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UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

Instituto de Ecología

Evolución de los mecanismos del desarrollo ontogenético de los fenotipos florales homeóticos/heterotópicos en las triuridales mexicanas *Lacandonia schismatica* y *Triuris brevistylis* (Triuridales: Liliopsida)

T E S I S

QUE PARA OBTENER EL GRADO DE

DOCTOR EN CIENCIAS BIOMEDICAS

P R E S E N T A

Biól. Francisco Roberto Vergara Silva

DIRECTORA DE TESIS: DRA. MARÍA ELENA ÁLVAREZ-BUYLLA ROCES

MÉXICO, D. F. DICIEMBRE, 2002

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FALLA
DE
ORIGEN

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UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMEDICAS

INSTITUTO DE ECOLOGIA

Pdcb/grad/049/Jur/nov/2002

ING. LEOPOLDO SILVA GUTIERREZ
Director General de
Administración Escolar
P r e s e n t e

Por medio de la presente, me permito informar a usted que en reunión del Subcomité Académico del Doctorado en Ciencias Biomédicas que se llevó a cabo el 24 de octubre del año en curso, se acordó designar el siguiente jurado para examen de Doctor en Ciencias Biomédicas del Biol. FRANCISCO ROBERTO VERGARA SILVA con número de cuenta 90522170 y número de expediente 3961669 con la tesis titulada **"Evolución de los mecanismos del desarrollo ontogenético de los fenotipos florales homeóticos/heterotópicos en las triuridales mexicanas *Lacandonia schismatica* y *Triuris brevistylis* (Triuridales: Liliopsida)"**, dirigida por la Dra. Elena Alvarez-Buylla Roces.

Presidente:	Dra.	Judith Márquez-Guzmán
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Suplente:	Dr.	Dr. Mario Zurita Ortega

Atentamente,
"Por mi raza hablará el espíritu"
Ciudad Universitaria, D. F., 4 de noviembre de 2002

DR. RODOLFO DIRZO MINJAREZ
Responsable de la Entidad Académica

DR. ABEL MORENO CARCAMO
Coordinador del Programa del PDCB

c.c.p. Secretaria de Asuntos Escolares.

VNIVERSIDAD NACIONAL AVENTA DE MEXICO

DOCTORADO EN CIENCIAS BIOMEDICAS
INSTITUTO DE ECOLOGIA

Pdcb/grad/049/dic/nov/2002

ING. LEOPOLDO SILVA GUTIERREZ
Director General de Administración Escolar
P r e s e n t e.

Por medio de la presente, le informamos que el candidato al grado de doctor FRANCISCO ROBERTO VERGARA SILVA con número de cuenta 90522170 y número de expediente 3961669, inscrito en el Doctorado en Ciencias Biomédicas, ha cubierto satisfactoriamente todas las actividades académicas establecidas en el plan de estudios del programa. En consecuencia y de acuerdo al artículo 7 de las normas operativas del programa y del artículo 33 del reglamento general de estudios de posgrado, se autoriza al candidato para solicitar su examen de grado Doctoral haciendo la defensa de la tesis: **"Evolución de los mecanismos del desarrollo ontogenético de los fenotipos florales homeóticos/heterotópicos en las triuridales mexicanas *Lacandonia schismatica* y *Triuris brevistylis* (Triuridales: Liliopsida)"**, dirigida por la Dra. Elena Alvarez-Buylla Roces.

Agradeciendo de antemano la atención que se sirva prestar a la presente, reiteramos nuestra consideración más distinguida.

Atentamente,
"Por mi raza hablará el espíritu"
Ciudad Universitaria, D. F., 4 de noviembre de 2002

DR. RODOLFO DIRZO MINJAREZ
Responsable de la Entidad Académica

DR. ABEL MORENO CARCAMO
Coordinador del Programa del PDCB

c.c.p. Biol. Francisco Javier Incera.- Jefe de la Unidad de Administración del Posgrado
c.c.p. Dra. Elena Alvarez-Buylla Roces.- Tutora.
c.c.p. Coordinación de Doctorado.

Introducción general

El proyecto de investigación correspondiente a la presente tesis es un estudio de biología evolutiva del desarrollo encaminado a explicar el origen del fenotipo floral autapomórfico de la monocotiledónea micoheterotrófica *Lacandonia schismatica*, un taxón endémico de la Selva Lacandona (Chiapas, México). Desde su descripción original, esta especie fue incluida dentro de su propia familia monotípica, Lacandoniaceae (Martínez y Ramos 1989). Este taxón, con una afinidad cercana con miembros del Orden Triuridales (*sensu* Dahlgren et al. 1985), ha sido considerada como el único linaje dentro de las angiospermas en el cual los órganos florales correspondientes al tercero y cuarto verticilos de una flor hermafrodita típica -los estambres y los carpelos, respectivamente- se encuentran invertidos espacialmente. Este arreglo estructural, en el cual los estambres son centrales y los carpelos periféricos, se ha considerado incluso una autapomorfía en el contexto de las espermatofitas (el grupo taxonómico que incluye a todas las plantas con semilla; Endress 2001). La rareza de este fenotipo floral, considerado a lo largo de este trabajo como un genuino caso de heterotopía completa -es decir, de homeosis- así como el *status* de las poblaciones naturales de la especie que lo posee, justifican no sólo el interés puramente científico por comprenderlo mejor, sino también los esfuerzos que el conjunto de investigadores participantes en diferentes aspectos del proyecto hemos puesto en su conservación a lo largo de los años.

Originalmente, el proyecto tuvo como interés primordial la evaluación de una hipótesis proximal, de naturaleza genético-molecular, para explicar el desarrollo ontogenético del arreglo floral de *L. schismatica*. Este estudio se hizo en conjunto con el grupo de investigación de Elena Alvarez-Buylla y de Elliot Meyerowitz (California Institute of Technology, EUA). En breve, esta hipótesis postula que el fenotipo homeótico floral de esta triuridal se debe a modificaciones en el patrón de expresión de secuencias homólogas a genes homeóticos florales selectos, pertenecientes a la familia multigénica MADS-box. Conforme se comenzaron a colectar datos para tratar de refutar dicha hipótesis, se hizo evidente la necesidad de realizar un estudio paralelo de sistemática que proporcionara hipótesis robustas adicionales acerca de las relaciones filogenéticas de *L. schismatica* y otras monocotiledóneas. La realización de este estudio -que incluye el análisis de matrices moleculares así como morfológicas y una colaboración cercana con grupos de investigación en el New York Botanical Garden y en la Universidad de Cornell (EUA)- llevó de manera natural a una tercera vertiente del proyecto, realizada en íntima colaboración con laboratorios de la Facultad de Ciencias y el Instituto de Biología (UNAM), que comprendió el análisis de la variación floral tanto en las poblaciones de *L. schismatica* como en las de su taxón hermano putativo, la triuridal *Triuris brevistylis* (Triuridaceae: Triuridales). *L. schismatica* y *T. brevistylis* son las dos únicas especies de triuridales para las cuales se conocen poblaciones en territorio mexicano (Martínez y Gómez 1994). Estas tres líneas de investigación comprenden el núcleo de la tesis y comprenden los capítulos segundo (sistemática de las triuridales mexicanas), tercero (variación floral en poblaciones naturales de las triuridales mexicanas; en prensa en *International Journal of Plant Sciences*) y cuarto (genética molecular del desarrollo floral en las triuridales mexicanas, incluyendo una revisión publicada en *Journal of Phycology*). En congruencia con el hecho de que el objetivo original del proyecto no se modificó, sino se expandió, el capítulo introductorio de la tesis es una panorámica general sobre los aspectos estructurales y funcionales básicos los genes homeóticos, no sólo los florales, sino también los pertenecientes a la familia multigénica homeobox, de suma importancia en la ontogenia de los animales.

Adicionalmente, los capítulos 5 (sobre la embriología de *T. brevistylis*) y 6 (sobre los patrones de expresión de genes de la familia MADS-box en estructuras no florales) representan colaboraciones del autor en proyectos independientes pero relacionados a la presente tesis, que han derivado en un artículo enviado a una revista internacional y otro ya publicado en *The Plant Journal*. Finalmente, el capítulo 7 es una perspectiva integrativa, desde el punto de vista de la filosofía de la biología, acerca de las implicaciones generales de los hallazgos comentados en la tesis. Este ensayo, ya aceptado para su publicación en la revista *Biology and Philosophy*, redondea la tesis al considerar de manera crítica hasta que punto el estudio de la biología evolutiva del desarrollo en plantas deberá influir en las generalizaciones hechas en su contraparte, la "evo-devo" en animales.

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Capítulo 1: generalidades

El presente capítulo pondera de manera crítica algunos aspectos históricos sobresalientes alrededor del descubrimiento de los genes homeóticos, tanto anteriores como posteriores a la caracterización de su identidad molecular en animales. El texto enfatiza los aspectos comparativos de la búsqueda de los mecanismos moleculares en los cuales participan los genes homeóticos para determinar la diferenciación celular durante la ontogenia, tanto en animales como en plantas, a la vez que abunda en detalles sobre la estructura molecular de los genes homeobox y MADS-box, así como las proteínas que codifican. La sección final del capítulo introduce consideraciones sobre la necesidad de realizar modelación matemática al estilo de la que se hace en teoría de sistemas complejos, con objeto de ayudar a cerrar la brecha entre nuestro conocimiento presente sobre los mecanismos celulares antes mencionados y el número de interacciones genéticas y epigenéticas que aún no han sido descubiertas con estrategias experimentales convencionales dentro del paradigma contemporáneo de investigación en genética molecular del desarrollo.

El capítulo será incluido como parte de una colección de contribuciones provenientes de laboratorios que hacen investigación relevante para la biología celular en nuestro país. El libro resultante ha sido editado por Luis Felipe Jiménez-García y Horacio Merchant-Larios, y está en vías de publicación en la editorial Addison-Wesley. El texto se presenta aquí en formato convencional, precedido de una portada correspondiente a la primera hoja de la segunda prueba de galera.

Genes homeóticos y mecanismos moleculares de diferenciación celular

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Resumen

Los genes homeóticos constituyen un grupo de secuencias codificantes de factores de transcripción que participan en numerosos eventos regulatorios de la diferenciación de territorios celulares que, en los adultos de una amplia diversidad de especies de plantas y animales, dan lugar a estructuras morfológicas de gran interés desde el punto de vista taxonómico, sistemático y ecológico. En virtud de las relaciones filogenéticas de *Homo sapiens*, el desarrollo ontogenético de muchas estructuras anatómicas del cuerpo humano depende de ellos de manera fundamental, por lo que su estudio también es de interés médico. El descubrimiento de los principios generales de su actividad en diferentes sistemas modelo y especies selectas en ambos grupos de eucariontes multicelulares ha tenido una influencia particular en la biología evolutiva contemporánea. Estos principios sustentan una base firme para establecer relaciones teóricas correctas entre las características estructurales y funcionales de los genomas, los mecanismos epigenéticos determinados por ellas, y los procesos involucrados en el desarrollo ontogenético, en el contexto proporcionado por el patrón de divergencia de las especies a lo largo del tiempo (Carroll et al. 2001, Arthur 2002). Sin duda, el entendimiento de aspectos fundamentales de la biología celular también se ha visto enriquecido de manera importante por los hallazgos moleculares alrededor de estos *loci* genéticos, toda vez que éstos indican que la elaboración morfológica que ha acompañado a dicha diversificación organísmica está basada en una impresionante conservación de mecanismos celulares (Gerhart y Kirschner 1997).

