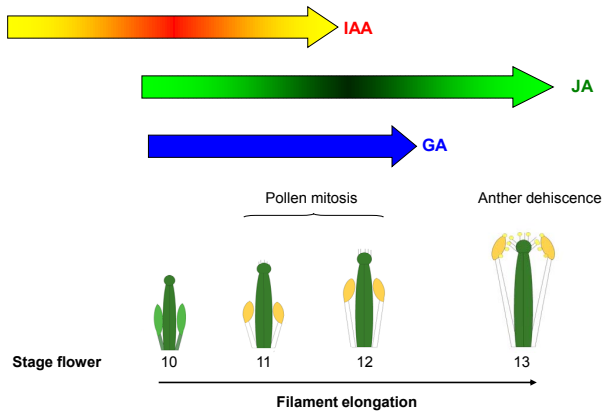


Figure 18. Hormones in late stages of stamen development.

At stage 10 of flower development, the auxin (IAA) concentration (yellow arrow) peaks (red gradient) in the stamens. During this period filaments start to elongate and auxin prevents premature dehiscence. Auxin also participates in later anther dehiscence, probably by inducing JA production (green arrow) that peaks (dark green gradient) at stages 11 and 12 (Nagpal et al., 2005). JA coordinates filament elongation, pollen maturation, anther dehiscence and flower opening (Ishiguro et al., 2001). Although it has not been quantified, GA (blue arrow) is involved in filament elongation and participates in microsporogenesis. Pollen development in anthers of GA-biosynthetic mutants is arrested before microspore mitosis (for details see section 3.4.6; Cheng et al., 2004; Iuchi et al., 2007).

form a finger-like structure that expresses *SPL* in its tips (Dinneny et al., 2006; Feng and Dickinson, 2007).

The correct number of microsporangial initials and the subsequent production of the tapetal cell and middle cell layer identities are properties specified by a putative LRR receptor kinase, EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS (EXS) (Canales et al., 2002). Until recently, the ligand for EMS1 was unknown, though it was hypothesized that it could be involved in the same signaling pathway as the *TAPETAL DETERMINANT1* (*TPD1*) gene. Both *tpd1* and *tpd1 ems1* mutants are similar to the single *ems1* mutant with arrested meiotic cytokinesis and degenerated microsporocytes (Yang et al., 2003). TPD1, is a small putatively secreted protein that interacts with EMS1 and induces its phosphorylation suggesting that TPD1 is the ligand of the EMS1 receptor that signals cell fate determination during sexual cell morphogenesis (Jia et al., 2008).

ROXY1 and *ROXY2* redundantly regulate anther development in Arabidopsis (Xing and Zachgo, 2008). Lateral and medial stamens of *roxy1* mutants might be fused and the former are sometimes missing (Xing et al., 2005). In these mutants, the adaxial anther lobes are affected in sporogenous cell formation during early differentiation steps, abaxial lobes develop normally but pollen mother cells degenerate, while the tapetum overgrows and occupies most of the locule space. Eventually, the tapetum degenerates too. *ROXY1* and *ROXY2* function downstream of *SPL* and upstream of *DYSFUNCTIONAL TAPETUM1* (*DYT1*). As with other glutaredoxins, they may need an interaction with glutathione to catalyze biosynthetic reactions, suggesting that they may have a role in redox regulation and/or plant stress defense mechanisms involved in the control of male gametogenesis (Xing and Zachgo, 2008).

After tapetal cells are specified, a range of genes are essential for subsequent development. *DYT1* encodes a putative bHLH transcription factor which functions downstream of *SPL* and *EMS1*. However *DYT1* is not able to complement the *spl* or *ems1* mutant phenotypes when it is overexpressed, indicating that it is required but not sufficient for normal tapetum development. *dyt1* exhibits abnormal anther morphology with largely vacuolated tapetal cells that eventually collapse. Several tapetum-expressed genes, such as *MALE STERILE 1* (*MS1*) and *ABORTED MICROSPORES* (*AMS*) are upregulated by *DYT1* (Zhang et al., 2006). In *ms1* mutants for example, tapetal cell abnormalities can be seen and pollen development is arrested just after microspores are released from the tetrads (Bowman, 1994; Wilson et al., 2001; Yang et al., 2007a). Other genes that participate in tapetum development include *RECEPTOR-LIKE PROTEIN KINASE2* (*RPK2*), *FAT TAPETUM* and *GUS-NEGATIVE1 and 2* (*GNE1*, *GNE2*). *RPK2* regulates tapetal function and middle layer differentiation (Mizuno et al., 2007). *FAT TAPETUM,* when mutated, has a middle layer that fails to collapse

after meiosis and shows tapetal-like behavior (Sanders et al., 1999; Ma, 2005). In *gne1* and *gne2* mutants the sporogenous cells enter meiosis, but cytokinesis is frequently arrested. The few highly aberrant tetrads formed degenerate early and microsporangia of mature anthers end up empty (Sorensen et al., 2002).

Several mutants affecting pollen development have been described: *pollenless3; three division mutant (tdm1); ms5, ms3* and *ms15*; *determinate infertile1* (*dif1*); *switch1* (*swi1*); *defective-pollen 1*; and *6492* among others (Bhatt et al., 1999; Sanders et al., 1999; Sorensen 2002). Meiotic cells in *pollenless3* anthers undergo a third division without DNA replication generating some cells with unbalanced chromosome numbers (Sanders et al., 1999) or "tetrads" with more than four microspores. *dif1* and *swi1* mutants have micro- and megaspores of uneven sizes because the encoded proteins are essential for sister chromatid cohesion in male and female meiosis and so mutants are totally infertile (Bhatt et al., 1999; Parisi et al., 1999; Mercier et al., 2001; Ma, 2005). Finally, other pollen mutants exhibit abnormal callose deposition (*ms32*, *ms31*, *ms37*, *7219*, and *7593*).

There are late-developmental anther mutants that affect anther dehiscence. In *non-dehiscence1* mutant plants, anthers contain apparently wild-type pollen but do not dehisce. It has been hypothesized that a cell death suppression program, which is normally responsible for dehiscence, might be inactive in this mutant (Sanders et al., 1999). *ms35* is also affected in anther dehiscence, because endothecial cells fail to develop the lignified secondary walls that after desiccation shrink differentially leading to the retraction of the anther wall and full opening of the stomium (Dawson et al., 1999; Scott et al., 2004). *MS35*, now *MYB26* (Steiner-Lange, 2003), is expressed during early anther development and may be a regulator of *NAC SECONDARY WALL-PROMOTING FACTOR 1* and 2 (*NST1*, *NST2*), which have also been linked to secondary thickening in the anther endothecium (Yang et al., 2007b). In *delayed-dehiscence* mutants (*dd1*, *dd2*, *dd3*, *dd4*, *dd5*) anther dehiscence and pollen release occurs after the stigma is no longer receptive preventing successful pollination, but stamens look wild-type and pollen is viable (Goldberg et al., 1993). On the contrary, in *defective-pollen1*, *2*, and *3*, anthers are able to dehisce, but the pollen is aberrant and unviable.

Recent publications have established that gibberellic acid (GA), jasmonic acid (JA), and auxins are involved during stamen development (Figure 18; Fleet and Sun, 2005; Nagpal et al., 2005; Wu et al., 2006; Cecchetti et al., 2008). The GA-deficient mutant, *ga1-3*, produces an abortive anther where microsporogenesis is arrested prior to pollen mitosis (Cheng et al., 2004). Mutations in two GA receptors, GA-INSENSITIVE DWARF1a and b (AtGID1a, b), affect the elongation of stamens, suggesting that these receptors have specific roles during stamen development (Iuchi et al., 2007). GA induces the degradation of the DELLA protein REPRESSOR OF GA1-3 (RGA) upon ubiquitination. Microarray analysis shows that 38% of the RGA downregulated genes are expressed in the male gametophyte at various stages of microsporogenesis (Hou et al., 2008).

Temporal coordination of the elongation of filaments, pollen maturation, and dehiscence of anthers is important for efficient fertilization. The expression overlap of RGA-regulated genes and jasmonate-responsive genes during stamen development suggest a crosstalk between GA and JA signaling pathways in these processes (Hou et al., 2008).

JA has been shown to be involved in at least three androecial developmental pathways: filament elongation, anther dehiscence and pollen production (Mandaokar et al., 2006). Different male sterile mutants have been found to be JA biosynthetic mutants (McConn and Browse, 1996; Sanders et al., 2000) including: the triple *fad* mutant (*fad3–2 fad7–2 fad8*), which lacks the fatty acid precursors of JA; *defective in anther dehiscence 1* (*dad1*), which encodes a phospholipase A1 that catalyzes the initial step of JA biosynthesis; and *dd1*, a member of the *12-OXOPHYTODIENOATE REDUCTASE* (*OPR3*) gene family (Stintzi and Browse, 2000; Ishiguro et al., 2001). *OPR3/DD1* is expressed in the stomium and in the septum cells of the anther that are involved in pollen release. All these mutant phenotypes can be rescued by exogenous application of JA, suggesting that this hormone plays an important role in controlling the timing of anther dehiscence. Interestingly, *DAD1* is a direct target of AG (Ito et al., 2007).

Similarly, the *coronatine insensitive 1* (*coi1*; JA receptor) mutant is defective in both pollen development and anther dehiscence. Stamens of *coi1* flowers have shorter filaments than those of wild-type flowers and anthers are indehiscent containing pollen grains with conspicuous vacuoles (Feys et al., 1994; Xie et al., 1998).

Three related polygalacturonases, enzymes involved in pectin degradation that promotes cell separation, are also involved in JA-regulated anther dehiscence. ARABIDOPSIS *DEHISCENCE ZONE POLYGALACTURONASE 1 (ADPG1) and 2 (ADPG2), and QUARTET2 (QRT 2)* gene expression are distinctly regulated by JA (Ogawa et al., 2009).

To determine the jasmonate-regulated stamen-specific transcriptome the expression profiles of JA-treated and untreated *opr3* mutants were compared (Mandaokar et al., 2006). It was found that 821 genes were induced (70% of them expressed in the sporophytic tissue) and 480 genes were repressed by JA and 13 transcription factors were identified that could be important for stamen maturation pathway(s). Of these, *MYB21*, *MYB24*, and *MYB28* are JA-responsive genes (Mandaokar et al., 2006). *myb21* mutants have short filaments, are late to dehisce and have reduced fertility. Though *myb24* mutants look like wild type, *myb21 myb24* double mutants have a more severe phenotype than *myb21*, suggesting that these two genes might be redundantly involved in important aspects of JA-dependent stamen development. *MYB28* is involved in amino acid metabolism and it is downregulated by both JA and RGA. This study also uncovered several other biochemical pathways that could be important during stamen and pollen maturation. Other results indicate that JA coordinates pollen maturation, anther dehiscence, and flower opening (Ishiguro et al., 2001). Auxins have also been proved to participate in these processes *arf6 arf8* double mutants are defective in anther dehiscence probably because they produce too little JA. Accordingly, this phenotype can be rescued by application of JA (Nagpal et al., 2005). However, auxins trigger filament elongation and prevent premature anther dehiscence and pollen maturation at earlier stages of stamen development. While JA production peaks at stages 11-12 of flower development (see Figure 6 and 18; Nagpal et al., 2005) auxin receptors (*TIR1* and *AFBs*) are already expressed at the end of meiosis. Mutants in these genes cause the release of mature pollen grains before filaments elongate. At later stages, the amount of JA decreases allowing these processes to continue (Figure 18; Cecchetti et al., 2008).

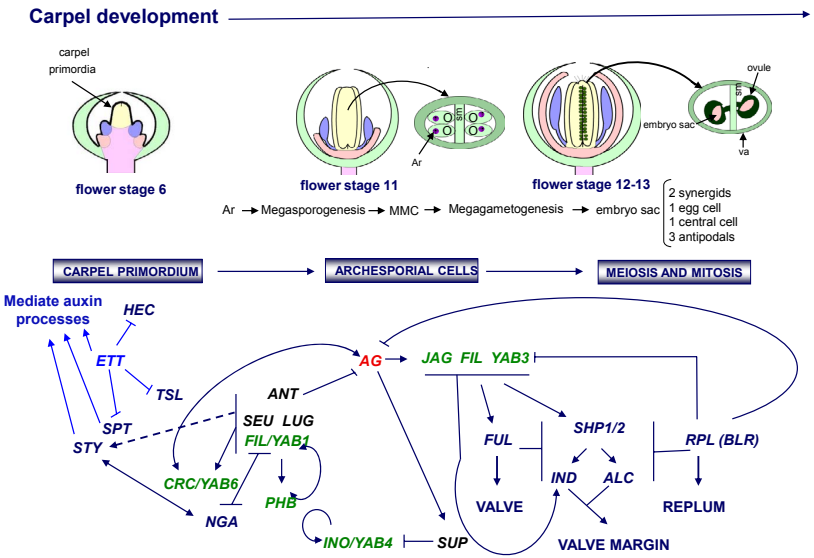


Figure 19. Main stages of carpel development and some genes involved.

Three different stages of carpel development are represented by the schemes in the upper part of the figure. Briefly, at stage 6, the central zone of the FM begins to grow upward and eventually will form the gynoecium. From stages 11 to 13, the ovule primordia (O) arise from the placenta flanking the medial ridges, and the Archesporial cell (Ar) develops from a single hypodermal cell at the ovule. The Ar then forms the megaspore mother cell (MMC) through megasporogenesis, and the MMC forms the embryo sac through megagametogenesis. The embryo sac consists of 2 synergids, 1 egg cell, 1 central cell and 3 antipodal cells. The medial ridges meet in the center of the fruit to form the septum (sm) which divides the gynoecium in two internal compartments. The mature gynoecium is externally formed by the fusion of two valves (va); internally, it also carries totally differentiated ovules each one containing its own embryo sac.

Carpel-specific gene networks are shown in blue. For genes and references not in the main text, see Table S1. Part of the network shown here was taken from Roeder and Yanofsky (2006) and Balanzá et al. (2006). Color codes of interactions and gene/floral organs are according to those of functional modules identified in Figure 15. Arrows and bars indicate positive and negative regulatory interactions, respectively.

Additional stamen or pollen microarray analyses have been performed recently. For example, a clear difference was found between the genes that are expressed in the sporophyte and in pollen with 39% of the expressed genes being pollen specific (Honys and Twell, 2003; Pina et al., 2005). The global gene expression profiles of wild-type reproductive axes have been compared to those of the floral mutants *ap3*, *spl/nzz*, and *ms1* in order to study gene expression during stamen development and microspore formation (Alves-Ferreira et al., 2007). The data suggest that different interconnected regulatory modules may control specific stages of anther and microspore development (for further details see: Amagai et al., 2003; Cnudde et al., 2003; Honys and Twell, 2003; Zik and Irish, 2003a; Wellmer et al., 2004; Pina et al., 2005; Alves-Ferreira et al., 2007; Verelst et al., 2007).

3.4.6 Carpels and ovules

Carpels are specified by the C gene *AG*, and the *SHP1, SHP2, and STK* genes (in an *AG* independent manner) together with the *SEP* genes (Bowman et al., 1989; Coen and Meyerowitz, 1991; Favaro et al., 2003; Pelaz et al., 2000; Pinyopich et al., 2003). They arise in the center of the flower meristem and when carpels are fully developed the floral meristematic cells are completely consumed. Carpels are the most complex structures within flowers and a GRN underlies their development (Figure 19; Table S1). Comprehensive reviews on carpel and fruit development can be found in Bowman et al., (1999), Ferrándiz et al., (1999), Balanzá et al., (2006) and in Roeder and Yanofsky (2006) in this book.

3.4.7 Nectaries

Little is known about the molecular genetics of nectary development. It is clear that nectaries are ABC-independent because *ap2-2 pi-1 ag-1* triple mutant flowers develop nectaries, although in these mutants nectaries are clearly smaller. However, ABC genes may play a role in nectary patterning as *pi-1 ag-1* and *ap3-3 ag-3* double mutants lack them (Baum *et al*., 2001). Also, single mutant *lfy* and *ufo* plants show reduced nectary formation (Lee *et al*., 2005a).

Several genes have been found to be expressed in the nectaries (e.g., *SHP1*, *ALC, SPL, MS35* and *XAL1*), but no detectable defect is observed in their respective mutants (Figure 5G; Schiefthaler et al., 1999; Roeder and Yanofsky, 2006; Yang et al., 2007b; Tapia-López et al., 2008). The only gene that has been clearly related to nectary development is *CRC,* which is also important for gynoecial development (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). The regulation of *CRC* in the nectaries seems to be independent of its expression in the gynoecium. Expression of this gene is already detectable at stage 6 of flower development in the area where the nectaries will be formed. Thus, *CRC* may be important for the early specification of nectary cells (Bowman and Smyth, 1999). *CRC* may also be necessary for maturation or maintenance of the nectaries, because it is expressed at high levels when they develop (Bowman and Smyth, 1999) and *crc* mutants have defects in nectary development. But *CRC* is not sufficient for nectary development, because its ectopic expression does not yield ectopic nectaries (Lee et al., 2005b). Bioinformatic and functional molecular genetic approaches have been taken to discover components of the regulatory network in which *CRC* participates during nectary and carpel development. A combination of floral homeotic gene activities acting redundantly with each other, involving *AP3*, *PI* and, *AG* and in combination with SEP proteins, activate *CRC* in both organs (Lee et al., 2005a). Interestingly in another study, *CRC* was also found to be a direct target gene of AG (Gómez-Mena et al., 2005) and to be indirectly regulated by *LFY* and *UFO* (Lee et al., 2005a). A model has been proposed in which *LFY* and *UFO* activate downstream MADS-box genes which could be working in conjunction with region-specific factors to activate *CRC* during nectary and carpel development (Lee et al., 2005a).

Evolutionary studies have indicated that the *CRC* gene may have been recruited as a regulator of nectary development in the core eudicot plant lineage, but its ancestral function could have been related to carpel development (Lee et al., 2005b).

4. THEORETICAL MODELS: INTEGRATIVE AND DYNAMIC TOOLS FOR UNDERSTANDING FLOWER DEVELOPMENT

As shown throughout this chapter, morphogenetic patterns underlying flower development arise from complex, often non-additive, interactions among several molecular and other kinds of components (e.g., cells) and factors (e.g., morphogen gradients, physical and geometrical constraints) at different levels of organization. Dynamical models can be used to study the concerted action of many elements at different spatio-temporal scales and levels of organization; an approach which is becoming both necessary and possible for understanding how morphogenetic patterns emerge and are maintained during development in general, and in particular, in flower development (for reviews Alvarez-Buylla et al., 2007; Grieneisen and Scheres, 2009). At the level of GRN, mathematical and computational models provide useful tools for integrating and interpreting molecular genetic information, or for detecting gaps and contradictions in the evidence for particular functional regulatory modules. At other levels, two or three-dimensional morphogenetic models of coupled GRNs within cells or among cells are useful for understanding spatiotemporal cell patterning in individual organs and overall plant architecture; and this enables novel insights into the mechanisms underlying developmental processes to be made. Such morphogenetic models are also a way of posing informed non-trivial predictions, testing hypotheses, uncovering potentially generic mechanisms underlying conserved features, and performing *in silico* investigations that guide novel experiments in biological development.

As the amount of experimental evidence increases and novel theoretical approaches and techniques develop, there continue to arise experimentally-grounded models of development and theoretical tools useful in posing predictions amenable to further experimental testing. These advances contribute to discussions of central issues in developmental and evolutionary biology (e.g., Kauffman, 1969; Berg et al., 2004; Milo et al., 2004; Wagner, 2005; Alvarez-Buylla et al., 2007; Balleza et al., 2008). In Arabidopsis, early flower development has been studied using dynamic gene regulatory network (GRN) models. Such models have helped capture the logic of gene regulation, mostly at the transcriptional level, during cell-type specification in various systems (e.g. von Dassow et al., 2000; Espinosa-Soto et al., 2004; Huang and Ingber, 2006; Li et al., 2006; Benítez et al., 2008). In this section we focus on this modeling approach and present some of the main results derived from network modeling in flower development.

4.1 Gene Regulatory Network Models

In this section we review some central notions in GRN theory and the main assumptions that are made and present some of the main results derived from network modeling in flower development. GRN models are composed of nodes, which stand for genes or proteins, and edges or connections, which represent the interactions among nodes (arrows for upregulation and bars for downregulation; for an example see Figure 20). Genes in the GRN model may take different activation states, depending on the activation states of their inputs. Given the architecture of the network and the sign of the interactions, it is possible to define a set of rules or kinetic functions that govern the GRN dynamics, that is, the way activation states of the genes change over time. These rules or kinetic functions may be defined and studied in the context of different mathematical frameworks, some of which have been thoroughly reviewed elsewhere (Gibson and Mjolsness, 2004; Alvarez-Buylla et al., 2007). In experimentally-based GRNs, the dynamic rules may be obtained from reported molecular genetics data as well as from functional genomics datasets.

The kinetic functions of gene activation depend on the states of the input nodes and are multivariate. These may be modeled with discrete or continuous functions. In the former, Boolean functions that allow only "0" (OFF; not expressed) or "1" (ON; expressed) values for the nodes have been successfully used

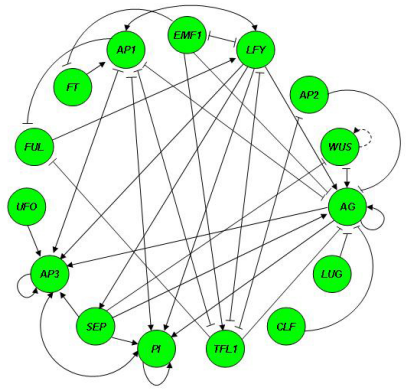


Figure 20. Floral organ specification gene regulatory network (FOS-GRN) model.

The diagram shows GRN topology where circles or nodes correspond to genes or proteins, and arrows and bars correspond to positive and negative regulatory interactions, respectively. The *SEP* node represents the *SEP1*, *2*, and *3* genes together. The interactions are updated with respect to previous publications (Espinosa-Soto et al., 2004; Chaos et al., 2006). The GRN attractors or steady states match the gene expression profiles that characterize inflorescence meristem regions and flower organ primordia. See text and Table 1 for details and experimental data supporting this model (and Table S2 for the dynamics truth tables). Dotted lines represent interactions predicted by the model.

to recover the key qualitative aspects of GRNs (e.g., Albert and Othmer, 2003; Espinosa-Soto et al., 2004). In Boolean networks, parameters of specific kinetic functions are not required. It is appropriate to assume that the GRN nodes are Boolean variables given that: (1) transcriptional regulation may be discrete and take place in the form of pulses, rather being continuous (Ross et al. 1994, Fiering et al., 2000, Ozbudak et al., 2002); (2) the experimental data at hand can be readily formalized as logical rules (see detailed discussions in Albert and Othmer, 2003; Espinosa-Soto et al., 2004; Chaos et al., 2006), while there are no or very few available data on parameters required to postulate continuous functions; and (3) in complex GRNs with many components interacting in non-linear manners, the overall topology of the GRN and the form of the logical rules of gene interaction, rather than the details of the kinetic functions, are what determine the qualitative network dynamics.

Independently of the mathematical formalism used, dynamical analyses of GRNs mostly focus on finding the steady gene activation profiles, that is, the configurations of the network that, once reached, remain in that configuration. These configurations are called *attractors*. The GRN model may be initialized on a particular gene-activation configuration known as an *initial condition* and then the elements of the GRN change their activation state according to the dynamic rules until they reach an attractor. Kauffman (1969) proposed that Boolean GRN attractors correspond to the activation profiles typical of different cell types and therefore that exploring the GRN architecture and dynamics is fundamental to understanding cell-type determination processes. This idea has now been verified experimentally and explored further (e.g. Albert and Othmer, 2003; Huang and Ingber, 2006; Alvarez-Buylla et al., 2007).

Another helpful notion in GRN dynamical studies is that of *basins of attraction*. Given the dynamic rules of the network, the set of initial conditions that lead to each of the attractors is known as its basin of attraction. As we discuss below, the concepts related to a GRN - attractor, initial condition and basin of attraction - may be useful in addressing some pertinent aspects of flower development.

4.1.2 Functional Modules in Flower Development

The functional data on genes involved in flower development reviewed in this Chapter suggest that several regulatory modules act at different stages and in different structures (Figures 9, 15-17 and 19). We define a regulatory module as a set of interacting genes that have more interactions among themselves than with other genes. These modules are semi-autonomous, meaning that their dynamic outcomes are fairly independent of other modules. In Figure 15 we have presented the best-studied modules associated with flower development. The approach described here for the functional module that includes the ABC genes could in principle be used for all of these modules as sufficient nodes have been identified and their regulatory interactions characterized. Eventually, models of coupled GRN that consider several such models together, both within and among cells, will be possible. For now, we have focused in just one such regulatory module.

In previous studies, we have proposed and analyzed the regulatory module, which includes the ABC genes as well as other components, that is sufficient to regulate the partitioning of the inflorescence and floral meristems into subregions of primordial cells. In the case of the flower meristem, each one of the four subregions is composed of the primordial cells that eventually give rise to each of the four types of floral organs: sepals, petals, stamens and carpels.

We use this functional module as a working example of the protocol that has been used in order to assemble an experimentally grounded gene regulatory network (GRN) model corresponding to a functional module. Then we demonstrate how once such a GRN model is postulated, it is possible to follow its dynamics, and explore how the concerted action of multiple interconnected molecular components eventually lead to stable gene expression profiles that may be compared to those characterizing different cell types. Then we delineate some theoretical approaches put forward to model coupled GRN dynamics that may underlie pattern formation and morphogenesis during the early stages of flower development, when the floral meristem is partitioned into four concentric rings of primordial cells. Finally, we review other modeling approaches that are useful to study signal transduction pathways.

4.2 Arabidopsis Flower Organ Specification GRN (FOS-GRN)

Soon after flowering is induced, the flower meristem is partioned into four concentric regions of primordial cells from which floral organs will later form. During the last decade, an experimentally-grounded GRN model for flower organ specification (FOS-GRN) has been built and investigated (Figure 20; Mendoza and Alvarez-Buylla 1998; Espinosa-Soto et al., 2004; Chaos et al., 2006; Alvarez-Buylla et al., 2008). This model incorporates the intricate regulatory interactions among ABC genes themselves and among ABC and non-ABC genes that are key to this process. This functional module includes: some key regulators underlying the transition from IM to FM (*FT, TFL, EMF, LFY, AP1, FUL*); the ABCs and some of their interacting genes (*AP1*, *AP3*, *PI*, *AP2*, *AG*, *SEP*); some genes that link floral organ specification to other modules regulating primordia formation and homeostasis (*AG*, *CLF* and *WUS)*; and some regulators of organ boundaries (*UFO* and *LUG*; Figures 9, 15 and 20).

The main result obtained from analyzing this GRN is that the postulated network converges to only ten attractors—even though it can be initialized in more than 130,000 different configurations of gene activation. Furthermore, the attractors—the stable configurations recovered—match gene activation profiles typical of the four inflorescence meristem regions (i.e., a region lacking *WUS* and *UFO*, two regions with either one of these two genes turned on, and a fourth region with both genes turned on; see Espinosa-Soto et al., 2004), as well as those of primordial sepal, petal, stamen and carpel cells (Figure 21). This shows that the FOS-GRN is sufficient to recover the gene activation profiles required to specify primordial cells during the first stages of flower organ development. Therefore the GRN itself constitutes a functional module that robustly leads to the gene configurations that characterize different regions of inflorescence and flower meristems during early flower development; and this independently of the activation states of additional genes that are connected to this elucidated regulatory module. Furthermore, various robustness analyses have been performed showing that the recovered attractors are also robust in response to permanent alterations in the logical functions of gene interactions and the inclusion of gene duplications. Therefore, these results (Espinosa-Soto, et al., 2004; Chaos et al., 2006) suggest that FOS dynamically and robustly emerges from complex networks of molecular components, rather than from a series of linear or hierarchical gene interactions or from the action of particular genes. The FOS-GRN model not only recovers the ABC gene combinations that are necessary for FOS, but it also provides a dynamic explanation for the formation of such gene combinations, and postulates a set of gene interactions with the ABC genes, that are also sufficient for FOS. The functions and interactions of the genes included are reviewed earlier in this chapter.

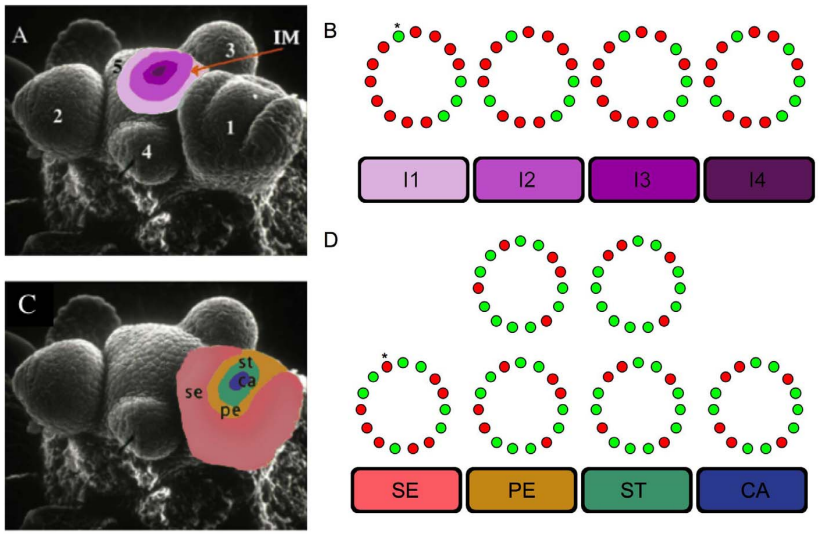


Figure 21. *Arabidopsis* inflorescence and flower development and FOS-GRN.

(A) SEM colored where four regions I1, I2, I3 and I4 are distinguished within the IM. FMs are also seen arising from the flanks of the IM,1 the oldest and 5 the youngest.
(B) I1, I2, I3 and I4 regions of the IM correspond to four of the FOS-GRN attractors. Expressed genes for each attractor are represented as green circles, while non-expressed genes correspond to red circles (nodes are in the same relative position as in Figure 20. * marks the position of the *EMF1* node for further reference). Note that this model recovers the respective regions in the IM with both *WUS* and *UFO*, with either one of these two genes, or with neither expressed.
(C) SEM colored to distinguish four types of primordial cells in young flower meristems. Each will eventually develop into the different flower organs, from the flower periphery to the center, sepals (se), petals (pe), stamens (st) and carpels (ca).
(D) The six attractors of the FOS-GRN model match gene expression profiles characteristic of sepal, petal (p1 and p2), stamen (st1 and st2) and carpel primordial cells. The gene activation profiles of the attractors are congruent with the combinatorial activities of A, B, and C genes described in the ABC model of floral organ determination (adapted from Alvarez-Buylla et al., 2008).

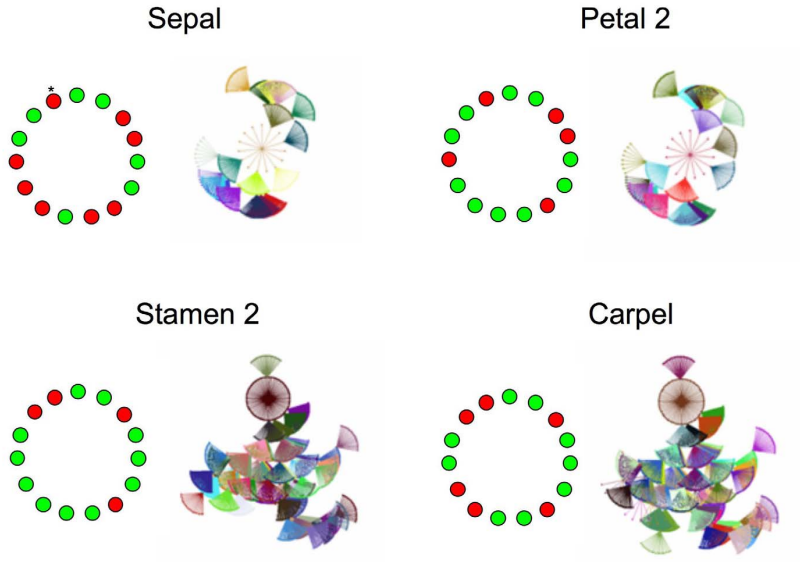


Figure 22. Basins of attraction for the four flower organ FOS-GRN attractors.

Attractors of FOS-GRN match the gene expression profiles of the four types of floral organ primordia of young floral buds (sepal, petal, stamen and carpel). The fan diagrams depict the GRN configurations (combinations of 0s and 1s corresponding to gene activation profiles) that lead to each of the attractors. Points in the outermost layers of these fan diagrams correspond to initial configurations of the network and they are linked to the transitory configurations. Petal2 and Stamen2 stand for one of the two possible attractors for each one of these organs. Relative position of nodes and their colors as in Figure 21. * marks the position of the *EMF1* node for further reference.

The FOS-GRN was validated by using this model to simulate the effect of loss-of-function mutations or overexpression, and comparing the results recovered from the model with the gene activation profiles determined experimentally in mutant or overexpressor lines. The mutants were simulated by fixing the state of the gene to 0 for loss of function, and to 1 for gain of function or overexpression (Figure 20; Table 1 and Table S2). In all cases tested, the simulated and empirically-reported profiles matched (Espinosa-Soto et al., 2004).

In addition, this GRN model has enabled investigations to be made into the sufficiency and necessity of particular gene regulatory interactions, which have led to novel predictions. For example, these analyses predicted that *AG* upregulated itself (Espinosa-Soto el al., 2004), which seemed somewhat counterintuitive at the time, but which was then verified by independent experiments

(Gómez-Mena et al., 2005). Also, computer simulations of the FOS-GRN that show that its attractors are robust to different types of perturbation and to duplications (Espinosa-Soto et al., 2004; Chaos et al., 2006) can account for the overall conservation of the flower structure throughout angiosperm (particularly eudicot) evolution (Rudall, 2007; Whipple et al., 2004; Adam et al., 2007).

Since the FOS-GRN model was based on thorough molecular data and is one of the few well-characterized regulatory modules, it has been used as a "model GRN" for further methodological, theoretical and conceptual developments in GRN and systems biology research (Table 2). The main conclusions obtained from the first versions of this GRN have been confirmed. New data regarding FOS are continuously being generated (novel data are also summarized in Table 1) and the FOS-GRN constitutes a basic theoretical framework in which to integrate it alongside previous data. Here, we have updated the FOS-GRN taking these novel data into account and conclude that the basic module originally put forward (Espinosa-Soto et al., 2004; Chaos et al., 2006) is robust to the addition of these newly discovered interactions. We consider, for instance, that *EMF1* downregulates *AG* (Calonje et al., 2008), and *AP3*/*PI* downregulate *AP1* (Sundström et al., 2006), so the postulated module seems to be robust to the addition of intermediary components or previously missing interactions.

Simulations of the updated FOS-GRN have been performed with the new software, ATALIA (http://www.ecologia.unam.mx/~achaos/Atalia/atalia.htm) developed in the Alvarez-Buylla laboratory by A. Chaos-Cador. This tool can be used to readily update this and other GRN models and explore their dynamics. We illustrate the use of this software with a visualization of the attractors' basins (Figure 22) and a simulation of the updated wild-type and certain mutant FOS-GRN dynamics (Figure 23).

In the simulated FOS-GRN, genes can take only two activation states: 0 for no expression and 1 for expression. Hence, by using combinations of 0s and 1s, we can describe all the possible initial conditions of the GRN. Figure 22 presents the so-called *fan diagrams* that show all the GRN configurations leading to each of the attractors. Knowing the relative sizes of the basins of attraction of each steady state is the key to exploring the robustness of each attractor in the face of perturbations.

ATALIA can also calculate the attractor that every possible initial condition will eventually reach and show this information in a tapestry of destinies. In such tapestries, each possible configuration of the GRN is represented by a square in a lattice and is colored according to the attractor it reaches. Moreover, ATALIA can draw a tapestry that represents the difference between the original wild-type tapestry and a mutant one (Figure 23). For example, if we want to know whether an *ap2* mutation has a more or less drastic effect in terms of the GRN dynamics than a *pi* mutation, we can analyze the tapestries of *ap2* and *pi* shown in Figure 23 and conclude that *ap2* mutation has stronger dynamic effects than *pi* given the GRN postulated up to now. Given the complexity of the network involved, such predictions are impossible to make without a tool like ATALIA. As the regulatory interactions in other modules that participate in flower development are gradually uncovered, for each one the experimental data can be exhaustively mined and formalized in the form of a GRN topology and logical rules governing its components' interactions. ATALIA can then be used to explore their dynamics, validate the proposed GRN models by simulating experimental reports of mutants or overexpressing lines, and to postulate novel interactions. Eventually, two or more functional modules may be interconnected via common components to postulate GRN aggregates. Such an approach will be useful in beginning to uncover the types of microtopological trait that characterize the nodes connecting different functional modules, for example.

We have illustrated the potential of using dynamic GRN models to understand cell differentiation using a relatively small and well-characterized module. Approaches used for small regulatory modules that are well-characterized in terms of molecular genetics, should feedback from functional genomic efforts that span the dynamics of a larger number of genes or proteins under diverse conditions and developmental stages or tissues.

4.2. Temporal and Spatial Patterns of Cell-fate Attainment During Early Flower Development

In real biological systems, populations of meristematic cells differentiate into different cell types in stereotyped temporal sequences and spatial patterns. The first primordial cells to be determined in the flower meristem are those of sepals, then those of petals, stamens and carpels going from the periphery to the center of the floral meristem. This suggests that in the population of meristematic cells the most probable temporal order in which each attractor is visited follows the same sequence (Alvarez-Buylla et al., 2008). Recent results from another theoretical approach show that the sequence of floral organ determination can be recovered by introducing some level of stochasticity (random noise) in the GRN dynamics, namely, a degree of error in the updating dynamical rules of the GRN (Alvarez-Buylla et al., 2008). These results are consistent with a handful of other recent studies showing that stochasticity at the molecular scale may contribute to the formation of spatiotemporal patterns in development (see review in Raj and van Oudenaarden, 2008). Studies with the stochastic version of the FOS-GRN also concluded that the relative position of the basins is important in determining the most probable temporal sequence of cell-fate attainment referred to above (Alvarez-Buylla et al., 2008). This fascinating result certainly suggests that the stereotypical temporal pattern of cell fate specification at early stages of flower development may be an emergent and robust consequence of the complex GRN underlying cell-fate determination and that, in principle, it could take place in the absence of inducing signals, emerging only as a result of the stochastic fluctuations that occur during transcriptional regulation (Alvarez-Buylla et al., 2008). Ongoing modeling efforts are explicitly focusing on spatial domains, and exploring the need and sufficiency of different cell-cell communication mechanisms or physical fields (e.g., created by curvature or tension forces) that could provide positional information for spatio-temporal cell patterning during early stages of flower development.