Aspectos históricos sobre el concepto de homeosis

Bateson: el inicio

The first question which the Study of Variation may be expected to answer relates to the origin of that Discontinuity of which Species is the objective expression. Such Discontinuity is not in the environment; may it not, then, be in the living thing itself?

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CAPÍTULO 5

GENES HOMEÓTICOS Y MECANISMOS MOLECULARES DE DIFERENCIACIÓN CELULAR

Francisco Vergara-Silva ■ Luis Mendoza
Caroline Burgeff ■ Alicia Gamboa de Buen
Rosalinda Tapia-López ■ Liz Izquierdo
Elena R. Álvarez-Buylla

Los genes homeóticos constituyen un grupo de secuencias codificantes de factores de transcripción

Los genes homeóticos constituyen un grupo de secuencias codificantes de factores de transcripción que participan en numerosos episodios regulatorios de la diferenciación de territorios celulares que, en los adultos de una amplia diversidad de especies de plantas y animales, dan lugar a estructuras morfológicas de gran interés desde el punto de vista taxonómico, sistemático y ecológico. En virtud de las relaciones filogenéticas de *Homo sapiens*, el desarrollo ontogenético de muchas estructuras anatómicas del cuerpo humano depende de ellos de manera fundamental, por lo que su estudio también es de interés médico. El descubrimiento de los principios generales de su actividad en diferentes sistemas modelo y especies selectas en ambos grupos de eucariontes multicelulares, ha tenido una influencia particular en la biología evolutiva contemporánea. Estos principios sustentan una base firme para establecer relaciones teóricas correctas entre las características estructurales y funcionales de los genomas, los mecanismos epigenéticos determinados por ellas y los procesos involucrados en el desarrollo ontogenético, en el contexto proporcionado por el patrón de divergencia de las especies, a lo largo del tiempo (Carroll y cols, 2001; Arthur, 2002). Sin duda, el entendimiento de aspectos fundamentales de la biología celular también se ha visto enriquecido de manera importante por los hallazgos moleculares alrededor de estos *loci* genéticos, toda vez que éstos indican que la elaboración morfológica que ha acompañado a dicha diversificación organísmica está basada en una impresionante conservación de mecanismos celulares (Gerhart y Kirschner, 1997).

Aspectos históricos sobre el concepto de homeosis

Bateson: el inicio

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William Bateson, *Materials* (1894), p. 17

En *Materials for the Study of Variation*, su obra de 1894, el investigador norteamericano William Bateson hizo uno de los recuentos generales más importantes de los modos en que la variación en la morfología de los organismos -anteriormente comprendida por Darwin (1859) como la base de una teoría sobre el origen de las especies- se presenta como un fenómeno de repetición serial y de organización de lo complejo con base en unidades discontinuas. En este libro se introduce el término homeosis como una modificacion del concepto de "metamorfía", que habia sido empleado previamente por M. T. Masters para referirse a las anormalidades en la formación de algunos tejidos y órganos; en palabras de Bateson, aquellas instancias en que "...el ojo de un crustáceo es sustituido por una pata, o un pétalo por un estambre...". Este investigador veía la necesidad de usar una nueva palabra para definir el rasgo general de sus observaciones al respecto, pues

(...) the essential phenomenon is not that there has merely been a change, but that something has been changed into the likeness of something else (p. 85).

El interés de Bateson en el estudio de los mecanismos que construyen las características invariables de cada clase de organismo a lo largo de las generaciones, lo llevó a darse cuenta de que los métodos embriológicos tendrían que orientarse por los principios de la incipiente teoría evolutiva. Con ello, sería posible superar las limitaciones que esa aproximación mecanicista había encontrado a principios del siglo XX para explicar la diversidad de la vida. El método de experimentación naturalmente complementario a esta búsqueda sería redescubierto unos cuantos años después de la publicación de *Materials*: nos referimos a la genética, también bautizada por Bateson.

Después del reconocimiento universal de la importancia de los hallazgos de Mendel, la visión batesoniana pudo haber desembocado en un programa concreto de investigación unificada. Pero, por desgracia, tanto la genética como la embriología desarrollarían a partir de los años 20s y 30s del siglo XX sus propias líneas de evidencia, sus propios experimentos paradigmáticos, sus propios sistemas modelo, sus propias publicaciones y sus propios vocabularios (Gilbert et al. 1996). En vista del inmenso poder de su enfoque, genetistas como T. H. Morgan se pronunciaron fuertemente por esta separación. Como una de varias consecuencias de esta postura, un grupo sobresaliente de biólogos y matemáticos redefinió a la evolución, hacia el final de la década de los 40s, como "el resultado de los cambios en las frecuencias génicas" (Dobzhansky 1937). Alrededor de esta premisa fundamental, estos investigadores constituyeron lo que conocemos actualmente como la Síntesis Moderna (Smocovitis 1996), asumiendo de que los mecanismos genéticos constituían la pieza de evidencia que le había hecho falta a Darwin, y dejando fuera de dicha síntesis a la embriología. A pesar de las voces disidentes de un puñado de heterodoxos, esta caracterización de la evolución como un epifenómeno de la genética de poblaciones permaneció prácticamente sin modificaciones durante cuarenta años, al menos, (ver, por ejemplo, Dobzhansky et al. 1977, Gould 1983).

Las primeras investigaciones experimentales

Evidentemente, no todos los estudios genéticos realizados a partir de entonces se concibieron como maneras de descubrir los mecanismos involucrados en la dinámica de los alelos en las poblaciones. En particular, la línea de investigación que sacaba ventaja de las bondades de *Drosophila melanogaster* -el sistema modelo inaugurado por el grupo de Morgan- habría de proporcionar los elementos para el descubrimiento, varias décadas más tarde, de la base material de las alteraciones homeóticas naturales descritas por Bateson. Estos experimentos se basaban fundamentalmente en el uso de agentes mutagénicos que producían efectos fenotípicos visibles en las moscas. Entre la gran variedad de sustancias empleadas en ellos, los mejores inductores de mutaciones resultaron ser los vapores de éter, el ácido bórico y los boratos de sodio y el 5-fluorouracilo, pues sus efectos eran fácilmente reproducibles (Ouweneel 1976).

Eventualmente, los investigadores notaron la adecuación del concepto de homeosis en la descripción del fenotipo resultante en muchas de las líneas mutantes, y éstas fueron llamadas homeóticas en consecuencia. Para entonces, era también evidente que las transformaciones en la diferenciación de órganos enteros de la mosca, y de algunos otros artrópodos que comenzaban a explorarse experimentalmente, seguían patrones muy interesantes. En algunos casos la homeosis entre las principales estructuras afectadas (los discos imaginales correpondientes a patas, antenas, labios, genitales, ojos, alas, halterios, abdomen y mesotórax) resultaba ser bidireccional, pero en otros únicamente procedía en un sentido. Asimismo, con el tiempo se encontró que ciertos tratamientos con calor, rayos X, luz ultravioleta y neutrones por bombardeo resultaban en fenotipos batesonianos. Las fenocopias homeóticas, como se les llamó a éstas mutaciones fenotípicas, no parecían tener la misma base genética que las homeosis naturales y las primeras homeosis experimentales, y se les consideraba como relacionadas a los fenómenos de transdeterminación (Hadorn 1968) y metaplasia (Yamada 1972).

Goldschmidt, Waddington y Davidson: la importancia de la embriología

¿Cómo explicar la homeosis? ¿Por qué el mismo resultado fenotípico experimental podía alcanzarse por medios aparentemente tan dispares como mutaciones directas en el material hereditario y cambios de temperatura? Asímismo, ¿por qué a veces se trata de un proceso espontáneo, como observó Bateson?

La primera hipótesis explicativa sobre la homeosis basada en un mecanismo explícito de diferenciación celular fue propuesta por Richard Goldschmidt en 1938. En su libro de 1940, *The Material Basis of Evolution*, este personaje lanzó un serio ataque en contra de la Síntesis Moderna, la ortodoxia reinante en la biología evolutiva, que menospreciaba el papel crucial del desarrollo embrionario en la diversificación organísmica. Los argumentos de Goldschmidt hacían referencia explícita a los fenotipos homeóticos y se basaban en experiencia personal, como lo demuestra su monografía sobre el mutante *podoptera* en *Drosophila* (Goldschmidt et al. 1951), en el cual se observa el crecimiento de patas en el lugar que normalmente habría alas. Goldschmidt sugería que en diferentes momentos durante el desarrollo de las larvas, una serie de "sustancias evocadoras" eran liberadas, determinando el destino adulto de aquellos discos imaginales que estaban "maduros" para tal proceso. Los evocadores, eminentemente extrínsecos según este modelo, circularían por la hemolinfa y afectarían únicamente las células de aquellas estructuras que ya habían alcanzado un estado de competencia. Esta hipótesis mecanística, sin embargo, estaba basada en evidencia preliminar y resultó ser errónea. Experimentos posteriores demostrarían que los discos imaginales pueden concluir su etapa larvaria separados de la irrigación hemolinfática, y que los discos homeóticos previamente establecidos como tales podían desarrollarse de manera autónoma dentro de larvas hospederas silvestres (Ouweneel 1976).

En general, las contribuciones de Goldschmidt relacionadas con los mecanismos del desarrollo embrionario cayeron en el olvido durante años debido a su insistencia en rechazar el modelo morganiano de los genes como "cuentas de collar" (Dietrich 2000a). Como es bien sabido, esta visión de los genes se aceptó universalmente a partir de las investigaciones sobre la naturaleza química del material hereditario que se derivaron del trabajo de Watson, Crick y sus colegas cristalógrafos y bioquímicos de bacteriófagos. En paralelo, sus ideas generales sobre el proceso evolutivo -en especial, su definición de las macromutaciones como modificaciones fenotípicas discontinuas que son el resultado de cambios a gran escala en procesos embriológicos (Dietrich 1992)- fueron constantemente combatidas e incluso ridiculizadas por los principales arquitectos de la Síntesis (particularmente por el ornitólogo alemán Ernst Mayr; ver, por ejemplo, Mayr 1942 y 1997). Irónicamente, el curso que actualmente lleva la re-construcción de la biología evolutiva a partir de la incorporación de los hallazgos moleculares sobre los genes homeóticos (Vergara Silva y Alvarez-Buylla 2001, Vergara Silva 2002) y muchas otras evidencias embriológicas modernas han promovido recientemente un serio reexamen de la importancia de su trabajo (Dietrich 1995). Este análisis ha desembocado en el reconocimiento de su lugar como uno de los pioneros en los esfuerzos por integrar a la genética, la embriología y la evolución (Dietrich 2000b).

De manera totalmente independiente, el embriólogo inglés Conrad Waddington también sugirió a los evolucionistas de la Síntesis voltear la atención hacia la ontogenia, y fue el autor de la segunda hipótesis relevante sobre los posibles mecanismos de la homeosis (Waddington 1940, 1966). Guiado por la evidencia previa a su propio trabajo en *Drosophila*, donde saltaba a la vista que los evocadores eran muy posiblemente de naturaleza intrínseca, este investigador pensaba que la causa de las transformaciones estaba en los mecanismos epigenéticos que operan dentro de los discos mismos (Waddington 1942).

Uno de los aspectos que encontraba más llamativos en los órganos homeóticos era su carácter entero y discreto, en virtud del cual nunca presentan una identidad intermedia, así como el hecho de que se ven modificados por otras mutaciones del mismo modo que los órganos normales que crecen en los lugares característicos del taxón natural. Por esta razón, él se imaginaba que las vías de diferenciación de las diferentes estructuras morfológicas eran también discretas y que los genes respectivos (cualesquiera que éstos fueran) formaban sistemas genéticos coherentes e intensamente canalizados, que a su vez se activarían en un campo de células embrionarias gracias a la actividad temprana de un "gen maestro". Evidentemente, para Waddington los mejores candidatos para cumplir dicha función eran los genes homeóticos (Waddington 1953). La noción de canalización –actualizada recientemente por Wilkins (1997) como "la estabilización de vías embriológicas mediante factores genéticos múltiples dentro del genoma"- es una de las herencias perdurables del pensamiento de Waddington. Sin duda, su comprensión aún espera estudios futuros que la relacionen apropiadamente con los conocimientos modernos sobre pleiotropía de *loci* cuantitativos y otros aspectos de la genética molecular contemporánea (Gibson y Wagner 2000).

Los trabajos de Roy Britten y Eric Davidson merecen una mención por su relación con la búsqueda propuesta en los textos de Waddington. En los modelos de estos autores ya no se hablaba de "estructuras

morfológicas" en general, sino específicamente de algunos de los segmentos de *Drosophila* que tradicionalmente mostraban la potencialidad de transformarse homeóticamente (Britten y Davidson 1969). En resumen, dichos modelos postulaban que para cada una de cuatro diferentes regiones del plan corporal de *Drosophila* -el metatórax (o tercer segmento torácico, T3), dividido en sus porciones anterior y posterior, y los segmentos abdominales primero y segundo (A1 y A2, respectivamente)- existía un conjunto particular de "genes expresores" activados por una proteína alostérica (P) producida por alguno de los miembros del "juego de genes maestros integradores" (Davidson y Britten 1971). Esta proteína tendría cuatro estados funcionales para la activación de cada uno de los conjuntos diferentes de expresores. Esta definición de estados se llevaría a cabo mediante la asociación de moléculas inductoras (I) a tres sitios alostéricos dentro de P. El elemento novedoso en esta idea es la postulación de un gradiente de I; en diferentes regiones del embrión, P se asociaría con una, dos ó tres moléculas I y de ese modo activaría al grupo de genes expresores correspondiente. En este punto de la investigación sobre el fenómeno, ya se preveía que el aislamiento de un ARN mensajero (ARNm) correspondiente a alguno de los genes, cuya mutación estaba claramente involucrada en los cambios de identidad de los segmentos, podría ser de mucha ayuda para definir los porqués de la homeosis. Sin embargo, los conocimientos necesarios para llevar esto a cabo -como por ejemplo, el mapeo de los cromosomas de las glándulas salivales- aún no existían de manera completa.

Varios años después, con el advenimiento de las tecnologías propias de la biología molecular -y gracias a algunos de los descubrimientos tratados más adelante en el presente capítulo- las ideas de Davidson tomaron la forma de una teoría sofisticada sobre la manera en la que el desarrollo está codificado en el ADN, a través de las ediciones de 1976 y 1986 de su libro *Gene Activity in Development*. A últimas fechas, Davidson ha desarrollado un marco conceptual idiosincrático -basado principalmente en sus propias investigaciones en el erizo de mar (ver, por ejemplo, Arnone y Davidson (1997)- para entender la manera en que redes regulatorias complejas han participado en aspectos del desarrollo embrionario, que en su opinión han sido críticos durante la evolución de los metazoarios (Davidson 2001). Es posible que su paradigma se vuelva aún más importante para las investigaciones futuras, hechas ya en el contexto de la secuenciación de genomas completos (Davidson et al. 2002).

Las ideas de Lewis Wolpert

De manera simultánea con las contribuciones de Davidson, se propusieron otros mecanismos para explicar mecanísticamente el proceso natural de diferenciación, que se veía afectado mediante la mutación experimental de los genes homeóticos. Entre estos mecanismos sobresale el de los gradientes químicos. Esta propuesta tenía una larga historia, que comenzó con el trabajo de Boveri y Driesch, publicado a principios del siglo, pero no había estado acompañada de experimentos bien diseñados que permitieran probar su existencia. En los modelos ya mencionados arriba que hacían uso de la idea de un gradiente, la homeosis en sentido estricto no estaba bien diferenciada de la actividad de tales gradientes hipotéticos. Esta situación cambió, sin embargo, con el trabajo de Lewis Wolpert, otro embriólogo británico quien habría de proponer, bajo la forma de un modelo entero y coherente, un mecanismo universal para la traducción de la información genética en patrones espaciales de diferenciación: la ahora célebre información posicional (Wolpert 1969, 1971 y 1994, Lawrence 1992). Su modelo es un ejemplo de la transformación de una proposición ambigua en una idea fértil, y su consecuencia principal es la distinción entre las bases del fenómeno homeótico como tal y las fases del desarrollo embrionario previas e indispensables para la definición de la modularidad de los planes corporales (Wolpert 1996).

El mecanismo propuesto por Wolpert se basaba en la especificación de la posición de las células con respecto a uno o más "puntos de referencia" en un sistema en desarrollo. Como en todo modelo, el concepto central contaba con sus auxiliares. Así por ejemplo, un *campo* fue definido por este autor como el conjunto de células que tiene especificada su información posicional con respecto a los mismos puntos de referencia, y la *polaridad* como la dirección en la cual la información posicional es medida o especificada. La versión más famosa del modelo de Wolpert es su solución al problema de cómo generar el patrón de colores de la bandera francesa a partir de una hilera de tamaño indefinido, compuesta por células indiferenciadas y totipotenciales (Wolpert 1971). Esta solución postula simplemente que las células de uno de los extremos de la hilera (por ejemplo, el izquierdo) responden a una concentración alta del morfógeno desarrollando el color azul; conforme ésta va disminuyendo, se acerca a un umbral de concentración a partir del cual las siguientes células -las del centro- responden de modo diferente formando el color blanco. Finalmente, un segundo umbral es alcanzado y traspasado, y las células que quedan delante de él desarrollan entonces el color rojo. La aplicación conjunta de los principios definidos en este ejemplo al organismo real resulta en una guía susceptible de confirmarse empíricamente, según la cual, los discos imaginales de *Drosophila* serían diferentes no en la especificación sino en la interpretación de la información posicional (Wolpert 1994).

El organismo que habría de proporcionar los datos experimentales necesarios para justificar la relevancia biológica del concepto de información posicional sería la misma mosca de la fruta, en una serie de

investigaciones conducidas por Christiane Nüsslein-Volhard y Eric Wieschaus (Nüsslein-Volhard y Wieschaus 1980). Experimentos de mutagénesis realizados por estos autores desde finales de los años 70s, demostraron la existencia de una serie de genes de origen materno cuyos productos funcionan exactamente como los morfógenos de Wolpert se comportarían (Lawrence 1992). Los ARNm de los genes maternos son especialmente importantes en este sistema biológico de información posicional, pues su actividad establece una serie de interacciones jerárquicas con los genes propios del cigoto, subdividiendo al embrión en unidades metaméricas cada vez más pequeñas a lo largo del eje anteroposterior, hasta llegar al número y forma características del adulto. A los genes responsables de esta fase del desarrollo se les conocería a partir de entonces como genes de polaridad del huevo, y su distinción con respecto a los homeóticos quedó entonces corroborada de manera definitiva (Lawrence 1992, Gerhart y Kirshner 1997, Carroll et al. 2001) .

Como fruto de la investigación sobre los morfógenos de polaridad del huevo, se descubrirían posteriormente que los gradientes de dichas sustancias controlan las primeras fases de la determinación de las cuatro distinciones fundamentales dentro del plan corporal de *Drosophila:* dorsal contra ventral, endodermo contra mesodermo y ectodermo, células germinales contra células somáticas y anterior contra posterior (Nüsslein-Volhard et al. 1987, Nüsslein-Volhard 1991, Lawrence 1992). También se encontró que entre la actividad de los genes de polaridad del huevo y los homeóticos, se encuentran niveles jerárquicos intermedios de genes a los cuales ahora conocemos como genes de segmentación (St Johnston y Nüsslein-Volhard 1992). Baste reiterar entonces que la importancia de mencionar dicho trabajo aquí radica en que se trata de la comprobación experimental del corolario principal del modelo de Wolpert: debido a que en los mutantes de polaridad del huevo se alteran las señales de posición a las cuales responden posteriormente las células embrionarias, es posible deducir que los genes homeóticos no son los responsables de dichas señales (Gerhart y Kirshner 1997, Gilbert 2000, Carroll et al. 2001). Por la misma razón, es posible inferir que el proceso epigenético esencial en el desarrollo, desenmascarado simultáneamente por las dos clases de mutantes, se compone de dos partes: la generación de las fronteras de los campos y la determinación interna de sus identidades. Correspondería a dos de los más importantes genetistas del siglo XX entender cabalmente la naturaleza de la segunda parte del proceso, es decir, la parte estrictamente homeótica, y exponerla del modo que se acepta universalmente.

Edward Lewis: de los mapeos cromosómicos a los modelos evolutivos

Edward B. Lewis, alumno de la segunda generación de la escuela de Morgan (Winchester 1996), fue uno entre varios de los investigadores que elaboraron hipótesis sobre los mecanismos genético-embriológicos en *Drosophila* con base en los fenotipos homeóticos. De hecho, él también elaboró una hipótesis preliminar basada en gradientes para explicar la homeosis (Lewis 1963). Sin embargo, a su profundo conocimiento sobre la localización cromosómica de los *loci* que codificaban para los genes homeóticos de la mosca, el genetista norteamericano añadió de manera explícita una consideración de la filogenia de los organismos relevantes -el grupo taxonómico de los insectos- a la ontogenia del sistema modelo. Efectivamente, Lewis fue el primer investigador que ordenó los datos de la genética en *Drosophila* con los que entonces se contaba, con el objeto de construir una especulación sobre la existencia de cambios en la expresión de los genes homeóticos en los artrópodos a lo largo de la evolución (Lewis 1963 y 1978).

Las investigaciones de Lewis se enfocaban en la región del cromosoma tres de *Drosophila* que conocemos con el nombre de complejo *bithorax* (BX-C). El nombre de la región proviene de uno de los fenotipos homeóticos clásicos, generado mediante la mutación de *Ultrabithorax*, uno de los tres genes que lo componen. En las moscas mutantes para este *locus*, el parasegmento 4 -abreviado P4, y correspondiente a la parte posterior de T1 y la anterior de T2, donde crecen las alas- se duplica en el lugar que correspondería normalmente a P5, donde normalmente crecen los halterios o balanceadores, dando como resultado una mosca con cuatro alas. Lewis había notado además que la eliminación del complejo en su totalidad producía una transformación homeótica generalizada, en la que todos los segmentos a partir de T3 se parecían a T2. Con estas evidencias, Lewis propuso en 1978 el modelo que le valdría la obtención del Premio Nobel de Medicina que en 1995 compartió con Nüsslein-Volhard y Wieschaus. En este modelo se postulaba que BX-C debería contener al menos un gen para cada segmento por debajo de T2. Esto significaba que el desarrollo de T3 involucraría la activación de todos los genes característicos de T2 -"el nivel basal"- además de la participación de uno o más genes responsables de las peculiaridades de T3. De manera similar, el desarrollo del primer segmento abdominal requeriría a su vez la expresión de los genes de T3, más aquellos específicos de A1, y así sucesivamente hasta llegar a A8 (Lewis 1978, Gilbert 1988; ver Figura 1).