It is important to mention that the FOS-GRN modeled up to now is an abstraction of the qualitative regulatory logic underlying the IM and FM subregionalization during early stages of flower development when the ABC patterns are established. However, other regulatory modules for meristem positioning, growth and polarity, among others, still need to be considered in order to fully understand spatiotemporal cell patterning and morphogenesis of IM and FM. Some genes interacting with FOS-GRN components

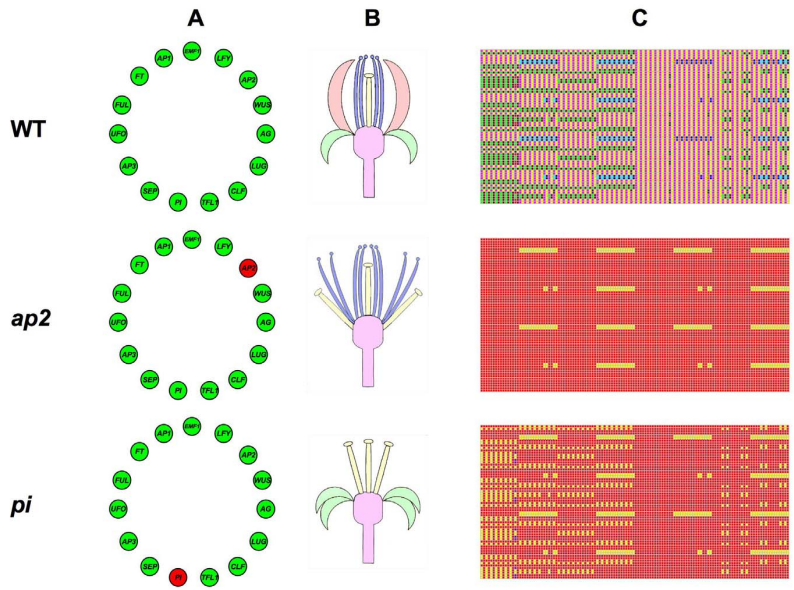


Figure 23. Simulation results for wild type (WT) and two mutants.

(A) Simplified representation of the FOS-GRN. The mutated genes are in red (nodes are in the same relative position as in Figure 20). Mutations were simulated by constitutively turning "off" (loss-of-function) mutated genes regardless of the dynamical rules.
(B) Floral diagrams showing floral organs of the simulated WT and mutant plants. These correspond to the steady-state gene expression arrays (attractors) attained in the simulation.
(C) Tapestries of gene configuration destinies corresponding to the simulated WT and mutant lines. In the WT simulation each square in the tapestry represents an initial condition and they are colored according to the attractor they eventually reach. In the mutant simulation for *ap2* and *pi*, the tapestries illustrate the difference between the WT tapestry of destinies and that obtained for the mutant simulations. Yellow squares, configuration attained is the same attractor as in the WT; red squares, configurations that reached a new attractor; purple squares, configurations that attained a pre-existing attractor but not the same one reached in the WT simulations. Images generated with ATALIA (http://www.ecologia.unam.mx/~achaos/Atalia/atalia.htm).

(e.g. *AGL24*, *BEL*, *RBE* and those described in the last section of Table 1) that do not seem to directly affect cell-type determination in the floral meristem, could link the FOS-GRN with: a) signaling pathways (e.g. Díaz and Alvarez-Buylla, 2006); b) genes involved in cell growth and proliferation both before and after the partitioning of the floral meristem into the four concentric regions; and c) other types of downstream genes or modules that are important during cell sub-differentiation and organogenesis at later stages of flower development.

A complete understanding of flower morphogenesis will continue to require multidisciplinary approaches and modeling tools that help unravel how such single-cell GRNs are coupled in explicit cellularized spatial domains and physicochemical fields (e.g. Jönsson et al., 2005, Savage et al., 2008; Benítez et al., 2008), including metabolism, signaling, and emergent gradients of morphogens (e.g., auxin), cell growth and proliferation, mechanical forces and cell-cell communication mechanisms. All of these are likely to feedback in non-linear ways from and to the GRNs underlying cell differentiation or proliferation (for example see Hamant et al., 2008).

It is important to keep in mind, for example, that plant cell growth in meristems is symplastic. This implies that the contacts

Table 1. Summary of evidence for the FOS-GRN gene interactions shown in Figures 20-23 (ChIP, chromosome immunoprecipitation; EMSA, electrophoretic mobility shift assays; arrows indicate gene induction and bars repression; Espinosa-Soto et al., 2004; Chaos et al., 2006).

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
AG (AT4G18960) → *AG*	ChIP shows that AG interacts *in vivo* with predicted regulatory sequences of *AG* .	Gómez-Mena et al., 2005.
AP1 (AT1G69120) --| *AG*	Sepals are replaced by carpels, and petals by stamens in *ap1* mutants. *AG* mRNA found in all flower primordia of *ap1-1* plants. First whorl organs are sometimes carpelloid, and second whorl organs are staminoid in *ap1* mutants.	Bowman et al., 1993; Weigel and Meyerowitz, 1993; Liu and Meyerowitz, 1995.
CLF (AT2G23380) --| *AG*	In *clf* mutants, first whorl sepals are frequently carpelloid, second whorl organs are staminoid petals and *AG* mRNA is detected in sepals. It is likely that CLF is part of a complex with EMF2, MSI1, and FIE that epigenetically regulate *AG*.	Goodrich et al., 1997; Calonje et al., 2008.
LFY (AT5G61850) → *AG*	Expression of *AG* is reduced in *lfy-6* flowers. The expression of *LFY* fused to a strong activation domain produces increased and ectopic AG expression. LFY binds to the first intron of *AG*, and cooperates with the WUS homeodomain to activate it.	Weigel and Meyerowitz, 1993; Parcy et al., 1998; Busch et al., 1999; Lohmann et al., 2001.
LUG (AT4G32551)--| *AG*	*AG* is ectopically expressed in *lug-1* mutants. LUG functions as a repressor of *AG* via its the second regulatory intron.	Liu and Meyerowitz, 1995; Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000; Gregis et al., 2006.
SEP3 (AT1G24260) → *AG*	There is *AG* expression in rosette leaves of *35S:SEP3* plants. In addition, *35S:AG 35S:SEP3* plants have more pronounced carpelloid features.	Castillejo et al., 2005.
TFL1 (AT5G03840) --| *AG*	Stigmas and styles of terminal flowers in *lfy ap1* double mutants are normal if the *tfl1* mutation is added.	Shannon and Meeks-Wagner, 1993.
WUS (AT2G17950) → *AG*	*wus* mutants lack carpels and most stamens. In *AP3:WUS* transgenic plants, second whorl organs are carpelloid stamens instead of petals, whereas in *AP3:WUS* ag plants, second and third whorl organs do not differentiate into carpelloid stamens.	Laux et al., 1996; Lenhard et al., 2001; Lohmann et al., 2001.
AG --| *AP1*	*AP1* mRNA accumulates uniformly in *ag-1* mutant flowers.	Gustafson-Brown et al., 1994.
FT (AT1G65480) → *AP1*	In *ft lfy* double mutants, there is no *AP1 mRNA* unlike in the respective single mutants, suggesting that at least one of these two genes needs to be active for *AP1* activation	Ruiz-García et al., 1997.
LFY → *AP1*	*AP1* expression is delayed in *lfy-6* null mutants, ectopic in *35S:LFY* plants and increased when *LFY-VP16* is induced. LFY directly binds the *AP1* promoter and activates this gene.	Parcy et al., 1998; Liljegren et al., 1999. Weigel and Nilsson, 1995; Wagner et al., 1999.
TFL1 --| *AP1*	In *tfl1* mutants, *AP1* is ectopically expressed in the basal lateral meristems and in terminal flowers. *AP1* expression is also retarded in *35S:TFL1*	Gustafson-Brown et al., 1994; Ratcliffe et al., 1998.
TFL1 --| *AP2* (AT4G36920)	The absence of petals in *tfl1 ap2* flowers and the presence of petals in *tfl1* single mutants suggest there is ectopic *AP2* activity in the terminal flowers of *tfl1* single mutants.	Shannon and Meeks-Wagner, 1993.
AG → *AP3* (AT3G54340)	There is weaker *GUS* expression in the third whorl of *ag-1 AP3:GUS* flowers than in the transgenic control. *AG* may maintain *AP3* expression because cauline leaves of *35S:PI 35S:AP3 35S:SEP3 35S:AG* are converted into stamen-like organs. ChIP shows that AG interacts *in vivo* with predicted regulatory sequences of *AP3*. Also, *AP3 RNA* is absent from the center of the *ag-1* meristem.	Hill et al., 1998; Honma and Goto, 2001; Gómez-Mena et al., 2005; Zhao et al., 2007.
AP1 → *AP3*	*AP3* expression is quite normal in *ap1* mutants but is almost undetectable in *lfy ap1* double mutants, indicating that *AP1* can act with *LFY* to regulate *AP3* expression. Futhermore, AP1 seems to bind *AP3 cis*-regulatory elements.	Weigel and Meyerowitz, 1993; Hill et al., 1998; Ng and Yanofsky, 2001; Lamb et al., 2002.
AP3 → *AP3*	Endogenous *AP3* is upregulated in *35S:AP3-GR* plants induced with dexamethasone, supporting the notion that *AP3* self-activates.	Hill et al., 1998; Honma and Goto, 2000.

(Continued)

Table 1. (continued)

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
LFY+UFO (AT1G30950) → *AP3*	Both the amount and the domain of *AP3* expression are reduced in *lfy-6* mutants. *ufo-2* plants have less *AP3* protein and less AP3 mRNA. Both *LFY* and *UFO* have to be overexpressed to induce ectopic expression of *AP3*. EMSA show that LFY binds directly to sequences in the *AP3* promoter. ChIP shows that UFO associates with the *AP3* promoter. This association was abolished when ChIP was performed using extracts from *lfy-26* plants harboring the *35S:UFO-Myc* transgene.	Weigel and Meyerowitz, 1993; Meyerowitz, 1995; Parcy et al., 1998; Lamb et al., 2002; Levin and Chae, 2008.
SEP (AT5G15800, AT3G02310, AT1G24260, AT2G03710) → *AP3*	In *AP3:GUS 35S:PI 35S:AP3 35S:AP1* mutants, AP3-GUS is expressed throughout the plant supporting the idea that full activation of the B-function genes requires tetramer formation to include SEP. The ectopic expression of *SEP3* resulted in the induction of ectopic *AP3* expression. Stronger *35S:SEP3* lines are also capable of activating *AP3:GUS* ectopically	Honma and Goto, 2001; Castillejo et al., 2005.
LFY --| *EMF1* (AT5G11530)	Ectopic *LFY* expression in *emfl-1* mutants increases the severity of the *emf* phenotype.	Chen et al., 1999.
EMF1 --| *FT*	*FT RNA* levels are higher in the *emf1-1* mutant and are detected earlier than in the wild type.	Moon et al., 2003.
AP1 --| *FUL* (AT5G60910)	*FRUITFULL* is ectopically expressed in *ap1* mutants.	Mandel and Yanofski, 1995b; Ferrándiz et al., 2000a.
TFL1 --| *FUL*	TFL1 has been postulated to be an inhibitor but it also is possible that other factors have this posttranscriptional inhibitory role. This interaction is necessary as when the negative posttranscriptional regulation of FUL by TFL1 is not considered, the nonfloral gene steady states disappear. No experimental evidence.	Espinosa-Soto et al., 2004.
AP1 → *LFY*	In *ap1* and *ap1 cal* double mutants, *LFY* expression is reduced. Additionally, *LFY* is activated earlier in *35S:AP1* plants than in the wild type.	Bowman et al., 1993; Kempin et al., 1995; Weigel and Nilson, 1995; Piñeiro and Coupland, 1998; Liljegren et al., 1999.
EMF1 --| *LFY*	Double mutants of the weak *emf1-1* allele and *lfy-1* bear *lfy*-like flowers suggesting that, for this trait, *lfy* is epistatic. These genes have antagonistic activities.	Yang et al., 1995.
FUL → *LFY*	Even though *LFY* expression is similar in wild type and *LFY:GUS ful-2* plants, there is less expression in *ful ap1 cal* triple mutants than in *ap1 cal* double mutants, suggesting that the role of *FUL* in *LFY* upregulation is only important when *AP1* is inactive.	Ferrándiz et al., 2000a.
TFL1 --| *LFY*	In *tfl1* mutant plants *LFY* is ectopically expressed in the shoot apex.	Weigel et al., 1992; Ratcliffe et al., 1999.
LFY→ *PI* (AT5G20240)	Amount and domain of *PI* expression are reduced in *lfy-6* mutants. There is no *GUS* expression in early *lfy PI:GUS* flowers.	Weigel and Meyerowitz, 1993; Honma and Goto, 2000.
PI → *PI*	AP3 and PI co-immunoprecipitate. *AP3* and *PI* mRNA levels are not maintained in *ap3-3 pi-1* double mutants. In *AP3:GUS 35S:PI 35S:AP3 35S:AP1* mutants, *AP3:GUS* is expressed throughout the plant supporting the idea that full activation of the B-function genes requires PI	Jack et al., 1992; Goto and Meyerowitz, 1994; Honma and Goto, 2001.
LFY → *SEP*	Microarray experiments show that the group of *LFY* dependent genes includes the homeotic cofactors *SEP1-3*.	Schmid et al., 2003.
AP1 --| *TFL1*	In *35S:AP1*, *TFL1* expression is greatly diminished. *TFL1* is ectopically expressed in *ap1 cal* double mutants.	Liljegren et al., 1999.
AP2 --| *TFL1*	The *tfl1-1* mutation partially suppresses the *ap2-1 ap1-1* inflorescence phenotype.	Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993.
EMF1 → *TFL1*	In *emf1-2 tfl1* double mutants, the *emf1-2* mutation is epistatic with respect to flower initiation. These genes do not have antagonistic activities. This suggests that *EMF1* upregulates *TFL1*.	Chen et al., 1997.
LFY --| *TFL1*	The *35S:LFY* plants resemble the *tfl1* mutant and have no *TFL1* expression. *LFY* can inhibit *TFL1* at the transcriptional level. *TFL1* is also ectopically expressed in *lfy* mutants.	Weigel and Nilsson, 1995; Liljegren et al., 1999; Ratcliffe et al., 1999.
AG --| *WUS*	There is strong *WUS* expression in the center of *ag* floral meristem.	Lenhard et al., 2001; Lohmann et al., 2001.

Table 1. (continued)

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
SEP --| *WUS*	*SEP* activity is required for *WUS* downregulation by *AG* because *sep1 sep2 sep3* triple mutant plants bear indeterminate flowers.	Pelaz et al., 2000.
WUS → *WUS*	No experimental evidence. Assumption of model.	Espinosa-Soto et al., 2004; Chaos et al., 2006
UPDATES (Chaos et al., 2006 and this chapter)		
EMF1 --| *AG*	In ChIP experiments, *EMF1* is associated with sites in the promoter and second intron of AG. *EMF1* interferes with transcription by RNA polymerase II and T7 RNA polymerase *in vitro*.	Calonje et al., 2008.
AP3 --| *AP1*	*AP1* transcript levels are significantly higher in *ap3-3* mutant plants than in both *WT* and *35S:AP3*.	Sundström et al., 2006.
PI --| *AP1*	ChIP shows that PI binds to target sequences in the *AP1* promoter	Sundström et al., 2006
MiR172 (AT2G28056, AT5G04275, AT3G11435) + *HEN1* (AT4G20910) --| *AP2*	Elevated *miR172* accumulation results in floral organ identity defects similar to those in loss-of-function *ap2* mutants. On the other hand, the *miR172* abundance depends on the activity of DICER-like protein HUA ENHANCER 1 (HEN1), which is expressed through the plant. This observation suggests that a cofactor expressed in the inner floral whorls is required to give specificity to the HEN1-dependent repression of *AP2*. The need for *AG* inactivity for *AP2* function is added to the *AP2* logical rules	Chen et al., 2002; Park et al., 2002; Chen et al., 2004; Zhao et al., 2007.
LFY → *SEP1-3*	Microarray experiments show that the group of *LFY* dependent genes includes the homeotic cofactors *SEP1-3*.	Schmid et al., 2003.
INTERACTIONS NOT INCLUDED IN THE MODEL		
AGL24 (AT4G24540) + *SVP* (AT2G22540) --| *AG*	In the *agl24 svp* double mutant, *AG* mRNAs are detected in the inflorescence and floral meristems as early as stage 1, indicative of early *AG* expression. In later stages, *AG* is still expressed in all floral organs. Probably, this interaction is part of a different GRN that ocurs before the cell fate determination	Gregis et al., 2006.
BLR (AT5G02030) --| *AG*	*AG* is expressed ectopically in *blr* mutants. *BLR* directly binds to *AG cis* elements (identified by EMSA). This interaction is probably important in organogenesis.	Bao et al., 2004.
RBE (AT5G06070) --| *AG*	In *rbe* mutants, there is ectopic expression of *AG* in second-whorl cells. This interaction may be important in organogenesis.	Krizek et al., 2006.
SEU (AT1G43850) --| *AG*	The direct *in vivo* association of SEUSS (SEU) with the *AG cis*-regulatory element was shown by ChIP. SEU interacts with LUG in a repressor complex to regulate *AG*, and LUG is already considered in the GRN model.	Sridhar et al., 2006.
AGL24+SVP --| *AP3*	An *in situ* analysis shows that in the *agl24 svp* double mutant, *AP3* is expressed in all parts of the floral meristem and later in all floral organs. Probably, this interaction is part of a different GRN occurring before the cell fate determination.	Gregis et al., 2006.
LFY → *CAL*(AT1G26310)	Using posttranslational activation of LFY-GR, it is demonstrated that CAL is a direct LFY target. cis-regulatory elements in the putative CAL promoter are bound by LFY. AP1 forms heterodimers with CAL and AP1 is already included.	William et al., 2004.
AP3 --| *FUL*	The domain of *FUL* expression is expanded to the third whorl in stage-3 *ap3* mutants, but no direct interaction is detected by ChIP analysis.	Mandel and Yanofsky, 1995b; Sundström et al., 2006.
FT --| *FUL*	*FUL* is expressed at higher levels in 35S:FT-VP16. It is not considered because this interaction could be mediated by TFL1 and LFY.	Teper-Bamnolker and Samach, 2005.
PNY (AT5G02030) → *LFY* *PNF* (AT2G27990) → *LFY*	The transcripts of *LFY* are substantially reduced in shoot apices of *pny pnf* double mutants after floral induction. *pny pnf* double mutants do not produce flowers but, *35S:LFY pny pnf* plants do produce flowers. This interaction is part of a different GRN.	Anrar et al., 2008.
AP2 → *PI*	*In situ* hybridization shows there is less *PI RNA* occupying a smaller area in *ap2-2* flowers than in wild type. Probably an indirect effect.	Zhao et al., 2007.
AG --- *SEP3*	ChIP shows that AG interacts *in vivo* with predicted regulatory sequences of *SEP3*. Insufficient experimental data.	Gómez-Mena et al., 2005.
FT → *SEP3*	Overexpression of *FT* causes ectopic expression of *SEP3* in leaves. No further experimental evidence.	Teper-Bamnolker and Samach, 2005.

Table 2. Some of the contributions that have used the flower organ specification GRN model in order to test, advance or discuss novel conceptual or methodological approaches.

Contribution	Reference
Logical analysis of the flower organ specification (FOS) GRN.	Mendoza et al., 1999
Introduction of the transsys formalism to represent GRN and implementation of FOS-GRN in this framework.	Kim, 2001
Method for gene network inference based on nonlinear differential equations and logical approaches. Predictions were tested using FOS-GRN.	Perkins et al., 2004
New method for automatically inferring gene regulation functions modeled as logical functions. The method is applied to FOS-GRN.	Bozek et al., 2006
Automatic Petri-net-based method, applied to FOS-GRN, for finding stationary states.	Gambin et al., 2006
Analysis of the dynamic role of feedback loops in networks including FOS-GRN.	Kwon and Cho, 2007
Application of the GenYsis software to model the discrete and multiple valued FOS-GRN.	Garg et al., 2007
Analysis of the effect of feedback loops on the robustness of Boolean networks, such as that of flower organ specification.	Kwon and Cho, 2008
Dynamic study of FOS-GRN and other GRNs with the finding that these exhibit a property known as criticality.	Balleza et al., 2008
Formal analysis of the main sources of perturbation and their effects in biological regulatory networks, with the FOS-GRN as example.	Demongeot et al., 2008

between cells are preserved because there is no displacement or sliding at middle lamellas that join neighboring cells (Priestley, 1930 and Erickson, 1986; cited in Kwiatkowska, 2008). Therefore, overall plant growth could be modeled using the principles of solid body mechanics (see review in Kwiatkowska, 2008). However plant cells also grow anisotropically which implies a variation in the directional growth rates at a given point (Baskin, 2005). Hence, meristem growth has rather been modeled using the principles of continuum mechanics, computing variables that characterize plastic strain (Goodall and Green, 1986; for review see Green, 1999).

Some quantitative mesoscopic models for flower development and growth in Arabidopsis and other angiosperms have been put forward (e.g., Rolland-Lagan et al., 2003; Lee et al., 2004; Skryabin et al., 2004; Mündermann et al., 2005). A finite element model of the SAM has also shown, for example, that lateral bulging of the meristem surface leading to the formation of a primordium results in a gradient of shear stresses with high shear stress at the point where the future primordium emerges (Selker et al., 1992; reviewed in Kwiatkowska 2008). More recently, it was shown that cells in the Arabidopsis SAM orient their cortical microtubules along lines of mechanical stress generated during tissue formation, and this then affects the mechanical properties of the cell, thus establishing a feedback loop (Hamant et al., 2008). This seems to be particularly relevant during the formation of the groove between the apical meristem and the primordium of lateral organs, but less so during growth and differentiation, because the lateral organ primordia are not affected when the microtubular network is disintegrated by a drug (Hamant et al., 2008). This implies that the mechanical feedback loop described is likely to act in parallel with the previously described auxin-mediated patterning mechanism (Laufs et al., 2009). Similar morphogenetic mechanisms are likely to be at work in flower meristem and floral organ development, and both morphogenetic mechanisms connected to the functional regulatory modules, including FOS-GRN and others that have been partly elucidated and reviewed in this Chapter.

5. CONCLUSIONS AND PERSPECTIVES

Arabidopsis has been indispensable in unraveling the molecular genetic bases of the stereotypical and most conserved aspects of flower development. It has also been used to resolve some basic mechanisms of floral meristem determination, as well as floral organ cell differentiation and morphogenesis. The challenge ahead will be to understand how modules regulating each aspect of flower development are interconnected among themselves and with signal transduction pathways that respond to environmental and internal cues to yield coupled GRN spatiotemporal dynamics during flower development. Such dynamics likely involve feedback from physical or mechanical forces, structural and geometric characteristics of domains of activity and from cell dynamics (cell growth and division) in complex ways still requiring multiple theoretical multilevel models and coordinated experimental research. Different functional modules are now being characterized (Figure 24 and Table S1) and shown to regulate some of the main processes involved in flower development. Some of these modules or their components may participate in one or more flower developmental process and data on the functions and interactions of genes are becoming available to enable new dynamic computational models of GRN and signaling pathways during flower development (Figure 24 and Table S1).

Computational models for the gene regulatory module that underlies patterning of the inflorescence meristem and determination of the primordial cell types during early stages of flower organ specification, have demonstrated the potential and need of theoretical dynamic approaches in understanding complex GRN underlying flower development. But information on each regulatory module and the interconnections between modules and with signal transduction pathways is still scanty.

It would be fascinating to unravel which molecular components, circuits, or sub-networks underlie the development and evolution of the diversity of flower forms and the variations

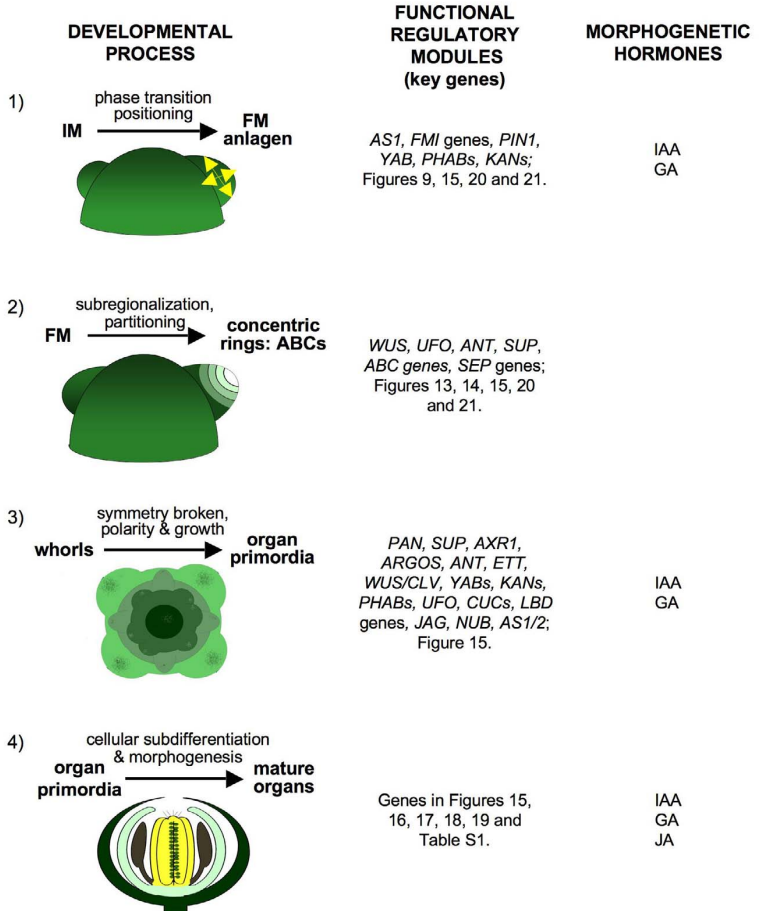
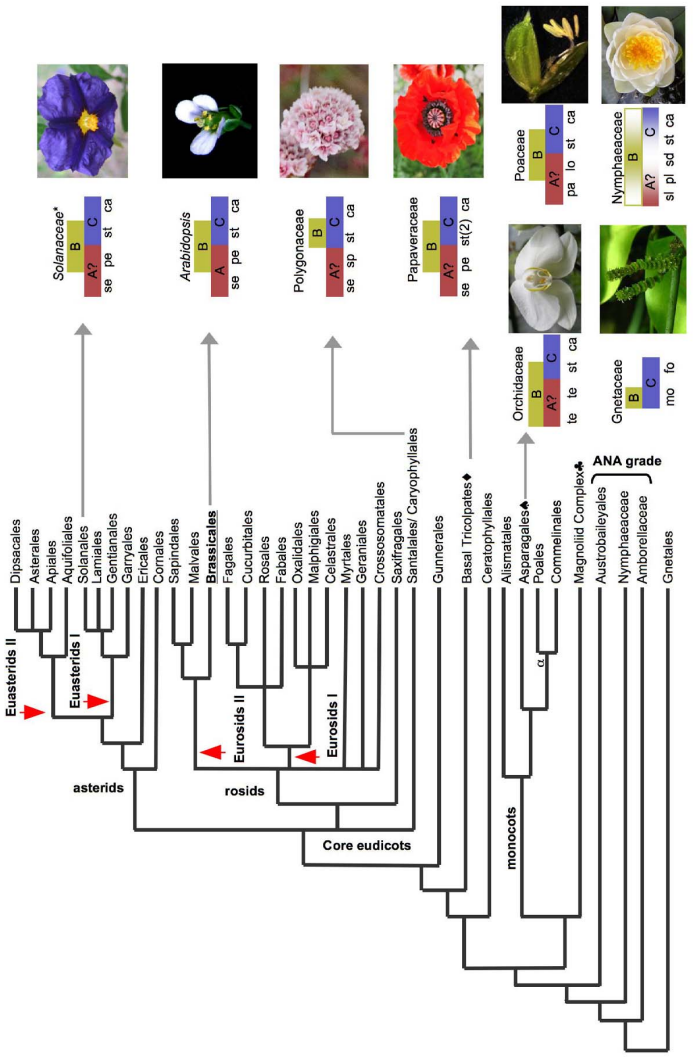


Figure 24. The main regulatory gene modules and hormone signaling pathways during flower developmental processes.

Four main developmental processes in flowers shown schematically from FM formation to mature flower formation. 1) Specification of the floral meristem anlagen. To initiate this process, FMI genes like *LFY* and *AP1* are upregulated. However the position and polarity of these meristems are determined by other gene families and hormones like auxin (IAA) and gibberellins (GA). 2) Specification of whorls of organ primordia. The ABC identity genes and *SEP* are necessary and, together with other genes, sufficient to specify floral organ primordial cells (FOS-GRN module). 3) Organ primordia cell proliferation, boundary establishment and organ polarity are regulated by additional modules that are presumably coordinated during floral organ primordia formation. 4) Cellular differentiation and organ morphogenesis yield the final shape, size and tissue composition of functional sepals, petals, stamens and carpels.



around the overall conserved "theme" of floral structure among angiosperms. This will be possible with integrated multidisciplinary approaches addressing pending questions. For example, in order to understand how a flower meristem forms will require knowledge of the regulatory mechanisms underlying mechanoreception and cell wall, microfibril and microtubule behaviour. How are such mechanisms interconnected or coordinated with the cell differentiation GRNs as well as with the morphogen-mediated patterning mechanisms? The challenge ahead consists in integrating mesoscopic mechanical and morphogen-gradient models with experimentally grounded models of the GRNs underlying cell behaviour, dynamics and differentiation. The aim is to build multilevel computational modeling frameworks that can be used to test the sufficiency and necessity of contrasting mechanisms, which scale from the biochemical and GRN level to the physical factors constraining plant growth (Hogeweg, 2002). Ideally, joint efforts in modeling, bioinformatics and experimentation continually feeding back on each other should give a better understanding of flower, and more generally, plant development and evolution.

Notwithstanding the usefulness of Arabidopsis, such a grand challenge will surely benefit or require comparative experimental and evolutionary studies of other angiosperms with divergent floral structures such as the monocots, other eudicots and basal angiosperms. Such an approach has been successful in understanding and interpreting morphological traits of plants (Kaplan, 2001). Recently, studies in non-model monocots such as orchids (Tsai et al., 2004; Xu et al., 2006) and commelinids, (Ochiai et al., 2004), in maize and rice (Whipple et al., 2004; Xu and Kong, 2007), in members of the Solanaceae, such as tomato (Hileman et al., 2006; de Martino et al., 2006), and in basal angiosperms (Soltis et al., 2007) among others, have started to demonstrate the power of coupling functional and evolutionary questions of a comparative approach with detailed molecular experimentation in several species.

Findings from diverse groups of angiosperms, mostly comparative analyses of ABC gene expression data among diverse angiosperm groups (especially basal angiosperm taxa), with emphasis on the A and B class genes, have already been used to account for the underlying genetic differences in the diversity of petal and stamen morphology among extant flowering plants (Kim et al., 2005; Rudall, 2007). Figure 25 shows a diagrammatic and very simplified angiosperm phylogeny and the variations observed in the domains of expression of the ABC class genes in selected species, representative of the morphological diversity present in their respective angiosperm lineages. Overall, these approaches are helping refine our knowledge of flower development, and will be instrumental in understanding the canonical GRN modules involved in flower formation and discovering variations.

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Figure 25. Angiosperm phylogeny and schematic representation of ABC gene expression patterns of selected taxa.

Schematic phylogeny based on APGII (2003) conventions with variations in the ABC model among angiosperm groups shown (see section 3.3). We present all rosids and asterids, but taxa comprising basal angiosperms, the magnoliid complex, monocots and core eudicots have been compacted and simplified. *Arabidopsis thaliana* belongs to the order Brassicales (bold and underlined). In the ABC model, the A function for sepal specification is maintained for all groups, although the class A genes involved in Arabidopsis are not functionally conserved for other taxa and may not be separable from floral meristem determination. The A function for all lineages was kept to enable comparison with Arabidopsis although a question mark was added to underline its dubious role. For B function, it should be noted that B class genes have undergone extensive duplications within different angiosperm lineages; while these duplications do not affect overall B function, on occasion they implicate subfunctionalization of the resulting paralogs (Irish and Litt, 2005; Soltis et al., 2007). For example, in species of Solanaceae such as tomato (de Martino et al., 2006) and petunia (Vandenbussche et al., 2004), and in the majority of eudicot taxa in which B function expression has been analyzed, two copies of the *AP3* gene are found that have undergone subfunctionalization, *AP3* and *TM6*. Specified floral organs are indicated underneath each ABC model (Theissen and Melzer, 2007). Abbreviations: male organs (mo); female organs (fo); sepal-like tepals (sl); petal-like tepals (pl); staminodes (sd); stamens (st); carpels (ca); petaloid tepals (te); petals (pe); palea/lemma (pa); lodicules (lo); sepals (se); sepaloid petals (sp). Symbols used to refer to compacted plant lineages are: Basal tricolpates (♦), including orders Ranunculales and Proteales and families Buxaceae, Sabiaceae and Trochodendraceae; Asparagales (▲) including Dioscorales, Liliales and Pandanales; (a) the Commelinid grade that, in addition to Poales and Commelinales, includes Dasipogonaeae, Arecales and Zingiberales; the Magnoliid complex (♣) including Canellales, Piperales, Laurales and Magnoliales. Images of rice spikelet, *Nymphaea alba* and the male *Gnetum gnemon* reproductive structure were taken from Yale Virtual Centre for Cellular Expression Profiling of Rice http://bioinformatics.med.yale.edu/riceatlas/anatomy.jspx; http://commons.wikimedia.org/wiki/Image:Nymphaea_alba.jpg and http://commons.wikimedia.org/wiki/Image:Gnetum_gnemon_male.jpg respectively.

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B-Function Expression in the Flower Center Underlies the Homeotic Phenotype of *Lacandonia schismatica* (Triuridaceae) [C][W][OA]

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Spontaneous homeotic transformations have been described in natural populations of both plants and animals, but little is known about the molecular-genetic mechanisms underlying these processes in plants. In the ABC model of floral organ identity in *Arabidopsis thaliana*, the B- and C-functions are necessary for stamen morphogenesis, and C alone is required for carpel identity. We provide ABC model-based molecular-genetic evidence that explains the unique inside-out homeotic floral organ arrangement of the monocotyledonous mycoheterotroph species *Lacandonia schismatica* (Triuridaceae) from Mexico. Whereas a quarter million flowering plant species bear central carpels surrounded by stamens, *L. schismatica* stamens occur in the center of the flower and are surrounded by carpels. The simplest explanation for this is that the B-function is displaced toward the flower center. Our analyses of the spatio-temporal pattern of B- and C-function gene expression are consistent with this hypothesis. The hypothesis is further supported by conservation between the B-function genes of *L. schismatica* and *Arabidopsis*, as the former are able to rescue stamens in *Arabidopsis* transgenic complementation lines, and Ls-AP3 and Ls-PI are able to interact with each other and with the corresponding *Arabidopsis* B-function proteins in yeast. Thus, relatively simple molecular modifications may underlie important morphological shifts in natural populations of extant plant taxa.

INTRODUCTION

An ABC Model–Based Hypothesis of the Developmental Genetic Factors Underlying the Unusual Reproductive Morphology of *Lacandonia schismatica*

The ABC model for the specification of floral organ identity (Bowman et al., 1991; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991) has played a critical role in the modern explanation of the molecular-genetic determinants of the ontogenetic development of reproductive structures in angiosperms. This combinatorial genetic model has guided diverse plant evolutionary developmental biology studies, especially during the formative years of this young field (Cronk, 2001; Cronk et al., 2002; Pruitt et al., 2003). As part of the larger discipline of evo-devo, the articulation of explanatory tools like the ABC model is considered essential for understanding the developmental mechanisms that underlie morphological innovation throughout evolutionary time (Cronk et al., 2002; Carroll et al., 2004; Gilbert, 2006; Wolpert et al., 2006).

The ABC model of flower development was based on the interpretation of floral homeotic phenotypes in *Arabidopsis thaliana* and *Antirrhinum majus* (Coen and Meyerowitz, 1991). However, the participation of homeotic transformations and other forms of heterotopy in the appearance of new organ arrangements during the evolution of angiosperms had already been considered in the botanical literature, long before the age of plant developmental genetics (e.g., Meyer, 1966; Sattler, 1984; Bowman et al., 1989; Weston, 2000). Outstanding instances of spontaneous floral homeotic phenotypes have continued to be recorded in well-characterized taxa (see, for instance, the examples presented in Bateman and DiMichele [2002] for the orchid genus *Ophrys*). So far, however, experimental data on the

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molecular mechanisms involved in the morphogenesis of plant species with naturally occurring homeotic morphologies is scarce.

In this artilce, we present molecular-genetic data collected from the Mexican endemic triurid *Lacandonia schismatica* (Triuridaceae), a mycoheterotrophic monocotyledonous plant with bisexual (i.e., hermaphroditic) reproductive axes, which might represent an unusual case of homeosis/heterotopy involving the male and female floral whorls. According to its original taxonomic description, *L. schismatica* represents the single example of a well-established angiosperm species in which the central floral whorl, composed of stamens, is surrounded by a peripheral gynoecium (Martínez and Ramos, 1989).

Observations of reproductive axes in the putative sister taxon of *L. schismatica*, the neotropical triurid, *Triuris brevistylis*, have also indicated the presence of this particular homeotic arrangement; however, such flowers are very rare and do not constitute an established floral morph in the wild (Vergara-Silva et al., 2003). To our knowledge, a peculiar teratology in the alismatid *Echinodorus amazonicus* (Sattler and Singh, 1978) is the only additional record of a floral homeotic/heterotopic phenotype where central stamens appear surrounded by carpels. Given that the *E. amazonicus* flowers described by Sattler and Singh (1978) have not been documented again, the floral organ arrangement of *L. schismatica* differs from the typical floral plan of thousands of known bisexual angiosperm species (72% of the ~250,000 species of extant flowering plants), all of which have central carpels.

The molecular-genetic research described in this study derives directly from our previous claim that the floral organ arrangement described by Martínez and Ramos (1989) for *L. schismatica* could be analyzed in the context of the ABC model of flower development (Vergara-Silva et al., 2000, 2003). The ABC model seemed to be of immediate application to the *L. schismatica* case, since it explicitly states that stamen identity is determined by the combined activities of two functions, labeled B and C, both of which are represented by one or more genes belonging to the MADS box multigene family (Coen and Meyerowitz, 1991). Based on this particular feature of the model, we postulated that a displacement of B-function gene expression toward the center of the floral meristem, where C-function genes are normally active, is the simplest explanation for the central position of the androecium in *L. schismatica* (Vergara-Silva et al., 2000).

Although our ABC model–based study of the reproductive morphology of *L. schismatica* could be interpreted as a test of the universality of the model in angiosperms, it depends on certain assumptions about its applicability outside *Arabidopsis*, *Antirrhinum*, and other taxa used in plant developmental genetic studies. Over the years, experimental evidence in certain nonmodel angiosperms has indicated that conserved features of floral organization are correlated with conserved ABC combinatorial gene functions (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2004, 2007). However, some divergent ABC gene expression patterns have also been documented in flowering plant species with symplesiomorphic (traits that are shared among extant taxa, as well as with their last common ancestor) phenotypic characters (within the so-called basal angiosperms), as well as in monocots or eudicots (Kramer and Irish, 1999; Kanno et al., 2003; Vandenbussche et al., 2004; Rijpkema et al., 2006; Xu et al., 2006). None of these divergent B-function gene expression patterns, however, include a case in which the B-function is restricted to stamens or is associated with a change in the relative positions of stamens and carpels in flowers.