En correspondencia con los fenotipos mutantes, y con base en las relaciones filogenéticas aceptadas para los diferentes tipos de insectos, Lewis propuso que algunos de los genes adicionales que se iban activando durante la ontogenia para suprimir las patas de los segmentos abdominales y formar halterios en posición posterior a las alas, eran los mismos que durante la filogenia habían aparecido para crear el plan corporal de un díptero a partir, primero, de artrópodos ancestrales milípedos y, posteriormente, de insectos

voladores con cuatro alas (Lewis 1978 y 1998). La característica más notable de este modelo -a saber, su conexión directa entre la función de los genes y el origen de estructuras morfológicas distribuidas de modo diferencial en las especies (Vergara Silva 2002)- es de fundamental importancia por haber animado a otros investigadores a pensar que tal vez el fenómeno homeótico era universal no sólo en los términos morfológicos en los que había sido documentado por Bateson, sino también en sentido molecular. En la actualidad, se reconoce además que el trabajo de Lewis en su conjunto ha proporcionado herramientas indispensables para comprender el desarrollo embrionario de *Drosophila* en el contexto de la genómica comparada (Rubin y Lewis 2000).

Antonio García-Bellido y el papel fundamental de las células

Mientras esto sucedía en Estados Unidos, el genetista español Antonio García-Bellido realizó contribuciones complementarias de enorme trascendencia para la comprensión de los mecanismos que determinan la diferenciación de los campos de células en *Drosophila* (García-Bellido 1968, 1975). Sus contribuciones se derivaron de sus experimentos con la mutación *engrailed* (García-Bellido 1998) Actualmente sabemos que la expresión del *locus* correspondiente a dicha mutación es muy importante para la división dentro de cada segmento de las porciones anterior y posterior, que son las verdaderas fronteras de los parasegmentos. A principios de los 70s, él y sus colaboradores observaron que, en respuesta a una señalización posicional de naturaleza aún desconocida, grupos enteros y clonales de células formaban una región cohesiva que no se mezclaba con regiones adyacentes ni permitía la entrada de células extrañas. Estas regiones, que bautizó como *compartimentos*, comenzaban con una sola célula que se dividía hasta formar la región entera (García-Bellido et al. 1973 y 1979).

Este investigador, quien durante una temporada colaboró con Lewis (García-Bellido y Lewis 1976) había observado además que las mutaciones homeóticas obtenidas por otros genetistas transformaban dominios de células que eran exactamente iguales en composición a los compartimentos definidos en el fenotipo silvestre de sus moscas. Con base en estas evidencias, propuso entonces que cada célula fundadora y sus descendientes expresaban una combinación única y particular (no necesariamente progresiva con respecto a un estado basal, como decía Lewis) de "genes (homeóticos) selectores", que se mantiene fija a lo largo del proceso de generación del campo, y que puede interpretarse como un "domicilio genético" (García-Bellido 1981). Esta interpretación equivaldría a una codificación binaria, es decir, consistiría en activaciones o inactivaciones de los genes como únicas alternativas, suficientes y necesarias. Este proceso de decisión binaria podría repetirse más tarde en el desarrollo, cuando de acuerdo con la ontogenia natural de los organismos, fuera necesario subdividir aún más un campo para formar una subestructura morfológicamente diferente de sus vecinas. Las elegantes concepciones de García-Bellido han perdurado prácticamente sin modificaciones hasta la fecha (ver, por ejemplo, Lawrence y Struhl 1996) como la mejor manera abstracta de representar la actividad conjunta de genes para la especificación de la diferenciación celular, y su trabajo experimental se considera fundamental para la articulación del "dogma central de la formación de patrones tisulares" (Irvine y Rauskolb 2001). Las contribuciones de García-Bellido son de gran interés para los biólogos celulares estudiosos de los mecanismos que subyacen al desarrollo embrionario, pero sus ideas sobre el proceso evolutivo –aunque menos conocidas- también son dignas de atención (ver, por ejemplo, García-Bellido 1984).

El estado de la investigación antes de 1984

En resumen, la investigación sobre la función de los genes de polaridad del huevo y los genes de segmentación eventualmente demostró que la expresión del segundo grupo de secuencias es necesaria y suficiente para determinar, independientemente de la influencia materna, las fronteras de las regiones naturales de transcripción de los genes homeóticos selectores en sentido estricto (Akam 1987; Ingham 1988). Así pues, éstos últimos pudieron definirse inequívocamente a partir de entonces -con base en la evidencia genética- como aquellos que están principalmente involucrados en establecer la identidad definitiva de las diferentes regiones previamente individualizadas en el embrión de *Drosophila* y posteriormente en el cuerpo del futuro adulto (García-Bellido 1975, García-Bellido et al. 1979). Como hemos visto, el mapeo genético de los homeóticos empezó en la época de Lewis, y su resultado final había reafirmado, aún antes de conocer su naturaleza molecular, que estos *loci* están organizados en dos complejos contiguos sobre el cromosoma 3 correspondientes a la región 84-89. Estos complejos son el ya mencionado BX-C y un segundo complejo conocido como *Antennapedia* (ANT-C; Lawrence 1992).

Ultrabithorax (*Ubx*) *abdominal A* (*abdA*) y *Abdominal B* (*AbdB*) son los genes que componen el primer complejo, mientras que ANT-C consiste de cinco unidades de transcripción -*labial* (*lab*), *proboscipedia* (*pbd*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) y *Antennapedia* (*Antp*; McGinnis y Krumlauf, 1992, Gilbert 2000). Las propiedades funcionales básicas de estos grupos de genes, estudiadas durante décadas con metodologías puramente genéticas, se habían ratificado ya en 1978, en el trabajo clásico de Lewis (ver también Lewis 1998): BX-C controla las diferencias entre los segmentos abdominales y torácicos, mientras que ANT-C determina las

que existen entre las regiones cefálica y torácica. Con García-Bellido, de manera complementaria se había confirmado, además, que cada gen homeótico selector tiene un dominio característico de acción, que se define como la región del cuerpo que se transforma homeóticamente como resultado de la mutación en ese gen. Finalmente, también se había establecido que las fronteras espaciales de estas regiones coinciden perfectamente con los límites de los parasegmentos, definidos a su vez por la actividad de dos subclases de genes de segmentación: los genes pair-rule y los genes de polaridad de segmentos (Gerhart y Kirshner 1997, Carroll et al. 2001).

Con la ventaja que proporciona la retrospectiva que ahora podemos hacer de la historia de la genética del desarrollo, es fácil comprender que muchos investigadores sospechaban que la jerarquía genética establecida entre todos los tipos distintos de genes de formación de patrones debería basarse en alguna característica funcional básica y común a todas o al menos a la mayoría de las proteínas codificadas. En otras palabras, los productos de los genes de formación de patrones, y de los homeóticos en particular, deberían tener la capacidad de regular de algún modo, a un número potencialmente gigantesco de genes, muchos de los cuales cumplirían las funciones bioquímicas básicas en las células. García-Bellido había bautizado ya a estos últimos como "genes realizadores" (García-Bellido 1975, 1984) y aún antes habían sido considerados por Britten y Davidson bajo el nombre de "genes expresores". A pesar de las incisivas intuiciones y de las cualidades de los modelos genéticos propuestos por estos autores sin embargo, el descubrimiento final de la base molecular de la función de los genes homeóticos y sus implicaciones en términos filogenéticos habrían de resultar completamente inesperadas.

Aspectos estructurales y funcionales de los *loci* homeóticos en plantas y animales

El descubrimiento de la caja homeótica

En una serie de trabajos publicados en el simbólico año de 1984 (aunque ya anunciados previamente en congresos durante 1983), los grupos de investigación de Walter Gehring (en Suiza) y de Matthew Scott (en Estados Unidos), reportaron el aislamiento y secuenciación de los primeros representantes de los genes componentes de los complejos homeóticos (Gehring 1994, McGinnis 1994). Estas investigaciones se sustentaron en el trabajo sistemático de mapeo de Lewis y del grupo de Thomas Kaufman (Kaufman et al. 1980), así como en las técnicas de caminata sobre cromosomas desarrolladas en el laboratorio de David Hogness (Bender et al. 1983) y en las técnicas de hibridización de ácidos nucleicos. La experiencia de estos investigadores en el campo de la homeosis era amplia: todos ellos habían participado desde tiempo atrás en la clonación de las regiones de mayor importancia en los cromosomas politénicos de acuerdo con el mapeo genético de ANT-C. En dos de estos artículos (McGinnis et al. 1984; Scott y Weiner 1984), los genes caracterizados provenían de *Drosophila*, pero en un tercero (realizado en la rana *Xenopus laevis*; Carrasco et al. 1984) se mostraba, por primera vez, que los genes involucrados en los procesos embriológicos mediante los cuales se establecen las identidades de las partes corporales características de un insecto, son miembros de la misma familia de genes que cumplen una función similar en un anfibio.

Después de hacer una comparación de las secuencias de ciertos fragmentos cuidadosamente mapeados de *Antp*, *Ubx* y *fushi tarazu* (*ftz*), Gehring y sus colaboradores notaron que todas ellas compartían una región muy similar de aproximadamente 180 pares de bases de longitud, bautizada primero por este investigador como "H" y posteriormente con el nombre de *homeobox* (caja homeótica) con que se le conoce universalmente (Duboule 1994). En sólo unos cuantos años a partir de la caracterización de esos primeros genes homeobox, se han encontrado ya un número cercano a 700 secuencias provenientes de animales con marcos abiertos de lectura que presentan una región homeobox homóloga por descendencia común (Spirov et al. 2002). Si bien las funciones embriológicas específicas de estos genes divergen en distintos aspectos conforme se muestrea la filogenia de los eucariontes multicelulares (Carroll et al. 2001), se ha demostrado que su distribución abarca no sólo al reino animal entero sino también a las plantas, los hongos y muy probablemente algunas especies de bacterias (Bürglin 1994, 2001).

La caja homeótica codifica para un segmento polipeptídico de 60 aminoácidos, al cual consecuentemente se le conoce como homeodominio. Una vez identificados los primeros homeodominios en términos puramente estructurales, rápidamente se postuló una hipótesis de trabajo según la cual los productos de los genes homeóticos serían justamente proteínas regulatorias, cuya actividad normal en las células sería la de comportarse como factores de transcripción que se asociarían a secuencias *cis*-regulatorias específicas de sus genes blanco (Gehring 1985 y 1994). La evidencia fundamental para construir esta hipótesis consistió en la notable similitud encontrada entre los homeodominios conocidos a la fecha y algunos motivos característicos de proteínas regulatorias de procariontes, en especial, el motivo hélice-vuelta-hélice de las proteínas MAT a1 y alfa2, que determinan el tipo de apareamiento en la levadura (Laughon y Scott 1984, Gehring et al. 1994). En virtud de que este motivo es precisamente una región de asociación al ADN, Gehring

supuso que el homeodominio tambien lo sería. Como decíamos arriba, una gran cantidad de datos ha confirmado esta hipótesis (McGinnis y Krumlauf 1992, Pabo y Sauer 1992, Gehring et al. 1994) para el caso de los productos de los genes homeóticos e incluso la ha extendido a los morfógenos que organizan la polaridad del huevo y los eventos iniciales de la segmentación.

Las secuencias con homeodominios se agrupan en más de 200 grupos homólogos o parálogos (Spirov et al. 2002). Algunas de estas clases están definidas con base en criterios adicionales, entre los que se cuentan los tipos de región que flanquean a la caja, la posición de los intrones y la asociación con otros dominios (Gehring et al. 1994). Así pues, se incluyen en dicha clasificación algunas agrupaciones de genes -como los *Pax* y los *POU*- que, además de contener una caja homeótica reconocible como tal, incluyen regiones adicionales de asociación a ADN, así como aquellos grupos que representan a los genes homeobox que no están organizados en complejos cromosómicos ordenados (De Robertis 1994). Los componentes principales del homeodominio son tres hélices alfa plegadas alrededor de un centro hidrofóbico y un brazo N-terminal flexible (Otting et al. 1990); en el caso del homeodominio de la proteína ANTP, también se puede reconocer una cuarta hélice flexible (Qian et al. 1993). Las hélices 2 y 3 forman el motivo hélice-vuelta-hélice ya mencionado (Qian et al. 1989, Gehring et al. 1994), y se ha visto que las tres hélices se ordenan únicamente cuando la proteína se pega al ADN (Wolberger 1996).

La información anterior proviene de estudios estructurales realizados con resonancia magnética nuclear (NMR) y cristalografía de rayos X de los complejos formados por el ADN y homeoproteínas escogidas, complementados en algunos casos con mutaciones puntuales en la secuencia de la caja homeótica (revisados en Laughon 1991, Gehring et al. 1994 y Sharkey et al. 1997). Estos trabajos han establecido además que los residuos de aminoácidos que participan en la formación del complejo -en particular, en el reconocimiento específico de las bases en ambas cadenas del ADN- están ubicados en la hélice 3 (Gehring 1992). La secuencia de consenso obtenida por Bürglin (1994) de la comparación de 346 homeodominios contiene siete posiciones que son invariantes en más del 95% de los casos; se trata de L16, F20, W48 y F49, correspondientes al centro hidrofóbico, y R5, N51 y R53, que se asocian directamente al ADN. En el homeodominio de ANTP, por ejemplo, es claro que estos sitios están distribuidos en las cuatro hélices. La primera abarca los residuos 10 a 21, la segunda va del 28 al 38 y la tercera de los residuos 42 al 52. Como ya se indicó, esta proteína contiene una cuarta hélice (residuos 53 a 59), más desordenada y flexible, que es inexistente en otros factores de transcripción, como FTZ, codificada por un de los genes de segmentación más importantes y mejor estudiados (Qian et al. 1994), o bien que es continua con la tercera, como sucede en MAT alfa2 (Wolberger et al. 1991). A pesar de estas diferencias, los estudios llevados a cabo con estas técnicas, han indicado que éstas tres proteínas, más el producto de *engrailed* (Kissinger et al. 1990, Draganescu y Tullius 1998), se unen al ADN de manera muy similar.

Los homeodominios mencionados anteriormente y otros (ver, por ejemplo, Klemm et al. 1994) se pegan al ADN como monómeros y lo hacen de manera especialmente favorable a secuencias de ADN que contienen el tetranucleótido ATTA, llamado también motivo central. La interacción requiere de la inserción de la tercera hélice en el surco mayor, el brazo N-terminal en el menor y de contactos entre el asa que une a las hélices 2 y 3 con el esqueleto del ADN. En opinión de algunos autores, las regiones de ADN que pegan homeodominios se pueden considerar de alta, media y baja afinidad, pues en algunos casos, en ausencia del sitio que contiene el tetranucleótido en el enhancer disminuye la especificidad en la asociación de la proteína, aunque la función de este elemento activador no se vea afectada de manera detectable (Gehring et al. 1994). Junto con estas evidencias que involucran la asociación de subunidades únicas, recientemente se ha establecido que las interacciones proteína-proteína juegan un papel importante en la modulación de la actividad de los homeodominios en condiciones fisiológicas (Wolberger 1996). De hecho, los monómeros sólo tienen 100 veces más afinidad por sitios específicos del ADN (de alta afinidad) en relación a sitios no específicos (de media y baja afinidad; Laughon 1991). Algunas homeoproteínas se unen al ADN como dímeros formados por subunidades iguales o diferentes en una asociación cooperativa, pues la afinidad aparente de cualquiera de ellas al ADN es mayor si se forma el dímero (Wilson y Desplan 1995, Passner et al. 1999). Las estructuras cristalinas del homodímero del homeodominio codificado por *paired* (Wilson et al. 1995) y las del heterodímero de MAT a1/alfa2 (Li et al. 1995) han sido importantes evidencias en favor de lo anterior. En el caso de esta última estructura se ha visto que cada subunidad se une a secuencias operadoras de ADN distintas -los nucleótidos TGT están conservados en el sitio de alfa2, y la secuencia CATC lo esta en el sitio de a1.

Finalmente, otros estudios de NMR y simulación de dinámicas moleculares, también en ANTP, indican que el agua solvente yace en una cavidad que forma la interfase entre la hélice de reconocimiento del homeodominio y las bases del surco mayor del ADN de doble cadena (Billeter et al. 1993, Fraenkel y Pabo 1998). Uno de los últimos homeodominios en estudiarse como cristal ha demostrado la participación conjunta del solvente y las interacciones entre homeodominios en la dinámica molecular de los mismos: la asociación de la proteína EVEN SKIPPED -también codificada por un gen de segmentación- al ADN es en realidad el pegado de dos homeodominios sobre caras opuestas de una sola vuelta de B-ADN, con puentes de hidrógeno

mediados por moléculas de agua en la interfase entre las bases y la hélice de reconocimiento (Hirsch y Aggarwal 1995).

Biología comparativa de los genes homeobox

Una cantidad enorme de estudios realizados en años recientes en varias especies de animales con muy diferentes planes básicos de organización corporal ha permitido establecer que no sólo el desarrollo de *Drosophila* está mediado por genes reguladores que poseen secuencias de asociación al ADN a través de las cuales controlan la expresión de otros genes (Slack et al. 1993, Carroll 1995, Carroll et al. 2001). Al mismo tiempo, dichas investigaciones han indicado que un porcentaje considerable de tales secuencias codificantes de factores de transcripción importantes durante el desarrollo pertenece a la familia de genes homeobox. En la rana, el ratón e incluso en el humano, los genes del tipo *Antp* –la categoría de genes homeobox que abarca los genes homeóticos *sensu stricto* (Gehring et al. 1994)- presentan parecidos asombrosos con los genes selectores de *Drosophila* más allá de las secuencias nucleotídicas: en los genomas de vertebrados estudiados en detalle, se encuentran cuatro "racimos" (clusters) o complejos cromosómicos conocidos como *Hox*, que corresponden a BX-C y ANT-C (denominados conjuntamente HOM-C por algunos autores) y que están distribuidos en cuatro cromosomas distintos (McGinnis y Krumlauf 1992, Maconochie et al. 1996) como producto de tres rondas de duplicación y la pérdida de cuatro racimos (Bailey et al. 1997) ocurridas a lo largo de los últimos cientos de millones de años de evolución (ver Figura 2).

A pesar de que la razón por la cual los genes *Hox* están agregados en racimos sigue siendo misteriosa (Mann 1997), este arreglo cromosómico da lugar a propiedades notables, como la colinearidad. Este rasgo funcional consiste en la correspondencia entre la posición de cada *locus* perteneciente a los complejos sobre cada cromosoma y el orden en el cual cada uno de ellos es expresado a lo largo del eje antero-posterior. Esto significa que los genes que se encuentran en el extremo 3' se expresan en la región anterior del embrión, y que sus fronteras de localización espacial se van recorriendo hacia atrás del cuerpo conforme se avanza sobre el cromosoma hacia el extremo 5'. Asímismo, dicha correspondencia incluye también la temporalidad de la expresión (los genes anteriores se expresan primero, los posteriores se expresan de forma tardía). Un elemento adicional de similitud entre los genes homeóticos homeobox provenientes de las especies estudiadas, es la correlación que existe entre la responsividad al ácido retinoico, que tanto en invertebrados como en vertebrados consiste en una alta sensibilidad mostrada por los genes del extremo 3' y un grado bajo de respuesta por los genes 5' (Duboule 1994). Gracias a la conclusión de algunos proyectos de secuenciación de genomas completos -por ejemplo, el de *Drosophila* (Adams et al. 2000) y el del nemátodo *Caenorhabditis elegans* (Kappen 2000)- actualmente es posible estimar con gran confiabilidad el número de genes pertenecientes a la familia homeobox presentes en diferentes clados de animales (Finnerty y Martindale 1998, De Rosa et al. 1999). Estas estimaciones se han visto complementadas por el hallazgo de grupos de genes homeobox que también forman racimos pero que no pertenecen a los complejos *Hox*; entre ellos sobresale el complejo *ParaHox*, descubierto originalmente en *Amphioxus* (Brooke et al. 1998) y posteriormente caracterizado en los cnidarios (Finnerty y Martindale 1999).

La aproximación comparativa ha permitido descubrir diferencias importantes en la función biológica de algunos genes homeóticos animales. En virtud de numerosas observaciones que indican que la extensa conservación de secuencia entre diferentes genes *Hox* no necesariamente se extiende a todos sus efectos sobre la especificación de la identidad de partes corporales, actualmente se tiene una visión equilibrada del peso que debe adjudicarse a los genes involucrados en el desarrollo embrionario en la elaboración de hipótesis de homología entre estructuras (Abouheif et al. 1997, Wray y Abouheif 1998). Por ejemplo, tanto en *Drosophila* como en *Tribolium castaneum* (otro insecto intensamente estudiado desde el punto de vista genético-molecular; ver, por ejemplo, Brown et al. 1999) los genes homeóticos son los determinantes de la identidad de patas, alas y antenas, y de ellos depende su localización en los segmentos correctos (Akam 1987 y 1995), pero el complejo de genes homeobox de *C. elegans* controla únicamente la identidad de linajes celulares particulares a lo largo del eje antero-posterior (Salser y Kenyon 1994). Esta misma especie presenta un gen (*egl-5*) cercanamente relacionado con *AbdB*, el cual a su vez es el gen más emparentado con el grupo funcional de genes homeobox de los vertebrados, que es activado de manera secuencial durante el desarrollo de las extremidades (Morgan y Tabin 1993; Capdevila e Izpisúa-Belmonte 2001). Sin embargo, como los nemátodos carecen de ellas, es muy probable que el gen ancestral de esta subfamilia haya antecedido al origen de las extremidades como tales, y que dicho gen haya cumplido en un principio con funciones diferentes de la especificación de dichas estructuras (Shubin et al. 1997).

Entre los ejemplos más interesantes y discutidos al respecto de la asignación de funciones diferenciales a lo largo de la evolución para los genes con cajas homeóticas y sus consecuencias sobre la estimación de hipótesis de homología se encuentra el caso del *locus Pax6* y su homólogo *eyeless* (*ey*), provenientes del ratón y la mosca, respectivamente. Estos genes parecen ser centrales en la actividad de una red regulatoria que controla la morfogénesis del ojo en varios grupos de animales con estructuras oculares divergentes (Gehring e

Ikeo 1999, Treisman 1999); entre otras funciones, *ey* es capaz de inducir el desarrollo de ojos ectópicos cuando se expresa en sitios donde normalmente no le corresponde (Halder et al. 1995). Un segundo grupo de evidencias en favor de esta hipótesis se relaciona con la conservación de secuencias y funciones en el humano (Quiring et al. 1994), los nemertinos (Loosli et al. 1996), los cefalópodos (Tomarev et al. 1997) y el anfioxo (Glardon et al. 1998); asimismo, se ha demostrado que la vía de señalización que controla la expresión de *ey* y que es organizada por el gen *Notch* está conservada tanto en la rana como en la mosca (Onuma et al. 2002).