Furthermore, B-function genes have been shown to be conserved in determining stamen identity in cases in which these genes have been functionally characterized (Ambrose et al., 2000; Nagasawa et al., 2003; Rijpkema et al., 2006). Several such studies have been performed in monocots (Ambrose et al., 2000; Nagasawa et al., 2003; Xiao et al., 2003; Xu et al., 2006). Grasses were reported to have flowers with distinctive structures in the two outer whorls (i.e., lemma and palea instead of sepals and lodicules instead of petals in the case of maize; Ambrose et al., 2000; Whipple et al., 2004). Interestingly, however, it seems that the orthologous B genes of these monocot species are also necessary for second and third whorl organ development, as in other angiosperms (Ambrose et al., 2000; Nagasawa et al., 2003; Xiao et al., 2003; Whipple et al., 2004; Xu et al., 2006). Moreover, several earlier studies of B gene expression show that *APETALA3*/*DEFICIENS* (*AP3*/*DEF*) and *PISTILLATA*/*GLOBOSA* (*PI*/*GLO*) orthologs are broadly expressed during early flower development but are restricted to second and third whorl organs later in flower development, when all organ primordial have been specified. *Arabidopsis PI* and its ortholog in the orchid *Dendrobium crumenatum* (Dc-*OPI*) are expressed in the fourth whorl (Goto and Meyerowitz, 1994; Xu et al., 2006), and other *PI*/*GLO* orthologs in monocots are expressed in both the fourth and the first whorls (Schwarz-Sommer et al., 1992; Chung et al., 1994; Goto and Meyerowitz, 1994; Xu et al., 2006; Ambrose et al., 2000; Lee et al., 2003). In *Arabidopsis*, *AP3* is expressed shortly after the sepal primordia begin to form in the precursor cells of the petals and stamens, which are located in a region between the sepals and the precursor cells of the gynoecium in the center of the flower (Jack et al., 1992). However, in *PI* overexpression lines, the first whorl is converted into chimeric sepal/petal organs, suggesting that AP3 should be present in this whorl (Krizek and Meyerowitz, 1996; Figure 4). Furthermore, it has been reported that some *AP3* orthologs in monocots are broadly expressed throughout the floral meristem (Chung et al., 1994; Moon et al., 1999; Ambrose et al., 2000; Xu et al., 2006). Regardless of differences in expression patterns, B-function is present only in the second and third whorl, where both *AP3* and *PI* are coexpressed.

Thus, our assumption that the ABC model can form the basis of a molecular-genetic hypothesis for the natural occurrence of homeotic transformation in flowers remains valid. We present the results of the molecular cloning of a group of MADS box genes from *L. schismatica*. We found these genes to be orthologous to B- and C-function genes in various model systems, after identifying conserved domains in automated alignments and conducting Bayesian phylogenetic analyses on a matrix of angiosperm MADS box genes. Furthermore, we determined the spatio-temporal pattern of expression of these genes in several developmental stages of the *L. schismatica* reproductive axes by means of radioactive in situ hybridization techniques. In addition, we assessed the capacity of the *L. schismatica* B-function genes to complement mutations in the *AP3* and *PI* genes in *Arabidopsis* using mutant transgenic lines. Finally, we tested for interactions

between the proteins encoded by the cloned *L. schismatica* MADS box genes using yeast two- and three-hybrid systems.

Given that some of the work presented here is related to the biochemical events occurring between the transcription factors encoded by B- and C-function MADS box genes, we also considered current knowledge of the floral quartet model, which is an extension of the ABC model (Theissen and Saedler, 2001). It has been known for some time that AP3/DEF and PI/GLO carry out the B-function of the model only as obligate AP3-PI heterodimers (Jack et al., 1992, 1994; Goto and Meyerowitz, 1994; Ambrose et al., 2000; Kanno et al., 2003; Zik and Irish, 2003; Whipple et al., 2004). In eudicots, heterodimerization apparently is required to move these proteins into the nucleus (McGonigle et al., 1996) as well as for DNA binding (Schwarz-Sommer et al., 1992; Riechmann et al., 1996). Later, it was discovered that the activity of AP3-PI requires an additional protein, SEPALLATA3 (SEP3), which imparts transactivation ability to the heterodimer, stabilizes it, and facilitates its migration to the nucleus (Honma and Goto, 2001; Immink et al., 2009).

On the other hand, it has been suggested that AP3 and PI from *Arabidopsis* are able to form homodimers that transiently localize to the nucleus in planta (Immink et al., 2009); however, the biological significance of this homodimerization is not known. Some B-class proteins from gymnosperms and monocots can also homodimerize and bind DNA, at least in vitro (Winter et al., 2002; Kanno et al., 2003; Tzeng et al., 2004; Whipple et al., 2004; Tsai et al., 2008), while those from other monocots, such as maize (*Zea mays*), must heterodimerize to bind DNA (Whipple et al., 2004). Since *L. schismatica* is a monocotyledon, these data are relevant for the interpretation of our in vivo assays of protein interactions in a heterologous system (yeast).

Morphological Identity of Reproductive Axes of *L. schismatica*: True Flowers or Reduced Inflorescences?

Several interpretations for the identity of the reproductive axes of *L. schismatica* have been proposed (Stevens, 1991; Rudall, 2003): (1) normal flowers in which the location of stamens and carpels are secondarily inverted during early flower development, (2) compressed inflorescences or pseudanthia that resemble true flowers and have apical male flowers surrounded by female flowers at more basal positions, and (3) true homeotic flowers or euanthia in which stamens and carpels arise at inverted locations from inception. The first interpretation is refuted by observations of early development (see below).

Support for the pseudanthial hypothesis was based on morphological comparisons of mature flowers of several genera within Triuridaceae (Rudall, 2003) as well as phylogenetic analyses. Triuridaceae was placed as the sister group of Pandanaceae, a family composed of three genera (*Freycinetia*, *Pandanus*, and *Sararanga*), all of which have bona fide pseudanthia (Cox, 1990; Stone, 1990; Chase et al., 2000). However, additional molecular systematic analyses have placed Triuridaceae in different positions within Pandanales (e.g., Davis et al., 2004). In addition, subsequent independent studies have supported the breakdown of the Pandanaceae-Triuridaceae group, which was the starting point for the proposal of the pseudanthia interpretation (Rudall and Bateman, 2006). These new phylogenetic data led Rudall and Bateman (2006) to modify Rudall's original pseudanthia hypothesis and to propose that carpel multiplication was involved in the origin of Triuridaceae and that this could have led to chimeric structures that exhibited a partial loss in floral determinacy, where the genetic programs underlying the development of flowers and inflorescences are mixed in a single reproductive structure (Rudall and Bateman, 2006). Even more recently, Rudall (2008) performed a comparative morphological analysis of members of the different tribes of Triuridaceae, finding a transition of centripetal to centrifugal carpel development (centrifugal carpel inception) in the tribe to which *L. schismatica* belongs. Assuming a euanthial understanding of triurid morphology, it was concluded that such a mode of carpel inception might indicate an ontogenetic predisposition for heterotopic change in the three central organ primordia (i.e., from carpels to stamens) of the flowers of *L. schismatica* (Rudall, 2008).

In parallel to the above studies, a detailed morphological analysis by Ambrose et al. (2006), in which the reproductive morphogenesis of *L. schismatica* was compared with that of its sister taxon, *T. brevistylis*, was performed. This study provided evidence in favor of the original morphological interpretation of the reproductive axes in Triuridaceae and specifically in *L. schismatica*. This renewed euanthial interpretation was supported by the observation that these two triurids have distinct floral meristems enclosed by a bract and that these floral buds arise from the flanks of clearly distinguishable naked inflorescence meristems. Also, the position of the protecting bract was similar to that of true flowers, namely, opposite the site where the first tepal arises. Furthermore, the temporal order of organ development recorded by Ambrose et al. (2006) was reminiscent of that commonly found in nonhomeotic flowers, where perianth organs occur in the basal-most (outermost) part of the flower, followed by stamens (in the center of the floral meristem in *L. schismatica*) and finally by carpels. Additionally, there was no evidence of aborted floral organs (Ambrose et al., 2006). Lastly, the fact that the organs of *L. schismatica* have a trimerous arrangement (i.e., three male elements in the center and six sterile organs in the periphery of the axis), as commonly happens in floral whorls of monocot species (although the latter is also true in some inflorescences), was interpreted as further evidence of the presence of true flowers in this species (Ambrose et al., 2006).

Admittedly, some of the morphological interpretations of Ambrose et al. (2006) can be contested on the grounds that not all of these characteristics are always found in true flowers and are sometimes present in inflorescences (for instance, the bracts subtending the lateral inflorescences of trimerous meristems). However, most of the data and the recently proposed alternatives to the structural interpretation of the reproductive axes in Triuridaceae support the original morphological proposal (Martínez and Ramos, 1989; Márquez-Guzmán et al., 1989), including the homeotic nature of the position of organs bearing male and female identities. Testing our ABC model–based hypothesis of the molecular-genetic basis of the reproductive phenotype in *L. schismatica* would thus advance our understanding of character evolution in Triuridaceae. Consequently, the experimental results presented here will be interpreted

largely in euanthial terms. However, we will briefly discuss some of the implications that our results have on noneuanthial proposals of the structure of reproductive axes in Triuridaceae.

RESULTS

B- and C-Function Orthologous Genes of *L. schismatica*

To test if the B-function of the ABC model is displaced toward the center of the reproductive meristem in *L. schismatica*, where male organs occur, and if the C-function genes are expressed in the male organs, we first cloned sequences putatively orthologous to the B-function (Ls-*AP3* and Ls-*PI*) and C-function (Ls-*AG*) MADS box floral organ identity genes. Orthology of the cloned *L. schismatica* sequences was supported by visual inspection of computer-based alignments of matrices of full-length angiosperm MADS box genes and by Bayesian phylogenetic inference methods applied to such matrices. To inspect the nucleotide and amino acid matrices used in our analyses, we used previously published cladograms and phenograms of plant MADS box genes as a reference (Kramer et al., 1998, 2004). Ls-AP3 and Ls-PI display the typical MIKC domain structure of Type II MADS domain proteins (Álvarez-Buylla et al., 2000) and group within their corresponding monocot *AP3* and *PI* lineages in the Bayesian analyses (Figure 1; see Supplemental Figure 1 and Supplemental Data Set 1 online). Furthermore, they possess the diagnostic paleoAP3 (NGFHDLRLA) and PI (IPVAFRVQPFQPNLQENK) motifs that characterize these gene families within monocotyledons (Kramer et al., 1998; Figure 1A).

Ls-AG also groups within the AGAMOUS (AG) monocot clade, bearing the two diagnostic motifs (FDSRNFLQVNMQD and YSHHQHIVFQLG) characteristic of AG angiosperm orthologs (Kramer et al., 2004) in its C-terminal region and thus supporting the view that this *L. schismatica* gene is an ortholog of *AG* from *Arabidopsis* (Figure 1B; see Supplemental Data Set 2 online). Another C-function-like gene was cloned during our search for MADS box–containing genes from *L. schismatica*. An additional Bayesian analysis including a greater number of MADS box sequences further supports the orthology of the genes cloned from *L. schismatica* with respect to their counterparts from *Arabidopsis.* In the Bayesian analyses, this gene was found to be orthologous to the *Arabidopsis* gene *SEEDSTICK* (*STK*) and has therefore been named Ls-*STK* (see Supplemental Figure 1 online).

The B-Function of the ABC Model of Flower Development Is Restricted to the Central Region of the Developing Reproductive (Floral) Meristem in *L. schismatica*

Once we tested the orthology of the cloned B and C *L. schismatica* genes, we determined the spatio-temporal expression patterns of Ls-*AP3* and Ls-*PI* in different stages of floral axis development using radioactive in situ hybridization (Figure 2; see Supplemental Figure 2 online). We found that the expression of these two genes coexists only at the center of the determinate (i.e., floral) developing meristem, which is the region where stamen primordia are later formed. The restriction of Ls-*AP3* to the apex of the flower meristem was observed from the early stages of flower development when stamen primordia are already apparent but no carpel primordia have differentiated (Figure 2A). Moreover, whereas Ls-*AP3* continues to be restricted to the apical regions of the later-formed compound primordia, coinciding with the position where stamen primordia will, in turn, be formed (Ambrose et al., 2006; Figures 2A to 2C and 2K to 2M), Ls-*PI* is expressed more broadly during early flower development, but it is also expressed in such compound primordia. In contrast with Ls-*AP3*, Ls-*PI* expression was also found in the basal positions of the compound primordia, where carpels later develop to constitute the second innermost floral whorl (i.e., the gynoecium; Figures 2D, 2E, and 2N). At even later ontogenetic stages, when carpel primordia are observable, expression of Ls-*AP3* remains absent in regions of the floral compound primordia that give rise to carpels, but Ls-*PI* expression is observed in such areas from which carpels continue to develop (Figures 2A to 2E and 2N; see Supplemental Figure 2 online). Therefore, the B-function of the ABC model of flower development, which requires the concerted expression of *PI* and *AP3* orthologs, is restricted to the center of the flower, where stamens appear and develop in *L. schismatica* flowers (Figures 2A and 2D).

In model plant systems, stamen development requires the expression of both B and C genes. Therefore, we also performed experiments to elucidate the pattern of expression of the C-function-like genes cloned from *L. schismatica*, namely, Ls-*AG* (the putative ortholog of At-*AG*) and Ls-*STK* (the putative ortholog of At-*STK*; see above). In situ hybridization showed that Ls-*AG* was expressed in both stamen and carpel primordia, with a pattern similar to that of Ls-*PI* (Figures 2D, 3A, and 3B), while Ls-*STK* was only expressed in carpels (Figure 3C).

Thus, both B- and C-functions are present in the center of the *L. schismatica* developing floral bud; however, in the second whorl, where carpels develop, only the C-function gene Ls-*AG* is expressed (Figures 2, 3A, and 3B; see Supplemental Figure 2 online). Except for Ls-*AP3*, the overall expression pattern of *L. schismatica* floral homeotic genes is very similar to the expression patterns that have been reported for *Arabidopsis* orthologs. Hence, the spatio-temporal patterns of in situ hybridization signals for the MADS box B- and C-function genes from *L. schismatica* are consistent with an ABC model–based explanation of its inside-out flower, provided that the B-function genes of this species are shown to be able to specify stamens.

L. schismatica B-Function Genes Are Able to Determine Stamens in *Arabidopsis*

L. schismatica is a mycoheterotrophic plant species, which cannot be grown in the laboratory as its survival depends on endophytic fungi (Martínez and Ramos, 1989; Vergara-Silva et al., 2003). Hence, we are unable to perform genetic experiments or induce mutations to experimentally test the roles of the B-function genes directly in this species. Therefore, as an indirect test of the ability of the coding sequences of Ls-*AP3* and Ls-*PI* to determine petal and stamen identity, we generated ectopic expression and complementation lines for Ls-*AP3* and Ls-*PI* in transgenic *Arabidopsis* plants with wild-type and mutant

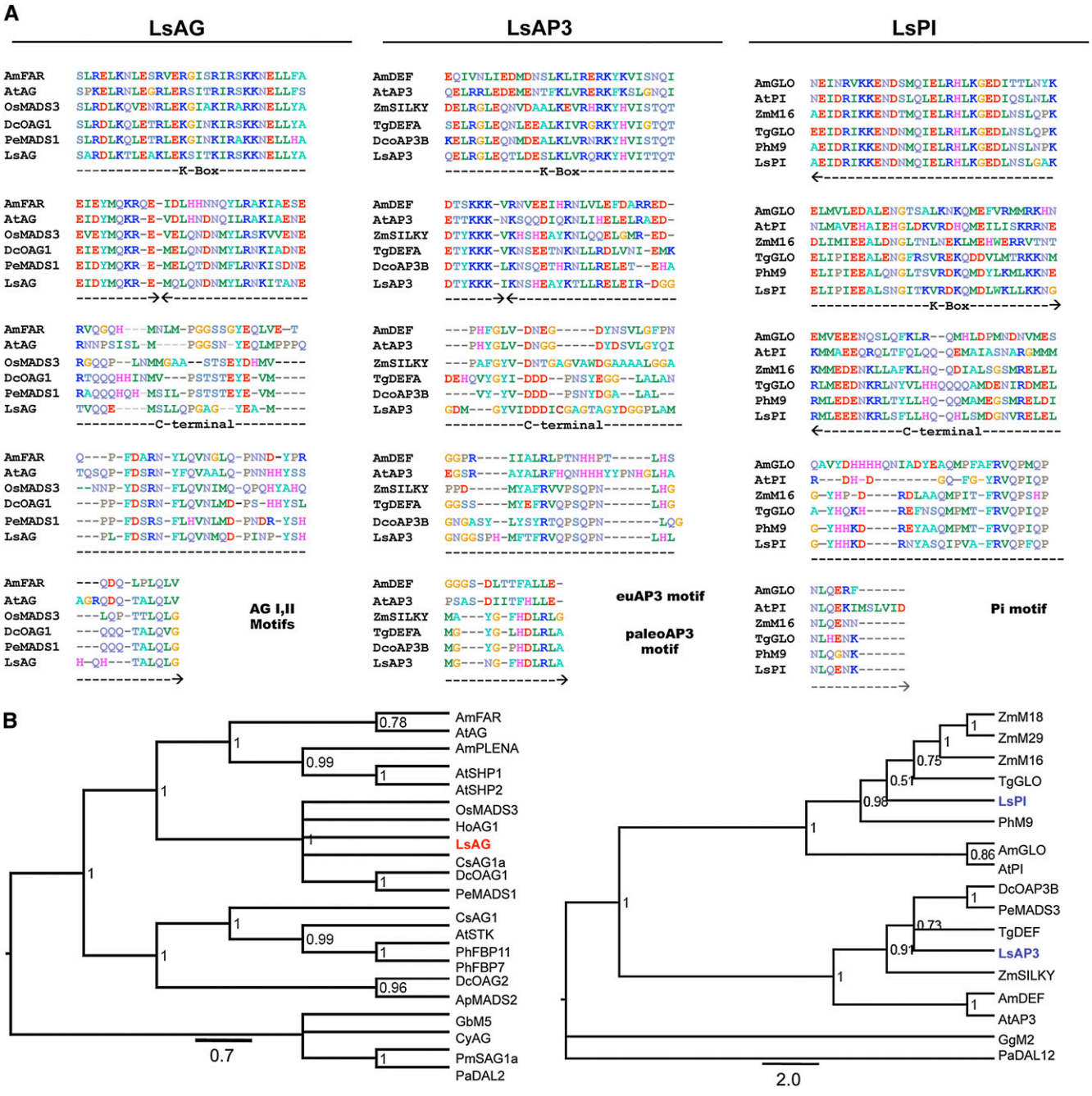

Figure 1. *L. schismatica* MADS Box B and C Orthologs.

(A) B and C MADS domain protein sequences of diverse angiosperms. *L. schismatica* orthologs possess diagnostic C-terminal motifs characteristic of each linage.

(B) Bayesian phylogenetic analysis (AG, left; B genes, right) corroborate the orthology of the *L. schismatica* genes (*AG* in red; *AP3* and *PI* in blue) to C and B genes of *Arabidopsis*, respectively. Trees are supported with high posterior probabilities showed for the main branches. The *L. schismatica* sequences were nested within monocots. The MADS box has been excluded from this analysis to align domains with the most informative sites. Gymnosperm genes were used as outgroups. Larger alignments confirmed these results (see Supplemental Figure 1 online).

[See online article for color version of this figure.]

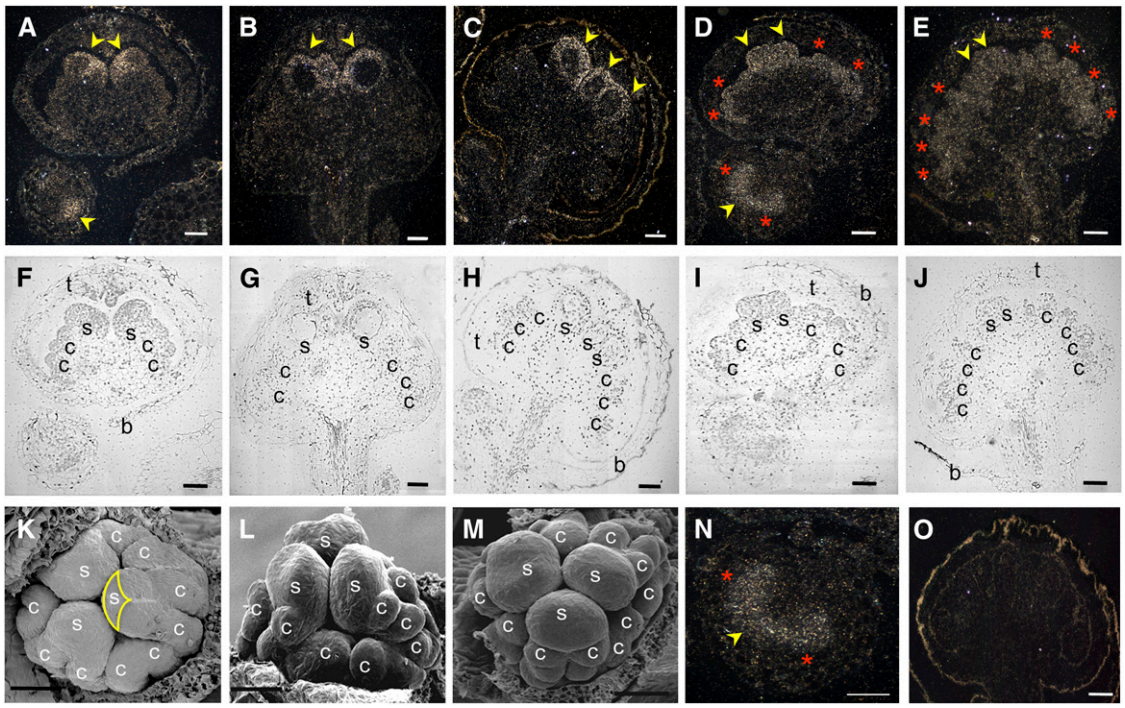

Figure 2. In Situ Localization of *L. schismatica* B Gene (Ls-*AP3* and Ls-*PI*) mRNA.

(A) to **(E)** Longitudinal sections of *L. schismatica* flower buds hybridized with Ls-*AP3* (**[A]** to **[C]**) and Ls-*PI* (**[D]** and **[E]**) probes. Expression of Ls-*AP3* is restricted to the apical zone (central zone in a cross section) of very early flower buds, where stamen primordia (yellow arrowhead in **[A]**) later arise and mature stamens will develop, while Ls-*PI* is expressed in a broader domain during the early stages of floral development and is then restricted to regions where both stamen (yellow arrowheads) and carpel (red asterisks) primordia develop. Ls-*AP3* expression is shown in four stages of floral development in **(A)** to **(C)**, and Ls-*PI* expression is shown for three stages in **(D)** and **(E)**.

(F) to **(J)** Light fields of **(A)** to **(E)**, respectively, for structural references.

(K) to **(M)** Scanning electron micrographs of three developmental stages of *L. schismatica* flower development for structural reference. Note that the form of the expression domain of Ls-*AP3* in **(A)** coincides with the form of the region within the compound primordia where stamen primordia will arise **(K)** (yellow line).

(N) Enlarged image of Ls-*PI* expression in the young meristem shown in **(D)**.

(O) In situ hybridization with an Ls-*AP3* sense probe.

b, bract; c, carpel; s, stamen; t, tepal organs. Bars = 100 μm.

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backgrounds, respectively, taking advantage of the known functional aspects of *AP3* and *PI* in this dicotyledonous species.

We generated *Arabidopsis* plants that overexpress *L. schismatica AP3* and *PI* orthologs (*35S:LsAP3* and *35S:LsPI*, respectively). Several independent lines were isolated for each genotype, and high expression *35S:LsAP3* and *35S:LsPI* lines were identified by RNA gel blots and RT-PCR analyses (see Supplemental Figure 3 online). Our results show that the *Arabidopsis* lines bearing 35S:*LsPI* in a wild-type background have similar gain-of-function phenotypes to those of *35S:AtPI* in *Arabidopsis* (Krizek and Meyerowitz, 1996; Figures 4A and 4Q). The most notable aspect of the *35S:LsPI* phenotype is a partial conversion of sepals into petals in the first floral whorl. To determine if Ls-*PI* could rescue organ identity in the absence of endogenous PI activity, we crossed *35S:LsPI* into a pi-1 mutant background. *35S:LsPI* was able to rescue petal identity in the second whorl and stamen identity in the third whorl, similar to the rescue phenotype observed in *pi-1 Arabidopsis* plants expressing *35S:PI* (Piwarzyk et al., 2007; Figures 4G to 4K and 4Q).

AP3 gain-of-function lines in *Arabidopsis* show a homeotic conversion of carpels to stamens (Jack et al., 1994). The *35S:LsAP3* overexpression lines did not show a conversion of carpels into stamens (Figure 4B), despite the high levels of Ls-*AP3* mRNA detected in them. However, flowers of 35S:*LsAP3 ap3-3* complementation lines had two outer whorls of sepals and a third whorl with stamens or staminoid organs that were able to produce functional pollen (Figures 4M to 4P and 4Q), demonstrating that Ls-*AP3* is able to rescue stamen identity in *Arabidopsis ap3-3* lines. However, petal identity was not rescued in these lines.

Given that MADS domain proteins corresponding to the B-function in *Arabidopsis* form functional heterodimers (Jack et al., 1994; Yang et al., 2003), we tested if the ectopic expression of both Ls-*PI* and Ls-*AP3* would result in central stamens in *Arabidopsis* by forming a conserved heterodimer. However, we

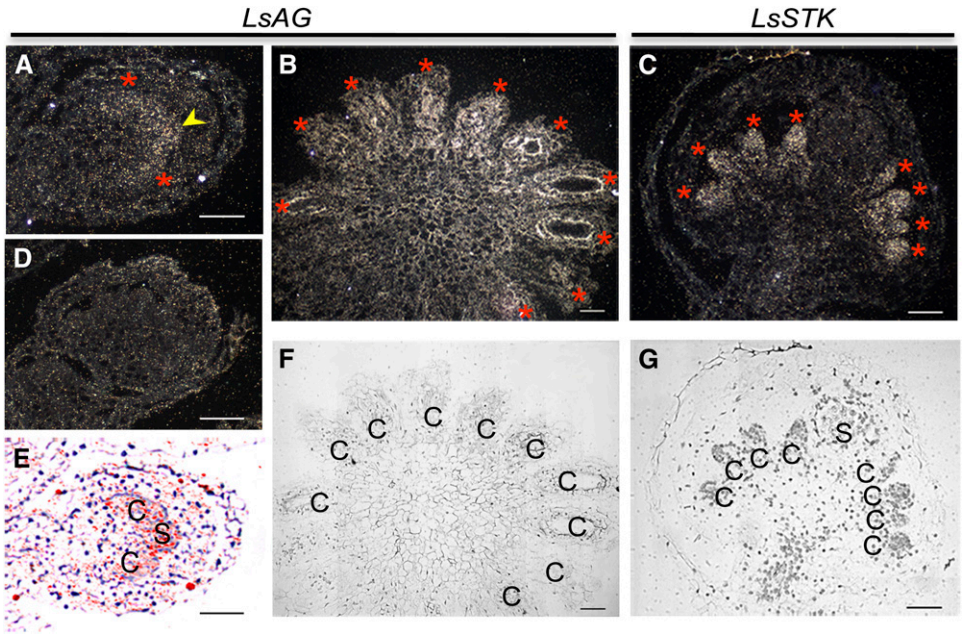


Figure 3. In Situ Localization of C Gene (Ls-*AG* and Ls-*STK*) mRNA in *L. schismatica* Flower.

(A) and **(B)** Longitudinal section of a young bud and of a noncentral section of an older meristem, respectively, hybridized with an antisense Ls-*AG* probe. Signal is observed in a pattern similar to that of Ls-*PI*, with a broad pattern during the early stages and in both stamen (yellow arrowhead) and carpel (red asterisks) primordia at later stages of flower development.
(C) Longitudinal section of an *L. schismatica* flower hybridized with the Ls-*STK* probe. Ls-*STK* mRNA is absent in stamen primordia and is strongly expressed in carpel primordia.
(D) Hybridization with a sense Ls-*AG* probe.
(E) Superposition of antisense Ls-*AG* signal (in red) shown in **(A)** over a bright-field view.
(F) and **(G)** Bright-field images of **(B)** and **(C)**, respectively, for structural reference.
c, carpel; s, stamen. Bars = 100 μm.
[See online article for color version of this figure.]

observed that the phenotype of *35S:LsPI 35S:LsAP3* double transgenic plants resembled that of plants ectopically expressing Ls-*PI*, where only sepals were converted into petals (Figures 4A and 4C). This indicates that the action of both Ls-PI and Ls-AP3 is not sufficient for proper stamen development (Figures 4C to 4E) in the context of *Arabidopsis* and that the formation of multimers might be necessary for proper Ls-AP3 function (Honma and Goto, 2001). To test for this secondary biochemical hypothesis, we performed yeast two-hybrid (Y2H) experiments with *AP3* and *PI* orthologs of both species, as well as yeast three-hybrid (Y3H) assays that comprised all combinations of B-function proteins. In the latter class of experiments, we tested for homo- and heterodimerization with SEP3, one of the prominent components of the floral quartet model of MADS domain protein interactions (Honma and Goto, 2001; Theissen and Saedler, 2001).

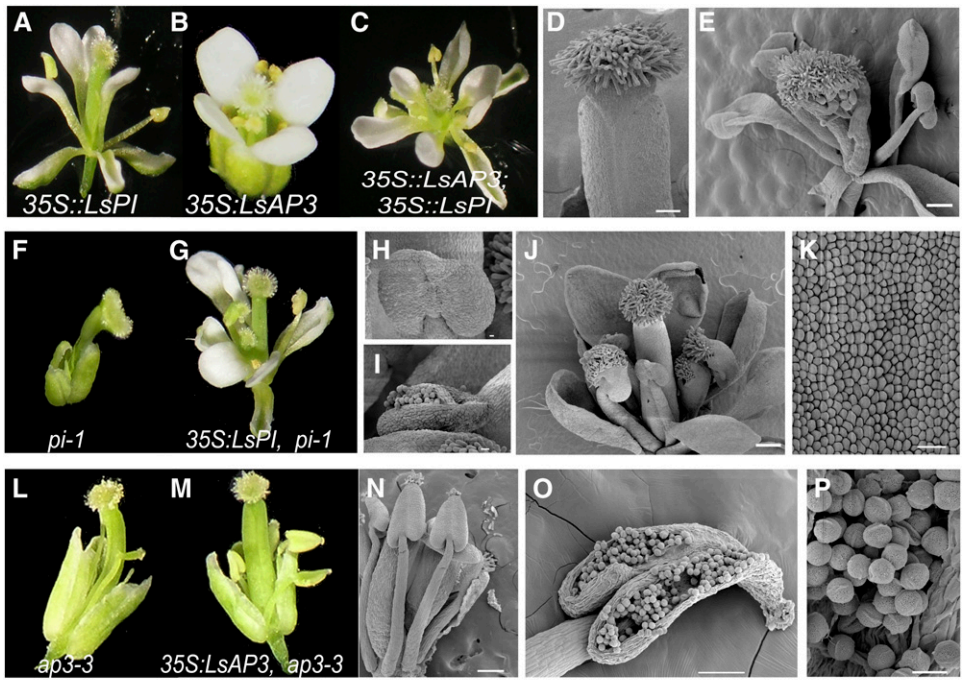
L. schismatica AP3 and PI Proteins Form Heterodimers with *Arabidopsis* SEP3 in the Yeast Three-Hybrid System

The yeast protein hybridization results showed that *L. schismatica* B-function proteins were not able to heterodimerize on their own, either when both proteins from this species are used, or when used in combination with *Arabidopsis* proteins (Table 1). As a positive control, only truncated *Arabidopsis* MADS box proteins with the IKC domains were used (Yang et al., 2003).

When the interactions between B proteins were tested in the Y3H system using SEP3, *L. schismatica* B proteins were found to form heterodimers with the corresponding proteins from *Arabidopsis* (Ls-AP3 with At-PI and At-SEP3 and Ls-PI with At-AP3 and At-SEP3). This might explain the capacity of Ls-AP3 and Ls-PI to complement the *ap3-3* and *pi-1* mutant lines (Figure 4). As expected, we also confirmed the interaction of all the heterodimers when using proteins from the same species (Table 1).

DISCUSSION

L. schismatica (Lacandoniaceae = Triuridaceae; Martínez and Ramos, 1989; Maas-van de Kamer, 1995; Mabberley, 1997; Kubitzki, 1998) is a mycoheterotrophic, monocotyledonous species endemic to the Mexican Lacandon rainforest (Vergara-Silva et al., 2003), and its flowers have a cleistogamic preanthesis mode of reproduction (i.e., fertilization occurs before the flower opens; Márquez-Guzmán et al., 1993). Along with its taxonomic description (Martínez and Ramos, 1989), initial detailed studies of the reproductive morphology and anatomy of the species (Márquez-Guzmán et al., 1989) emphasized that there is an



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A. thaliana background	*A. thaliana* B genes overexpression				*L. schismatica* B genes overexpression			
Wt	*35S:AtPI*				*35S:LsPI*			
	SE/PE	PE	ST	CA	SE/PE	PE	ST	CA
Wt	*35S:AtAP3*				*35S:LsAP3*			
	SE	PE	ST	ST	SE	PE	ST	CA
Wt	*35S:AtAP3 35S:AtPI*				*35S:LsAP3 35S:LsPI*			
	PE	PE	ST	ST	SE/PE	PE	ST	CA/ST
*pi-1**	*35S:AtPI*				*35S:LsPI*			
	SE/PE	SE/PE	ST/CA	CA	SE/PE	SE/PE	ST/CA	CA
ap3-3	*35S:AtAP3*				*35S:LsAP3*			
	SE	SE/PE	ST	ST	SE	SE	ST/CA	CA

Figure 4. *L. schismatica* B Genes Are Able to Specify Petal and Stamen Cell Identity in *Arabidopsis* Transgenic Lines.

(A) The Ls-*PI* overexpression line, *35S:LsPI*, in a wild-type background.
(B) The Ls-*AP3* overexpression line, *35S:LsAP3*, in a wild-type background.
(C) to **(E)** Flowers of the F2 descendants of the double overexpressor line *35S:LsAP3 35S:LsPI*. The flower in **(C)** is similar to the single Ls-*PI* overexpression line in **(A)**. Carpels do not show conversion to stamens **(D)**. However, aberrant filamentous carpels occasionally are observed **(E)**.
(F) *pi-1* homozygous flower.
(G) *35S:LsPI* in the *pi-1* mutant background, showing the ability of Ls-*PI* overexpression to partially rescue petals and stamens of the *pi-1* mutant flower.
(H) to **(K)** Scanning electron microscopy images of the line in **(G)** showing immature stamens **(H)** and mature stamens with viable pollen **(I)**. Flower with a chimeric stamen/carpel organ in the third whorl **(J)**. Second whorl petal-like cells from **(G)** are shown in **(K)**.
(L) An *ap3-3* homozygous flower.
(M) to **(P)** Examples of progeny from *35S:LsAP3* lines crossed to *ap3-3* plants.
(N) Scanning electron microscopy image of the dissected perianth organs of a flower, showing at least four stamens.
(O) and **(P)** Scanning electron microscopy images of an opened anther.
(P) Enlargement of viable pollen. Bars = 1 mm.
(Q) Summary of floral organ phenotypes observed in overexpression lines harboring *L. schismatica* B genes in *Arabidopsis* wild-type (Wt) backgrounds and B mutant plants compared with the corresponding phenotypes in lines harboring *Arabidopsis* B genes (Jack et al., 1994; Krizek and Meyerowitz, 1996). Asterisk indicates the phenotype reported for the *pi* mutant line complemented with the *Arabidopsis* cDNA corresponds to data using the *pi-4* allele (Piwarzyk et al., 2007). CA, carpels; PE, petals; SE, sepals; ST, stamens.
[See online article for color version of this figure.]

Table 1. Interactions between *Arabidopsis* AP3 and PI and *L. schismatica* AP3 and PI in the Y2H and Y3H Hybrid Systems

Interactors	HIS	HIS/1 mM 3AT	HIS/3 mM 3AT	HIS/5 mM 3AT
Y2H (AD/BD)[a]				
At-AP3/At-AP3	–	–	–	–
At-PI/At-PI	–	–	–	–
Ls-AP3/Ls-AP3	–	–	–	–
Ls-PI/Ls-PI	–	–	–	–
Ls-AP3/At-AP3	–	–	–	–
Ls-PI/At-PI	–	–	–	–
Ls-AP3(IKC)/At-AP3	–	–	–	–
Ls-AP3(IKC)/Ls-AP3	–	–	–	–
Ls-PI(IKC)/At-PI	–	–	–	–
Ls-PI(IKC)/Ls-PI	–	–	–	–
*At-AP3/At-PI	+++	+++	+++	+++
Ls-PI/Ls-AP3	–	–	–	–
Ls-AP3(IKC)/Ls-PI	–	–	–	–
Ls-AP3/At-PI	–	–	–	–
Ls-PI/At-AP3	–	–	–	–
Ls-AP3(IKC)/At-PI	–	–	–	–
Ls-PI(IKC)/At-AP3	–	–	–	–
Ls-AP3(IKC)/Ls-PI(IKC)	–	–	–	–
Ls-AP3(IKC)/Ls-AP3(IKC)	–	–	–	–
Ls-AP3/Ls-PI(IKC)	–	–	–	–
Ls-PI(IKC)/Ls-PI(IKC)	–	–	–	–
Y3H (AD/BD/SEP3)[b]				
At-AP3/At-AP3	+	+/–	–	–
At-PI/At-PI	+	–	–	–
Ls-AP3/Ls-AP3	+++	+++	+++	+++
Ls-PI/Ls-PI	++	+/–	–	–
*At-AP3/Ls-AP3	+++	+++	+++	+++
Ls-PI/At-PI	++	–	–	–
*At-AP3/Ls-AP3(IKC)	+++	+++	+++	+++
*Ls-AP3/Ls-AP3(IKC)	–	–	–	–
Ls-PI(IKC)/At-PI	+	+/–	–	–
Ls-PI(IKC)/Ls-PI	++	+/–	–	–
*PI/AP3	+++	+++	+++	+++
Ls-PI/Ls-AP3	+	+	–	–
Ls-AP3(IKC)/Ls-PI	++	–	–	–
Ls-AP3/At-PI	++	–	–	–
Ls-PI/At-AP3	++	+	+/–	–
*At-PI/Ls-AP3(IKC)	+/–	–	–	–
Ls-PI(IKC)/At-AP3	++	+/–	–	–

Asterisk indicates the interaction took place if the genes were cloned either in the AD or BD vector.