Otros hallazgos, sin embargo, apuntan a que la participación central de los homólogos de *Pax6* en la ontogenia del ojo no es tan generalizada –por ejemplo, el desarrollo por regeneración de los ojos en las planarias es *Pax6*-independiente (Pineda et al. 2002). Por este motivo, algunos autores actualmente piensan que en el ancestro común de protostomados y deuterostomados –que no tenía aún ojos complejos, sino únicamente receptores fotosensibles- el homólogo de *Pax6* estaba dedicado a determinar el destino de células sensoriales en tejido ectodermal, pero no de especificar ojos como tales (Nilsson 1996). Recientemente, Arendt y Wittbrodt (2001) han sugerido que Urbilateria -el ancestro común hipotético de todos los animales bilaterales- ya poseía ojos simples, pero mientras la homología de los ojos cerebrales en Protostomia puede sustentarse, es probable que dichas estructuras no provengan de un ancestro común –al menos, la comparación hecha entre animales cordados y no cordados. El caso de los genes *Pax* y la determinación de la identidad de estructuras oculares ilustra, pues, que los genes homeóticos no hacen exactamente lo mismo en todas las especies en las cuales su función se ha estudiado en detalle. No obstante, esto no elimina la validez del consenso actual que afirma que los genes homeóticos, en virtud de sus funciones celulares, siempre han participado en algún aspecto del desarrollo de los planes corporales (Knoll y Carroll 1999, Carroll et al. 2001).

La aplicación generalizada de una de las técnicas más comunes de la biología molecular -la reacción en cadena de la polimerasa (PCR)- ha permitido la detección de genes *Hox* en cientos de especies, algunas de las cuales nunca serán accesibles a la aplicación de metodologías genéticas convencionales (ver, por ejemplo, De Rosa et al. 1999). Aunque a veces la PCR no proporciona información inequívoca respecto al número de genes presente, las búsquedas realizadas con esta técnica en muy diversos genomas, interpretadas adicionalmente con evidencias paleobiológicas, han demostrado que la presencia de genes *Hox* precede a la aparición de los animales como tales (Valentine et al. 1996, Erwin et al. 1997, Valentine et al. 1999) y que existe una correlación general entre el número de genes *Hox* estimado y la complejidad de los planes corporales (Gellon y McGinnis 1998). Con ayuda de esta técnica, se sabe que el racimo de genes homeóticos homeobox de *C. elegans* tiene cuatro genes debido a pérdidas masivas ocasionadas por la condición de parasitismo (Kenyon 1994), pero que en general todos los protostomados estudiados a la fecha presentan un solo racimo de genes *Hox* (Finnerty y Martindale 1998; ver Figura 2).

En contraste, la situación en los cordados (Holland y García-Fernández 1996) es diversa: los mamíferos tienen 39 genes, repartidos en los 13 (McGinnis y Krumlauf 1992, Maconochie et al. 1996) o 14 (Pollard y Holland 2000, Popovici et al 2001), grupos de genes parálogos a los miembros de BX-C y ANT-C; algunos peces tienen genes adicionales debido a duplicaciones génicas exclusivas (Amores et al. 1998); mientras que el anfioxo tiene sólo un complejo (Ferrier et al. 2000). El panorama general al respecto indica que cada *phylum* o clase taxonómica en la que se han caracterizado los complejos *Hox* exhibe un patrón único de duplicación o pérdida de genes, relativo a otros phyla y clases (Finnerty y Martindale 1998, Martindale y Kourakis 1999, De Rosa et al. 1999); y si bien cada uno de los complejos de los vertebrados es diferente de los demás, se ha podido rastrear de manera consistente su origen a un racimo ancestral único (Bailey et al. 1997, Ferrier y Holland 2001).

Finalmente, es importante recalcar que actualmente la interpretación correcta de la información que proporcionan la PCR y las técnicas genéticas y embriológicas tradicionales sobre los genes homeóticos homeobox depende de modo indispensable del uso de las herramientas de la sistemática, especialmente de la cladística (Carroll et al. 2001, Arthur 2002). Esta relación entre areas diversas que confluyen en la biología evolutiva está plenamente justificada: finalmente son estos genes los principales responsables de la determinación de la identidad de las estructuras morfológicas características de cada grupo, las cuales a su vez han sido tradicionalmente la fuente de caracteres de la sistemática en el pasado. De modo sobresaliente, los datos de secuencia de genes *Hox* no sólo han podido ser mapeados en reconstrucciones obtenidas con *loci* independientes, sino que ellos mismos han contribuido para la modificación de las hipótesis filogenéticas tradicionales de los animales en su conjunto (ver, por ejemplo, Adoutte et al. 1999, De Rosa et al. 1999; Figura 2). La adición de evidencias sobre las modalidades de evolución molecular de los genes *Hox* (ver, por ejemplo, Schubert et al. 1993, Gauchat et al. 2000) a los análisis anteriores está apoyando la unificación de la genetica molecular del desarrollo con las otras áreas de la biología relacionadas con el estudio de la macroevolución: tal unificación es, en nuestra opinión, la inevitable culminación de las observaciones de Bateson y una vía para la superación de las limitaciones de la teoría evolutiva tradicional (Vergara Silva 2002).

Las plantas también tienen *loci* homeóticos: la familia MADS-box

El resumen que hemos hecho de la historia del descubrimiento de los genes homeóticos en los animales y la descripción de algunos de los aspectos moleculares más relevantes sobre ellos bien pueden crear la impresión de que los términos homeótico y homeobox son sinónimos, y que la reunión de la genética, la embriología y la sistemática sólo pueden ayudar a resolver preguntas acerca del origen y divesificación de los metazoarios. Lo anterior está lejos de ser cierto: la aplicación de la metodología genético-molecular a algunas especies modelo de dicotiledóneas -*Arabidopsis thaliana* y *Antirrhinum majus*, en especial- ha permitido el hallazgo de una segunda familia multigénica, cuyas funciones conjuntas bien pueden considerarse análogas a las de los genes homeóticos en los animales. Al igual que los genes homeobox, la agrupación de genes MADS-box (Theissen et al. 2000, Vergara-Silva et al. 2000) tiene una amplia distribución taxonómica, e incluso, además de su papel fundamental en la morfogénesis de las estructuras de las plantas terrestres, algunas de las proteínas codificadas por estos genes participan en la respuesta a feromonas y en el metabolismo de arginina en levadura (Herskowitz 1989, Dubois y Messenguy 1991), así como en el desarrollo de los tejidos musculares en animales (Buckingham 1994).

La familia multigénica MADS-box deriva su nombre de las iniciales de sus cuatro miembros fundadores -*MCM1* (proveniente de *Saccharomyces*; Jarvis et al. 1989), *AGAMOUS* (*AG*; un gen hallado en *Arabidopsis*; Yanofsky et al. 1990), *DEFICIENS* (que se encuentra en *Antirrhinum*; Sommer et al. 1990) y *SERUM RESPONSE FACTOR* (*SRF*; un gen del humano, Norman et al. 1988), y agrupa a las secuencias codificadoras de factores de transcripción que presentan un dominio formado por 60 aminoácidos denominado MADS (Schwarz-Sommer et al. 1990). En un principio, se consideraba que la unidad mínima de unión específica al ADN estaba constituída por este dominio -el más conservado de toda la proteína- más 30-40 aminoácidos en la región carboxilo terminal (Mueller y Nordheim 1991). Gracias al trabajo cristalográfico realizado con la proteína codificada por *SRF*, se sabe ahora que el dominio MADS se pliega en un motivo estructural nuevo para la interacción con el ADN y para la dimerización que consiste en un rizo enrrollado antiparalelo de dos hélices alfa anfipáticas, cada una proveniente de una subunidad diferente (Pellegrini et al. 1995).

Al igual que los homeodominios, el dominio MADS presenta una serie de sitios muy conservados dentro de su secuencia. El recuento de 107 proteínas pertenecientes a plantas, hongos y animales ha permitido identificar que I11, K23, R24, K30, K31, E34 y L38 son residuos absolutamente invariantes, así como 9 residuos más, donde los cambios de aminoácido han sido muy raros y conservativos, y finalmente 16 sitios de cambios no conservativos en menos del 5% de los casos (Theissen et al. 1996).

Las proteínas con dominios MADS de plantas son de naturaleza modular. Además de este dominio, existe en ellas una región parcialmente conservada, de aproximadamente 70 residuos, denominada K (por su similitud con el dominio de rizo enrollado presente en la queratina), que se localiza en la dirección 3' con respecto al dominio MADS y se separa de esta última por una región de secuencia muy variable denominada I ("intermediary") o L ("linker"). Aunque la secuencia de aminoácidos de la región K no está muy conservada, su estructura secundaria sí lo está (Ma et al. 1991; Purugganan et al. 1995). Por último, las regiones más variables en términos de tamaño y secuencia -tanto en animales como en plantas y hongos- corresponden a la región amino y carboxilo terminal. De éstas, la primera región sólo se halla en un pequeño grupo de proteínas MADS, pues en la mayoría de ellas el codón de metionina de inicio coincide exactamente con el principio de la región del mismo nombre, mientras que en todas ellas se encuentra el extremo carboxilo (Theissen et al. 1996).

Empleando la información del genoma completo de *Arabidopsis* (The *Arabidopsis* Genome Initiative 2000) y el de especies selectas de animales, en nuestro laboratorio hemos podido reconstruir la historia evolutiva de la familia de los genes MADS-box en los eucariontes. En particular, hemos demostrado que antes de la divergencia de los animales y las plantas ocurrió al menos una duplicación de los genes MADS-box que dió lugar a dos linajes -los genes Tipo I y los Tipo II- dentro de la familia (Alvarez-Buylla *et al.*, 2000a). Estos dos tipos de genes corresponden, respectivamente, a dos agrupaciones de loci animales conocidas como tipo SRF y tipo MEF, ninguna de los cuales tiene funciones homeóticas. Por un lado, los genes de animales tipo SRF se agrupan con un gran número de genes de plantas de los cuales aún no conocemos su función. Por el otro, es interesante notar que los genes tipo MEF -entre los que se encuentran genes que regulan la diferenciación del corazón y otros músculos estriados- forman un grupo monofilético con un grupo grande de genes MADS-box de plantas, dentro del cual se encuentran los genes homeóticos florales.

Es común que las proteínas con dominio MADS sean activas como pares, tanto homodímeros como heterodímeros (Riechmann et al. 1996b, Riechmann y Meyerowitz 1997, Winter et al. 2002). Se ha establecido, por ejemplo, que los productos de los genes *APETALA3* (*AP3*) y *PISTILLATA* (*PI*), caracterizados en *Arabidopsis* (Jack et al. 1992 y 1994; Goto y Meyerowitz 1994, Bouhidel y Irish 1996) pueden formar heterodímeros, así como sus contrapartes en *Anthirrinum majus* (las proteínas deficiens y globosa) que requieren específicamente del dominio K, además del MADS, para interactuar (Davies et al. 1996). Todas estas

proteínas son fundamentales para la determinación de la identidad de algunos de los órganos florales característicos de las especies correspondientes. Trabajos recientes han demostrado, sin embargo, que no sólo los dominios MADS y K juegan un papel en la función celular normal de los factores de transcripción florales. La construcción de genes híbridos acoplada con algunos experimentos de expresión ectópica en plantas transgénicas de *Arabidopsis* han demostrado que el único dominio que siempre participa en la determinación de la especificidad funcional de las proteínas es la región I (Krizek y Meyerowitz 1996). Probablemente, esto se deba a que dicho dominio se requiere para la interacción con otras proteínas accesorias que formarían entonces un complejo múltiple de proteínas-DNA que activa la transcripción (Riechmann y Meyerowitz 1996a).

Tal como ha sucedido con las proteínas con homeodominios, diversas estrategias experimentales han permitido demostrar que existe una secuencia conservada en los promotores y otras regiones activadoras de los genes blanco de las proteínas con dominio MADS y funciones homeóticas, a la cual éstas se asocian con diferentes grados de afinidad. Dicha secuencia de nucleótidos es un motivo palindrómico denominado caja CArG, que consiste en la secuencia consenso CC(A/T)6GG (Shore y Sharrocks 1995). Si bien es común que se piense que la especificidad funcional de los factores de transcripción reside básicamente en su especificidad de asociación al ADN, se ha observado que muchas proteínas MADS-box reconocen prácticamente los mismos sitios CArG en el ADN (Nurrish y Treisman 1995, Huang et al. 1995) a pesar de que su efecto en el desarrollo sea muy diferente (Riechmann y Meyerowitz 1997). Curiosamente, esta paradójica situación se ha venido descubriendo también para las proteínas homeóticas con homeodominios de los animales (Gross y McGinnis 1996).

La importancia de determinar las causas de la especificidad de los factores de transcripción codificados por genes homeobox y MADS-box en estos términos es enorme: en principio, ésta es la única manera de acceder al siguiente nivel de las interacciones regulatorias en el desarrollo, es decir, el de los genes realizadores (ver, por ejemplo, Parcy et al. 1998, Benfey y Weigel 2001). Por esta razón, una gran parte de los proyectos de investigación sobre la base molecular de la homeosis -tanto en animales como en plantas- ahora se enfocan en la caracterización de procesos que, junto con la regulación de la transcripción *per se*, controlen de manera fina la actividad de las proteínas homeóticas dentro de las células (Gerhart y Kirschner 1997). Entre los procesos descubiertos durante estos proyectos sobresalen la degradación selectiva de las proteínas mismas, el transporte del citoplasma al núcleo, el movimiento de célula a célula y otras modificaciones post-transcripcionales y post-traduccionales como la fosforilación, así como la participación de proteínas accesorias y cofactores en los complejos activadores de la transcripción, ya mencionada arriba, se perfila como la regla (Graba et al, 1997, Riechmann y Meyerowitz 1997, Egea Gutérrez-Cortines y Davies 2001).

Los genes MADS-box y la evolución de las estructuras reproductivas de las plantas terrestres

El rápido avance en la caracterización molecular de las proteínas con dominio MADS de las plantas ha puesto las bases para la comparación de los detalles moleculares de la función de ésta y otras familias de factores de transcripción que también son importantes durante el desarrollo ontogenético (Riechmann et al. 2000). A su vez, estos análisis comparativos han creado la posibilidad de analizar los eventos evolutivos más importantes en la historia de las plantas terrestres en términos de la expresión de los genes durante el desarrollo, a semejanza de lo realizado para el reino animal (Cronk 2001). También, esta nueva manera de entender los procesos evolutivos vegetales es guiada por el conocimiento que se obtiene en los sistemas modelo. Tanto en *Arabidopsis* como en *Antirrhinum*, la estructura cuya morfogénesis se conoce mejor en términos moleculares es la flor -la estructura reproductiva diagnóstica del clado de las angiospermas. Dilucidar las razones del origen de esta novedad evolutiva es uno de los problemas clásicos de la biología desde tiempos de Darwin, quien le llamaba "el misterio abominable" (Crepet 1998).

Las flores silvestres hermafroditas -es decir, las que presentan simultáneamente órganos masculinos y femeninos- de *Arabidopsis* y *Antirrhinum* se componen de cuatro verticilos concéntricos, dispuestos en la siguiente secuencia desde la periferia hasta el centro: sépalos, pétalos, estambres y carpelos (ver Figura 3). Un número considerable de mutaciones en genes que codifican para factores de transcripción de la familia MADS-box cambian el arreglo de los órganos florales de manera homeótica, y se ha encontrado que sus correspondientes genes se requieren para una o más de tres diferentes funciones: (a) el control directo de la identidad de los órganos, probablemente mediado por la activación de los genes realizadores característicos de las células de cada órgano, (b) la regulación espacial de la expresión de los genes que controlan la identidad del órgano, que puede entenderse como la regulación de la posición, y (c) la inducción inicial de los genes que especifican la identidad de los órganos. Los genes del primer tipo -de identidad de los órganos- son equivalentes a los genes homeóticos selectores de los animales, y el producto de sus mutaciones es una drástica modificación de la fórmula floral. Por su parte, los genes con la segunda actividad se llaman catastrales, porque establecen límites espaciales para los genes anteriores, impidiendo su expresión ectópica.

Por último, los genes que inducen positivamente a los del primer tipo se llaman genes de identidad meristemática, porque la ausencia de su actividad causa una conversión parcial o completa de las flores hacia vástagos vegetativos (Weigel y Meyerowitz, 1994, Weigel 1995, Yanofsky 1995).

Las mutaciones en los genes de identidad de los órganos son especialmente interesantes. Estas caen en tres clases diferentes, cada una de las cuales altera la identidad de los órganos en dos verticilos adyacentes, de acuerdo con el modelo combinatorio conocido universalmente como ABC (Coen y Meyerowitz 1991, Meyerowitz 1994; ver Figura 4). Este modelo es un resumen de la evidencia genética y molecular sobre el desarrollo floral en los dos sistemas modelo ya mencionados. Según el modelo ABC, a cada una de las tres funciones de identidad de órganos, A, B y C, le corresponde una clase de genes particular. La inactivación de los genes de la clase A, es decir, la pérdida de la función marcada con la misma letra, causa la transformación de los sépalos en carpelos, y de los pétalos en estambres. En los mutantes de pérdida de la función B, los pétalos son reemplazados por más sépalos, y los estambres por más carpelos. Finalmente, la pérdida de la actividad C transforma a los estambres (tercer verticilo) en pétalos, y a los carpelos (cuarto verticilo) en sépalos. En el contexto del mismo modelo, el estudio de los dobles mutantes posibles ha revelado que la actividad B es independiente de A y C, y que cuando desaparece la función C de las células que formarán los verticilos 3 y 4, A se expresa ectópicamente en esas mismas células y viceversa -es decir, las actividades A y C se inhiben mutuamente. Finalmente, el modelo también establece que los efectos de las diferentes actividades son independientes de su posición relativa dentro de la flor (Alvarez-Buylla 2002). Sin duda, el modelo ABC se ha convertido en la mejor herramienta predictiva de los patrones de expresión de los genes MADS-box en prácticamente todas las flores de las angiospermas conocidas, así como en las estructuras reproductivas de las gimnospermas (Vergara-Silva et al. 2000).

Recientemente, se ha reportado la existencia de un nuevo grupo funcional de genes de la familia MADS-box que tiene especial relevancia para profundizar en el conocimiento de los mecanismos de especificación de la identidad de los órganos florales. Este grupo de genes, agrupados en un clado monofilético, se ha denominado *SEPALLATA* (*SEP*) porque la mutación simultánea de todos ellos resulta en una conversión de los cuatro tipos de órganos florales a sépalos (Pelaz et al. 2001). Adicionalmente, la sobreexpresión de estos genes, en combinación con la de los ABC, origina la transformación de hojas de la roseta u hojas caulinares en diferentes órganos florales, dependiendo de los genes ABC coexpresados. Por ejemplo, si se sobreexpresan dos de los tres genes *SEP* con los genes A y B se transforman hojas en pétalos (Pelaz et al. 2000), pero la combinación de cualquier par de *SEP* y los genes de las funciones B y C especifica la diferenciación de estambres o carpelos (Honma y Goto 2001, Pelaz et al. 2001).

Las evidencias resumidas arriba indican que los genes *SEP* son indispensables para redirigir el programa de desarrollo de hojas a órganos florales. Si bien se sabía ya que los genes ABC eran indispensables para el desarrollo de los órganos de la flor, ahora sabemos que éstos genes no son suficientes y que requieren de la función conjunta de los genes *SEP*. Estos interesantes genes MADS-box, sin embargo, no regulan transcripcionalmente a los genes ABC. De hecho, la localización de sus ARNm es normal en los mutantes de los *SEP*. Experimentos recientes han permitido demostrar, en contraste, que éstos actúan de manera redundante en la formación de cuartetos de proteínas junto con diferenctes productos proteicos de los genes ABC, para así pegarse a sitios de los promotores de los genes blanco que regulan (Honma y Goto 2001). Estos genes blanco no se han identificado aún, pero sabemos ahora que los genes ABC necesitan interactuar con los genes *SEP* para funcionar como factores transcripcionales. Con estos datos se ha integrado lo que se conoce como el "modelo de los cuartetos" del desarrollo floral, que plantea que los genes ABC actúan en tetrámeros como factores de transcripción (Theissen y Saedler 2001). Datos empíricos en Antirrhinum, donde los genes ortólogos de los SEOP han sido llamados "intermediarios de identidad", también apoyan este modelo (Egea-Cortines 1999, Egea Gutiérrez-Cortines y Davies 2000).

Las etapas de la ontogenia de las plantas que ocurren previamente al desarrollo floral son notablemente plásticas, y responde constantemente a factores ambientales como la luz y la temperatura. A nivel molecular, ya se tiene información acerca de las conexiones entre diferentes redes de regulación transcripcional que controlan la floración y las vías de transducción de señales que responden a estos factores ambientales (ver, por ejemplo, Blázquez 2000). Sin embargo, en términos bioquímicos es poco lo que se sabe acerca de otros factores que no pertenecen a la familia de proteínas con dominio MADS y que podrían regular la función transcripcional y postr-trasncripcional de éstas. En relación a este interesante aspecto de la ontogenia vegetal, en nuestro laboratorio hemos emprendido una búsqueda de proteínas que puedan regular post-transcripcionalmente a los genes MADS-box durante el desarrollo floral y que eventualmente se puedan convertir en herramientas moleculares para entender la manera en que se regula el desarrollo vegetal por las condiciones externas. Hasta el momento, hemos aislado dos proteínas: una de ellas es rica en motivos de leucina y la otra es una fosfatasa. Ambas interactúan *in vitro* de manera específica con *AG*, que es el gen correspondiente a la función C en *Arabidopsis* (Gamboa et al. 2002). Este es el primer reporte de proteínas codificadas por genes que no son de la famila MADS-box que interactúan con una proteína con dominio MADS. Las proteínas ricas en motivos de leucina se han asociado a funciones del desarrollo y funcionan como

cinasas y/o como receptores (ver, por ejemplo, Clark et al. 1997). Ahora nos queda averiguar la función de esta nueva proteína, que bautizamos FLOR1.