[a]In the Y2H assay, B-class proteins from *L. schismatica* and *Arabidopsis* were assayed for heterodimerization. *Arabidopsis* proteins lacked the MADS domain, and both full-length *L. schismatica* proteins and *L. schismatica* IKC domains were tested. Heterodimerization was only observed in the positive control, AP3/PI from *Arabidopsis*.

[b]In the Y3H experiment, all combinations were tested between full-length AP3, PI, and SEP3 from *Arabidopsis* and both full-length proteins and IKC domains from *L. schismatica*.

inversion in the expected position of the androecium with respect to the gynoecium. Subsequent work on the natural variation in the reproductive morphology of this rare monocot has followed the original euanthial interpretation (i.e., *L. schismatica* reproductive units are true bisexual flowers baring an inversion on stamen position) of its reproductive axes (Vergara-Silva et al., 2003; Ambrose et al., 2006). The aforementioned publications discuss some of the implications of alternative morphological proposals that depart from a euanthial interpretation of the reproductive axes in the entire family Triuridaceae. According to these proposals, triurid flowers should be construed instead either as pseudanthia (Rudall, 2003) or as reproductive axes where the boundary between flower and inflorescence identity is ambiguous (Rudall and Bateman, 2006; see also Rudall, 2008).

On the basis of evidence presented in Ambrose et al. (2006) and the argument set forth at the end of the Introduction in this study, we adopted a euanthial view of the reproductive axes of *L. schismatica*. According to this morphological perspective, our main result is describing the evolutionary shift in the spatio-temporal expression patterns of a single transcription factor (Ls-*AP3*) at both early and, importantly, later stages of flower

development, when such patterns are compared between *Arabidopsis* and *L. schismatica* (Figures 2A to 2C and 5). In *L. schismatica*, we found that Ls-*AP3* expression is restricted to the center (most apical zones) of the flower meristem from early stages of flower development, before stamen primordia are distinguished. As *L. schismatica* flower development proceeds, Ls-*AP3* remains restricted to the central portion of the meristem, and at later stages it is only expressed in the area within the compound primordia, where stamens arise and develop (Figures 2A to 2C, 5B, and 5C). This pattern differs from the expression profiles of *AP3* documented during the early and late developmental stages of *Arabidopsis* flowers (Jack et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996; Figure 5A). However, as in *L. schismatica*, in several angiosperms (Tsai et al., 2004; Kim et al., 2005), including some model plant systems, such as *Antirrhinum* (Zachgo et al., 1995), the *AP3* ortholog *DEF* is also expressed in the center during the early developmental stages. Nevertheless, expression of the *PI* ortholog *GLO* does not coincide with that of *DEF* in the flower center of *Antirrhinum*; hence, it is not associated with stamen development in the central whorl.

In a broader comparative framework, although the domain of expression of *AP3* and *PI* orthologs varied over the evolution of flowering plants, it is important to note that the BC combinatory function has consistently remained restricted to the third whorl, where stamens develop in hermaphroditic flowers (Figure 6; Xiao et al., 2003; Irish and Litt, 2005; Kim et al., 2005; Soltis et al., 2007; Theissen and Melzer, 2007; Whipple et al., 2007; Mondragón-Palomino and Theissen, 2009; Álvarez-Buylla et al., 2010b). Furthermore, to the best of our knowledge, in no other case, including the one presented here, has the BC combinatory function been displaced to the flower center in bisexual flowers, resulting in central stamen development (Figure 6 and references therein).

The Ls-*AP3* expression pattern during flower development revealed in this study is, in turn, consistent with the euanthial molecular-genetic hypothesis previously proposed (Vergara-Silva et al., 2000) for the homeotic arrangement of male and female floral whorls in this Mexican triurid species. The additional observation that both *L. schismatica* B-function gene orthologs Ls-*AP3* and Ls-*PI* are capable of rescuing stamen identity in *Arabidopsis* plants with *AP3* or *PI* mutations, respectively, is also consistent with the euanthial molecular-genetic hypothesis. Furthermore, the fact that the observed expression pattern of Ls-*AG* is also detected in stamens, as in other flowering plants, strengthens the coherence between the molecular-genetic data and the ABC model–based molecular-genetic explanation of the homeotic floral phenotype in *L. schismatica*.

The data presented here could be interpreted as being discordant with the pseudanthial hypothesis to explain the reproductive structures of *L. schismatica* (Rudall, 2003). In our in situ hybridization analysis, we did not detect a partitioned expression pattern in the floral primordium for any of the MADS box genes assayed (Figures 2 and 3). Such a partitioned pattern of expression for ABC-like MADS box genes could correspond to minute aborted organs within multiple flowers of a pseudanthium. Such a pattern would be expected under the pseudanthial hypothesis and is supported by existing information on the early morphogenesis of reproductive structures in selected genera of Pandanaceae, where true pseudanthia occur (e.g., *Freycinetia*; Stone, 1990).

The in situ hybridization data presented here are less relevant to the most recent noneuanthial hypotheses proposed by Rudall and Bateman (2006) and Rudall (2008). Therefore, we suggest that additional in situ hybridization experiments should be conducted for *L. schismatica* homologs of other transcription factor–encoding genes that participate in flower and inflorescence development in the model systems (e.g., *LEAFY* [*LFY*]). However, in the context of the evidence presented here, the genetic alterations underlying central B-function gene expression in *L. schismatica* could be preliminarily understood in a phylogenetic context. Morphological analyses of the sister taxon of *L. schismatica*, *T. brevistylis*, a dioecious member of the family Triuridaceae also distributed in the Lacandon rainforest (Vergara-Silva et al., 2003), are worth mentioning. Female flowers of *T. brevistylis* have a strikingly similar developmental pattern to those of *L. schismatica* (Ambrose et al., 2006). Moreover, careful screening of the natural variation among female flowers of *T. brevistylis* identified the presence of central staminoids with viable pollen in a few individuals of this species (Vergara-Silva et al., 2003). Given these findings, if the common ancestor of the Mexican Triuridaceae had flowers similar to current *T. brevistylis* female individuals, the de novo activation of B-class genes (*AP3* in particular) in the central whorl would have required a single mutational step. Alternatively, a less parsimonious scenario would involve a hermaphroditic ancestor in which the stamen identity genes underwent deactivation in an outer whorl, along with a secondary reactivation in the center of the flower meristem.

Further exploration of the implications of our data under euanthial assumptions indicates that a deeper understanding of the molecular mechanisms involved in the origin of heterotopic B-function gene expression in *L. schismatica* will require careful comparisons of *cis*-regulatory sequences of both B genes of *L. schismatica* and *T. brevistylis*, as well as analysis of the factors that regulate *AP3* genes in trans in several triurids with contrasting floral arrangements. For example, transactivators of *AP3* in *Arabidopsis* include the F-box protein UNUSUAL FLORAL ORGANS (UFO), which is an interactor of LFY. The LFY-UFO complex binds directly to the *AP3* promoter and thereby induces *AP3* expression specifically in floral whorls 2 and 3 (Lee et al., 1997). Moreover, it has been shown that *UFO* orthologs present in other angiosperms have divergent expression patterns (Souer et al., 2008), highlighting the importance of determining the expression patterns of *UFO* and *LFY* orthologs in *L. schismatica*.

Another MADS box gene that should be further analyzed in *L. schismatica* and related triurids with contrasting flower arrangements is *SEP3*. Our results in the Y3H analysis suggest that SEP3 is important for the formation and functioning of the AP3-PI dimer. Also, recent findings have shown that SEP3 functions not only as an overall regulator of floral meristem patterning that, besides activating B and C genes in wild-type plants (Liu et al., 2009), can induce ectopic B and C gene expression when ectopically expressed (Castillejo et al., 2005; Liu et al., 2009) but also as an element that forms ternary complexes with AP3 and PI in planta and colocalizes these proteins to the cell nucleus (Immink et al., 2009). *SEP3* is also an important regulator of the

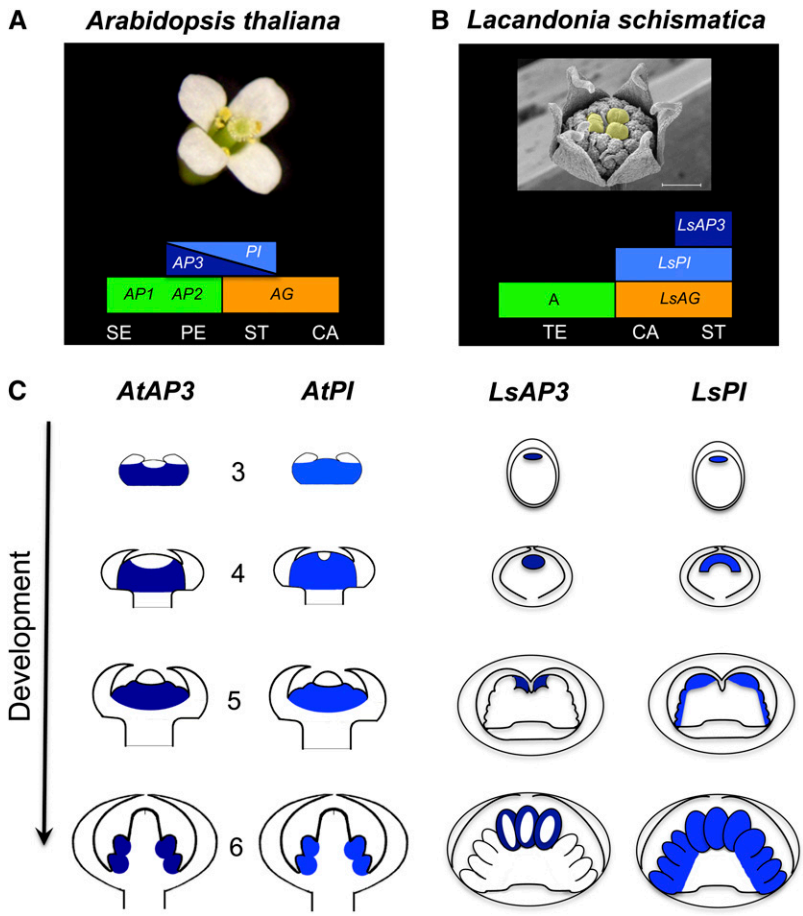

Figure 5. Modified ABC Model for *L. schismatica* Based on Expression Data Reported Here and Compared with the ABC Model of *Arabidopsis.*

(A) The combinatorial ABC model of flower organ identity conserved among eudicots, with B function restricted to the petal and stamen primordia (Coen and Meyerowitz, 1991).

(B) Proposed molecular genetics hypothesis to explain the inside-out flower of *L. schismatica*: *AP3-PI* dimer function is restricted to the flower center, where stamens initiate and develop. A (green), B (AP3 dark blue and PI light blue), and C (orange) function. CA, carpels; PE, petals; SE, sepals; ST, stamens; TE, tepals. Bar = 500 μm.

(C) Changes in the in situ expression patterns of B gene mRNA along flower developmental series comparing *Arabidopsis* (Jack et al., 1992; Goto and Meyerowitz, 1994; Krizek and Meyerowitz, 1996) and *L. schismatica* (this article). The differences between these two species represent two instances of the evolutionary shifts of such patterns during angiosperm evolution. Schematic representation of the developmental stages of *Arabidopsis* and *L. schismatica* flowers, starting at stage 3 of *Arabidopsis* flower development when sepal primordia are already visible. Sepal primordia continue to grow until they enclose the flower meristem, from stage 4 to 6. Meanwhile, at stage 5, petal and stamen primordia start to appear, and the gynoecium starts to form at stage 6. Similar stages of *L. schismatica* flower development were selected based on morphological traits.

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transition from inflorescence to flower meristem (Liu et al., 2009). Therefore, analyses of the spatio-temporal expression patterns of putative *L. schismatica SEP3* homologs, as well as of SEP3 protein complexes and regulatory interactions, should be helpful in understanding the unique expression pattern of Ls-*AP3* during flower development.

As in other monocotyledons, the function of *PI* orthologs in *L. schismatica* seems to be more conserved than that of *AP3*-like genes (Xu et al., 2006). The *35S:LsAP3* construct was insufficient to complement a loss of petal identity in *ap3-3* plants (Figure 4M) (Xu et al., 2006; Su et al., 2008). However, the relatively well-conserved MIK domains of Ls-AP3 correlate with the capacity of this protein to specify stamen identity in *Arabidopsis*. The so-called paleoAP3 domain of LsAP3 is also conserved (Figure 1A), although the functional role of this protein region is contentious (Lamb and Irish, 2003; Piwarzyk et al., 2007; Su et al., 2008). Conservation of the biochemical role of B-function genes/proteins and homology of petals among angiosperms is supported by functional experiments both in eudicots (Sommer et al., 1990) and in other monocots (Whipple et al., 2004, 2007). Since petal identity requires A- plus B-function in model species, the lack of B-function gene expression in the perianth organs of *L. schismatica* and the inability of Ls-*AP3* to specify petals in *Arabidopsis* suggest that the perianth

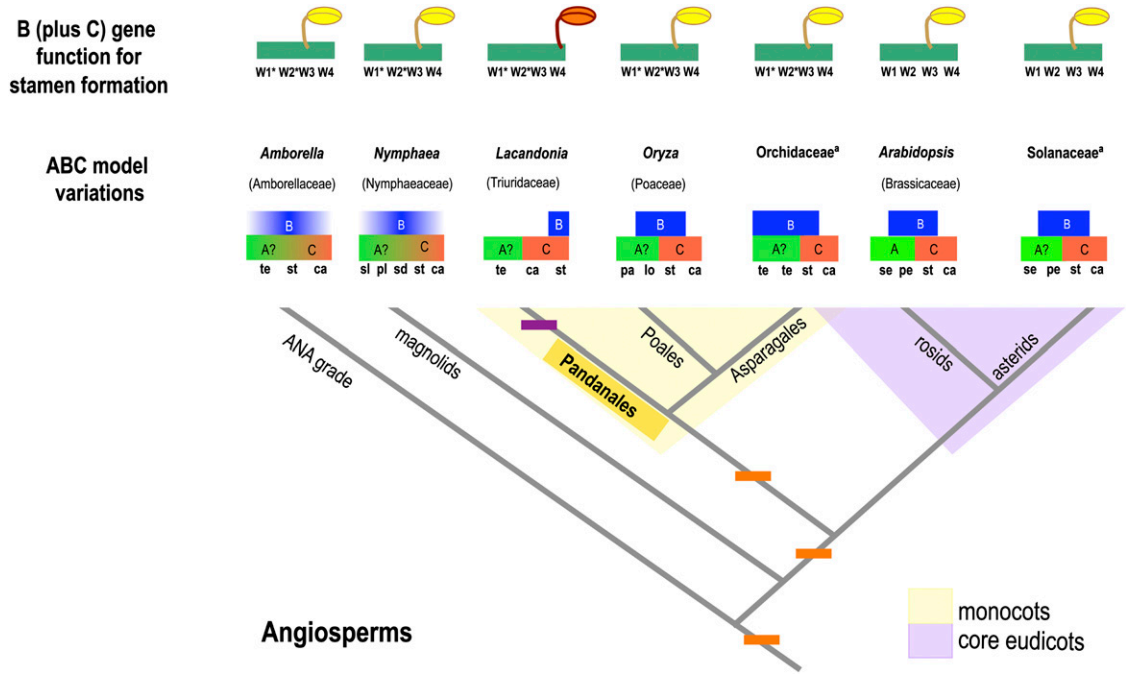

Figure 6. The Evolution of ABC Genes in Angiosperms.

Simplified angiosperm phylogeny (based on the Angiosperm Phylogeny Group III, 2009). Variations in the ABC model of floral organ formation for selected taxa, as well as the site where the BC combinatory function defines the development of stamens, are mapped. Representative angiosperm groups are shown in the branches of the tree with their taxonomic family in parentheses, except for Orchidaceae and Solanaceae ([a]), for which the ABC model depicted stands for several species that have been experimentally analyzed (for Orchidaceae, *Phalaenopsis* spp [Tsai et al., 2004] and *D. crumenatum* [Xu et al., 2006]; for Solanaceae, *Petunia hybrida* [Vandenbussche et al., 2004] and tomato [*Solanum lycopersicum*] de Martino et al., 2006). The A function for all lineages is included for all taxa to enable comparison with *Arabidopsis*, although a question mark was added to emphasize its dubious role in other flowering plants, where it has not been shown to be necessary for perianth formation; rather, it is fundamental for floral meristem determination (Soltis et al., 2007). Along the phylogeny branches, the moments when the variants of the ABC genes could have evolved are indicated with orange rectangles (synapomorphies; characters shared by several lineages derived from a common ancestor). Purple rectangles indicate the appearance of traits that favored homeosis in the Triuridaceae lineage (an autapomorphy; a derived character unique to a single lineage/taxon). This autapomorphy pertains to *L. schismatica AP3* expression and stamen development, which are constrained to the center of the flower during early and late developmental stages, in contrast with the early expression of *AP3* orthologs in other hermaphroditic flowering plants, which can encompass several whorls, although the combined B (joint expression of *AP3* and *PI* orthologs) and C functions are restricted to the third whorl, where stamens usually develop. ca, carpels; lo, lodicules; pe, petals; pl, petal-like tepals; ps, palea/lemma; sd, staminodes; se, sepals; sl, sepal-like tepals; st, stamens; te, petaloid tepals; sp, sepaloid petals; W1-W4, whorls within the flower primordia numbered from the outermost position to the center; asterisk, while in numerous angiosperms, W1 would stand for the sepal whorl, W2 for the petals, W3 for stamens, and W4 for the carpels, several angiosperm lineages differ from this organization either because they have a spiral arrangement (*Amborella* and *Nimphaea*), by having very modified perianth organs (*Oryza*), or by lacking a defined sepal/petal identity (tepals; *Amborella*, *Lacandonia*, and orchids). ABC model modified from Figure 25 of Álvarez-Buylla et al. (2010b) (data summarized from Xiao et al., 2003; Irish and Litt, 2005; Kim et al., 2005; Soltis et al., 2007; Theissen and Melzer, 2007; Whipple et al., 2007; Mondragón-Palomino and Theissen, 2009).
[See online article for color version of this figure.]

organs of the Mexican triurid are developmentally closer to sepals than to petals, which is in agreement with their appearance (Figure 5B). Such putative organ-type loss could be related to the presence of cleistogamy in *L. schismatica*, which might be associated with an absence of Darwinian selection for organs whose putative function is related to pollinator attraction.

In contrast with previous reports on Y2H AP3-PI interaction experiments for other monocots (Xu et al., 2006; Su et al., 2008), the fact that the proteins of *L. schismatica* form heterodimers only in the presence of At-SEP3 confirms the importance of other MADS domain proteins (AG and SEP proteins) in stabilizing the PI-AP3 interaction and specifying stamen formation (Honma and Goto, 2001). Moreover, it is also important to explain why the Ls-AP3 complementation of the *Arabidopsis ap3-3* mutant lines was poorer than that of Ls-PI in *Arabidopsis pi-1* mutants. Coincidently, the interaction of Ls-PI with At-AP3 or At-SEP3 was stronger than that of Ls-AP3 with At-PI/At-SEP3 (Table 1). Moreover, the Ls-AP3 protein is able to form dimers with At-AP3 as well as homodimers with itself, as found in other monocots, such as *Phalaenopsis* spp (Tsai et al., 2008). On the other hand, the Ls-PI/At-PI dimers and Ls-PI/Ls-PI homodimers are weaker than those of AP3 (all of these dimers were found in the presence of SEP3). These data suggest that the formation of an Ls-AP3/At-AP3/At-SEP3 complex in planta could compete with the

functional Ls-AP3/At-PI/At-SEP3 heterodimeric complex, and this may explain why the *Arabidopsis ap3-3* lines were not complemented as well as those of *pi-1*. Interestingly, the Ls-PI/Ls-AP3 dimer is weaker than the dimers formed between Ls-AP3 and either Ls-AP3 or At-AP3, and this could explain why the double overexpression line (*35S:LsAP3 35S:LsPI*) in a wild-type background does not present stamens in the fourth whorl as in *Arabidopsis* (Figures 4C and 4Q).

Finally, we propose that future studies on the expression and function of ABC model-related genes/proteins in the Mexican triurids should address the identity of the *cis*- or *trans*-acting factors underlying the expression of Ls-*AP3* in the center of the flower in *L. schismatica* (Figure 2). If a *cis*-alteration is involved, and it turns out to be sufficient to explain the expression pattern of this gene, the homeotic floral organ arrangement of this species might be considered a strong candidate of morphological (evolutionary) saltation, in which a phenotypic autapomorphy (i.e., a new character state only present in a particular lineage) became fixed among natural populations through rather simple changes at the molecular level. However, additional *trans*-acting factors may have been involved in establishing the unique floral phenotype of this species. We suggest that research related to these questions should also be pursued in other species within families of the order Pandanales and that the use of formal modeling tools (Álvarez-Buylla et al., 2008, 2010a) might be helpful to complement such experimental investigations.

METHODS

Collections

Samples of *Lacandonia schismatica* were collected and processed for in situ localizations, scanning electron micrography, and DNA or RNA extraction, according to protocols previously described (Vergara-Silva et al., 2003; Ambrose et al., 2006) during Summer and Winter seasons of 1995 to 2005.

Cloning of Ls-*AP3*, Ls-*PI*, and Ls-*AG*

RNA was isolated from fresh or nitrogen frozen inflorescences using a standard Trizol (Invitrogen) extraction method. cDNA synthesis was followed by 3′ and 5′ rapid amplification of cDNA ends experiments, and final amplifications were based on 5′ and 3′ untranslated region sequences. Primer sequences are presented in Supplemental Table 1 online. Fragments were cloned into pGEM-T Easy vectors (Promega).

Alignment and Phylogenetic Analyses

Amino acid matrices were initially aligned using ClustalX and later aligned manually using Se-Al sequence alignment editor (http://evolve.zoo.ox.ac.uk). Bayesian phylogenetic analysis for B and C MADS box lineages was performed using unambiguously conserved domains in Mr. Bayes 3.2 (Huelsenbeck and Ronquist, 2001). In both cases, we used the mixed model option (aamodelpr=mixed) for a better estimation of the amino acid fixed rate model. Each analysis was run until the average deviation of split sequences went below 0.01 (from 1,000,000 to 1,500,000 generations). The first 10% of the total number of saved trees (1000 to 1500) was discarded to allow for the burn-in phase. Posterior probability values are indicated in each branch leading to a particular lineage. Trees were rooted using previously corroborated gymnosperm B and C genes as outgroups (Sundström et al., 1999).

In Situ Hybridization

In situ RNA hybridization was performed as previously described (Jackson, 1991). Sections (7 μm) were hybridized to ^{35}S-labeled RNA probes obtained using the SP6/T7 transcription kit (Roche). To obtain probes, we used PCR and primers with T7 extensions to amplify fragments of ~150 bp. Primer sequences are presented in Supplemental Table 2 online. The slides were coated with NBT2 emulsion (Kodak) and exposed for 2 months. After development, the sections were stained in 20% Gills hematoxylin (British Drug House). The sections were photographed using a Leica microscope coupled to a Leica DFC 490 camera, and the contrast of the photomicrographs was adjusted using Adobe Photoshop 8.0 software.

Complementation Lines

Both Ls-*PI* and Ls-*AP3* full-length cDNAs were amplified with primers (see Supplemental Table 3 online) that conferred *Xho*I and *Bam*HI restriction sites to the 5′ and 3′ ends, respectively, to allow directional cloning. PCR products were cut with the same enzymes and inserted into pART7, a shuttle vector carrying a single copy of the cauliflower mosaic virus 35S promoter and unique *Not*I sites flanking the promoter and OCS terminator. These constructs were cut with *Not*I and subcloned into a *Not*I-digested cut pART27, a binary vector that confers kanamycin resistance to plants (Gleave, 1992). Wild-type *Arabidopsis thaliana* plants (Columbia ecotype) were transformed with the *35S:LsAP3* or *35S:LsPI* constructs using the standard floral dipping procedure and selected on kanamycin plates (Clough and Bent, 1998). RNA gel blot hybridization was performed using 5 μg of total RNA from leaves and gene-specific 3′ probes (see Supplemental Table 4 online) to verify mRNA expression of B genes (see Supplemental Figure 3 online). At least four independent lines showed consistent phenotypes for *35S:LsPI*, while nine independent lines were obtained for *35S:LsAP3. 35S:LsPI* (ABA30) and *35S:LsAP3* (ABA13.2) F2 lines were crossed with *pi-1/pi-1* and *ap3-3/ap3-3* mutants, respectively. The F2 of such crosses [ABA 64-4(X) for *35S:LsPI;pi-1/pi-1* and ABA101-1(X), ABA101-3(X), and ABA102-3(X) for *35S:LsAP3;ap3-3/ap3-3*] segregated individuals with wild-type, mutant, ectopic expression, and complementation phenotypes according to Mendelian ratios (see ratios in Supplemental Table 5 online). Double transgenic crosses *35S:LsAP3* × *35S:LsPI* were performed, and the F2 plants [71-2(X)] were analyzed. RT-PCR was performed using specific primers (see Supplemental Table 4 online) and cDNA from leaf tissue (see Supplemental Figure 3 online). The phenotype of plants was analyzed using light microscopy and scanning electron microscopy in a Jeol microscope model JSM-5310LV, as previously described (Ambrose et al., 2006).

Y2H and Y3H Assays

All of the genes that were used in Y2H assays were cloned both into the AD vector (pDEST22) and into the BD vector (pDEST32). We transformed yeast by the Li-acetate method, as described initially by Ito et al. (1983) and modified by Lauermann (1991). All the genes in the pDEST22 vector were transformed into yeast strain Y187 (*MAT*α; Harper et al., 1993), and all of the inserts in pDEST32 into strain PJ69-4 (*MATa*; James et al., 1996) and selected on SD plates lacking Trp and Leu, respectively. Cells were routinely grown on YPD propagation medium (1% yeast extract [Difco], 2% bacto-peptone [Difco], and 2% glucose) or SD selection medium (2% glucose, 0.7% yeast nitrogen base without amino acids [Difco], and 0.05 M MES [Sigma-Aldrich], pH 6) supplemented with different compounds: adenine (Ade; 30 mg/mL), uracil (Ura; 30 mg/mL), His (30 mg/mL), Trp (100 mg/mL), and Leu (100 mg/mL) at 30°C. Solid medium contained 2% agar (Difco).

Each transformant was grown on SD medium. Transformants were mated with each other by sowing each colony over the other on SD

medium containing all the requirements and incubated at 30°C. The resulting diploid yeast colony was transferred to SD plates lacking both Leu and Trp and, after 3 d of growth, yeast cells were transferred to different selection plates containing SD medium lacking His, Leu, and Trp (HIS) and SD medium lacking His, Leu, and Trp, supplemented with 1 (HIS/1 mM 3AT), 3 (HIS/3 mM 3AT), and 5 mM (HIS/5 mM 3AT) 3-amino-1,2,4-triazole (3AT). These plates were incubated at 28°C and scored for growth over a 10-d period to detect protein–protein interactions.

For the Y3H experiment, all of the AD clones were crossed with the TFT vector and selected on SD medium lacking Trp and Ade and then crossed with each of the yeast lines containing the BD constructs. After this point, the same SD selection medium supplemented with various compounds was used as for Y2H, but without Ade. All of the combinations generated in the Y2H experiment were assayed, and almost all of the diploids were assayed as both AD and BD in the Y3H assay, except for LsPI(IKC)/AtSEP3/LsPI(IKC), LsAP3FL/AtSEP3/LsPI(IKC), LsPI(IKC)/AtSEP3/LsAP3(IKC), and LsAP3(IKC)/AtSEP3/LsAP3(IKC), where the first construct was in the AD vector and the third construct was in the BD vector. The results of the Y2H and Y3H assays (Table 1) were reported with the first gene in the AD vector and the second gene in the BD vector.

Accession Numbers

Sequence data from this article can be found in the National Center for Biotechnology Information/GenBank/nucleotide data libraries under accession numbers GQ214161 (Ls-*AP3*), GQ214162 (Ls-*PI*), GQ214163 (Ls-*AG*), and GQ214164 (Ls-*STK*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Bayesian Analysis of Members of the AG Lineage.

Supplemental Figure 2. Ls-*PI* in Situ Localization.

Supplemental Figure 3. Overexpression of Ls-*PI* and Ls-*AP3* in F2 Segregating Lines in Their Respective Mutant Backgrounds.

Supplemental Table 1. Primers Sequences Used for 5′ and 3′ RACE.

Supplemental Table 2. Primer Sequences for 3′ Specific in Situ Hybridization Probes.

Supplemental Table 3. Primers Sequences Used for Amplified Ls-*AP3* and Ls-*PI* Full-Length cDNAs.

Supplemental Table 4. Specific Primers for Ls-*AP3* and Ls-*PI* RT-PCR and RNA Gel Blot Probes.

Supplemental Table 5. Phenotypic Frequencies of an F2 Segregating Population of a Cross between the Ectopic Expression Line of *L. schismatica* B Genes and an Endogenous B Gene Mutant in *Arabidopsis.*

Supplemental Data Set 1. Gene Alignments of *L. schismatica PI* and *AP3.*

Supplemental Data Set 2. Gene Alignments of *L. schismatica AG* and *STK.*

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Journal of Experimental Botany, Page 1 of 19
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INVITED REVIEW

When ABC becomes ACB

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Abstract

Understanding how the information contained in genes is mapped onto the phenotypes, and deriving formal frameworks to search for generic aspects of developmental constraints and evolution remains one of the main challenges of contemporary biological research. The Mexican endemic triurid *Lacandonia schismatica* (Lacandoniaceae), a mycoheterotrophic monocotyledonous plant with hermaphroditic reproductive axes is alone among 250 000 species of angiosperms, as it has central stamens surrounded by a peripheral gynoecium, representing a natural instance of a homeotic mutant. Based on the classical ABC model of flower development, it has recently been shown that the B-function gene *APETALA3* (*AP3*), essential for stamen identity, was displaced toward the flower centre in *L. schismatica* (ABC to ACB) from the early stages of flower development. A functional conservation of B-function genes from *L. schismatica* through the rescue of B-gene mutants in *Arabidopsis thaliana*, as well as conserved protein interactions, has also been demonstrated. Thus, it has been shown that relatively simple genetic alterations may underlie large morphological shifts fixed in extant natural populations. Nevertheless, critical questions remain in order to have a full and sufficient explanation of the molecular genetic mechanisms underlying *L. schismatica*'s unique floral arrangement. Evolutionary approaches to developmental mechanisms and systems biology, including high-throughput functional genomic studies and models of complex developmental gene regulatory networks, constitute two main approaches to meet such a challenge. In this review, the aim is to address some of the pending questions with the ultimate goal of investigating further the mechanisms of *L. schismatica*'s unique homeotic flower arrangement and its evolution.

Key words: ABC model, angiosperms, flower development, gene regulatory networks, homeotic mutations, *Lacandonia schismatica*, molecular genetics, stamen and carpel development, tropical forests.

Introduction

The importance of development for modern evolutionary biology was not fully recognized until the late 20th century because it was left out of the 'Modern Synthesis', even though its significance as a central mechanism of transformation and generation of biodiversity had been postulated in the mid-19th century (Friedman and Diggle, 2011). Evolutionary developmental biology (*evo-devo*) arises from a fusion of different scientific fields such as embryology, taxonomy, morphology, anatomy, and developmental genetics to explain various aspects of the historical origin of different morphological traits. The contemporary field of *evo-devo* has incorporated molecular biology tools and has been behind the fundamental finding that relatively small gene modifications can produce major phenotypic shifts

(García-Bellido and Robbins, 1983; Álvarez-Buylla *et al.*, 2010*b*).

Lacandonia schismatica (Lacandoniaceae) is a mycoheterotrophic monocotyledonous plant with hermaphroditic homeotic reproductive axes; alone among 250 000 species of angiosperms, it has central stamens surrounded by a peripheral gynoecium (Martínez and Ramos, 1989). This outstanding feature of the reproductive axes of *L. schismatica* has been regarded as a natural homeotic mutant that is fixed in natural populations (Vergara-Silva *et al.*, 2003). It has been shown that relatively simple genetic alterations may underlie this large morphological shift fixed in natural populations of extant plant taxa (Alvarez-Buylla *et al.*, 2010*b*). Critical issues related to the molecular genetics and evolution of *L. schismatica* flower development and morphology include: which changes in the underlying Gene Regulatory Network (GRN) controlling flower morphogenesis enabled the emergence of this plant's singular floral phenotype? What is the identity of the reproductive axes of this species? Additional questions that remain to be addressed in order to have a full and sufficient explanation of the molecular genetic mechanisms underlying *L. schismatica*'s unique floral arrangement are indicated. Finally, the question of whether an understanding of the particular modifications present in *L. schismatica* at the functional/genetic levels can help expand our view of the mechanisms and processes implicated in flower and plant *evo-devo* is also addressed.

The homeotic flowers of *Lacandonia schismatica* constitute a morphological saltation.

Flower structure and reproductive biology of L. schismatica

The flower is one of the defining structures of angiosperms and it is essential for their reproduction. Still, its origin and the rapid radiation of this group of plants are currently unresolved mysteries. Angiosperms include the early or basal angiosperms (ANITA grade and the magnoliids), the monocots, and the eudicots. The eudicots comprise about 75% of the angiosperm species known to science and have relatively standard flowers consisting of four different organs arranged in whorls: sepals, petals, stamens (androecium), and carpels (gynoecium; from the outermost to the innermost whorl; see Fig. 1). By contrast, *L. schismatica* has three floral whorls with a heterotopic arrangement of carpels and stamens (Martínez and Ramos, 1989). The androecium with three anthers (occasionally four) is located in the centre of the flower and surrounded by the gynoecium that is occupied by numerous (60 to 80) apocarpous carpels (Fig. 1). Each carpel has a sessile, anatropous, bitegmic, and basal ovule (Martínez and Ramos, 1989; Márquez-Guzmán *et al.*, 1989; Vázquez-Santana *et al.*, 1998). The outermost whorl is occupied by a basally fused perianth comprised of six tepals on average. Thus, to

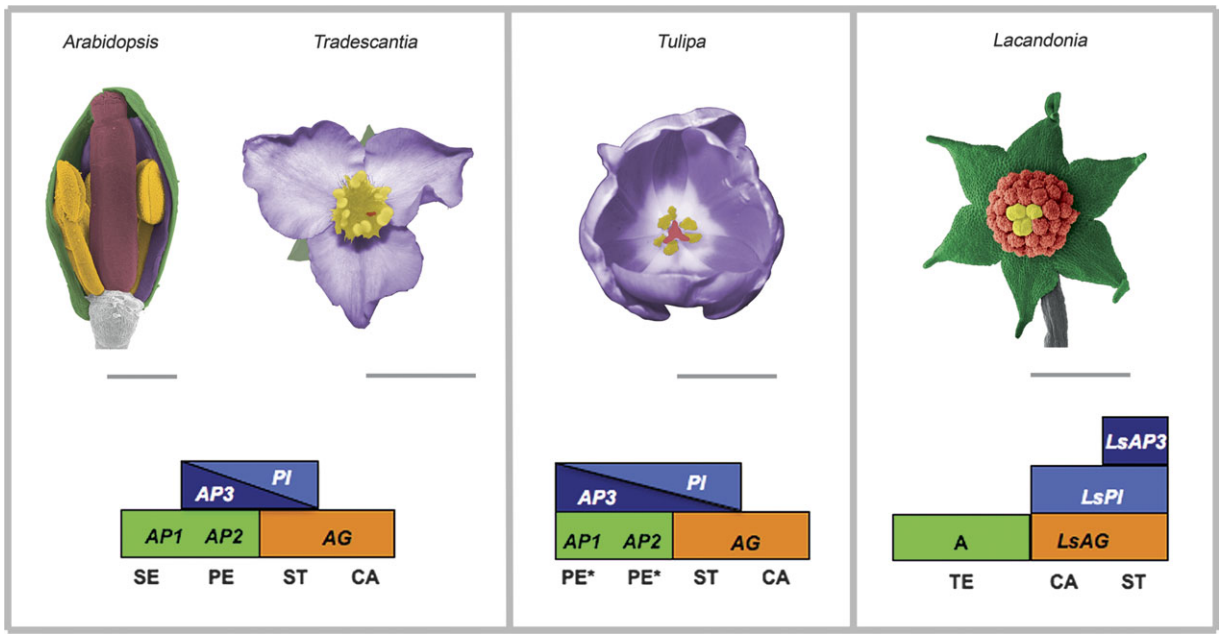

Fig. 1. Variations of the ABC model of floral organ development in monocots, in contrast with the canonical model proposed for *Arabidopsis*. Some monocots such as *Tradescantia* (Commelinaceae) share the same underlying genetic regulation of B genes as *Arabidopsis*, while the tulip (*Tulipa*) conforms to the 'shifting boundaries ABC model' where B function has expanded to the first whorl and petaloid tepals develop. By contrast, the floral plan in *Lacandonia* differs from all other angiosperms in having the B function displaced to the centre of the floral meristem (rendering an ACB model), where central stamens develop. Green: sepals; purple: petals; yellow: stamens; red: carpels. (This figure is available in colour at *JXB* online.)

date, this is the only bisexual angiosperm species with central stamens surrounded by carpels.

Since the *L. schismatica* flower is cleistogamous, that is, it self fertilizes before anthesis, it does not require pollinators for its reproduction and anthesis becomes relevant only for fruit dehiscence and dispersal. The unit of dispersal is a unicarpellate one-seeded indehiscent fruit (achene); the maturation of these fruits within a single flower is synchronous and each seed has an immature embryo when it dehisces from the receptacle (Vázquez-Santana *et al.*, 1998). This autogamic fertilization mode, together with the fact that the inflorescences from this species are commonly located below organic litter, may explain the lack of genetic variation found in one of the populations of *L. schismatica* that was studied through isozyme variation (Coello *et al.*, 1993).

L. schismatica *reproductive structures are true flowers*

While *L. schismatica* is currently in its own family (Lacandoniaceae; Martínez and Ramos, 1989), there is strong evidence that suggests that this species is part of the Triuridaceae family. It shares with all Triuridaceae species a mycoheterotrophic achlorophyllous perennial herbaceous habit with reduced vegetative and reproductive parts (Maas and Rubsamen, 1986; Rubsamen-Weustenfeld, 1991; Leake, 1994). Due to this reduction and peculiar floral structure, there have been several interpretations of the reproductive axes of *L. schismatica* (Stevens, 1991; Rudall, 2003). Initially, it was proposed that *L. schismatica* had true flowers with a homeotic mutation fixed in natural populations (Martínez and Ramos, 1989). This interpretation was contended firstly on the grounds that the central stamens observed in *L. schismatica* could be a by-product of a secondary torsion event that would put the otherwise external stamens in the centre of the floral meristem (Stevens, 1991).

Later, an alternative interpretation of the reproductive axes of *L. schismatica* was proposed, based on a phylogenetic analysis that established that the Triuridaceae was a sister family to Pandanaceae, a family with bona fide inflorescences (Chase *et al.*, 2000; Rudall, 2003). In this alternative interpretation, the reproductive axes of *L. schismatica* were reinterpreted as reduced inflorescences (pseudanthia) with distal male flowers (reduced to three single, naked stamens) and proximal female flowers (also reduced to single carpels), arranged in such a manner that they superficially resembled an inverted bisexual flower (Rudall, 2003). This hypothesis was further supported by comparative morphological analysis of mature reproductive units of Pandanaceae, Triuridaceae, and other members of the order Pandanales (Rudall, 2003). In subsequent studies, this hypothesis was refined, suggesting that triurid flowers should be interpreted either as inflorescences that have undergone an extensive process of compression and organ reduction or as reproductive axes where the flower and inflorescence meristem boundaries are not clear and yield a single reproductive structure, thus, rendering an ambiguous morphological identity (Rudall and Bateman, 2006; Rudall, 2008).

In parallel, the identity of the reproductive axes of two species of Triuridaceae was addressed with a comparative developmental series of the reproductive structures of *L. schismatica* and *Triuris brevistylis*. Our evidence supported the interpretation that the reproductive axes of *L. schismatica* are true flowers (euanthia; Ambrose *et al.*, 2006). Some of the key features that supported our euanthial interpretation are: (i) the enclosed floral buds arise from the flanks of clearly distinguishable naked inflorescence meristems; (ii) each floral meristem is enclosed by a bract that is located opposite to the site where the first tepal arises; (iii) the temporal order of organ development is as follows: perianth organs develop first (in the outermost whorl), followed by stamens (in the centre of the floral meristem) and lastly the carpels develop (in the whorl between the tepals and the stamens); (iv) there was no evidence of aborted floral organs or mechanical shifts of organs during development, (v) stamens and carpels arise from compound primordia that are characteristic of flowers, and (vi) the organs of *L. schismatica* have a trimerous arrangement (i.e. three elements or its multiples in the different floral organs), which is a unifying trait in monocot flowers. Although the latter is also true in some inflorescences, all six characteristics together strongly suggest the reproductive axes of *L. schismatica* and *T. brevistylis* are true flowers (Ambrose *et al.*, 2006). Hence, the inside-out flower of *L. schismatica*, as a case of a morphological saltation (i.e. a homeosis), has been analysed in the context of flower angiosperm evolution.

Molecular genetic mechanisms of the inside-out flower in *Lacandonia schismatica*: the ABCs and beyond

The ABC model of flower development and the MADS-box genes

In the late 1980s, the study of floral homeotic mutants of *Antirrhinum majus* and *Arabidopsis thaliana* (*Arabidopsis*) lead to the now classical ABC model of floral organ determination (Bowman *et al.*, 1989; Coen and Meyerowitz, 1991). The homeotic mutations used to postulate this model were categorized in three different classes: in the A class mutants, sepals are replaced by carpels in the first whorl and petals are replaced by stamens in the second whorl; B class mutants have sepals instead of petals in the second whorl and carpels instead of stamens in the third whorl while C class mutants have petals in place of stamens and sepals in place of carpels, in addition, they are indeterminate, generating a flower within a flower (Bowman *et al.*, 1989). Based on these mutants, the ABC model postulates that: the A genes are necessary to specify sepals; A plus B genes to specify petals; B and C genes to specify stamens, while the C genes alone are necessary to specify carpels. In addition, A and C genes act in an antagonistic fashion, repressing each other in the whorls where they function (Coen and Meyerowitz, 1991; Fig. 1). The genes responsible for these

phenotypes were cloned and it was shown that all of them encoded transcription factors and that four out of five belonged to the MADS-box transcription family (MADS box Type II genes with a plant-specific MIKC protein structure) while the non-MADS-box gene (*AP2*) is part of the AP2/ERF transcription factor family. In *Arabidopsis*, class A genes are named *APETALA1* (*AP1*) and *APETALA2* (*AP2*); class B genes are *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), and the class C gene is *AGAMOUS* (*AG*).

In 2001 it was shown that the ABC genes were necessary but not sufficient to transform leaves into floral organs, and that other MADS-box genes called *SEPALLATA* (*SEP*), genes were also required as a *sep1*/*sep2*/*sep3*/*sep4* quadruple mutant produced floral structures with leaf-like organs in all whorls (Honma and Goto, 2001; Pelaz *et al.*, 2001; Robles and Pelaz, 2005).

Since its proposal, the ABC model (Coen and Meyerowitz, 1991), has been instrumental in establishing a link between developmental genetics and evolutionary studies of flower development, as it provided a straightforward framework for comparison of expression patterns of orthologous genes that could potentially determine peculiar floral or inflorescence features in specific taxa (Fig. 1; Table 1). With regard to the role of B genes in floral specification, the perianth is a structure that has received much attention, especially regarding the petaloid organs present in many flowering plants. The emphasis on this structure and not other floral organs is based on two issues: firstly, the perianth is thought to have evolved several times independently in different angiosperm groups and thus, this structure is particularly informative of the evolutionary processes occurring in angiosperm evolution. Secondly, this structure is important for pollinator attraction in many lineages and, as such, it has been considered of nodal importance to elucidate the process of angiosperm adaptive radiation (Irish, 2009). Furthermore, the role of the A class genes in specifying sepal development has not been observed in many flowering plants other than *Arabidopsis*; analyses of *AP1* and *AP2* orthologues in other species have shown expression patterns that differ from those predicted by the ABC model (Soltis *et al.*, 2007), although both genes play a fundamental role in floral meristem determination. On the other hand, the C (and derived D) class genes have been found to be broadly conserved, and thus are generally regarded as less informative from a phylogenetic point of view (Irish, 2009). These considerations have made the B class genes good candidates to undertake comparative developmental and lineage evolution studies in angiosperms (Irish, 2009).

The function of the B genes in monocotyledons and the exceptions to the ABC model in monocot flower development

Class B genes (*AP3* and *PI*) characterized in *Arabidopsis* are both required to specify stamens and petals: the *ap3* and *pi* single mutants have homeotic transformation of both petals and stamens into sepals and carpels, respectively (Bowman *et al.*, 1989; Jack *et al.*, 1992). A number of publications analysing different basal angiosperms, non-model eudicot, and monocot species have appeared in recent years, with important implications on the applicability and variation of the ABC model in members of this latter group (Kanno *et al.*, 2007; Mondragón-Palomino and Theißen, 2009).

Monocots comprise a diverse group of plants that incorporate some 'petaloid' species (such as those present in the Commelinids, Alismatales, and Orchidaceae, among others) which can have a perianth structure composed by undifferentiated organs (tepals; for example, the tulip) to well-differentiated sepal and petal whorls (*Tradescantia reflexa*, a commelinid; Ochiai *et al.*, 2004; Fig. 1; Table 1) similar to those found in eudicot species, as well as species with unique perianth organs such as the labellum in orchids (Mondragón-Palomino *et al.*, 2009) and some members of the Costaceae and Zingiberaceae (Bartlett and Specht, 2010).

Other monocots have a highly modified floral structure, such as the grasses (Poaceae; Whipple *et al.*, 2004). Several early studies of B gene expression in monocots showed that, in some grass species, the B-class genes were generally conserved with respect to *Arabidopsis*, determining stamen and petal identity (in the case of maize, the second whorl organs, the lodicules, have been homologized to petals; Whipple *et al.*, 2004). On the other hand, other studies found that *AP3* and *PI* orthologues are broadly expressed in all whorls during the early flower development of monocots such as orchids (Chung *et al.*, 1994; Xu *et al.*, 2006; Kanno *et al.*, 2007; Table 1). These observations supported a proposition that the formation of petaloid-tepals in the first and second whorls of some non-grass monocot species or petaloid-sepals in two dicot species, was due to the expansion of the B genes expression domain into the first whorl (Kanno *et al.*, 2003, 2007). Thus, in those monocots where B-class genes are expressed in the first whorl, a modified 'shifting boundaries' ABC model has been proposed (Bowman, 1997; Kanno *et al.*, 2007).

An additional finding that has complicated the elucidation of B-class gene function in plants other than *Arabidopsis*, is the fact that several duplications within the *AP3* and *PI* genes have taken place throughout the angiosperms (Kim *et al.*, 2004; Hernández-Hernández *et al.*, 2007; Mondragón-Palomino *et al.*, 2009). Such duplications could have an important evolutionary effect, as duplicated genes can be subject to differential selective pressures, enabling the acquisition of new functions via subfunctionalization (a restriction in the domain of expression of the paralogues compared with what has been documented in a functionally homologous single copy gene) and/or neofunctionalization, (through either a shift of the domain of expression into another whorl or organ, or acquiring a new function relative to its paralogous gene; Vandenbussche *et al.*, 2004).

All of the examples listed so far are consistent with a "shifting boundaries" ABC model; however, there are some examples in monocot species with floral phenotypes and B expression patterns that do not fit this modified model (Table 1). Some of these contradictions could be explained by the existence of additional duplicates of each of the

Table 1. Expression pattern of B (DEF/AP3 and GLO/PI) and C (PLE/AG) genes of several monocot plant species
Grey colour boxes represent gene expression patterns (mRNA) in different floral whorls (W) of each species included in the table. Light grey boxes indicate a lower gene expression than dark grey. Boxes with hatched lines represent the whorl(s) where B function is present. B function is predicted from the observed flower morphology, i.e. the presence of petaloid perianth organs or stamens and stamen derivatives such as the columella in orchids and labellum in Zingiberaceae and Costaceae. The dimerization pattern between the B genes of the same species is shown in column Ho/He. Ho, homodimerization; He, heterodimerization. (a) Expression of B genes without B function; (b) B function without B gene expression, as inferred from the phenotype; (c) No expression data for these PI orthologues in the second whorl are available but functional studies using antisense lines indicate that these genes participate in lodicule development (Kang *et al*., 1998); (d) Inferred expression based on the observed expression of these B genes in adjacent whorls. OT, outer tepal; IT, inner tepal; Te, tepal; St, stamen; Ca:, carpel; Le/Pa, lemma/palea; Lo, lodicules; col, column; pol, pollinia; N/A, Not Analyzed.

Family	Species	Gene name	W1 W2 W3 W4 OT IT St Ca	Ho/He	Reference
Liliaceae	*Tulipa gesneriana*	*TGDEFA* *TGDEFB* *TGGLO*		He He Ho	Kanno *et al.*,2003
	B function				
	Lilium regale	*LRDEF* *LRGLOA* *LRGLOB*	N/A N/A N/A	He Ho/He Ho/He	Winter *et al.*,2002
	B function				
	Lilium longiflorum	*LMADS1(DEF)* Prot LMADS1 *LLAG1*		Ho/He(PI)* Ho	Tzeng and Yang, 2001 Benedito *et al.*, 2004 Hsu *et al.*, 2010
	B function				
	Tricyrtis affinis	*TriaDEF* *TriaGLO*			Kanno *et al.*,2007
	B function				
Amaryllidaceae	*Agapanthus praecox*	*ApDEF* *ApGLO*			Nakamura *et al.*,2005
	B function				
Alstroemeriaceae	*Alstroemeria ligtu*	*AlsDEFa* *AlsDEFb* *AlsGLO*	a		Hirai *et al.*, 2007
	B function				
Hyacinthaceae	*Muscari armeniacum*	*MaDEF* *MaDEF2* *MaGLOA1* *MaGLOA2* *MaGLOB*	a		Nakada *et al.*,2006 Kanno *et al.*,2007
	B function				
Asparagaceae	*Asparagus officilianis*	*AODEF* *AOGLOA* *AOGLOB*	b		Park *et al.*,2003 Park *et al.*,2004
	B function				
Iridaceae	*Crocus sativus*	*CsatAP3* *CsatPIc* *CsatAG*	a		Tsaftaris *et al.*,2005 Tsaftaris *et al.*,2006 Kalivas *et al.*,2007
	B function				

Table 1. continued

Family	Species	Gene name	W1	W2	W3	W4	Ho/He	Reference
Arecaceae	*Elaeis guineensis*	*EgGLO1*	N/A					Adam *et al.*,2007
		EgGLO2						
		EgDEF						
	B function							
Commelinaceae	*Commelina communis*	*CcGLO*						Ochiai *et al.*,2004
		CcDEF						
	B function							
	Tradescantia reflexa	*TrGLOA*						Ochiai *et al.*,2004
		TrGLOB						
		TrDEF						
	B function							
			Le/Pa	**Lo**	**St**	**Ca**		
Poaceae	*Zea mays*	*Si1 (DEF)*					He	Schmidt *et al.,* 1993
		Zmm16 (GLO)					He	Mena *et al.*, 1995
		Zmm18 (GLO)						Mena *et al.*, 1996
		Zmm29 (GLO)						Munster *et al.*, 2001
		ZAG1(AG)						Whipple *et al.*, 2004
		Zmm2(AG)						
	B function							
	Oryza sativa	*OSMADS16 (DEF)*					He	Chung *et al.*, 1995
		OSMADS2 (GLO)		[c]				Kang *et al.*, 1995
		OSMADS4 (GLO)		[c]			He	Moon *et al.*,1999
		OSMADS3 (AG)						Yamaguchi
		OSMADS58 (AG)						*et al.*, 2006
	B function							
			W1	**W2**	**W2**	**W3**	**W3**	**W4**
			Se	**pe**	**lab**	**col**	**pol**	**ca**
Orchidaceae	*Phalaenopsis equestris*	*PeMADS2 (DEF)*						He
		PeMADS3 (DEF)						He
		PeMADS4 (DEF)						Ho/He
		PeMADS5 (DEF)						He
		PeMADS6 (GLO)					[d]	Ho/He
		PhalAG1 (AG)						
	B function							
	Oncidium G. Ramsey	*OMADS3 (DEF)*						Ho
		OMADS2 (AG)						Ho
	B function							
	Dendrobium crumantum	*DcOAP3A*						He
		DcOAP3B					[d]	He
		DcOPI						He
		DcAG1						
		DcAG2						
	B function							

Table 1. continued

Family	Species	Gene name	W1	W2	W3	W4	Ho/He	Reference
			W1 Se	W2 Pe	W3 Lab	W3 St	W4 ca	
Zingiberales	*Alpinia oblongifolia*	*AoPI* *AoAP3* *AoAG*						Gao *et al.*,2006 Xia *et al.*, 2009
	B function							
	Alpinia hainanensis	*AhMADS5 (GLO)* *AhMADS6 (DEF)* *AhMADS6 (AG)*						Song *et al.*,2010
	B function							
	Musa basjoo	*MbGLO1*						Bartlett and Specht, 2010
	Costus spicatus	*CsGLO1* *CsGLO2*						Bartlett and Specht, 2010
	B function							
				W1/W2 Te	W3 Ca	W4 St		
Triuridaceae	*Lacandonia schismatica*	*LsPI* *LsAP3* *LsAG*						Álvarez-Buylla *et al.*,2010b
	B function							

B genes and with anomalous B class gene expression patterns (as in *Asparagus officinalis*, *Dendrobium crumenatum*, *Crocus sativus*, and *Muscari armeniacum*; Table 1; Stellari *et al.*, 2004). Other explanation of these 'rare' phenotypes would be the existence of post-transcriptional regulation on these genes and/or the absence of the protein of one or both B genes. Historically, it was assumed that the MADS genes exerted their function in the places where their mRNA was expressed, however, this is not always the case (Tzeng and Yang, 2001; Table 1). For instance, in *Arabidopsis*, *PI* is expressed in all three inner whorls, while *AP3* is expressed only in the precursor cells of petals and stamens (Jack *et al.*, 1992) but it has been inferred that AP3 is also present in the first whorl, as plants that overexpress *PI*, show a conversion of sepals into sepals/petals organs (Krizek and Meyerowitz, 1996). Nonetheless, B proteins coincide only in whorls two and three, where they form a functional heterodimer and exert the B function (Krizek and Meyerowitz, 1996; Riechmann *et al.*, 1996). Thus, in the case of the monocots, in order to clarify the molecular mechanisms that have led to the appearance of whorls with mixed or novel characteristics, more studies that address the *in vivo* localization of B class MADS proteins, as well as their functionality in heterologous systems, are needed. In addition, in contrast to eudicot plants, B proteins from some monocots can bind CArG boxes as homodimers (Table 1) although it is not known if such homodimers are able to perform the B function.

It is important to note, however, that, with the exception of *L. schismatica*, the divergences documented to date have never been associated with a homeotic inversion on the position of stamens and carpels caused by a displacement of the B function to the flower centre (Kramer and Irish, 1999; Kanno *et al.*, 2003; Vandenbussche *et al.*, 2004; Rijpkema *et al.*, 2006; Xu *et al.*, 2006).

Higher order MADS protein complex formation

The experiments reported by Riechmann *et al.* (1996) and Egea-Cortines *et al.* (1999) in *Arabidopsis* and *Antirrhinum majus*, respectively, demonstrated that MADS proteins could only bind to DNA (CArG boxes) as dimers. Moreover, it was shown that these transcription factors are able to form tetramers with a stronger interaction to DNA

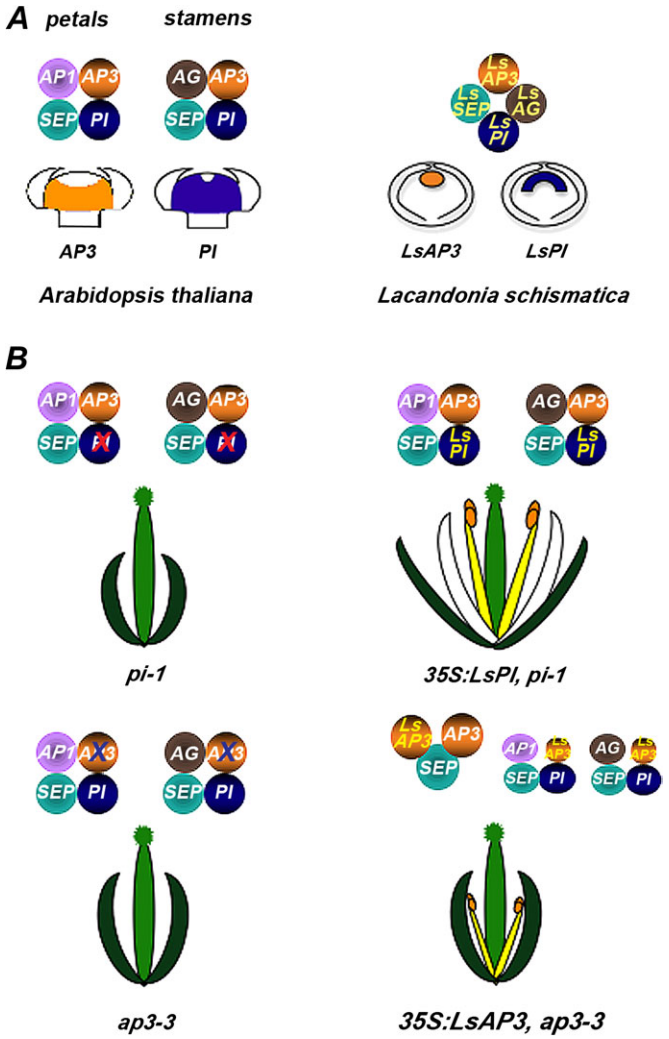

Fig. 2. Protein complexes in *Arabidopsis* and *L. schismatica* involved in specifying B function. (A) Left panel: protein tetramers involved in B function in *Arabidopsis* and their schematic representation at floral stage 4, together with the expression pattern of *AP3* (orange) and *PI* (blue) (Jack *et al.*, 1992; Goto and Meyerowitz, 1994). Right panel: protein tetramer involved in B function in *L. schismatica*, based on Y3H assays (Álvarez-Buylla *et al.*, 2010*b*); LsAG is included in the protein complex [as there is no case where stamens can be specified without the action of the C gene (AG)] although this interaction has not been corroborated experimentally. Below are the documented *in situ* domains of expression of *LsAP3* and *LsPI* at stage 4 (stage categorization was based on morphological traits) in a *L. schismatica* floral meristem. (B) On the left side is a representation of the protein complexes and phenotypes of the B mutants *pi-1* and *ap3-3*, where only sepals and carpels develop. On the right side is the observed phenotype and putative protein complexes formed in the complementation lines of *35S::LsPI*, *pi-1*, and *35S::LsAP3*, *ap3-3*, respectively. *LsPI* has a relatively more conserved function with respect to *PI* in *Arabidopsis*, as it rescues both petals and stamens, while *LsAP3* only rescues stamens. Based on our results from Y3H assays, the latter could be due to a competitive effect of a SEP3/LsAP3/AP3 or a SEP3/LsAP3/LsAP3 protein complex that effectively sequesters part of the available LsAP3 protein in the floral meristem (the dominant tetramer shown larger in the figure), leaving less LsAP3 available for heterodimerization with PI and thus form the expected protein tetramers that exert B function in *Arabidopsis*. (This figure is available in colour at *JXB* online.)

that the one observed for dimers (Egea-Cortines *et al.*, 1999); this observation was later confirmed and generalized by Theissen (2001) in the 'Quartet model'. Currently, it is accepted that MADS-domain transcription factors regulate specific sets of target genes through the formation of dimers and multimeric protein complexes that bind to DNA and activate or repress their targets. The 'Quartet model' proposes that MADS domain proteins form whorl-specific tetrameric complexes during floral organ determination (Theissen, 2001; Theissen and Saedler, 2001; Becker and Theissen, 2003; Jack, 2004; Melzer and Theissen, 2009; Fig. 2). Within each transcriptional complex, there would be two MADS dimers; one of these dimers could function as the activation domain of the tetramer (Honma and Goto, 2001). Each of these dimers, would bind to a single CArG-binding site causing the DNA of the promoter region to bend, enabling the MADS dimers to act co-operatively in a tetrameric complex to regulate the target gene (Honma and Goto, 2001).

Interestingly, several dimers and potential tetramers have been documented in the complete *Arabidopsis* MADS-box type II family protein–protein interactome, using the yeast two-hybrid system (Y2H; de Folter *et al.*, 2005). This database has been updated with a yeast three-hybrid (Y3H) screen for MADS-domain proteins (Immink *et al.*, 2009) using SEP3 as the third interactor. Furthermore, the protein interaction between SEP3, AP3, and PI and the formation of 'quartets' has been corroborated *in vitro* (Melzer and Theissen, 2009), while, to date, approximately 20 potential higher-order complexes involving *Arabidopsis* MADS box proteins have been reported in a heterologous *in vivo* system (Immink *et al.*, 2009). In the case of SEP3, it has been shown that this protein functions not only as an overall regulator of floral meristem development, activating B and C genes, but can also form ternary complexes with AP3 and PI *in planta* and co-localizes these proteins to the cell nucleus (Immink *et al.*, 2009). These qualities, plus the fact that the SEP3 protein contains four different motifs in the carboxy-terminal which are known to function as transcription activation domains, have led some authors to suggest that SEP3 can function as the 'glue' in MADS-box protein interactions and regulation (Immink *et al.*, 2009), at least for the ternary complex of AP3–PI–SEP3 (Honma and Goto, 2001). The constitutive expression of these three proteins is sufficient to convert leaves into petals (Honma and Goto, 2001; Pelaz *et al.*, 2001) in contrast with the constitutive overexpression of AP3 and PI fused to the VP16 *trans*-activation domain, that does not show such conversion (Immink *et al.*, 2009), although this dimer can bind to the *AP3* promoter (Goto *et al.*, 2001). These results suggest that the SEP3 protein is more than a *trans*-activator and is probably needed for petal development (Immink *et al.*,

2009). It will be instrumental to study the protein interactions of SEP3 with other MADS-box proteins in monocots, in order to elucidate their role in the particular expression patterns previously documented for different *AP3* paralogues (Table 1) and to evaluate if a variable copy number of *SEP* genes is present in different species and has a functional impact on the construction of different floral morphologies in this plant lineage.

Molecular genetic data on L. schismatica *floral development*

Based on the ABC model of flower organ determination (Coen and Meyerowitz, 1991), it is proposed that the homeotic inversion of stamens and carpels in *L. schismatica* could be explained by the shift of the B function to the flower centre (Vergara-Silva *et al.*, 2003). Indeed, in a recent paper, it has been shown that the expression of *LsAP3*, essential for stamen identity, has been displaced toward the flower centre in *L. schismatica* from the very early stages of development until stamen development is almost fully completed. *LsAP3* is absent in sections of the compound primordia that will give way to carpels (Álvarez-Buylla *et al.*, 2010*b*) in contrast to *LsPI* gene expression which is observed in both the carpel and stamen whorls, as it occurs in *Arabidopsis*. These results imply that the dimer necessary for B function can only be formed in the *L. schismatica* flower centre, where stamens develop. It was also found that C class gene function, *AG,* is expressed in the third and fourth whorls of the developing flower primordia. Thus, B plus C genes expression only coexists in the region where stamen primordia develop in the centre of *L. schismatica* flowers (Álvarez-Buylla *et al.*, 2010*b*).

The A-class genes remain to be investigated in *L. schismatica*, however, it is clear that the expression domains of the B and C genes documented for the flowers of this plant, suggest that the classical ABC model of floral organ determination is effectively switched to a unique ACB model (Álvarez-Buylla *et al.*, 2010*b*).

To evaluate the functional conservation between the B-function genes of *L. schismatica* and *Arabidopsis* further, complementation assays were performed generating over-expression lines of *LsAP3* and *LsPI* in wild-type and mutant *ap3-3* and *pi-1 Arabidopsis* lines, respectively. It was found that the B genes from *L. schismatica* were able to rescue stamens in these mutants (Álvarez-Buylla *et al.*, 2010*b*). As documented for other monocot species (Xu *et al.*, 2006), *LsAP3* complementation of the *Arabidopsis ap3-3* mutant lines could recover stamens but not petals and the degree of complementation was lower than that of *LsPI* in *Arabidopsis pi-1* mutants, where similar gain-of-function phenotypes to those of 35S::*AtPI* in *Arabidopsis* were observed (Jack *et al.*, 1994; Krizek and Meyerowitz, 1996; Álvarez-Buylla *et al.*, 2010*b*; Fig. 2). In addition, complementation lines overexpressing both *LsPI* and *LsAP3* in a double mutant background *ap3-3 pi-1*, showed similar phenotypes to those of plants ectopically over-expressing *LsPI*, where only sepals were converted into petals, suggesting that proper *LsAP3* function may need the formation of multimers with native L. schismatica proteins (Honma and Goto, 2001; Álvarez-Buylla *et al.*, 2010*b*).

Higher order MADS-box protein complex formation in L. schismatica

The B-class proteins function as obligate heterodimers to bind to DNA in core eudicots and some monocots such as maize (*Zea mays*) (Davies *et al.*, 1996; Riechmann *et al.*, 1996; Egea-Cortines *et al.*, 1999; Whipple *et al.*, 2004; Immink *et al.*, 2010), while in gymnosperms and other monocots these proteins are able to bind DNA as homodimers, at least *in vitro* (Winter *et al.*, 2002; Kanno *et al.*, 2003; Tzeng *et al.*, 2004; Tsai *et al.*, 2008). In two-hybrid assays using LsPI and LsAP3 from *L. schismatica,* it was found that B-function proteins were able to form a complex only when SEP3 was introduced and were not able to homodimerize as previously reported for other monocots (Xu *et al.*, 2006). Facultative heterodimerization of LsAP3 and LsPI proteins was also found with AP3 and PI proteins of *Arabidopsis,* which explains the capacity of LsAP3 and LsPI to complement the *ap3-3* and *pi-1* mutants *in vivo* (Alvarez-Buylla *et al.*, 2010*b*; Fig. 2). Moreover, it was observed that *Lacandonia* B proteins were able to form dimers with their *Arabidopsis* orthologue, as well as homodimers with themselves, with the particularity that the AP3 interactions are stronger than those of PI (all of these dimers were found in the presence of SEP3). These data suggest that the formation of an LsAP3/AtAP3/AtSEP3 complex *in planta* could compete with the functional LsAP3/AtPI/AtSEP3 heterodimeric complex, explaining why the *Arabidopsis ap3-3* lines were not complemented as well as those of *pi-1* (Álvarez-Buylla *et al.*, 2010*b*, Fig. 2).

In addition, these results suggest that SEP3 is fundamental for the formation and functioning of the LsAP3/LsPI dimer. As previously mentioned, the SEP3 protein in *Arabidopsis* is a key factor for the co-localization of AP3 and PI proteins to the cell nucleus and can induce ectopic B and C gene expression when ectopically expressed (Castillejo *et al.*, 2005). SEP3 binds to the *AP3* promoter, enhancing its expression, although loss-of-function mutants of *SEP3* do not seem to affect stamen formation significantly (but petals are lacking; Kaufmann *et al.*, 2009), perhaps due to redundancy with the other *SEP* genes (Kaufmann *et al.*, 2009). It will be useful to analyse the spatio-temporal expression patterns and protein interactions of putative *L. schismatica* SEP3 orthologue(s), as this protein may be fundamental in understanding the molecular basis of the unique expression pattern of *LsAP3* during flower development and its unique inside-out flower arrangement.

Alterations of cis *versus* trans *factors in the regulation of B genes underlying the inside-out flower of* L. schismatica

It has been shown that relatively simple molecular genetic alterations may underlie the unique spatial inversion of

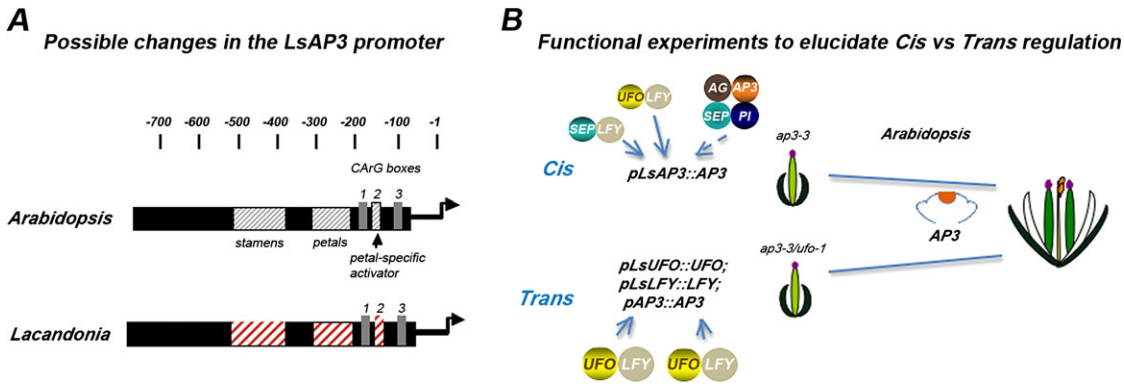

Fig. 3. Putative *cis* and *trans* regulation mechanisms involved in the central expression of LsAP3. (A) Regions of the *LsAP3* promoter that could have been subject to nucleotide changes that affect the *LsAP3* domain of expression, as compared with the documented functional modules of the *Arabidopsis AP3* promoter (Tilly *et al.*, 1998), are presented in red hatched squares. (B) A scheme of functional experiments to unravel the regulatory mechanisms of *LsAP3*: *in cis* regulation, critical motifs in the promoter of *LsAP3* direct the expression to the centre of the flower. *In trans* regulation, *LsAP3* could be displaced to the centre of the meristem due to a 'pre-pattern' determined by *trans*-regulator factors, such as *LsUFO*. In both cases it is expected that a flower with central stamens will be recovered. (This figure is available in colour at *JXB* online.)

stamens and carpels in *L. schismatica* flowers (Álvarez-Buylla *et al.*, 2010*b*). However, it is still not known to what extent other aspects of the GRN, where the ABC genes are embedded, are altered in this species with respect to model organisms. For example, it is still not known which factors displace the expression of *LsAP3* towards the centre of the *L. schismatica* flower, as well as if the atypical expression pattern of *LsAP3* is due to changes in the *cis*-regulatory sequences of this gene, while the rest of the GRN is conserved, or are alterations in other *trans*-regulatory components also involved?

Changes *in cis* involve particular mutations in promoter and other regulatory sequences like introns, which could affect the binding affinities of transcription factors that regulate the expression of *LsAP3* (Fig. 3A). In plants, comparative studies using phylogenetic footprinting of the type to be undertaken in *LsAP3-like* promoters have shown that the particular inputs of *cis*-motifs can be dissected by comparing promoter or intron sequences of developmentally relevant genes of closely related species (Hong *et al.*, 2003; Lee *et al.*, 2005). For instance, comparisons of 5′ upstream regulatory regions between *Arabidopsis*, *Lepidium*, and *Brassica* of the transcription factor *CRABS CLAW* (*CRC*), showed conserved modules that have both positive and negative regulatory elements. It is of note that each particular motif has the ability to drive reporter gene expression in specific sites, as if the overall summation of inputs gave rise to the complete wild-type expression profile (Lee *et al.*, 2005). In a related paper, the comparison of the second intron of *AG* among 29 species of *Brassica* allowed the dissection of two conserved binding sites for WUS and LFY; furthermore, these sites seem to be specific to the Brassicales (Hong *et al.*, 2003). The upstream and intronic regulatory sequences of *L. schismatica* and *T. brevistylis AP3* orthologues are currently being characterized in order to compare putative regulatory regions of these genes to previously characterized regions from other angiosperm *AP3* orthologues.

In a conceptually connected but still alternative explanation, divergences in the types, interactions or binding capacities of *trans* activating factors could also render the atypical expression pattern of *LsAP3* (Fig. 3B). Under such a scenario, alternative compositions of MADS-domain protein tetramers could have different affinities for specific regulatory sequences in the *LsAP3* transcriptional regulatory regions. In addition, proteins forming those complexes could also have experienced spatial or temporal modifications in their expression domains. One such floral regulatory gene that could be concomitantly affected with a change in the LFY domain of expression could be *UFO* (UNUSUAL FLORAL ORGANS), an F-box protein that in *Arabidopsis*, is a necessary cofactor of LFY required to activate *AP3* expression in the primordia of floral whorls 2 and 3 of this plant (Lee *et al.*, 1997). This is achieved by a physical interaction between LFY and UFO in a complex that binds directly to the *AP3* promoter, seemingly tagging an as yet unknown *AP3* repressor for degradation or favoring a rapid turnover of *LFY* that could enhance its transcriptional activity (Lee *et al.*, 1997; Chae *et al.*, 2008). It is proposed that the displacement of *LsAP3* towards the centre of the flower meristem of *L. schismatica* could be related to *UFO* remaining in the central whorl of this flower during a longer period of its development, resembling the pattern of *UFO* observed during early *Arabidopsis* flower development and thus, effectively biasing where *LsAP3* can ultimately be expressed (Fig. 2A). This would imply that the homeotic phenotype of *L. schismatica* could be due to a neotenic expression pattern of *UFO*, compared with that observed in *Arabidopsis.* Furthermore, the fact that *UFO* orthologues from other angiosperms show divergent expression patterns, suggests that alterations in the domain of expression of UFO could be involved in the inversion of stamens and carpels in *L. schismatica* (Souer *et al.*, 2008).

It is important to note, as well, that with respect to MADS-box genes, the *SQUAMOSA*-like *FRUITFUL*

(*FUL*), and *AP3* seem to have the greatest degree of conservation in their promoter sequences, at least between poplar and *Arabidopsis* (De Bodt *et al.*, 2006). Whether this responds to a particular developmental or historical constraint is yet to be tested. Overall, the structure of the promoters of *AP3* in angiosperms should be related to the following factors: initial recognition by UFO and LFY and later-developmental state recognition by the AP3–PI heterodimer (coupled with AP1/SEP3 in whorl 2 and AG/SEP3 in whorl 3; Fig. 3) which keeps the *AP3* active in a positive feedback loop. Furthermore, it was recently shown that SEP3 is an activator of *AP1*, *AP3*, *AG*, and *SEP* genes (Immink *et al.*, 2009; see section 'Higher order of MADS box function'). Molecular techniques such as Yeast One Hybrid analysis that allow identification of proteins interacting with a specific DNA sequence, or ChiP-SEQ, that allow identification of DNA sequences to which transcription factors bind, should be used once the *LsAP3* promoter and genetic sequences are characterized in order to dissect the genetic basis of the inside-out floral phenotype of *L. schismatica* further.

Evolutionary developmental biology of *Lacandonia schismatica*

Why is L. schismatica *so interesting from an* evo-devo *perspective?*

L. schismatica dazzled botanists and developmental geneticists due to the possession of its unique 'inside-out' bisexual flowers. Aside from being a botanical rarity, it was also a unique case where a 'hopeful monster' (a species with a radical and discrete change in the position of its floral organs) had survived and established as a species in the wild. This case proves that organisms that were subject to extreme and discontinuous changes, instead of continuous, cumulative, and gradual changes, as assumed by the current theory of evolution, could and did exist in nature; this supports Richard Goldschmidt's theory of what we would now call a biological saltation (Dietrich, 2000). Furthermore, this particular heterotopy (central stamens surrounded by carpels) has not been re-created in the laboratory either in *Arabidopsis* mutants (Álvarez-Buylla *et al.*, 2010*b*; see section on Molecular genetic mechanisms') or in spontaneous mutants in *Arabidopsis* and other plant species. Thus, to the best of our knowledge, no simple homeotic mutant reported to date, regardless of the model system employed or the gene family involved has been able to mimic this particular change in the position of the reproductive organs of a bisexual flower.

Given these observations, a series of questions that need to be addressed in a comparative evolutionary framework, arise. For instance, the scientific debate regarding the euanthial or pseudanthial origin of the flowers of *L. schismatica* that has been addressed through morphological and genetic analyses (see sections, '*L. schismatica* reproductive structures are true flowers' and 'Molecular genetic data on *L. schismatica* floral development'), can also be addressed from an evolutionary comparative perspective in terms of the number of character changes required to achieve the reproductive structures of *L. schismatica* if these are considered flowers or inflorescences. In addition, one could address if the unique floral phenotype recorded in *L. schismatica* is indeed a discrete morphological saltation, or a continuum of intermediate floral morphs that can be found in natural populations. Furthermore, microevolutionary and phylogeographic analyses both within *L. schismatica* and closely related Triuridaceae (Chase *et al.*, 2000, Chase, 2004; Rudall and Bateman, 2006), could be used to distinguish between the alternative evolutionary scenarios and the role of different evolutionary forces in the origin of this species and its unique floral structure. Lastly, investigating the gene expression patterns and molecular evolution of the B and C class genes in several triurid species, as well as other relevant genetic members and the dynamics of the underlying GRNs, will most likely shed light on the genetic configurations necessary for the inception of central stamens in *L. schismatica* (Álvarez-Buylla *et al.*, 2010*a*, Barrio *et al.*, 2010).

Floral structure variation in natural populations of L. schismatica*: phylogeographic and morphogenetic hypotheses on the origin of this taxon*

An exhaustive analysis of over 1000 inflorescences of *L. schismatica*, found that 98% of the flowers studied had central stamens and peripheral carpels, that is, the majority of flowers complied with the heterotypic arrangement described originally by Martínez and Ramos (1989). Furthermore, of the remaining 2% of flowers with a different sexual organ organization, these were either unisexual or bisexual with varying numbers of stamens (different from the three stamens which are typically present) and carpels. Bisexual flowers with central carpels and lateral stamens were never recorded (Vergara-Silva *et al.*, 2003).

In this same study, the flowers of another dioecious Triuridaceae species that grows in the Lacandon rainforest (*Triuris brevistylis*) were also analysed. Of the approximately 1000 inflorescences studied, only 1% were male and 0.5% were bisexual, either monoic or with carpels and stamens in the same flower. In the latter, a wide range of variation regarding the position of the ectopic stamens and carpels in the receptacle or androphore was observed. Within the floral variants documented, only one bisexual flower with a central stamen arrangement was found (see Fig. 10 in Vergara-Silva *et al.*, 2003). Interestingly, no clear floral series going from unisexual to bisexual flowers could be organized either for *L. schismatica* or for *T. brevistylis*. Findings in these taxa led the authors (Vergara-Silva *et al.*, 2003) to suggest that: (i) the homeotic phenotype was discrete and, thus, genetically controlled by one or a few mutations, and (ii) the genetic modules governing female and male sexual organ determination are independent.

While the reproductive mechanisms in *T. brevistylis* have not been fully studied, the fact that almost all carpels in a female flower develop into seeds despite the scarcity of male individuals is suggestive of apomixis and/or cryptic hermaphroditism in female flowers (the latter could lead to cleistogamy *sensu L. schismatica*). Apomixis has been suggested to occur in other Triuridaceae (Wirz, 1910, cited by Maas and Rubsamen, 1986), while the possibility of cryptic hermaphroditism is supported by the observation of staminoid tissue embedded within female-like flowers, as well as carpeloid tissue in male-like flowers of *T. brevistylis* (Martínez and Gómez, 1994).

The findings presented in the study by Vergara-Silva *et al.* (2003) also suggested that the predisposition to homeosis should have to be present in the ancestor of both taxa, which could be a dioecious *Triuris*-like species. The dioic nature of the putative ancestor of *L. schismatica* and *T. brevistylis* is supported by the fact that while the Triuridaceae are mostly monoecious, all known *Triuris* species are dioecious. Furthermore, in constrast to *L. schismatica,* which is endemic to the Lacandon rainforest, the *Triuris* genus has a larger distribution that spans several rainforests from Northeastern Brazil to Southeast Mexico but is exclusive of the American continent (Maas and Rubsamen, 1986).

Under the above scenario, the ancestor of *L. schismatica* and *T. brevistylis* could have dwelled in the Lacandon rainforest during the last glaciation (*c.* 5 million years before present), occupying the rim of an ancestral lake that would overlap with the territory where extant *L. schismatica* and *T. brevistylis* occur. In that period, individuals from the putative ancestor species could have acquired the phenotype present in *L. schismatica* while others could keep the *Triuris*-like characteristics. At the end of this glacial period, when the ancestral lake retracted, the populations of *L. schismatica* could have established in the lower part of the rainforest, while the populations of *T. brevistylis* could have stayed in the upper, cooler part of the rainforest. Now separated, these triurids could have eventually differentiated and established as distinct species. This hypothesis is additionally supported by the fact that although *L. schismatica* populations occupy lower lands that are 6–8 °C warmer than the sites where *T. brevistylis* dwell, the overall floristic composition of the sites where these two species live is quite similar (Vergara-Silva *et al.*, 2003). This hypothesis had been postulated based on the fact that the distribution of *L. schismatica* and *T. brevistylis* was thought to be restricted to the South of Mexico and to Central America (Lacandon rainforest and Guatemala), respectively. Now, recent evidence supports the presence of a new *Lacandonia* species (*Lacandonia brasiliana*) in the Atlantic Forest of Northeastern Brazil (Melo and Alves, 2012). This new finding suggests an older origin for *Lacandonia* and opens new fascinating questions concerning the evolution of this genus. Comparative genetic, morphological and biogeographic analyses of other South American species of Triuridaceae, particularly those in the closely related Triurideae tribe (*Peltophyllum, Triuris, Triuridopsis*; Maas and Rubsamen, 1986; Maas-van der Kamer and Maas, 1994) will help unravel the evolutionary forces involved in the emergence and fixation of *Lacandonia*'s unique inside-out-flowers.

From a morphogenetic standpoint, a restriction of the B function (i.e., the overlaping expression of *LsAP3* and *LsPI*) to the centre of the floral meristem in a female flower of a Triurideae-like ancestor could explain the central stamens observed in *L. schismatica*. This novelty could have been favoured and fixed in *L. schismatica* populations due to a selective advantage of hermaphrodites versus dioic individuals in a context where male flowers are scarce. Alternatively, a restriction of B-function to the flower centre of a male flower of the Triurideae-like ancestor can also explain the emergence of the *L. schismatica* flower structure. This last scenario would be simpler as B and C genes would already have been expressed in the floral meristem of a male flower, while in a female flower, re-activation of B-gene expression would have to occur in order to attain a *L. schismatica*-like flower structure. B and C gene *in situ* expression during flower development of male and female flowers of *T. brevistylis* are being investigated to address if B-gene expression is present in female flowers at the early stages, thus supporting the male-ancestor hypothesis.

Systematic approaches and morphological mapping

L. schismatica was originally placed in its own family—Lacandoniaceae—due to its singular homeotic flowers, as well as for its peculiar megagametophyte development (Martínez and Ramos, 1989). However, the available evidence—morphological, histological, developmental genetics, and systematic studies—strongly supports that *L. schismatica* may be considered a genus within the Triuridaceae. Thus, the position of Triuridaceae within the Pandanales order becomes important to unravel the macro evolutionary history of the lineage that led to the evolution of *L. schismatica*.

Triuridaceae is currently placed in the order Pandanales (APG III, 2009). The recent inclusion of this family within this order was based on molecular data (Chase *et al.*, 2000). Such grouping was unexpected given the divergent morphology of *L. schismatica* and Triuridaceae with respect to the Pandanales, which are, in turn, quite divergent morphologically. The families within Pandanales are: Cyclanthaceae, Pandanaceae, Stemonaceae, Triuridaceae, and Velloziaceae. All of these families, with the exception of the Triuridaceae, are autotrophic green plants and include families with palm-like resemblance and complex reduced inflorescences or pseudanthia (Cyclanthaceae and Pandanaceae), as well as a family amenable to a 'stereotypic' monocot *bauplan* (Velloziaceae) and a family with dimerous and pentamerous flowers, unique within monocots (Stemonaceae). The peculiarities of Triuridaceae have been addressed in the section on 'The homeotic flowers of *L. schismatica*', and many of them are probably associated with their heterotrophic (mycoheterotropic) habit.

Due to the extreme variation in morphology among members of this order, resolving the affinities of Triuridaceae within Pandanales is a challenge and implies the use of molecular as well as morphological traits. A resolved phylogenetic framework of Pandanales will be fundamental to perform comparative analysis and evaluate alternative hypotheses concerning the nature of the reproductive axes of *L. schismatica*. To date, several phylogenetic analyses have been undertaken. In these, while the members of this order have remained stable, the relationships among families have varied depending on the molecular marker used and the number of ingroup taxa sampled (Rudall and Bateman, 2006). In the case of Triuridaceae, this family has been variously placed as sister to Pandanaceae (Chase *et al.*, 2000) suggesting that the Triuridaceae reproductive units are pseudanthia. By contrast, other analyses place Triuridaceae as sister to Velloziaceae or sister to a clade composed by Stemonaceae, Cyclanthaceae, and Pandanaceae (Davis *et al.*, 2004). In a morphology-based analysis, Triuridaceae forms a paraphyletic group with Stemonaceae (Rudall and Bateman, 2006).

Given these contrasting results, a combined molecular and morphological analysis is being completed to resolve these seemingly contradictory topologies (A Piñeyro-Nelson *et al.*, unpublished results). Furthermore, once a well-supported phylogenetic reconstruction is attained, the information at hand will be interpretable within a strong historical, phylogenetic context, while the topology attained will be instrumental to map the putative character states in the most recent common ancestor of Pandanales and in the lineage that gave way to Triuridaceae. This exercise will enable the proposition of a transformation series that could aid in unravelling the evolutionary origin of the heterotopic flower of *L. schismatica*, as well as refining the position of this taxon within Triuridaceae, which could lead to a change in the taxonomic rank of *L. schismatica* from being an independent family to a genus within Triuridaceae or potentially, becoming *Triuris schismatica*.

Molecular evolution of B-class genes

The relevant genes involved in floral meristem determination, as well as carpel and stamen formation have been reviewed in the section on 'Molecular genetic mechanisms', where the available data for B and C genes in *L. schismatica* have also been described. The B-class genes have been amply investigated, as these are necessary for (petaloid) perianth formation, a structure that has played an important role in angiosperm diversification and speciation (Irish, 2009). In recent years, a plethora of information regarding B-class genes cloned from non-model species—basal angiosperms, non-graminoid monocots, basal dicots, etc.—has been published. The *in situ* expression patterns of these genes has been evaluated for some angiosperm species, impelling modifications to the ABC model of floral organ determination, in order to fit the empirical observations for a given species or lineage (see the section on 'The function of the B genes in monocotyledons'). To our knowledge, the most extreme change in an expression pattern of an *AP3* orthologue is the pattern reported for *LsAP3* (Álvarez-Buylla *et al.*, 2010*b*; see the section 'Molecular genetic data on *L. schismatica* floral development'; Table 1). Expression pattern data should ideally be coupled with functional experiments that can clarify if changes in the amino acid sequences of proteins originated from MADS-box genes impact the functionality of a protein *in vitro* (EMSA), in protein–protein interactions assessed in heterologous systems (Y2H/Y3H), or *in planta* (FRET), plus the individual analysis of differential substitution rates in particular amino acid residues along a given protein can shed light on sites that have been under selective pressures (Hernández-Hernández *et al.*, 2007).

Using methods that enable site and lineage specific substitution analyses, a study for the complete MADS-box *Arabidopsis* gene family (Martínez-Castilla and Álvarez-Buylla, 2003), suggested that positive natural selection has been important in fixing certain residues within regions that codify for protein–protein interaction domains and at particular gene family lineages, notably those related to flowering control. Later, Hernández-Hernández *et al.* (2007) performed a molecular evolutionary analysis of all amino acid residues of B-class genes from gymnosperms (where only one B-like gene has been documented) and all major lineages of angiosperms. In this latter study, positive selection was detected in specific amino acid residues within the K domain, a protein domain that is important for MADS-box protein heterodimerization (Hernández-Hernández *et al.*, 2007). Interestingly, signals for positive selection were detected in particular evolutionary moments of the B genes across the angiosperm clade: after the duplication of the ancestral B-gene present in gymnosperms, which gave rise to the AP3 and PI lineages in angiosperms, and in the TM6 lineage, after the duplication of AP3 in the core eudicots (Hernández-Hernández *et al.*, 2007). This study was one of the first papers to perform a thorough analysis of substitution rates in B-class genes, providing important insights pertaining the evolution of these MADS-box genes in flowering plants. Nevertheless, it had a poor sampling of monocot B-class gene sequences/groups, where little analysis of the B-gene evolution among members of this lineage could be performed.

In recent years, many more monocot sequences and functional studies have become available, enabling an assessment of B-class gene evolution in this lineage. In monocots, not only specific duplications of *AP3* and *PI* genes have occurred but also, protein homodimerization and divergent patterns of B-class gene mRNA *in situ* expression with respect to eudicots have also been observed [for instance, homodimerization of an AP3 orthologue has been documented *in vitro* for *Lilium regale* (Winter *et al.*, 2002) and ubiquitous expression of AP3 paralogues, detected in all floral whorls, has been recorded in several orchid species (Tsai *et al.*, 2004; Pan *et al.*, 2011); see Table 1]. A recent analysis on B gene evolution in orchids and grasses (Mondragón-Palomino *et al.*, 2009), has evaluated the role of purifying and positive selection on B orthologues present in orchids (with four lineage-specific DEF-like clades and one GLO-like clade,

except for one subfamily with two GLO-like genes) and grasses (one or two DEF-like clades, with one or two GLO-like clades). The main findings of this study were that strong purifying selection could have led to differential neofuncionalization of each of the DEF-like genes in orchids, which could impact the *trans*-regulatory interactions of these genes and their proteins, rendering not only novel expression patterns but also protein interactions that could underlie the singular perianth organs in these plants. Furthermore, the particular combinatory qualities of each DEF-like gene with its paralogues (favoured by specific deletions that were also documented in the C-domain of some of the DEF-like genes) and other MADS-box genes could have enabled a 'modularization' of the perianth, facilitating the independent evolution of each perianth whorl, in clear contrast with other petaloid monocots with single DEF and GLO-like genes (Mondragón-Palomino and Theißen. 2009; Mondragón-Palomino *et al.*, 2009).

In the case of the grasses (Poales) positive selection in residues within the K domain were in accordance with the general findings of Hernández-Hernández *et al.* (2007). Recent molecular data in the Zingiberales, where up to four PI-like genes exist in certain families, supports the modularization hypothesis postulated by Mondragón-Palomino and colleagues for the orchids (Bartlett and Specht, 2010), although in all cases, protein–protein interactions and functional experiments will be necessary to compare such an hypothesis with empirical data. Molecular evolution analyses of the B and C genes including those of the Triuridaceae and other Pandanales will probably shed light on the role of the patterns and processes of molecular evolution of key genes in stamen and carpel development on the evolution of the floral structures in Triuridaceae.

Transcriptome analysis of *Lacandonia schismatica* flowers

Massive transcriptome data as a means to improve our capacity to answer evolutionary questions

The availability of ubiquitous transcriptome (cDNA) data through massive sequencing technologies will accelerate the rate at which novel relevant genes involved in the developmental processes under analysis can be experimentally isolated. With such data at hand, the inference of GRNs involved in the stable genetic configurations underlying floral organ formation in non-model species such as *L. schismatica* and others will be amenable to analyses that have been undertaken for *Arabidopsis* (see for instance: Espinosa-Soto *et al.*, 2004; Álvarez-Buylla *et al.*, 2010*c*). Also, comparative transcriptome-profiling between closely related and distant species in order to assess gene expression under particular developmental stages, stress conditions, etc, can potentially surpass the inference power of microarray experiments, while evolutionary studies such as the inference of whole genome duplications through the investigation of substitution rates among paralogues (K_s rates; Lynch and Connery, 2000) and the reconstruction of ancestral gene order among members of one or several angiosperm lineages, are already being used as a means to investigate genomic stabilization processes among taxa derived from whole genome duplications (Sankoff *et al.*, 2009).

Transcriptome data for L. schismatica *and* T. brevistylis*: prospects and future plans*

In the particular case of *L. schismatica* and *T. brevistylis*, a collaborative effort with the Monocot Tree of Life initiative has been established that has enabled us to sequence the transcriptomes of *L. schismatica* and the two morphs (male and female) of *T. brevistylis.* The Illumina-Solexa sequencing technology has been used and, after a *de novo* assembly process and curation of the data, it was possible to obtain good-sized contigs for all taxa (N_{50}=1632 bp for *L. schismatica*; N_{50}=1143 bp for male inflorescences of *T. brevistylis*; N_{50}=1410 bp for female inflorescences, based on the K31; Cappello *et al.*, unpublished results). This transcriptome data will be used for molecular evolution analyses aimed at detecting gene orthologues, as well as putative paralogues of different MADS-box genes, and hence enable phylogenetic analyses of MADS-box genes and site-specific/lineage-specific hypothesis-testing for the role of Darwinian selection at particular amino acids (Álvarez-Buylla *et al.*, 2000; Martínez-Castilla and Álvarez-Buylla, 2003, Hernández-Hernández *et al.*, 2007; Bartlett and Specht, 2010, 2011).

Furthermore, using the transcriptome data of *L. schismatica* and its sister taxon, *T. brevistylis*, there is an aim to recover all the genes that have been shown to constitute a GRN module sufficient to determine floral organ primordial cell specification in *Arabidopsis* (Espinosa-Soto *et al.*, 2004) and to compare expression patterns to identify genes that have expression differences between the two species. Such *in silico* approaches, combined with quantitative PCR assays and other experimental assays, will be instrumental in addressing several of the functional and evolutionary questions that have been presented in this review and which have emerged during the course of our research with *L. schismatica* and *T. brevistylis*. For instance, the transcriptome data has yielded a single *AP3* gene for *L. schismatica* (the same as the one previously cloned experimentally in our laboratory), while *T. brevistylis* seems to have two *AP3* genes (Cappello *et al.*, unpublished results). The implications of such duplication events or the possible loss of one paralogue in *L. schismatica*, could well be fundamental events to better understand flower structure evolution in the Triuridaceae.

The dynamics of the gene regulatory network of *Lacandonia schismatica* flower development

Investigating the Epigenetic Landscape in L. schismatica

The *L. schismatica* unique floral phenotype constitutes a wonderful natural system to both validate and challenge,

as well as to develop further the models for GRN that our group has proposed to explain the stable genetic configuration underlying cell differentiation and morphogenesis in *Arabidopsis* flowers (Espinosa-Soto *et al.*, 2004; Barrios *et al.*, 2010). Crucial aspects of how genes are mapped to phenotypes depend upon the topological and dynamical characteristics of a GRN, but recovering the spatial realizations of the steady-states of such networks that characterize the gene configurations of different cell types have been poorly explored. A general approach to study the relationship between morphogenetic events and GRNs would imply deriving and quantifying what CH Waddington (1957) called the Epigenetic Landscape (EL). This conceptual approach is a useful tool for generating a theoretical framework to explicitly and generally recover the spatial and temporal constraints imposed during morphogenesis. Among such constraints those imposed by the complex gene interactions that regulate cell differentiation and patterning are particularly important. Epigenetic Landscapes were initially presented by Waddington as an image of morphogenesis in which a developmental process is represented by a ball rolling down along a landscape with peaks and valleys. In the EL that emerges from a GRN, the steady-state multigenic profiles are found at the bottom of the basins, referred to as attractors. These profiles characterize each cell type or cellular condition, with as many dimensions in the landscape as the number of genes in the underlying GRN. Only recently, have data accumulated in order to enable formal and quantitative derivations of ELs from experimentally grounded gene regulatory networks (GRN). With the help of data retrieved from the whole transcriptome sequencing effort described above, information regarding the expression of the genes involved in the GNR of *Arabidopsis* in *L. schismatica* are starting to be generated.

Conclusions

L. schismatica constitutes an important model system in plant developmental genetics and evolution to address the proximal (molecular genetic) and ultimate (evolutionary) causes underlying the emergence of innovations or unique phenotypes. In the case of the homeotic *L. schismatica* floral structure, it has been shown that a relatively simple and small molecular change underlies its unique phenotype: a shift of the spatio-temporal pattern of mRNA expression of one of the B genes (*LsAP3*) to the centre of the floral meristem from the early stages of development. This particular shift has not been documented in any other angiosperm, and it remains to be investigated what *cis* and *trans* regulatory mechanisms govern the displacement of *LsAP3*. Furthermore, ongoing transcriptomic studies in *L. schismatica* and *T. brevistylis* hint at the possibility that duplications of the B genes in the latter may be involved in subfunctionalization or neofunctionalization events that will continue to help in elucidating the functional role of the B proteins and their residues during flower development of this lineage. Future molecular research should also assess further the role of protein–protein interactions among B-class members, their mobility among cells or modes of B-gene postranscriptional regulation in monocot flower development.

L. schismatica also provides a unique system to investigate the processes through which a rare and large morphological change can be fixed in a natural population, as well as the ultimate causes explaining why the unique floral structure of *L. schismatica* has only emerged in the Triuridaceae lineage and which evolutionary events might have been involved in such a rare case. One possible scenario could involve a synergistic interplay between floral organ reduction due to the mycoheterotrophic habit of this family and the presence of autogamous reproduction in several triurid species (such as the observed cleistogamy, cryptic cleistogamy, and potential self-fertilization in *L. schismatica*, *T. brevistylis*, and monoic genera such as *Sciaphila* and *Soridium*, respectively; Maas and Rubsamen, 1986, Martínez and Gómez, 1994). These phenomena could have allowed for a larger flexibility in the number and position of sexual organs, increasing a predisposition to homeosis. Evolutionary analyses addressing the emergence of *L. schismatica*'s unique floral plan should include phylogenetic and comparative morphological analyses of the Triuridaceae within Pandanales. Insights from these investigations will enable testing alternative hypotheses and most parsimonious scenarios of trait transitions in the evolutionary history of *L. schismatica*.

A more complete picture to the evolutionary emergence of the singular floral structure of *L. schismatica,* that will link the molecular proximal mechanisms to the ultimate evolutionary explanations, will rely, however, on integrative and formal approaches to postulate GRN and multicellular/multilevel dynamic and epigenetic landscape models (Alvarez-Buylla *et al.*, 2010*a*). For example, it will be fundamental to understand the mechanisms governing the de-coupling between the spatial and temporal inception of floral organs in *L. schismatica*, where the ontogeny of floral organs (perianth, stamens, carpels) is clearly independent from the spatial disposition of such organs (perianth, carpels, stamens), effectively rendering the unique ACB floral plan of this species.

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Phylogenetic inference of Pandanales and ancestral character reconstruction analyses support an euanthial hypothesis for Truiridaceae

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ABSTRACT

Inclusion of the mycoheterotophic monocot family Triuridaceae within the order Pandanales has raised a series of questions regarding the evolutionary developmental paths of this group as well as its affinities with the families within this order. Pandanales include five families with very divergent morphologies, in which the functional reproductive units are either condensed partial inflorescences (pseudanthia; Cyclanthaceae, Pandanaceae) or true flowers (euanthia; Velloziaceae, Stemonaceae) and one family that has *bauplans* that in some respects are at odds with either category (Triuridaceae). The affinities of Triuridaceae within the order have remained unresolved in previous works using either molecular or morphological characters, hindering comparative analyses of the putative floral state of the Most Recent Common Ancestor (MRCA) of Triuridaceae. In this work, we use two molecular markers (fragments of the nuclear *atpA* and the ribosomal 18S subunit) plus an expanded morphological matrix to resolve the position of Triuridaceae within Pandanales, using Maximum Parsimony (MP; paup) and Bayesian Inference analysis (BI; Mr.Bayes). Both approaches recover a monophyletic Triuridaceae clade that is either sister to all other Pandanales (MP) or a sister group to Velloziaceae (BI), which has true flowers. Besides, in both topologies, the families with pseudanthia (Cyclanthaceae and Pandanaceae) are grouped together. Furthermore,, the character-state reconstruction analysis of relevant floral traits at internal nodes of the resolved BI phylogeny suggests that the MRCA of both Pandanales in general and Triuridaceae-Velloziaceae in particular had euanthia. Conditional upon whether Triuridaceae reproductive structures are coded as true flowers or as reduced inflorescences, the probable MRCA of Triuridaceae seems to be a monoecious plant with unisexual true floral units.. Within this family, the tribe leading to the genus *Lacandonia* has a complex evolutionary history concerning floral structures and contrasting reconstructions of the MRCA of this tribe are recovered depending on whether a polyaxial or an uniaxial interpretation is used.

KEYWORDS: Pandanales, Triuridaceae, MRCA, 18S, atpA, morphology

INTRODUCTION

To understand how genetic variation maps onto morphological traits remains a significant challenge in biology. Studies that combine molecular and morphological characters for phylogenetic or comparative analyses provide the foundation for *evo-devo* studies (Doebley and Lukens, 1998; Erbar, 2007). The monocot order Pandanales constitutes an interesting case for such studies since it encompasses a set of morphologically contrasting families, which nevertheless for a monophyletic order in monocot-wide molecular phylogenies (Chase et al., 1993, 2000; Davis et al., 2004; Davies et al., 2004). However, the affinities among the families comprising the Pandanales have remained incongruent (Chase et al., 1993, 2000 and 2004; Davis et al., 2004; Davies et al., 2004; Rudall and Bateman, 2006).

The family Triuridaceae (Gardner 1843), which consists of mycoheterotrophic, achlorophyllous, morphologically reduced herbs, has been the subject of much debate among scholars regarding the interpretation of its reproductive units (Stephens, 1991; Rudall, 2003; Vergara-Silva et al., 2003; Ambrose et al., 2006; Rudall and Bateman, 2006; Rudall, 2008; Álvarez-Buylla et al., 2010). This controversy ignited after the inclusion of Triuridaceae into Pandanales in 2000 by Chase and collaborators. While *Lacandonia* was initially placed in its own family, Lacandoniaceae (Martínez and Ramos, 1989), is now widely regarded as a monotypic member of Triuridaceae (e.g. Maas-van de Kamer and Weustenfeld, 1998). *Lacandonia schismatica* possesses an unusual floral structure comprising reproductive units with central stamens surrounded by carpels (Martínez and Ramos, 1989). Some scholars have regarded the reproductive structures of *L. schismatica* and other Triuridaceae as being uniaxial structures that are readily assigned to true flowers or euanthia (Martínez and Ramos, 1989; Vergara-Silva et al., 2003, Ambrose et al., 2006; Álvarez-Buylla et al., 2010), whereas others have interpreted them as condensed inflorescences, sometimes termed pseudanthia (Stevens, 1991; Rudall, 2003) or at least polyaxial structures, possibly derived from true flowers (Rudall and Bateman, 2006; Rudall, 2008). This debate has been addressed through a set of recent studies based on data from both comparative morphology and developmental genetics.

Morphological data have demonstrated the existence of anomalous ontogenetic patterns of floral development and homeosis in *Lacandonia* and other Triuridaceae (Rudall, 2003; Vergara-Silva et al., 2003; Ambrose et al., 2006; Rudall, 2008). Recent developmental genetic analyses have indicated that one factor underlying the shift in

organ patterning (to central stamens surrounded by carpels) in the *L. schismatica* flower is correlated with a shift to the center of the developing flower primordium of one of the transcription factors (LsAP3) involved in stamen formation; *APETALA3* of the B-class genes within the ABC model of floral organ specification (Bowman et al., 1989; Álvarez-Buylla et al., 2010). Comparative analyses of gene expression among other Triuridaceae would be useful to address the evolution of the developmental mechanisms underlying the evolution of the unique floral structure of *Lacandonia*. Such analyses and other types of comparative approaches require a well-resolved phylogenetic tree of Triuridaceae within Pandanales.

The available phylogenetic analyses addressing the affinities of Triuridaceae within Pandanales have generated contrasting hypotheses of relationships. In molecular analyses, Triuridaceae were placed either as sister to Pandanaceae (Chase et al., 2000), sister to all other Pandanales (Davies et al., 2004; Chase et al., 2004), sister to Velloziaceae (Chase et al., 2004; Davis et al., 2004) or sister to Stemonaceae (Vergara-Silva et al., 2003b), though with little statistical support for any of these contrasting placements. In a morphology-based phylogenetic analysis of Pandanales at the genus level, Triuridaceae formed a paraphyletic group with Stemonaceae (Rudall and Bateman, 2006). While all molecular analyses are inconclusive and the only available morphological analysis renders two of the Pandanales paraphyletic: Stemonaceae and Triuridaceae (Rudall and Bateman, 2006).

Morphology-based phylogenetic analyses are problematic because the five families included in Pandanales: Cyclanthaceae (Poit. Ex A. Rich 1824); Pandanaceae (R.Br. 1810); Stemonaceae (Caruel 1878, nom. cons.); Triuridaceae (Gardner 1843) and Velloziaceae (Endler 1841), have a significant morphological divergence and homology is difficult to establish. For instance, Cyclanthaceae and Pandanaceae have either bushy or arborescent palm-like representatives with bona fide inflorescences; Stemonaceae and Velloziaceae include herbaceous species with true flowers and Triuridaceae comprises reduced mycoheterotrophic plants. Therefore, establishing putative ontogenetic trends and homologies among the reproductive structures of Pandanales is a challenge. Additionally, all Pandanales except Velloziaceae, have floral morphologies that are at odds with what is considered the *stereotypical* monocot floral ground plan, which includes a trimerous merism, five floral whorls, ontogeny of reproductive structures, and leaf phyllotaxy, among others (Endress, 1995; Remizowa et al., 2010). Given these complexities, a "total evidence" approach using both

morphological and genetic markers as well as an extended taxon sampling, could render a better understanding of the phylogenetic relationships and evolutionary paths in Pandanales (Rudall and Bateman, 2006; Davis et al., 2004).

In the last two decades new taxa and genera have been added to Triuridaceae, it has contributed with more information about this group but it also complicated the elucidation of its evolutionary history. The new genera are *Triuridopsis* (Maas-van de Kamer H. and Maas P. J. M; 1994; Franke et al., 2000), *Kupea* and *Kihansia* (Cheek et al., 2003). *Triuridopsis* has been placed as part of the tribe Triurideae (Maas-van de Kamer H. and Maas P. J. M; 1994). The other two genera, which are distributed exclusively in restricted areas within tropical rainforests in Africa, have been proposed to comprise a separate tribe (Kupeae), due to their particular disposition of reproductive structures on the inflorescence axis; their placentation and the presence of two ovary locules (Cheek 2003). Thus, this pantropical family is now organized in three tribes: Triurideae, Sciaphileae and Kupeae (Cheek 2003; Rudall and Bateman 2006; APG III 2009). The biggest tribe is Sciaphileae, with over forty species distributed in five genera (*Seychellaria, Andruris, Hyalisma, Soridium and Sciaphila*), where *Sciaphila* is the genus with more species, approximately 30 different representatives distributed across the tropics. Triurideae has four neotropical genera (*Peltophyllum, Triuris, Triuridopsis* and *Lacandonia* [if we consider the latter as part of the Triuridaceae]) with about eight species (Maas-van de Kamer, H. and Weustenfeld, T.; 1998; Franke et al., 2000).

It must be highlighted that a new *Lacandonia* species was recently discovered in the Atlantic Forest of northeastern Brazil: *L. brasiliana* (Melo and Alves, 2012), suggesting a much more complex evolutionary history for this genus which until then was considered endemic to Mexico (Vergara-Silva et al., 2003; Garay-Arroyo et al., 2012). Furthermore, in recent years, representatives of all Triuridaceae tribes have been the subject of several morphological analyses which have yielded insights regarding the ontogeny of the reproductive units of several species, as well as of the development of unique structures such as centrifugal carpels and filamentous appendices (Rudall 2003, 2005, Vergara-Silva et al., 2003; Ambrose et al., 2006; Furness and Rudall, 2005; Rudall and Bateman, 2006; Rudall 2008). Phylogenetic analyses using combined markers that address the relationship among members of the Triuridaceae and of this family with respect to the remaining Pandanales is still pending.

In this paper, we explore the phylogenetic position of Triuridaceae within

Pandanales using both morphological and molecular analyses. The latter were done with fragments of the rDNA *18S* ribosomal subunit and of the *atpA* gene. For the morphological analysis, we expanded and updated the data matrix of Rudall and Bateman (2006). Phylogenetic analyses are performed with both Maximum Parsimony and Bayesian Inference approaches to evaluate whether the *a priori* assumptions engrained within a Bayesian Inference framework aid in the resolution of the phylogenetic relationships within Pandanales. Finally, based on the resolved (our final) BI topology, we map floral characters in order to address the most likely transformation series in the MRCA of Triuridaceae, exploring the evolutionary scenarios that could have lead to the unique homeotic (inside-out??) flower of *Lacandonia.*

MATERIALS AND METHODS

Taxa and characters analyzed

We used data from 22 species of Pandanales plus nine species from two other related monocot orders, Dioscoreales (Nartheciaceae, Thismiaceae, Burmanniaceae, Dioscoreaceae) and Petrosaviales (Petrosaviaceae), which were included for outgroup comparison (following Davis et al, 2004). Of a total of 60 partial sequences of *atpA* (872 nucleotides in length) and 18S (1614 nucleotides in length) used for the analyses presented here, 25 were obtained at the EAB laboratory; three were provided by VM* and the remaining sequences were retrieved from GenBank. Taxa included in our analyses and GenBank accession numbers for mitochondrial *atpA* and ribosomal 18S are listed in Table 1. For the morphological analysis, a total of 53 characters were used, with two alternative scorings of reproductive units for Triuridaceae (see sections "Morphological data" and "Appendix 1" for details).

Molecular data

DNA extraction

Samples used for this study were collected in the field and immediately frozen in liquid N_2 and transported to the laboratory where they were stored at -70ºC in an ultrafreezer. Alternatively, samples were dehydrated in silica gel and stored at room temperature. For DNA extraction, 75−100 mg of fresh-frozen tissue or up to 30 mg of dehydrated tissue was ground with liquid N_2 in a pre-cooled (in liquid N_2) mortar and pestle. The resulting powder was then transferred to a pre-cooled 2 ml microtube to avoid thawing of the sample during handling. DNA extraction was adapted from CIMMYT's laboratory

protocols for maize gDNA extraction: (http://www.cimmyt.org/spanish/docs/manual/protocols/Lab-geneticaMolecular.pdf). In summary, 1000 µL of CTAB extraction buffer pre-warmed to 65ºC was added to each tube of ground tissue. Each sample was vortexed at mid speed and incubated for 90 minutes at 65ºC, mixing by inversion every 15 minutes during the incubation period. Samples were left to cool down for 10 - 15 minutes and a 500 µL aliquot of a 24:1 chloroform: octanol mix was added, vortexing at low speed and placing in an orbital shaker for 15 minutes to mix. Samples were centrifuged for 15 minutes at 3600 rpm in a tabletop centrifuge to separate the organic and aqueous phases. The aqueous phase was transferred to a fresh 2 ml microtube and precipitated with 0.7 volumes of ice-cold isopropyl alcohol and left to precipitate all night at -20°C. Samples were centrifuged at 4ºC for 15 minutes at 13,000 rpm and supernatant was discarded. The gDNA pellet was washed as indicated in the CIMMYT protocol; afterwards, samples were left to dry at room temperature. Extracted DNA was eluted in 30 to 50 µL of molecular-grade water with a 1 to 1000 dilution of RNAse A stock solution (100mg/ml). DNA quality was verified through visual inspection of 1 or 2 µL gDNA aliquot run in a 1% agarose/Ethidium bromide gel; gDNA concentration was estimated using a Nanodrop 2000 spectrophotometer (®Thermo Scientific). DNA was stored at -20ºC until used.

A change with respect to the original protocol was that once the aqueous phase was recovered from the chloroform-octanol extraction, the samples were re-extracted adding a new 1000µL aliquot of pre-warmed CTAB extraction buffer to the remaining cellular debris/ chloroform-octanol residue. The samples were mixed by inversion and incubated for another 90 minutes. Samples were left to cool for 15 minutes at room temperature and centrifuged for 15 minutes at 3600 rpm. The second aqueous phase was transferred to a new microtube, precipitated and washed as mentioned above. With this procedure we could extract more gDNA from all samples (equivalent to approximately 10 to 20% of the first extraction). Furthermore, for *Barbacenia purpurea* the majority of gDNA was recovered in the second, rather than the first extraction.

PCR and Sequencing

***atpA* and *18S* sequences**

From total genomic DNA, we obtained a mitochondrial fragment of the *atpA* gene and a ribosomal fragment of 18S via polymerase chain reaction amplifications with newly designed primers to obtain amplification products: the primers *atpA* F_1 (5’

AAGTGGATGAGATCGGTCGAG 3') and *atpA* R_1 (5' GGCATTCGATCACAGA 3') were used. For the 18S sequence, two sets of primers were used 18S-N_1F (5' GTAGTCATATGCTTGTCTC 3') with primer 18S 854R (5' ATCATTACTCCGATCCCG 3') and 18S-9299F (5' **) with 18S-1769R (5' CACCTACGGAAACCTTGTT3').

PCR for *atpA* was carried out in 50 µl reaction volumes using the PCR Core Kit (Boehringer, Ingelheim, Germany), with the following thermocycler conditions: 3 min at 94°C, then 35 cycles of: 1 min at 94°C, 30 s at 50°C, and 2 min at 72°C, with a final extension of 10 min at 72°C. PCR for 18S was carried out following the same profiles except the final extension, which was performed for 7 min at 72°C. PCR products were gel-purified and extracted from agarose with Concert Gel Extraction Systems (Gibco Life TechnologiesTM, Gaithersburg MD, USA). PCR fragments were sequenced directly in an Applied Biosystems Automatic DNA Sequencer ABI Prism 3100 (Foster City, CA, USA), using a BigDye Terminator Cycle Sequencing Reaction Kit (Foster City). We obtained 29 sequences; *atpA* has 1107 bp and 18S with 1615 bp (see Table 1). We assured that our amplifications were the right ones by obtaining clean and unambiguous electropherograms and by comparing our sequences with other *atpA* and 18S sequences from GenBank. All sequences amplified in EAB laboratory were ligated into a pGEMT-Easy vector using the pGEMT-Easy vector kit according to the manufacturer's instructions. The pGEMT-Easy vector was transformed into either electro or thermocompetent DH5∞ *E. coli* cells and selected clones containing the insert were sequenced. pGEMT-Easy clones for each sequence amplified were kept in DH5∞ *E. coli* strains stored in 40% glycerol at -70ºC.

Sequence assembly and alignment

All sequences were edited and aligned using CLUSTALX (version 2.1). Afterwards sequences were checked and edited manually in MacClade 4.08 OS X (Maddison and Maddison, 2003). In the case of the *atpA* gene, we edited this sequence to 872 nucleotides so that we could compare our data with sequences provided by VM, which were approximately of this size.

Morphological data

We included a total of 53 characters for the morphological data matrix. Of these characters, 39 were taken from a previous work (Rudall and Bateman, 2006), while 14

characters are new (numbers 2, 9, 10, 13, 14, 32, 34, 39, 40, 42, 43, 46, 48, 51 and 53). Characters 4, 5 and 6 were scored differently with respect to the original paper (Triuridaceae and Burmanniaceae, although achlorophyllous, have reduced leaves) and character 41 was recodified to include a new character state: a biovular carpel, which is a synapomorphy for *Kupea martinetugei* and *Kihansia jengiensis* [Triuridaceae]). For all characters, the codification was reviewed in light of available morphological and anatomical publications of the taxa under study, as well as direct observations of herbarium and spirit specimens (for details on voucher information of specimens analyzed, character states and matrices analyzed, see Appendix 1). We used the binary coding system (Pleijel, 1995) for most of our characters unless there was a compelling reason to use multistate coding; cases when there were clearly three different states of a character (multistate); when one or more taxa lacked a component of the character definition (inapplicable); or when the information was not available (missing) were also considered during character scoring.

Two morphological matrices were analyzed in this study, with alternative interpretations of the reproductive units of Triuridaceae. Matrix I scores all floral characters for Triuridaceae (specifically, 11, 12, 13, 14, 15, 37, 46) using an interpretation of the axis as unbranched, while in Matrix II such characters were scored as equivocal, following a previous analysis (Rudall and Bateman, 2006). In Matrix II these three characters were left with the original codification made by Rudall and Bateman (2006). The polarity of the character state transformation was set by using outgroup comparison.

Phylogenetic analyses

Phylogenetic analyses using either the molecular data matrix or the morphological matrices as well as combined data matrices (18S + *atpA* + morphological Matrix I or II) were performed under a Maximum Parsimony and a Bayesian Inference framework.

The molecular phylogeny comprised 31 terminals and was reconstructed using equally weighted parsimony (MP) with paup* (Swofford 2001) version4.0b10 for Macintosh (PPC) for each molecular data set and a combined 18S + *atpA* matrix. The analysis included all codon positions of the *atpA* mitochondrial coding-protein fragment because the highly divergent third codon positions appeared to contain phylogenetic information and produce better estimates (Yoder and Yang 2000), while their exclusion produces poorly resolved trees. All characters were unordered.

The transformation series for the data set of morphological characters was analyzed separately with paup*, using equally weighted parsimony (MP) under the same parameters used for the molecular data. Of the 53 morphological characters, 46 were parsimony-informative.

For outgroup rooting, we used one species of Nartheciaceae (*Narthecium ossifragum*); three Burmanniaceae (*Gymnosiphon divaricatus*, *Burmannia capitata* and *Apteria aphylla*); one Dioscoreaceae (*Trichopus sempervirens*) and two Petrosaviaceae (*Petrosavia stellaris* and *Japonolirion osense*), following Davis et al. (2004). We also explored the occurrence of artifactual hypotheses of relationship between Triuridaceae and distantly related mycoheterotrophs (i.e. two species of Burmanniaceae) due to morphological convergence or to a differential rate of evolution for each molecular marker (as documented for Thismiaceae by Merckx et al., 2009).

We performed parsimony analyses using a heuristic search algorithm via stepwise addition, with 1000 random additional sequence replicates saving 100 trees per replicate and using tree-bisection and reconnection (TBR) branch swapping. To assess branch support, we obtained bootstrap values with 1000 replicates using simple addition sequence and TBR swapping with 100 trees saved per replicate. For a total-evidence approach (18S + *atpA* plus morphological Matrix I or II), we combined all our data in a single matrix. We included a total of 2772 characters for 31 terminals with the same outgroup taxa. We analysed this combined data set with paup* under the same parameters used in the molecular analysis.

A Bayesian Inference approach as implemented in the program Mr.Bayes v3.0b4 (Huelsenbeck and Ronquist 2001) was performed with the combined matrix. Searches were based on five data partitions: *atpA* sequences for three codon positions, 18S sequences and the morphological characters. We ran 10 000 000 generations with one cold and three heated chains, a temperature of 0.5, while trees were sampled every 10,000 th generation, starting from random trees and a burn-in of 1000 trees. The log-likelihood scores were plotted against generation time and the Markov chains were assumed to be stationary when the likelihood value had converged (around 50 000 generations). Sample points prior to stationary chains were discarded as burn-in values and the remaining values were used to generate a 50% majority consensus tree.

The program JModelTest 3.06 (Posada and Crandal 1998) with the Akaike Information Criterion (AIC) inferred the Tamura-Nei 1993 model with equal base frequencies with invariant sites and a gamma correction (TrNef+I+G) for the 18S data

set and the Felsenstein 1981 model with gamma correction (F81+G) for the *atpA* data set as the best-fit evolutionary models of DNA substitution. For the discrete morphological data, we used a Mk generalization of the Jukes-Cantor model (1969: JC69) assuming a discrete gamma distribution for the relative rates, where 'M' stands for Markov and 'k' refers to the numbers of states observed (k ≥2) with no state considered plesiomorphic or apomorphic *a priori* (Yang 1994; Lewis 2001). The prior assumption of overall rate heterogeneity across partitions was set at variable to allow our partitions to evolve under different rates. The MCMC sampling was run three times to verify that the same topology was recovered for each.

In both MP and BI analyses, the two Petrosaviaceae species were set as outgroups. To explore whether the inclusion of mycoheterotrophic taxa other than Triuridaceae (*G. divaricatus* and *A. aphylla*; Burmanniaceae) significantly affected the topology of Pandanales as well as the statistical support of this clade, we repeated all analyses selectively excluding all Burmanniaceae.

Estimation of ancestral character states

The recovered BI phylogenies based only on molecular characters or with the combined sets of characters were used to estimate the likelihood of occurrence of alternative character states for the internal nodes (assumed to be the Most Recent Common Ancestor; MRCA) leading to Pandanales and to Triuridaceae. The *ace* library implemented in the R platform was used (Paradis, 2006). The floral characters mapped were: reproductive habit (11); floral unit (12); number of carpels per floral unit (36); carpel fusion (37) and number of ovules per carpel (45). These traits were mapped considering the nature of the reproductive axes of Triuridaceae to be uniaxial (Matrix I) or strictly polyaxial (Matrix II). In the case of Matrix II, the inference program in R did not allow character state estimation based on equivocal or unknown character states (originally encoded as "?" in Matrix II) there, in order to contrast the effect of different interpretations of relevant floral characters contained in Matrices I *vs* II, all characters under analysis were rescored as inflorescences for all Triuridaceae in Matrix II.

RESULTS

Relationships of Triuridaceae within Pandanales

The phylogenies recovered using only the combined molecular markers are consistent with previous studies in that they retrieve a monophyletic Pandanales clade (e.g. Chase

et al., 2000; Davis et al., 2004). Besides, this topology has a high statistical support under both analyses (92% Bootstrap [BS] and 1.00 of Posterior Probability [PP], for MP and BI analyses, respectively; see Figure 1). Furthermore, all families within Pandanales are monophyletic, though the relationships between them remain unresolved, both approaches (Fig. 1) In the MP consensus tree, Triuridaceae are sister to another weakly-supported clade comprising the rest of Pandanales (Fig. 1A), while in the BI analysis, Triuridaceae are part of a basal polytomy where three defined groups (each with 1.00 PP) collapse: Velloziaceae, Triuridaceae and a group comprising Stemonaceae as sister to Cyclanthaceae plus Pandanaceae (see Fig. 1B). Thus, it seems that the phylogenies recovered based on the analyses of our molecular data alone are not enough to resolve the relationships within Pandanales

In the phylogenetic analyses using only the morphological matrices, when all taxa are included in either Matrix I or II under a MP or BI approach, a paraphyletic Pandanales is recovered, due to the inclusion of the two mycoheterotrophic species of Burmanniaceae as sister to an otherwise monophyletic Triuridaceae (data not shown). When all Burmanniaceae were eliminated from the analysis, Pandanales persisted as a paraphyletic clade, as *Barbaceniopsis sp.* (Velloziaceae) grouped with *T. sempervirens* (Dioscoreaceae). Thus, when the morphological matrices were analyzed alone, artifactual topologies were recovered due to convergent traits related to mycoheterotrophy between Triuridaceae and the Burmanniaceae, as well as convergent traits between a poorly studied species of Velloziaceae (*Barbaceniopsis sp.)* and a Dioscoreaceae (*T. sempervirens).*

With the combined data matrix (molecular plus morphological data for Matrices I or II), two contrasting topologies within a monophyletic Pandanales are recovered. (1) Under a MP approach (Fig. 2A), in the strict consensus tree, Triuridaceae represent the first family to branch from a weakly supported Pandanales (less than 50% BS); among the other families, Velloziaceae are sister to a clade comprising (Stemonaceae, ((Cyclanthaceae), (Pandanaceae))). The statistical support for this clade is also low, although individual bootstrap values for each family are high (around 80% BS). It should be pointed out that the position of Velloziaceae is unstable in some of the 15 most-parsimonious trees recovered, affecting the resolution among families in the latter clade (data not shown). (2) In contrast, the BI phylogeny (Fig. 2B) of Pandanales has a high PP (1.00), where two well-supported clades are recovered: one comprising

Triuridaceae as sister to Velloziaceae (PP = 0.80) (.80 para PP es bajo) and another (PP = 1.00) consisting of (Stemonaceae, ((Cyclanthaceae), (Pandanaceae))). Additionally, each individual family has a high PP (1.00). The same topologies are recovered regardless of which morphological matrix is used.

While the contrasting topologies generated under MP or BI are likely to be affected by the *a priori* hypotheses included in each inference method, it is clear that Pandanales is a monophyletic group and within this order, a monophyletic Triuridaceae is always recovered.

In summary, Triuridaceae takes one of three positions: it is either grouped as sister to all other Pandanales (Figs. 1A and 2A), monophyletic within an unresolved Pandanales (Fig. 1B) or sister to Velloziaceae (Fig. 2B). Furthermore, in the two BI phylogenies presented here (Figs 1B, 2B), as well as in the MP phylogeny reconstructed using the combined data matrix (Fig. 2), Cyclanthaceae and Pandanaceae are always embedded within a clade that has Stemonaceae as sister group.

In the following section, we use the phylogenetic hypothesis based on a BI approach using a combined data matrix (Fig. 2B) to map the evolution of relevant floral characters in the MRCA of Pandanales and the different nodes leading to the MRCA of Triuridaceae. (In this hypothesis, Velloziaceae and Triuridaceae are sister groups, while Stemonaceae, Pandanaceae and Cyclanthaceae are included in a separate clade).

Exploring morphological character evolution

Five floral characters were selected because they are important in the discussion on whether the unusual reproductive units of Triuridaceae are derived from flowers or inflorescences. These characters are: reproductive habit (character 11); floral units (character 12) and number of carpels per floral unit (character 36). number of ovules per carpel (character 45) and placentation (character 46)~~. ?? The~~ reproductive habit (11), did not change under Matrices I or II (Table 2). In contrast, the most likely character state and therefore, the evolutionary progression in the MRCA of Pandanales and the MRCA of Triuridaceae (nodes 5 and 17, respectively; see figure 3) did change with the floral units (12) and the number of carpels per floral unit (36), based on the *a priori* assumptions made for Triuridaceae (i.e. codings in Matrix I or II; see Table 2).

For the number of carpels per floral unit (45), two different scenarios are derived from the character mapping based on Matrices I or II. Figure 3A depicts these

alternative hypotheses for this trait and for the reproductive habit (Figure 3B). Thus, when Triuridaceae are coded as having numerous carpels per flower (Matrix I), the MRCA ancestor of Pandanales has an estimated probability of 64% of having numerous carpels. The MRCA of the clade composed of Velloziaceae plus Triuridaeae has a 60% probability of having three carpels per floral unit (although it has a 35% probability of having numerous carpels). Independently, the node that depicts the MRCA of Cyclanthaceae plus Pandanaceae has a 78% probabilityof having numerous carpels. In contrast, the probabilities for these nodes change significantly when mapping is based on Matrix II. Under this scenario, the MRCA of Pandanales has a single carpel as the most likely character state (80%). Furthermore, given that under Matrix II Triuridaceae are considered as inflorescences where each reduced flower bears a single carpel (or stamen), the occurrence of numerous carpels evolves only in the clade that includes Cyclanthaceae plus Pandanaceae (78%; see Figure 3). Besides, the likelihood of occurrence of a particular character state at internal nodes also changed when two alternative characters states for placentation in a single taxon were explored (*Acanthochlamys bracteata*; see Table 2).

In addition to the number of carpels per floral unit, the mapping of other characters suggests that several other traits could have evolved convergently among the Cyclanthaceae, Pandanaceae and Triuridaceae; it seems that these three families have independently acquired the same reproductive habit (see Figure 3B). In the MRCA of Pandanales, the most likely character state seems to be bisexual (this inference is the same regardless of the morphological character matrix used; see Figure 4) and this character state continues to be the most likely in both the MRCA of Velloziaceae plus Triuridaceae and in the clade that includes Stemonaceae, Cyclanthaceae and Pandanaceae, respectively. This evolutionary trend changes in the MRCA of Triuridaceae where the most likely reproductive habit is monoecious as well as in the MRCA of Cyclanthaceae and Pandanaceae. This phenomenon also applies for the case of the partially redundant character of floral units (12), where these two clades (Triuridaceae and Cyclanthaceae plus Pandanaceae) bear unisexual flowers, a characteristic that does not change regardless of the morphological matrix used. The only important difference between character progression using Matrix I vs II is that in the Triuridaceae, the MRCA of *T. brevistylis* and *L. schismatica* (tribe Triurideae; see Figure 5) has a higher probability of having had bisexual flowers under Matrix I, although the statistical difference between this hypothesis and an alternative one that

suggests a monoic plant with unisexual flowers, is low. In contrast, this MRCA under Matrix II has a higher probability of being monoecious with unisexual floral units, which is consistent with the character state present in the MRCA of the Sciaphileae tribe, which also is seemingly monoecious (see Figure 5).

In conclusion, under a uniaxial interpretation of the Triuridaceae reproductive unit, bisexuality would have to evolve *de novo* in the MRCA of the Triurideae tribe. Given that some species of the genus *Sciaphila* (tribe Sciaphileae) such as *S. picta* or *S. rubra,* have bisexual flowers, and instances of bisexual flowers in the otherwise monoecious *Soridium spruceanum* have been reported (Espinosa-Sánchez, 2009), this would suggest that this feature could have been present not only in the MRCA of a particular tribe, but also in the ancestor of Triurideae and Sciaphileae, although our analyses suggest that this ancestor was more likely to be a monoecious plant with unisexual flowers (see Table 2).

In the alternative scenario examined here, in which the floral units of Triuridaceae are interpreted as condensed polyaxial structures (Matrix II), both the MRCA of the two tribes and the MRCA of tribe Triurideae in particular, would have been monoecious plants with unisexual floral units (see Table 2 and Figure 5).
It is intriguing that the extant genera of Triurideae are dioecious (*Triuris, Triuridopsis, Pelthophyllum;* Maas-van de Kamer and Weustenfeld, *1998*), with the exception of *Lacandonia*, which bears either true flowers (Matrix I) or bisexual inflorescences (Matrix II). In the context of the evolution of tribe Triurideae, both scenarios imply different challenges for the interpretation and reconstruction of the ontogenetic series leading to the known extant species in this group. In the first case, dioecious species would have to be derived from an originally hermaphrodite ancestor (see left panel in Figure 5), suggesting that the hermaphroditism of the *Lacandonia* species is an ancestral trait within the tribe. Although comparative analyses are lacking for most of the species in this tribe, two phenomena give indirect support to the hermaphrodite ancestor hypothesis; the first is the sporadical presence of "cryptic" hermaphrodite flowers found in *T. brevistylis* (Martínez and Gómez, 1994; Vergara-Silva et al., 2003), which suggests that unisexualism is not entirely canalized in this species. The second is the very recent report of a new *Lacandonia* species in Northeast Brazil, indicating that this genus is older than previously thought (Vergara-Silva et al., 2003; Melo and Alves, 2012). In the second case, the transition from monoecy to dioecy would have to be

explained in the context of a tribe where all its members are dioecious (except for *Lacandonia*, if interpreted as an inflorescence). To substantiate both propositions thorough research of the systematic relationships and developmental series of extant members of this and the other two tribes of Triuridaceae are still required.

DISCUSSION

Phylogenetic topologies recovered for Pandanales and character mapping

While the total evidence approach under MP recovers a monophyletic Triuridaceae as sister to all other Pandanales, whose affinities remain unresolved due to the instability of Velloziaceae, this approach using a BI method recovers two clearly distinct monophyletic clades within this order: one composed of Stemonaceae, Pandanaceae and Cyclanthaceae, and the other of Velloziaceae and Triuridaceae.

With respect to Triuridaceae, the analyses presented here confirm the result of the earlier morphological analysis (Rudall and Bateman 2006) in suggesting that this family is derived from a MRCA that had true flowers. This assertion is supported by the character mapping exercise of relevant reproductive characters performed on the resolved BI phylogeny (see Figures 2B and 3; Table 2), where under either an uniaxial or a branched (polyaxial) interpretation of the Triuridaceae reproductive structures (Matrix I or II, respectively), the MRCA of Velloziaceae and Triuridaceae is reconstructed as having true flowers (see Table 2 and Figure 4). Furthermore, in the molecular and combined phylogenetic hypotheses recovered from both MP and BI, a monophyletic Triuridaceae are placed either as sister to a clade that encompasses all other Pandanales (Figure 1A and 2A); as monophyletic in an unresolved Pandanales families (Figure 1B) or as sister to Velloziaceae (Figure 2B). In no instance is Triuridaceae recovered as sister to Stemonaceae, Pandanaceae or Cyclanthaceae, in contrast with a previous molecular analysis that placed Triuridaceae as sister to Pandanaceae (Chase et al., 2000) and a morphological analysis that placed Triuridaceae as embedded within Stemonaceae (Rudall and Bateman, 2006). In addition, the two families that have uncontroversial inflorescences, Cyclanthaceae and Pandanaceae, are placed as sister to each other within a clade that includes Stemonaceae (See Figures 1 and 2), suggesting that taxa with inflorescences are derived from an ancestor that had euanthia.

Furthermore, while the MRCA of Triuridaceae is a monoecious plant with unisexual flowers, the reconstruction of the most likely character states present in the MRCA of the three tribes that compose this family suggests a complex evolutionary history for this pantropical lineage of mycoheterotrophs. In these reconstructions, the MRCA of Triurideae under M-I is recovered as having bisexual flowers (see Figure 5). This result is unexpected given that the majority of extant genera in this tribe are

dioecious. In contrast, the reconstruction based on M-II is more conservative as it recovers a MRCA of this tribe with the same character states as those present in the MRCA of all the Triuridaceae (i.e., a monoecious plant with unisexual flowers). While this analysis is based on few taxa from each of the tribes and will benefit from the inclusion of additional Triuridaceae species, it suggests a new evolutionary scenario for the emergence of the unique bisexual inside-out flowers present in *Lacandonia*, a group that until now, had been regarded as likely probably being derived from a dioecious ancestor (Vergara-Silva et al., 2003, Garay-Arroyo et al., 2012).

Morphogenetic trends in Pandanales based on recovered phylogenies

Rudall and Bateman (2006) suggested a series of morphological steps that could have lead to the anomalous reproductive structures present among the different families of Pandanales other than Velloziaceae, which is the only group with flowers that conform to a typical monocot *bauplan* (following the criteria of Remizowa et al., 2010). Given the topology of their most parsimonious tree, in which Triuridaceae are paraphyletic with respect to Stemonaceae and Triuridaceae, and both families as sister to Cyclanthaceae plus Pandanaceae, Rudall and Bateman (2006) hypothesized a serial sequence of losses and gains. These included the loss of septal nectaries and trimery in the clade that encompasses Pandanaceae, Cyclanthaceae, Stemonaceae and Triuridaceae; reduction to a single carpel in Stemonaceae (excluding *Pentastemona*) and finally, carpel multiplication in Triuridaceae. In the work presented here, the hypothesis of a paraphyletic Triuridaceae associated with Stemonaceae is not supported, while the pseudanthial nature of Triuridaceae is difficult to sustain, given the position of this family within Pandanales in either of the topologies recovered (see Figures 1 and 2).

With respect to the ontogenetic phenomena affecting the development of the floral units of Triuridaceae, Rudall and Bateman (2006) proposed three possible factors in a polyaxial interpretation (see also Rudall and Bateman, 2010): (1) paedomorphic heterochrony (i.e. increasingly precocious initiation of nodes), (2) loss of determination between an inflorescence and a flower meristem, and (3) differential progression of carpel formation. While events 1 and 3 are still possible, given the phylogenies recovered in this work, and paedomorphy has been suggested as a possible explanation for the pattern of expression of a stamen-specification gene in the case of *L. schismatica* (LsAP3; see section below and Álvarez-Buylla et al., 2010, Piñeyro-Nelson et al.,

2010), event 2 is unwarranted, given the position of Triuridaceae as sister to a family with clearly defined inflorescence and floral meristems (Velloziaceae). Furthermore, the developmental series published for *L. schismatica* and *T. brevistylis*, show a clear differentiation of a naked inflorescence meristem with respect to a bract-covered floral meristem, as well as no abortion of organs within the flowers of these taxa or other morphological criteria that would suggest a polyaxial identity (Ambrose et al., 2006). Interestingly, while taxa that have flowers with reduced or bractless floral meristems have been documented in several angiosperm lineages, all specimens of Triuridaceae analyzed for this work presented distinct bracts subtending each reproductive unit, a phenomenon that is very suggestive of uniaxial identity.

Another piece of evidence that lends support for a uniaxial interpretation of the Triuridaceae reproductive unit is the character mapping and MRCA reconstruction presented here (see Table 2 and Figure 4). Under a polyaxial interpretation (M-II) more transitions are required, while greater uncertainty of the floral state of the MRCA of the Pandanales subsists under this hypothesis (see Figure 4). On the other hand, the discovery in Triuridaceae of highly unusual (perhaps unique) carpel fascicles that strongly resemble individual axes (Ambrose et al. 2006, Rudall, 2008) could be interpreted as evidence for a polyaxial structure. Thus, the phylogenetic placement of Triuridaceae and the character mapping exercise presented here suggest that Triuridaceae should be regarded as having true flowers where one of its genera; *Lacandonia*, has a unique floral organ inversion not previously documented in nature.

Molecular genetic basis for the morphogenesis of Triuridaceae reproductive structures

While the ancestral state reconstruction discussed in the previous section broadens our ideas of the putative evolutionary paths that could have been followed by the ancestors of *Lacandonia*, it does not explain how the inversion in the position of the stamens of this lineage took place. Molecular genetic studies of flower development in *L. schismatica* have addressed the proximal causes of this phenotype, demonstrating that that the so-called B and C function genes (APETALA3 [AP3]/PISTILLATA [PI] and AGAMOUS [AG], respectively) that are necessary and sufficient for floral organ determination (Bowman et al., 1989) are conserved in this species. Additionally, it was shown that one of the B genes orthologues, *LsAPETALA3*, in *L. schismatica* is displaced since early stages of development to the flower center. This gene in conjunction with

Arabidopsis and/or *L. schismatica* are able to specify stamens in *Arabidopsis thaliana;* and thus this functional data constitute a sufficient mechanistic explanation of the inside-out flowers of *L. schismatica* with central stamens surrounded by carpels (Álvarez-Buylla et al., 2010).

Two phenomena that are observed in other extant Triuridaceae could explain how the displacement of the B function to the flower center of *L. schismatica* evolved: the lack of canalization in the number of carpels per flower, and the occurrence of ectopic stamens or carpels in otherwise unisexual flowers (as suggested by Rudall and Bateman, 2006; Rudall, 2008). In the first case, it has been observed that for some Triuridaceae studied, the number of carpels present in a particular flower seem to be related more closely with the size of the floral primordia and underlying receptacle than with a genetic program (Maas and Rübsamen 1986; Martínez and Ramos, 1989, Vergara-Silva et al., 2003; Rudall, 2008; Alves and Melo, 2012). In the second case, the documentation of occasional ectopic sexual organs in flowers of some Triuridaceae, such as *T. brevistylis* and *S. spruceanum* (Martínez and Gómez, 1994; Vergara-Silva et al., 2003; Espinosa Sánchez, 2009) suggests the possibility that the regulation of molecular components (single proteins and/or higher order complexes, miRNAs, etc.), that delimit the boundaries of gene or protein expression of the B and C function genes, could be relatively unstable or have a more transient expression in Triuridaceae than in other angiosperm lineages (Álvarez-Buylla et al., 2010). Under such a scenario, some of the transcription factors that could have a different spatio-temporal domain of expression are those dealing with the pre-patterning of the location where stamens develop in floral meristems (ie., UNUSUAL FLORAL ORGANS and LEAFY genes; Rudall and Bateman, 2006; Rudall, 2008; Rudall et al., 2009; Piñeyro-Nelson et al., 2010; Álvarez-Buylla et al., 2010; Rudall and Bateman, 2010); the delimitation of stamen and carpel boundaries (SUPERMAN; Bowman et al., 1992); carpel boundaries and development (CRABS CLAW, Bowman and Smyth, 1999) or overall floral meristem determination and higher order transcription factor complexes (involving multifunctional proteins such as SEPALLATA3; Theissen and Sadler, 2001; Castillejo et al., 2005; Immink et al., 2009).

Furthermore, in order to better understand the ontogenetic processes involved in the peculiar development of carpels in Triurids, the positive and negative regulators of AG which have been shown to involve numerous transcription factors and proteins from diverse biochemical families such as APETALA1, SEUSS and SEPALLATA3 (Sridhar

et al., 2006); APETALA2 and miRNA172 (Wollman et al., 2010), PERIANTHIA (Maier et al., 2009), among many others (for a recent review and compendium of gene families involved, see Liu and Mara, 2010 and Álvarez-Buylla et al., 2010b, respectively) should be further investigated, as this gene is necessary not only in stamen and carpel specification (Yanovsky et al., 1990; Riechmann, et al., 1996), but is also fundamental for floral meristem exhaustion through the direct and indirect repression of WUSHEL (Liu et al., 2012). In fact, more than twenty genes have been characterized to be involved in the regulation of *AGAMOUS*, including members of the YABBY, KNOX, bZIP families, among others (*refs).

Additionally, the outcomes of these investigations will be instrumental to address whether a blurred boundary between the inflorescence and floral meristems actually exists in this family (Rudall and Bateman, 2006; Rudall 2008).

Compound primordia meristems involved in floral organogenesis in Triuridaceae?

Compound stamen-carpel primordia at early stages of floral meristem development are present in *L. schismatica* and *Triuris brevistylis* (Martínez and Ramos, 1989; Márquez-Guzmán et al., 1989; Ambrose et al., 2006), and compound carpel primordia in other Triridaceae (Rudall, 2008). In addition, such primordia are followed by the emergence in the carpel whorl of a structure that resembles a ring meristem (Ambrose et al., 2006; Rudall, 2008, 2010). Both types of floral organ primordia could also facilitate the inversion of stamen and carpel positions during development. The fascicles of Triuridaceae strongly resemble individual axes, and could therefore be interpreted as evidence for a polyaxial structure.

Compound primordia are present in various angiosperm lineages, being the most common type those where stamen and petal initials arise from a common meristematic section and afterwards migrate to a third and second whorl, respectively (Tucker 2003a; Ronse Decraene and Smets, 1993; Endress 1995, 2006). However, compound primordia that involve both carpel and stamens initials are rare (Rudall 2008, 2009). Likewise, ring meristems involving polyandry have been documented in a diverse set of angiosperms (Ronse Decraene and Smets, 1993; Rudall, 2009), but ring meristems involving carpels are unusual (documented in some Alismatales; Endress, 1995; Rudall 2008; and in *Nelumbo nucifera*; Hayes et al., 2000).

Interestingly, stamen fascicles can have either a whorled, spiral or “chaotic”

pattern and in the latter, they commonly develop centrifugally (Tucker, 2003b; Hayes et al., 2000; Ronse Decraene and Smets, 1993). Furthermore, while some species in Triuridaceae additionally posses filamentous structures on the tepal tips as well as projections from the center of the receptacle (for instance, in male *Seychellaria* specimens; Rudall, 2008), no instances of proper "reversal", understood as either organ abortion, mosaic organs that appear late in ontogeny, development of intercalating phyllomes (as documented for some floral units of the basal monocot family Hydatellaceae; Rudall et al., 2009) or ectopic flowers have been reported for this group. In order to discern the molecular bases of both the compound/ring meristems and filamentous structures in this lineages, the investigation of the expression patterns of inflorescence to floral meristem regulators such as LEAFY should be performed but also, the investigation of floral meristem key interactors such as SEP3 and the regulators of the spatio-temporal expression of AG and WUS. Also, it is possible that molecular interactions of genes involved in maintaining ring meristems in Triuridaceae, where a "time lag" in processes that are downstream of floral organ determination (involving organ polarity, number, maturation), could be the underlying phenomena that favor the appearance of homeotic changes in this lineage, rather than a blurred boundary between an inflorescence and floral meristem.

In a broader context, encompassing representatives of all Pandanales, it would be desirable to perform comparative analyses of early floral meristem and floral organ development, investigating the existence of compound/ring primordia, as a means to investigate if these phenomena are widespread in the order and can account for several unique features such as complex patterns of inflorescences (Cyclanthaceae and Pandanaceae); carpel reduction and changes in merism (Stemonaceae); complex development of stamen structures (Velloziaceae) and altered merism behavior, as well as the occurrence of homeotic changes in flowers (Triuridaceae). In the case of the Triuridaceae, it will be interesting to investigate if the presence of fascicles could have been a trait already present in the MRCA of this family and Velloziaceae, as stamen ring meristems have been documented in some species of the latter family (Menezes, 1980; Sajo et al., 2010).

Finally, the investigation of the presence of both compound and ring meristems in non inside-out bisexual flowers in Triuridaceae (for instance, *S. picta or S. rubra*; Maas and Rubsamen, 1986) could shed light on the question of the possibilities of these structures favoring the displacement of B function to the center of the floral meristem,

as found in the *Lacandonia* genus. It would also be informative concerning the likelihood that the MRCA of the Triurideae bared bisexual flowers, as our character state reconstructions under a uniaxial hypothesis for Triuridaceae, suggest (Figure 5).

The above molecular genetic hypotheses need to be experimentally tested, ideally under a comparative framework that includes comparative morphology, developmental series and developmental genetic studies. Furthermore, it is clear from this work that the evolutionary history of the Pandanales order and of Triuridaceae in particular, that could have favored the appearance and fixation of the floral homeosis documented in the *Lacandonia* genus, is much more complex than previously thought.

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FIGURE LEGENDS

Figure 1. Inferred MP and BI phylogenies using the 18S and *atpA* sequences

Figure 2. Inferred MP and BI phylogenies using a combined data matrix (morphological and molecular characters).

Figure 3. Character state reconstructions under an uniaxial (M-I) or polyaxial (M-II) interpretation of the floral units of Triuridaceae.

Figure 4. Simplified floral diagrams of the transformation series for the MRCAs leading to Triuridaceae

Figure 5. Simplified floral diagrams of the MRCA ancestors of the three tribes within Triuridaceae .

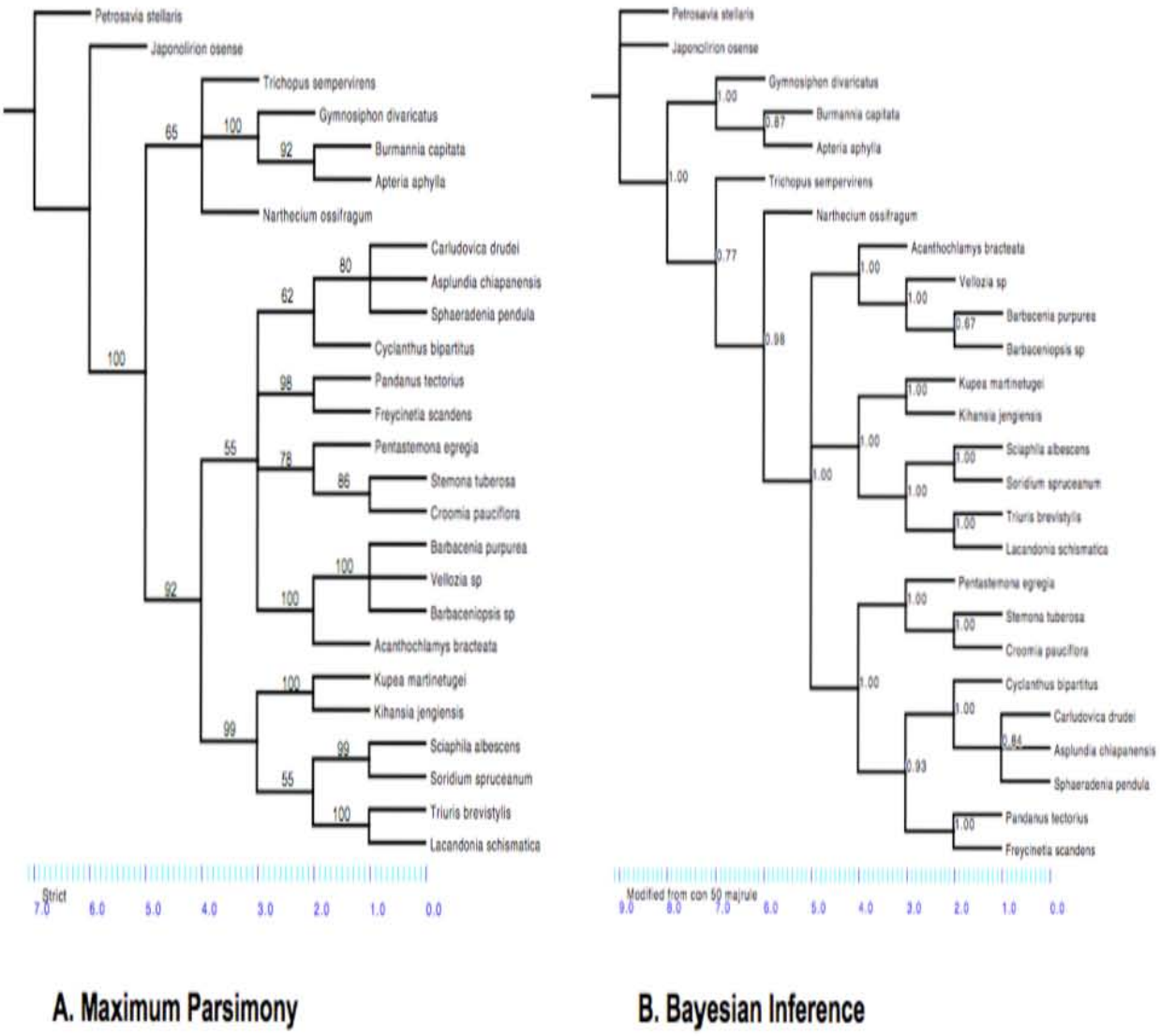
Appendix 1

List of sampled species, Genbank accession numbers and voucher information arranged into the major Angiosperm groups. Newly obtained 18S rDNA sequences for this study are indicated with an asterisk. Sequences obtained from Genbank do not list voucher information.

Taxon-Genbank accession; Voucher.

FIGURES

Figure 1. Inferred MP and BI phylogenies using the 18S and *atpA* sequences



A. Maximum Parsimony **B. Bayesian Inference**

Figure 2. Inferred MP and BI phylogenies using a combined data matrix (morphological and molecular characters).

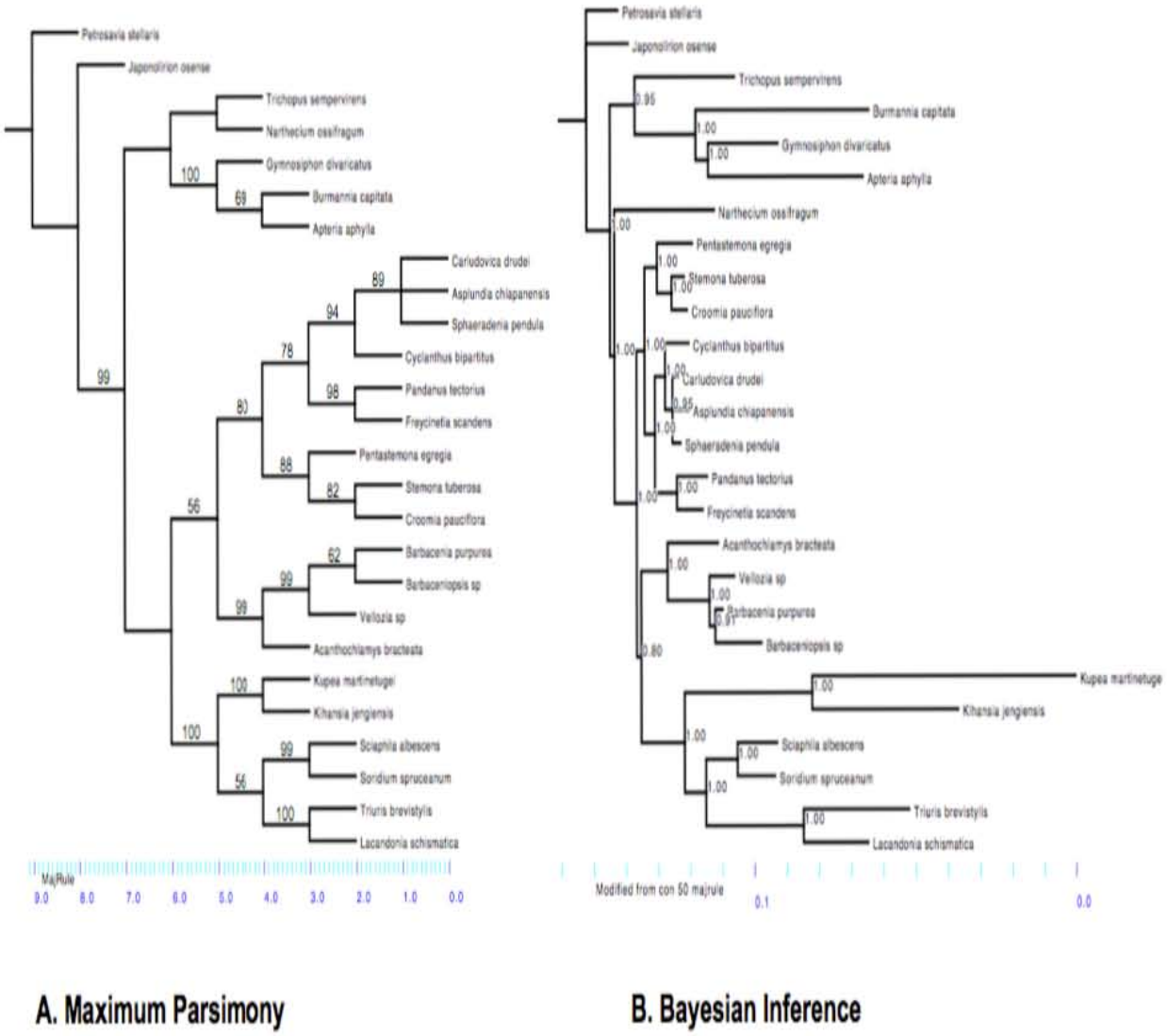
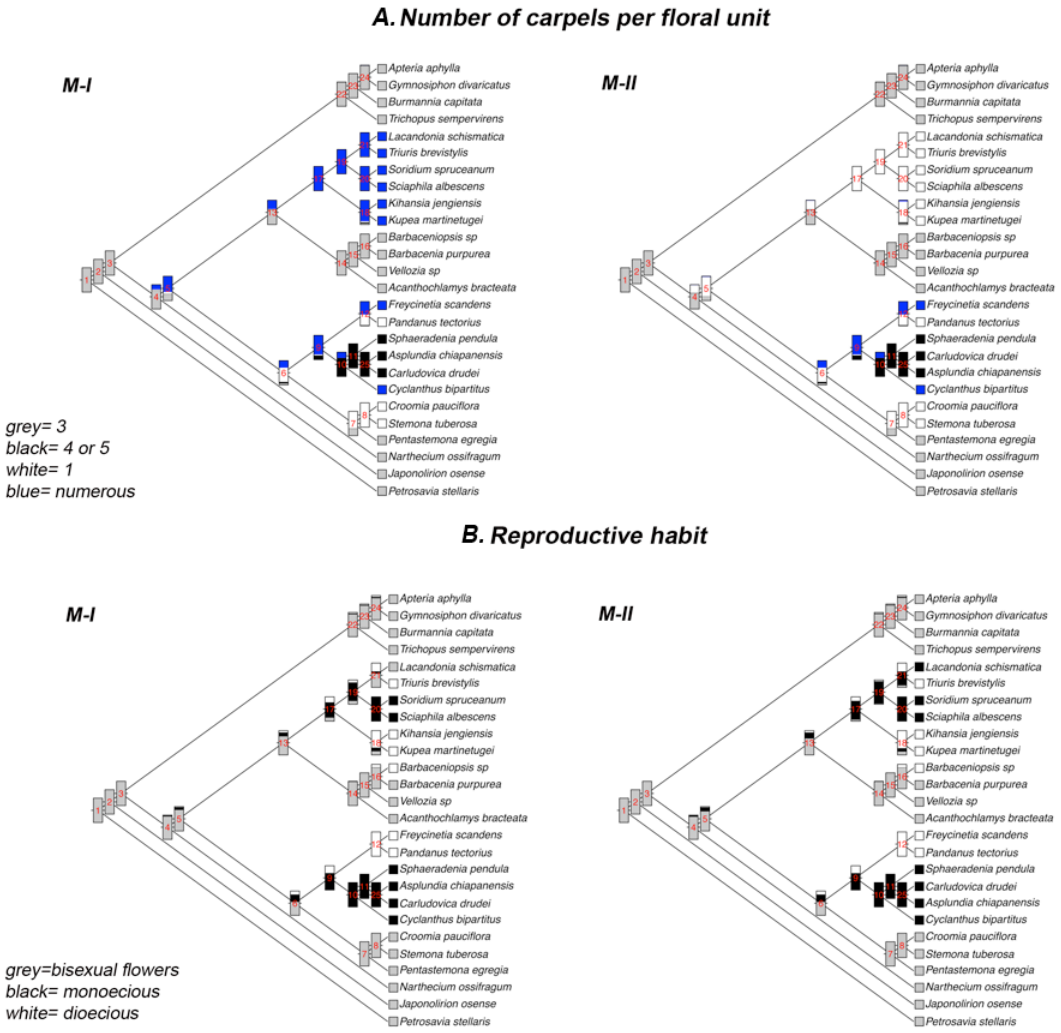
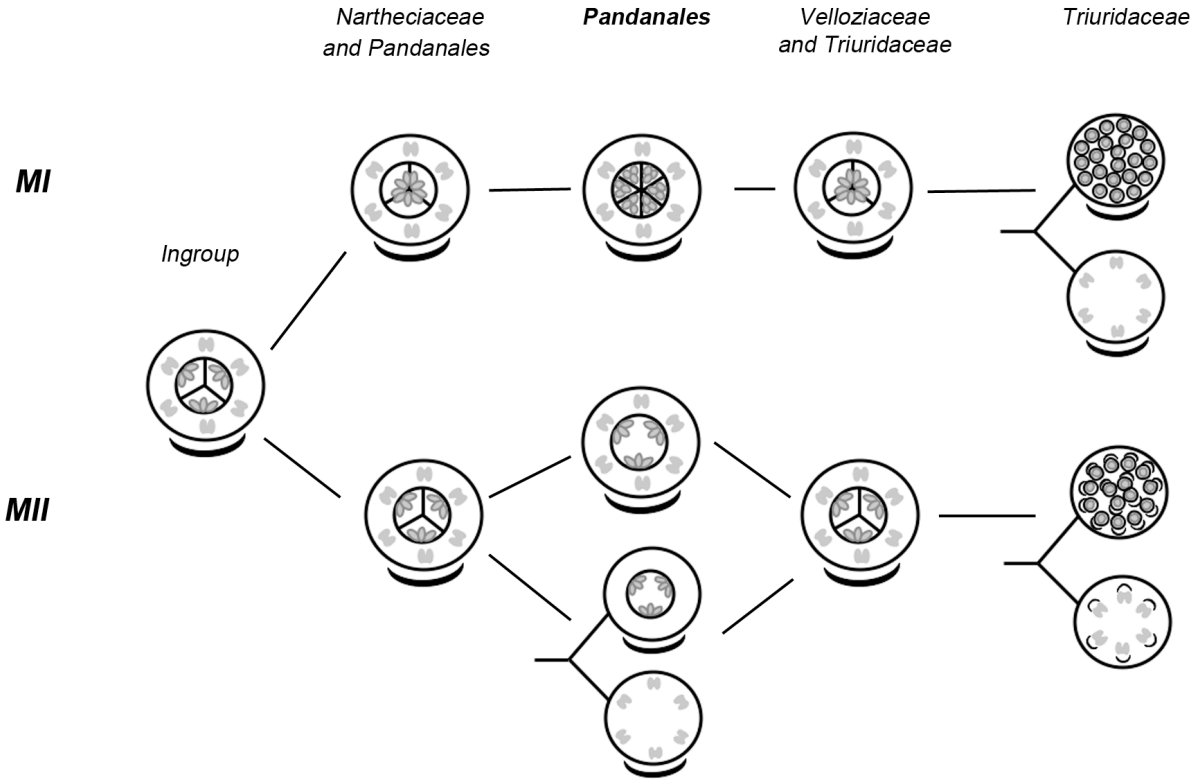


Figure 3. Character state reconstructions under an uniaxial (M-I) or polyaxial (M-II) interpretation of the floral units of Triuridaceae.



A. The probability of having floral units with numerous carpels *vs* a single carpel changes significantly in the MRCA of Triuridaceae (node 17), depending on the codification of the terminal taxa. **B.** In contrast, the probability of occurrence of a monoecious floral habit in the MRCA of Triuridaceae (node 17) does not change significantly under the MI vs MII hypotheses. The proportion of each color in the squares plotted in the internal nodes depicts the probability of occurrence of a particular character state. The number of each internal node is depicted in red.

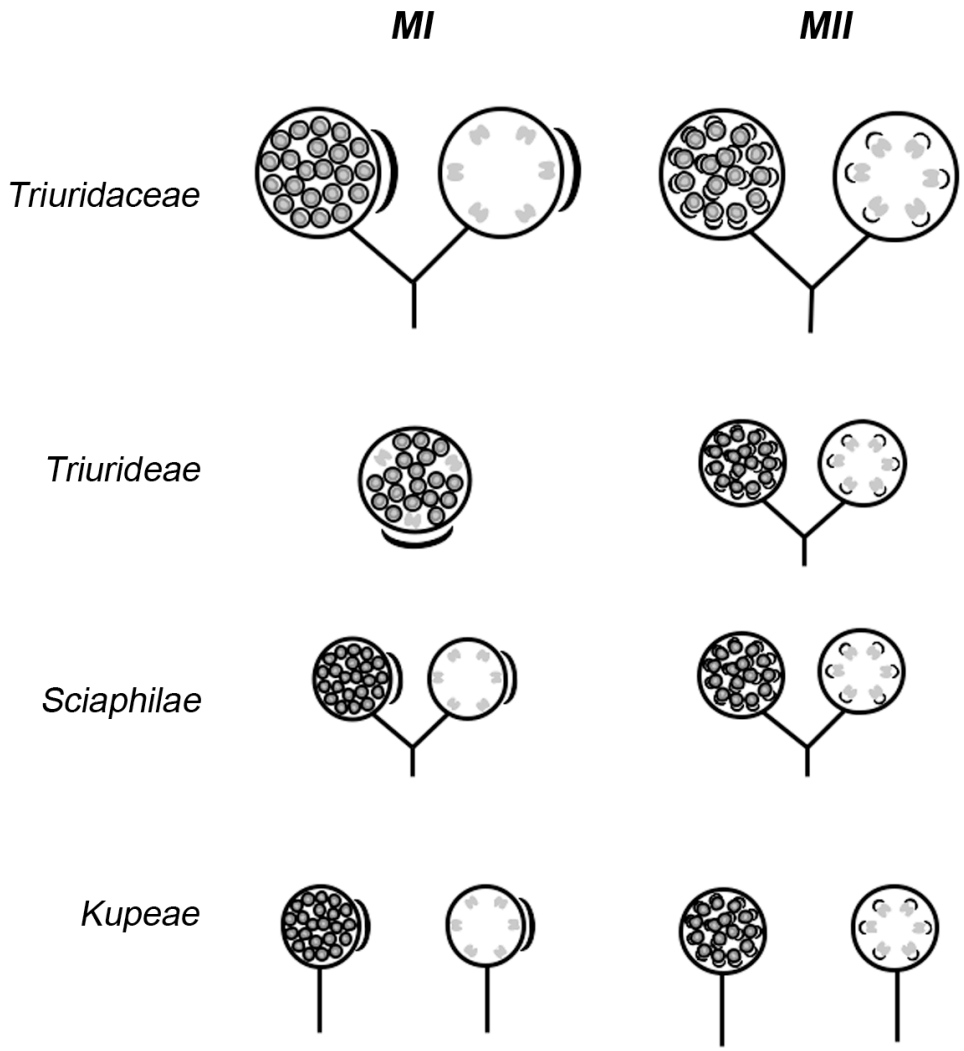
Figure 4. Simplified floral diagrams proposing a transformation series for the MRCAs leading to Triuridaceae



In this diagram the perianth structures were dismissed and only a subtending bract was drawn to separate a "true flower" or euanthia, from an inflorescence or pseudanthia (the perianth is assumed to have the trimerous merism characteristic of monocots). The reconstruction using M-I suggests that the MRCA of several internal nodes leading to the Triuridaceae possessed a true flower where the number of carpels and disposition of ovules within (placentation) varied. The MRCA of Triuridaceae (last column on the right) was a monoic plant with unisexual flowers with numerous carpels in the female flowers. In the reconstruction based on the M-II the floral state of the MRCA of Pandanales is unclear, suggesting this ancestor could have bared either bisexual flowers or unisexual ones. Nevertheless, the MRCA of Velloziaceae plus Triuridaceae under M-II is also reconstructed as having true flowers. Thus, the MRCA of Triuridaceae in this scenario would have to undergo several transformations that included not only monoecism, but also flower reduction, ovule reduction and multiplication of very simplified flowers in compressed unisexual inflorescences (these simplified unisexual flowers are drawn as having individual subtending bracts as a proxy to distinguish them

from the euanthial scenario in M-I).

Figure 5. Simplified floral diagrams of the MRCA of each of the tribes within Triuridaceae .



The floral states of the MRCA of each of the three tribes within Triuridaceae change significantly depending on the morphological matrix used. In the case of M-I (left panel), each individual tribe would have evolved a different floral habit from a monoecious ancestor. In contrast, under M-II, only the Kupeae would have evolved dioecism from a monoecious ancestor. Floral diagrams are as in Figure 4.

Tables

Table 1. Species sampled for this study with their GenBank accession numbers

Table 2. Most likely character states for relevant floral characters at selected internal nodes (MRCA) of the BI phylogeny from figure 2B. Comparisons of results between M-I and M-II are presented.

Table 1. Species sampled for this study with their GenBank accession numbers

Family	***Taxa***	***18S***	***atpA***
PETR	*Petrosavia stellaris*	AF206987*	AY299821*
PETR	*Japonolirion osense*	AF206942*	AY299790*
DIOS	*Trichopus sempervirens*	AF309395*	AY299724*
DIOS	*Dioscorea bartlettii*	EAB	EAB
BURM	*Gymnosiphon divaricatus*	EAB	EAB
BURM	*Burmannia capitata*	DQ786065*	EU421009*
BURM	*Aletris farinosa*	EU186221*	AY299706*
THIS	*Thismia panamensis*	DQ786081*	EU421050*
NART	*Narthecium ossifragum*	AF309411*	AY299809*
CYCL	*Carludovica palmata*	AF293756*	EAB
CYCL	*Carludovica drudei*	EAB	EAB
CYCL	*Cyclanthus bipartitus*	AF168837*	AY299754*
CYCL	*Asplundia chiapanensis*	EAB	EAB
PAND	*Pandanus tectorius*	AY952391*	EU421052*
PAND	*Freycinetia scandens*	AF206915*	EAB
PAND	*Martellidendron hornei*	EAB	-
PAND	*Sararanga sinuosa*	EAB	-
STEM	*Pentastemona egregia*	EAB	EAB
STEM	*Stemona tuberosa*	EAB	EAB
STEM	*Croomia pauciflora*	AF168835*	AF197708*
VELL	*Barbacenia purpurea*	EAB	EAB
VELL	*Acanthochlamys bracteata*	AY952411*	AY299698*
VELL	*Vellozia sp.*	EAB	EAB
VELL	*Barbaceniopsis sp.*	EAB	AY299725*
TRIU	*Kupea martinetugei*	EU816706*	GQ469520*
TRIU	*Kihansia jengiensis*	VM	VM
TRIU	*Seychellaria*	VM	VM
TRIU	*Sciaphila albescens*	EU816705*	AY299835*
TRIU	*Soridium spruceanum*	EAB	EAB
TRIU	*Triuris brevistylis*	EAB	AY299854
TRIU	*Lacandonia schismatica*	EAB	AY299794

The accession numbers marked with an asterisk were obtained from GenBank. - we could not obtain good quality *atpA* sequences for these taxa.

Table 2. Most likely character states for relevant floral characters at selected internal nodes (MRCA) of the BI phylogeny in figure 2B. Comparisons of results between MI and MII are presented.

Node number	*Reproductive habit (11)*	*Floral Units (12)*	*No. of carpels per floral unit (36)*	*No. of ovules per carpel (45)*	*Placentation (46)*	*Inferred character states of MRCA:*
1 MRCA of all taxa	0/0	0/0	0/0	0/0	1/1	Monoecious plant with bisexual flowers with 3 carpels per floral unit, numerous ovules per carpel with parietal placentation
4 MRCA of Nart and PAND	0/0	0/0	0/0*	0/0	**0*/1***	Monoecious plant with bisexual flowers with 3 carpels per floral unit, numerous ovules per carpel and **axile (parietal) placentation**
5 MRCA of PAND	0/0	**0*/?**	**3*/2**	0/0	**?/1***	Monoecious plant with bisexual flowers with **numerous (single) carpels** per floral unit, numerous ovules per carpel and **(?) parietal placentation**
6	0*/0*	?/?	2*/2*	0/0	1*/1*	Monoecious plant with unisexual flowers

MRCA of Ste (Cycl+Pand)						with 1 carpel per floral unit, numerous ovules per carpel and parietal placentation
13 MRCA of Vell+Triu	0/0*	0*/0*	0*/0*	0/0*	**0*/1***	Monoecious plant with bisexual flowers with 3 carpels per floral unit, numerous ovules per carpel and **axile (parietal) placentation**
17 MRCA of Triu	1*/1	1/1	**3/2**	1/1	1*/1*	Monoecious plant with unisexual flowers with **numerous (single) carpels** per floral unit; 1 ovule per carpel and parietal placentation
18 MRCA Kupeae	2/2	1/1	**3/2**	0/0	0*/0*	Dioecious plant with unisexual flowers and **numerous (single) carpels** per floral unit, more than 1 ovule per carpel and axile? placentation.
19 MRCA tribes	1/1	1/1	**3/2**	1/1	1*/1*	Monoecious plant with unisexual flowers with **numerous (single) carpels** per floral unit; 1 ovule per carpel and parietal

Tri+Scia						placentation
20 MRCA Sciaphileae	1/1	1/1	**3/2**	1/1	1/1	Monoecious plant with unisexual flowers, **numerous (one) carpels** per floral unit, 1 ovule per carpel and parietal placentation
21 MRCA Triurideae	0*/1*	0*/1	**3/2**	1/1	0/0	**Hermaphrodite plant (monoecious)** with **bisexual (unisexual) flowers**, **numerous (single) carpels** per floral unit, one ovule per carpel and axile placentation.

Node numbers are the same as those in Figure 3. In this table, the character states with highest probabilities of occurring are presented, with results from MI/MII. Characters in underlined and in **bold** had different characters states depending if mapping was based on MI or MII. In the last column, when two alternative character states were inferred using MI or MII, these are in bold and the alternative character state generated using MII is additionally in parentheses. : Placentation only changes for *A. bracteata*, where this character was coded either as 0 or 1. A particular character state was assigned if a character had at least a 75% likelihood, in a two-state character; * = character state was assigned that had the highest likelihood, although > 75% (this criterion was applied only to characters with more than 2 character states). ? = an ambiguous character (approximately 50-50% probability). Nart = Nartheciaceae; PAND = Pandanales; Ste = Stemonaceae; Cycl = Cyclanthaceae; Pand = Pandanaceae; Triu = Triuridaceae; Tri = tribe Triurideae; Scia = tribe Sciaphileae.

APPENDIX 1

Morphological characters scored for the morphological matrices I and II. Characters in bold were added in this analysis. Underlined characters are those that make *a priori* assumptions of floral character state and that are thus scored as equivocal (?) in Matrix II.

1.Habit

0: rhizomatous herb or climber, seasonal

1: perennial

2: rhizomatous perennial

2. Source of nutrients

0: endogenous (fotosynthetic)

1: exogenous (parasitic/ saprophytic)

3.scale leaves on rhizome

0: absent

1: present

4.foliage leaves

0: present

1: absent

5.foliage leaf margins

0: entire

1: spiny

6.foliage leaf morphology

0: linear

1: petiolate

7.Raphide crystals

0: absent

1: present

8.Styloid crystals

0: absent

1: present

9.stomata

0: present

1: absent

10. Photosynthetic pigments in sporophyte

0: present

1: absent

11.reproductive habit

0: bisexual flowers

1: monoecious

2: dioecious

12.floral units

0: hermaphrodite

1: unisexual

13.Presence of floral bracts

0: present

1: absent

14.Floral symmetry*

0: actinomorphic

1: zygomorphic

15.tepals

0: present

1: absent

16.tepal thickness

0: not fleshy

1: fleshy

17.tepal inner surface

0: smooth

1: papillate

18.filamentous structures on tepals

0: absent

1: present

19.corona

0: absent

1: present

20.stamen filaments

0: slender

1: thick, short or absent

21.stamen filament surface

0: smooth

1: papillate

22.connective stamen appendages

0: absent

1: present

23.staminodes

0: absent

1: present

24.number of stamens

0: based on multiples of 3 (mostly 3 or 6)

1: 4 or 5

2:numerous

3: 2

25.pollen type

0: monosulcate

1: innaperturate

26.pollen apertures 1

0: elliptical

1: ulcerate

27.pollen apertures 2

0: without operculum

1: with operculum

28.pollen shape

0: oval or spherical

1: reniform

29.pollen exine morphology

0: reticulate or psilate

1: spinulate or gemmate

2: spiny-gemmate

30.exine

0: columellate

1: single layer

31.endexine lamellae

0: present

1: absent

32.Number of pollen nuclei

0: 2

1: 3

33.tapetum type

0: secretory

1: plasmodial

2: invasive non-syncytial

34.Anther thecae

0: bilocular

1: unilocular

35.ovary position

0: superior or semi-inferior

1: inferior

2: sunken into axis

36.number of carpels per floral unit

0: three

1: four or five

2: one

3: numerous

37.carpel fusion

0: fused

1: free

38.Carpel initiation

0: initiated and arranged separately

1: organized in fascicles

39.carpel progression

0: centripetal (or single whorl)

1: centrifugal

40.Carpel vascularization

0: absent

1: present

41.number of ovules per carpel

0: one per carpel

1: unilocular in syncarp

2: bilocular carpel ***(Kupea, Kihansia)***

42.Ovule type

0: Anatropous

1: Orthotropous

2: Campylotropous

43.Embryo sac

0: Polygonum-type

1: Lacandonia-type

2: Fritillaria-type

44.ovules

0: crassinucellate

1: tenuincellate

45.number of ovules per carpel

0: more than one

1: one

46.placentation

0: axile

1: parietal

2: basal

3: apical

47.style insertion

0: (sub-)apical

1: (sub-)basally inserted on carpel

48.pistillodes

0: absent

1: present

49.septal nectaries

0: absent

1: present

50.seed aril

0: absent

1: present

51.Seed tannins

0: absent

1: present

52.Late dev. sterile fil. struc. Center male fl.

0: absent

1: present

53.Mode of fruit dispersion

0: biotic

1: abiotic (wind, water, etc)

Anexo C: Publicaciones de divulgación relacionadas con el tema de la tesis de doctorado en los que participé como co-autora (sólo portadas).

García-Ponce et al., 2011. *Oikos.*

Garay-Arroyo et al., 2011. *Ciencia y Desarrollo*.

Lacandonia schismatica: Ventana a la evolución del desarrollo

BERENICE GARCÍA PONCE DE LEÓN, ADRIANA GARAY-ARROYO, ALMA PIÑEYRO NELSON, MARÍA DE LA PAZ SÁNCHEZ, ESTEBAN MARTÍNEZ Y ELENA R. ÁLVAREZ-BUYLLA

La genética de la flor y la sexualidad de las plantas

Adriana Garay-Arroyo, Berenice García-Ponce, Rigoberto V. Pérez-Ruiz, Alma Piñeyro-Nelson y María De La Paz Sánchez

La sexualidad y la evolución de la mayor parte de las plantas que nos rodean y alimentan (las angiospermas) gira alrededor de sus flores; sin embargo, aún quedan muchas interrogantes sobre las bases genéticas que favorecen que las flores sean al mismo tiempo tan conservadas en su estructura básica y tan variables en sus detalles. Nuestra curiosidad por entender a las flores no es nada nuevo; desde tiempos remotos el ser humano se ha preguntado sobre su origen, su modo de desarrollo y las fuerzas naturales que dieron paso a la gran diversidad vegetal existente. Al inicio, el interés del humano se concentraba en conocer las plantas en función de su valor de uso, pero luego le fueron surgiendo preguntas en torno a su desarrollo y relaciones de parentesco. Dichas preguntas datan desde la época de los griegos y diferentes culturas fueron creando sus propios sistemas de organización y descripción de la flora con la que convivían. Este fenómeno se exacerbó en Europa desde el Renacimiento, cuando se comenzó a tener registro de la flora y fauna exótica procedente de otros continentes. Sin embargo, la flor como estructura no adquirió la importancia botánica que tiene ahora sino hasta que Carl von Linné estableció, en 1753, un sistema de clasificación taxonómica (*Species Plantarum*) con base fundamentalmente en la estructura de la flor. A partir de ese momento, el entender cómo se generaba esta estructura y sus variantes en diferentes linajes de angiospermas ocupó el tiempo y mente de los morfólogos de los siguientes dos siglos, entre ellos, de Johann Wolfgang von Goethe.

Foto: Alma Piñeyro Nelson

De todas las plantas, sólo las espermatofitas, que agrupan a las gimnospermas y a las angiospermas, tienen estructuras reproductivas con semillas en sentido estricto y de éstas, sólo las angiospermas tienen flores. Las flores tienen un conjunto de características que las hacen únicas, como son: 1) la presencia de un carpelo, conocido también como gineceo o pistilo, que constituye el órgano femenino de las flores y dentro del cual se desarrollan primero los óvulos y después de la fertilización, los embriones (Fig. 1). Este carpelo tiene como característica fundamental el estar conformado por dos integumentos que protegen a los óvulos. Otras caracterís-

Anexo D: Otras publicaciones arbitradas realizadas durante el doctorado en los que participé como co-autora (sólo portadas).

Apéndice VII: Wegier et al., 2011. *Molecular Ecology*.

Apéndice VIII: Piñeyro-Nelson et al., 2009[a]. *Molecular Ecology*.

Apéndice IX: Piñeyro-Nelson et al., 2009[b]. *Molecular Ecology*.

Apéndice X: Dyer et al., 2009. *PLoS one*.

Apéndice XI: Artículo de difusión: Álvarez-Buylla, E.R. y Piñeyro Nelson, A. (2009) *Revista Ciencias* 92-93.

MOLECULAR ECOLOGY

Molecular Ecology (2011) **20**, 4182–4194 doi: 10.1111/j.1365-294X.2011.05258.x

Recent long-distance transgene flow into wild populations conforms to historical patterns of gene flow in cotton (*Gossypium hirsutum*) at its centre of origin

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Abstract

Over 95% of the currently cultivated cotton was domesticated from *Gossypium hirsutum*, which originated and diversified in Mexico. Demographic and genetic studies of this species at its centre of origin and diversification are lacking, although they are critical for cotton conservation and breeding. We investigated the actual and potential distribution of wild cotton populations, as well as the contribution of historical and recent gene flow in shaping cotton genetic diversity and structure. We evaluated historical gene flow using chloroplast microsatellites and recent gene flow through the assessment of transgene presence in wild cotton populations, exploiting the fact that genetically modified cotton has been planted in the North of Mexico since 1996. Assessment of geographic structure through Bayesian spatial analysis, BAPS and Genetic Algorithm for Rule-set Production (GARP), suggests that *G. hirsutum* seems to conform to a metapopulation scheme, with eight distinct metapopulations. Despite evidence for long-distance gene flow, genetic variation among the metapopulations of *G. hirsutum* is high (He = 0.894 ± 0.01). We identified 46 different haplotypes, 78% of which are unique to a particular metapopulation, in contrast to a single haplotype detected in cotton cultivars. Recent gene flow was also detected (m = 66/270 = 0.24), with four out of eight metapopulations having transgenes. We discuss the implications of the data presented here with respect to the conservation and future breeding of cotton populations and genetic diversity at its centre of crop origin.

Keywords: *Gossypium hirsutum*, long distance gene flow, metapopulations, Mexico, transgene flow

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Introduction

The complexes of wild and cultivated varieties of crop plants at their centres of crop origin and/or diversity (hereafter, CCO) provide useful systems for addressing fundamental questions on population structure, genetics, and specifically, gene flow dynamics (e.g. maize to teosinte; Baltazar *et al.* 2005; Ellstrand *et al.* 2007; the beet family; Bartsch *et al.* 1999; Viard *et al.* 2004; Fénart *et al.* 2007; Arnaud *et al.* 2009; or *Brassica* spp. Jørgensen & Andersen 1994; Snow *et al.* 1999). In cases where genetically modified varieties have been released at the CCO, transgenes become useful markers for addressing ongoing patterns, dynamics, and pervasiveness of gene flow (maize, van Heerwaarden *et al.* 2009; *Cucurbita*, Sasu *et al.* 2009; *Sorghum*, Sahoo *et al.* 2010). At the same time, these cases become particularly relevant for assessing the

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Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations

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Abstract

A possible consequence of planting genetically modified organisms (GMOs) in centres of crop origin is unintended gene flow into traditional landraces. In 2001, a study reported the presence of the transgenic 35S promoter in maize landraces sampled in 2000 from the Sierra Juarez of Oaxaca, Mexico. Analysis of a large sample taken from the same region in 2003 and 2004 could not confirm the existence of transgenes, thereby casting doubt on the earlier results. These two studies were based on different sampling and analytical procedures and are thus hard to compare. Here, we present new molecular data for this region that confirm the presence of transgenes in three of 23 localities sampled in 2001. Transgene sequences were not detected in samples taken in 2002 from nine localities, while directed samples taken in 2004 from two of the positive 2001 localities were again found to contain transgenic sequences. These findings suggest the persistence or re-introduction of transgenes up until 2004 in this area. We address variability in recombinant sequence detection by analyzing the consistency of current molecular assays. We also present theoretical results on the limitations of estimating the probability of transgene detection in samples taken from landraces. The inclusion of a limited number of female gametes and, more importantly, aggregated transgene distributions may significantly lower detection probabilities. Our analytical and sampling considerations help explain discrepancies among different detection efforts, including the one presented here, and provide considerations for the establishment of monitoring protocols to detect the presence of transgenes among structured populations of landraces.

Keywords: centers of origin, landraces, maize, Mexico, transgene flow

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Introduction

Mexico is the centre of origin and diversification of maize (*Zea mays* L.) and is home to about 60 domesticated landraces (Sánchez *et al*. 2000) as well as several wild relatives with which domesticated maize can cross-pollinate (Doebley 2004). Maize is a staple food in Mexico, with a pivotal place in the country's past and present economic, cultural and agricultural spheres. In contrast to the United

MOLECULAR ECOLOGY

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NEWS AND VIEWS

REPLY

Resolution of the Mexican transgene detection controversy: error sources and scientific practice in commercial and ecological contexts

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Keywords: biomonitoring, maize landraces, transgene flow

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Bernd Schoel and John Fagan (Vice-President and Founder/CEO, respectively, of Genetic ID, henceforth BS&JF) criticize and dismiss our recent publication in *Molecular Ecology* by focusing on our use of the Polymerase Chain Reaction (PCR) to detect specific DNA sequences. They raise important questions about the standards required to use PCR in various environmental conditions, pointing to the well-known fact that this delicate method may lead an unskilled operator to false results. They further suggest that our observations of transgenic DNA sequences in Mexican landrace maize should be attributed to false positives, i.e. a type I error. After considering their challenge and reviewing the evidence, we find their arguments seriously lacking in substance, and their practice permissive of false negatives, a type II error.

We seem to have attracted BS&JF's attention because, in an effort to corroborate our own results, we utilized the services of Genetic ID as full-paying customers. We established that Genetic ID failed on occasion to detect positive blind samples, which should not be surprising given the known vagaries of the PCR method. Yet for BS&JF this detection failure is not a factual possibility; instead, to explain our observations they would have us both (i) contaminating our samples and (ii) lying about the origin and nature of our materials. Specifically, BS&JF state:

1 'We contend that results such as these are incorrectly interpreted as positive and are more likely to be indicative of contamination in the laboratory.' and
2 'We would argue that the leaf sample provided by the authors did not contain the claimed NK603 event and, furthermore, does not contain material from any commercialized transgenic single plant.'

Other charges include an implication that we used false evidence and/or withheld inconvenient data (BS&JF, p.5, lines 5–11) to reach our conclusions.

All of these are indeed very serious challenges to our technical capacity and expertise, as well as our professional and personal integrity.

PCR contamination or false negatives (type II error)?

BS&JF declare their suspicion that all of our PCR positive results arose from systematic contamination. They note the presence of bands in the PCR gels that are weaker than they would expect for a '100% (homozygous) or 50% (heterozygous) GMO level', the only evidence that they would take as a positive result. Such a view is based on the unwarranted expectation that an end-point PCR could be used as a quantitative method.

In our experience and that of other independent laboratories, the PCR amplification of transgenic sequences in landrace maize backgrounds tends to produce relatively faint bands of variable intensity in end-point reactions visualized on agarose gels, which so far has been the standard approach in the field (Quist & Chapela 2001; Alvarez-Morales 2002; Piñeyro-Nelson *et al.* 2009). Genetic ID's own gels (their standard to screen-out 'negatives') show this kind of variability, even for repeats of a single sample in a single assay, or for different assays performed for the same sample at different times [see Fig. S1 (Supporting information)].

Such results should not surprise anyone versed in the PCR method. Although early cycles in the PCR assay may reflect stoichiometric molecular relationships, end-point

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Dispersal of Transgenes through Maize Seed Systems in Mexico

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Abstract

Objectives: Current models of transgene dispersal focus on gene flow via pollen while neglecting seed, a vital vehicle for gene flow in centers of crop origin and diversity. We analyze the dispersal of maize transgenes via seeds in Mexico, the crop's cradle.

Methods: We use immunoassays (ELISA) to screen for the activity of recombinant proteins in a nationwide sample of farmer seed stocks. We estimate critical parameters of seed population dynamics using household survey data and combine these estimates with analytical results to examine presumed sources and mechanisms of dispersal.

Results: Recombinant proteins Cry1Ab/Ac and CP4/EPSPS were found in 3.1% and 1.8% of samples, respectively. They are most abundant in southeast Mexico but also present in the west-central region. Diffusion of seed and grain imported from the United States might explain the frequency and distribution of transgenes in west-central Mexico but not in the southeast.

Conclusions: Understanding the potential for transgene survival and dispersal should help design methods to regulate the diffusion of germplasm into local seed stocks. Further research is needed on the interactions between formal and informal seed systems and grain markets in centers of crop origin and diversification.

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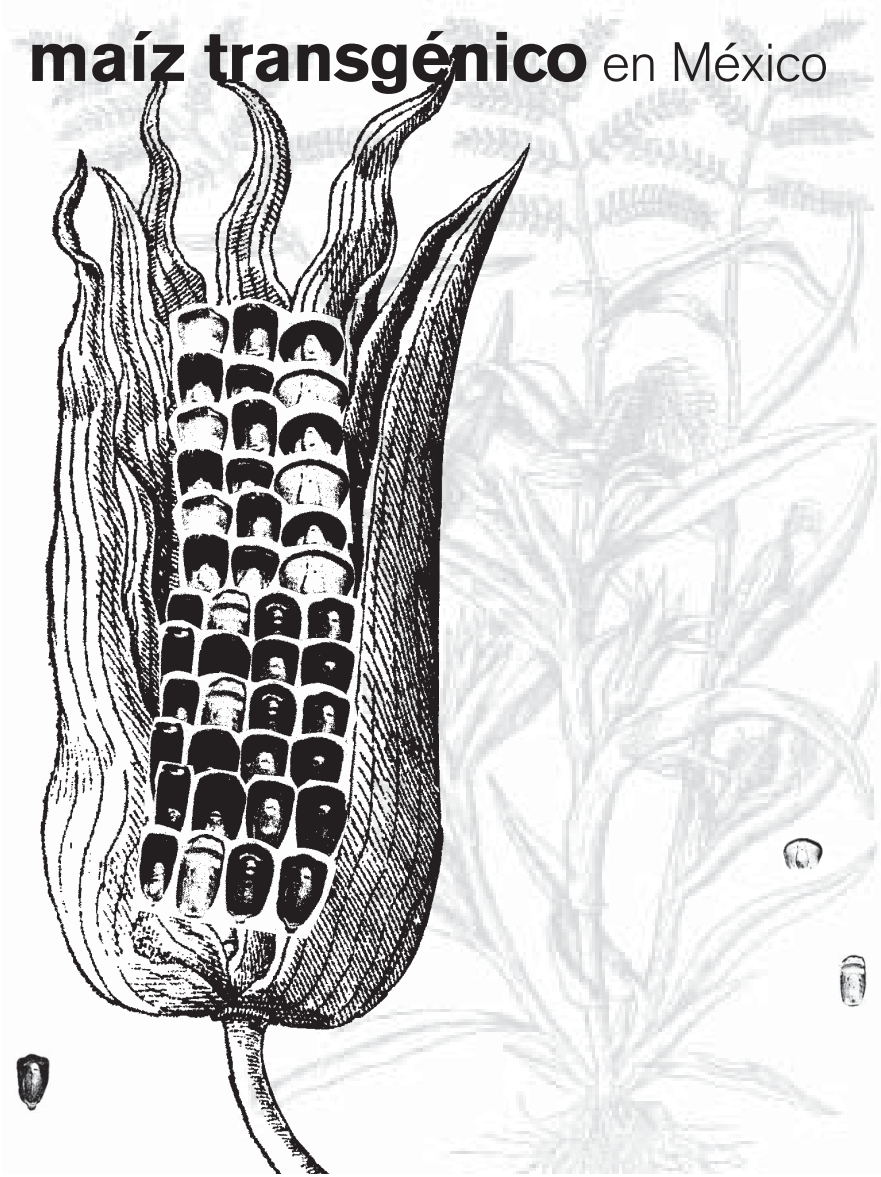
Introduction

As increasing numbers of genetically modified crops are released into the environment, the likelihood of unintended ecological effects on both agricultural and natural systems increases. These effects become particularly relevant in centers of crop origin and diversity [1]. In Mexico, a country that harbors over 60% of maize's (*Zea mays* L.) genetic variation, gene flow among landrace and teosinte (wild *Z. mays*) populations has occurred readily since maize's domestication 9,000 years ago [2,3]. But unlike domestication genes, which often represent a loss of function that decreases a plants' ability to survive without human intervention, many transgenes (*e.g.*, Cry genes) represent a gain of function that could enhance the survival or even the weediness of wild relatives [4,5].

Assessing the potential for the dispersal of transgenes into crop landrace and wild populations is critical [6,7]. The presence of transgenes in Mexican maize landraces was first reported in 2001 in the state of Oaxaca [8], but the extent of their dispersal is still in question. A subsequent study reported the presence of transgenes [9], while a third failed to detect them [10]. Some suggested that transgenes had disappeared, but recent studies have confirmed their presence in Oaxaca and found them in a new area of Mexico [11,12]. Inconsistencies across studies might be due to differences in the analytical methods used or to narrow geographic sampling [12,13]. Most analyses to date have been based on haphazard sampling of fields and seed stocks in a restricted number of localities; results are not representative of a well-defined population. Discrepancies might also be due to the dynamics of seed populations [13,14]. However, the absence of proper data on seed dynamics and a formal framework to interpret these data has lead to widespread speculation.

In this paper, we analyze the implications of seed dynamics on the dispersal of maize transgenes across Mexico. There have been

Riesgos y **peligros** de la dispersión de **maíz transgénico** en México



Elena Álvarez-Buylla Roces y Alma Piñeyro Nelson

Actualmente estamos frente a la posibilidad de que se apruebe la liberación de líneas de maíz transgénico en el campo mexicano. Las consideraciones sobre lo deseable y seguro de esta tecnología para nuestro país han sido guiadas por intereses políticos y económicos privados, más que por estudios científicos concluyentes, dejando de lado además las consideraciones sociales y ambientales. Estos intereses han moldeado y apresurado un marco regulatorio encaminado a posibilitar la liberación de las líneas comerciales disponibles de maíz transgénico en el campo mexicano, lo cual desencadenaría un conjunto de riesgos y peligros.

Lo que es peor, en el caso particular del maíz transgénico se sabe ya que estos desarrollos son obsoletos en términos tanto científicos como tecnológicos, aun para las condiciones de agricultura industrializada para los que fueron creados originalmente, debido a que se basan en un paradigma científico ya superado: un gen determina un rasgo visible —fenotípico— de manera simple y prácticamente independiente del resto de los genes del organismo y del ambiente en donde se desarrolla dicho organismo. Mientras se creaban los primeros organismos genéticamente modificados (OGM), este paradigma ya era cuestionado con base en innumerables datos experimentales y modelos formales. Sin embargo, se siguen desarrollando transgénicos con base en este paradigma y se promueve su comercialización y dispersión en el ambiente sin medir las consecuencias. ¿Cuáles son los riesgos de dicha liberación?

Para evaluar los riesgos e incertidumbres del uso de una tecnología se han elaborado diferentes protocolos de evaluación que analizan diferentes niveles en los cuales un desarrollo tecnológico puede presentar peligros, riesgos e incertidumbres. En el caso de los organismos genéticamente modificados, uno de los protocolos más acabados presentados hasta el momento por una autoridad nacional o supranacional es el elaborado por el panel científico consultado por la Autoridad Europea de Seguridad de los Alimentos. Este protocolo contempla que el análisis de bioseguridad de un OGM específico debe hacerse en varios niveles y mínimamente incluir: las características biológicas del(os) organismo(s) de donde se obtuvieron las secuencias transgénicas; las características biológicas del organismo receptor; el proceso de transformación genética; las características de la(s) proteína(s) recombinante(s), tanto su toxicidad para el hombre y los animales como la posibilidad de transferencia horizontal de los (trans)genes que las codifican hacia otros organismos, así como los posibles riesgos de su liberación al ambiente en diversos contextos.

Lo notable de este documento es que hace referencia explícita a que la evaluación de los posibles efectos negativos o peligros de la liberación de un OGM particular debe hacerse caso por caso, en donde un "caso" está conformado por el OGM mismo y sus características, pero también por el ambiente y el contexto agrícola en el cual se usará, así como por sus posibles usos. Para la liberación al ambiente de un OGM es necesario evaluar los posibles peligros