Así como en *Drosophila*, en *Arabidopsis* también existen una serie de niveles de regulación de los genes homeóticos selectores que se activan antes que éstos últimos. Entre ellos, se encuentran los genes *CONSTANS* (*CO*; Putterill et al. 1995) y *GIGANTEA* (*GI*; Fowler et al. 1999), que junto con otros *loci* se conocen como genes de floración tardía (Weigel y Meyerowitz 1994; Blázquez 2000). Estos genes, a su vez, regulan la actividad transcripcional de los genes de identidad de meristemo *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) y *TERMINAL FLOWER 1* (*TFL1*; Weigel y Meyerowitz 1994, Coupland 1995, Blázquez 2000). Estos últimos son los que controlan la actividad de los genes catastrales *CLAVATA1* (*CLV1*), *LEUNIG* (*LUG*) y *UNUSUAL FLORAL ORGANS* (*UFO*; Levin y Meyerowitz 1995, Liu y Meyerowitz 1995, Blázquez 2000). Por último, tanto los genes de identidad de meristemo como los catastrales regulan la expresión de los genes de identidad de órgano *APETALA2* (*AP2*), *AP3*, *PI*, y *AG* (Weigel y Meyerowitz 1994). Este esquema temporal de expresión tiene sus particularidades, sin embargo; por ejemplo, el gen *SUPERMAN* (*SUP*) se activa después que los genes de identidad de órgano, a pesar de ser considerado formalmente como un gen catastral (Sakai et al 1995). Por su parte, el gen *AP1* realiza funciones de especificación de la identidad del meristemo floral, además de determinar la identidad de sépalos y pétalos (Weigel 1995).

El trabajo en angiospermas que no son sistemas modelo -basado en técnicas de biología molecular similares a las empleadas en animales para muestrear en especies donde no se puede hacer genética- ha permitido la caracterización de decenas de genes MADS-box que también están involucrados en la morfogénesis de las estructuras reproductivas correspondientes. Entre estas especies podemos contar al arroz (*Oryza sativa*, Chung et al. 1994), la coliflor (*Brassica napus*, Mandel et al. 1992), el maíz (*Zea mays*, Schmidt et al. 1993, Ambrose et al. 2000), la papa (*Solanum tuberosum*, Kang y Hannapel 1995), la petunia (*Petunia hybrida*, Angenent et al. 1992, Immink et al. 1999 y 2002), *Rumex acetosa* (Ainsworth et al. 1995), el tabaco (*Nicotiana tabacum*, Mandel et al. 1994) y el tomate (*Lycopersicon esculentum*, Pnueli et al. 1994), así como algunas dicotiledóneas basales (Kramer et al. 1998, Kramer y Irish 1999).

Por otra parte, en gimnospermas como la conífera *Picea abies* (Tandre et al. 1995, Tandre et al. 1998, Sündstrom et al. 1999) y las gnetales *Gnetum parvifolium* (Shindo et al. 1999) y *Gnetum gnemon* (Winter et al. 1999) ya se han encontrado secuencias con caja MADS ortólogas de los genes homeóticos florales canónicos. Este último estudio, además de sugerir que la conservación de aspectos importantes de la regulación génica de la morfogénesis de órganos reproductivos se extiende más allá de las angiospermas, han reforzado los cambios que ha sufrido recientemente la hipótesis de relaciones filogenéticas más aceptada para el clado de las espermatofitas (Winter et al. 1999). El más notable de estos cambios es la agrupación de gimnospermas y angiospermas como grupos monofiléticos y hermanos entre sí (ver, por ejemplo, Chaw et al. 2000). A este respecto, no sólo los genes MADS-box han sido de utilidad, sino también los que codifican a los factores de transcripción del tipo *LFY*. En especial, se ha encontrado que las gimnospermas poseen un gen adicional perteneciente a esta familia denominado *NEEDLY* (*NLY*; Mouradov et al. 1998). La distribución de este locus en las gimnospermas apoya la monofilia de este grupo (Frohlich y Parker 2000).

Los trabajos de evolución molecular del clado de genes MADS-box (Purugannan et al. 1995, Purugganan 1997, Purugannan 2000) han predicho que los tiempos de aparición de las subfamilias con funciones durante el desarrollo son más antiguos que la aparición de las estructuras morfológicas cuya ontogenia controlan. Esto supondría que los helechos, las briofitas y otros linajes vegetales también poseerían genes MADS-box con distintos grados de parentesco con los loci homeóticos florales. Esta expectativa se ha corroborado en ambos grupos de plantas terrestres (ver, por ejemplo, Münster et al. 1997, Ashton y Krogan 2000, Svensson et al. 2000), e incluso ya se han encontrado representantes de la familia en algunos grupos de algas cercanamente relacionados a las primeras plantas terrestres: las carofitas (Tanabe et al. 1999).

Otra contribución de nuestro laboratorio al estudio de la familia multigénica MADS-box en plantas está relacionada con la coopción de funciones para algunos de los genes MADS-box en *Arabidopsis*, enfatizando que no todos ellos son florales y homeóticos (Alvarez-Buylla et al. 2000b). Recientemente encontramos que hay clados de genes de esta familia que agrupan a loci que se expresan de manera específica o primordial en tejidos vegetativos. Al mapear los patrones de expresión sobre la filogenia de los genes MADS-box, nos damos cuenta de que los genes ancestrales hipotéticos tenían patrones de expresión generalizados, los cuales evolucionaron hacia dominios espaciales de expresión más específicos, tanto en estructuras reproductivas como vegetativas. Los estudios preliminares de los genes que se expresan de manera primordial en raíz sugieren que éstos no tienen funciones homeóticas (Burgeff et al. 2002). Aún nos queda un largo camino para entender a plenitud la función de estos genes MADS-box durante el desarrollo de la raíz.

Redes de regulación genética

Como hemos visto, el descubrimiento de los genes homeóticos ha ayudado enormente en el entendimiento de la relación entre los genes, el desarrollo ontogenético y la evolución de estructuras complejas en los organismos multicelulares. Tanto en animales como en plantas, las mutaciones homeóticas son fácilmente distinguibles en términos generales, y se han podido explicar con base en modelos sencillos de combinaciones de actividades de un puñado de genes, muchos de ellos pertenecientes únicamente a dos familias multigénicas que codifican para factores de transcripción: los genes homeobox y los genes MADS-box. Sin embargo, análisis más profundos de los mecanismos moleculares y celulares que subyacen a los mismos mutantes homeóticos, así como a la amplia variedad de tamaños, formas y colores de los diferentes tipos de planes corporales y órganos que los constituyen, están demostrando consistentemente que hay muchos más genes e interacciones regulatorias involucrados en la morfogénesis de las estructuras complejas (Gerhart y Kirschner 1997).

Tomemos como ejemplo la flor. Mutantes nulos de *AG*, que constituye la función C en el modelo ABC expuesto arriba, tienen restos de tejido carpelar; en ellos las mutaciones homeóticas no son totales. Esto indica que hay otros genes importantes en determinar la diferenciación en el cuarto verticilo de las flores. A pesar de su utilidad, los modelos genéticos clásicos que hemos revisado tienen limitaciones, porque son estáticos y no incorporan propuestas que integren los mecanismos e interacciones regulatorias dinámicas de los genes caracterizados hasta ahora. Por ejemplo, aunque se sabe que mutaciones en *AG* son las responsables de anular, en gran medida, la función C, este gen cumple otras funciones pues tiene interacciones regulatorias con muchos más genes. En base a la experiencia acumulada durante décadas de investigación empírica, es razonable estimar que el tiempo que llevaría llevar a cabo estudios detallados de todas estas interacciones adicionales podría ser demasiado largo como para que nuestra generación pudiera ver sus resultados. Entre otras, ésta es una fuerte razón por la que el interés por aplicar herramientas formales y computacionales que permitan hacer inferencias funcionales integrativas acerca de tales interacciones está creciendo a una gran velocidad. De hecho, esta inquietud generalizada alimenta de modo importante la empresa científica multinacional que se conoce como genómica comparativa –dentro de la cual se incluye, por supuesto, *Arabidopsis* (Riechmann et al. 2000, The *Arabidopsis* Genome Initiative 2000)- así como la puesta al día de la teoría de sistemas para los organismos modelo en la era molecular (ver, por ejemplo, Kitano 2002).

La existencia de múltiples relaciones transcripcionales entre los genes tiene como consecuencia inmediata que la actividad transcripcional de uno o varios genes puede modificar la dinámica transcripcional de genes involucrados en más de una vía de regulación. Esta regulación cruzada entre distintos grupos de genes, tiene como resultado el que la magnitud de una modificación genotípica no corresponda a la magnitud del cambio fenotípico asociado; en otras palabras, la relación genotipo-fenotipo se vuelve no lineal (ver, por ejemplo, Moreno 1994). Traducido al contexto de la diferenciación celular, esto es equivalente a la antigua y paradójica observación de que, mientras que los oprganismos poseen diferentes tipos celulares, éstos son especificados a partir del mismo genotipo. ¿Cómo puede explicarse esto? Para entender el fenómeno, es importante explorar qué tipo de arquitectura de interacciones podría permitir la aparición de los tipos de perfiles de actividades genéticas que caracterizan a cada uno de los diversos tipos celulares.

En la actualidad se reconoce que la arquitectura adecuada para resolver el problema anterior puede ser obtenida a partir de la modelación formal de las redes de regulación genética (ver, por ejemplo, Mjolsness et al. 1991, Clarket et al. 1993, Thieffry et al. 1993, Zuckerkandl 1994, Salazar-Ciudad et al. 2001). En la naturaleza, dichas redes se establecen cuando los productos de algunos genes controlan la transcripción de otros genes, cuyos productos a su vez regulan la expresión de otros genes. Las redes de regulación, a diferencia de una "cascada" o una jerarquía, presentan además la posibilidad de retroalimentación, directa o indirecta. La retroalimentación funciona de manera análoga a lo que sucede en las rutas metabólicas, en donde la producción de un sustrato regula la tasa de su misma producción, o en los casos en que el producto de cierto gen puede regular indirectamente su propia transcripción.

Existe una vasta literatura sobre la constitución y propiedades de las redes formales que representarían a las redes genéticas reales. Uno de los teóricos más sobresalientes en el area es Stuart Kauffman, un médico interesado desde los 70s en aplicar herramientas formales como el álgebra de Boole al hallazgo de restricciones estructurales a los modos en que ocurre el desarrollo embrionario en diferentes organismos. Curiosamente, su trabajo comenzó como una interpretación de los mismos mutantes homeóticos que analizaba García-Bellido (ver, por ejemplo, Kauffman 1971, 1973, 1975, 1981 y 1987). A partir de sus contribuciones se han definido los rasgos fundamentales que las redes formales deben tener para aplicarse al estudio de la ontogenia y la citodiferenciación.

Las redes de regulación genética modeladas matemáticamente están constituidas por elementos (nodos) interconectados, y cada nodo tiene la capacidad de adquirir más de un estado de activación. El estado de activación de un nodo en particular es una función del estado de activación del conjunto de los nodos de los cuales recibe algún estímulo. No existen restricciones sobre el tipo de función que determina la activación de los nodos, pero por su simplicidad en muchas ocasiones se ha preferido la postulación de redes con

elementos que sólo pueden adquirir dos estados de activación. Estas son las llamadas redes booleanas en sentido estricto (Kauffman 1993).

En la idealización de los genes como nodos en una red, la tasa de transcripción puede interpretarse como el estado de activación de los nodos. Al mismo tiempo, se puede proponer que los genes siempre se encuentran en uno de dos estados posibles: "apagados", es decir con una tasa transcripcional de cero, o "encendidos", con una tasa constante positiva de transcripción. La ventaja de modelar a grupos de genes como redes es que se puede aprovechar el amplio conocimiento que se tiene de las dinámicas de activación de los genes. Las redes son sistemas dinámicos que pueden adquirir diversos patrones de activación. Esto permite modelar a diferentes dinámicas de activación/inactivación de los nodos de una red como si esta fuera genética. Dado que los diferentes tipos celulares de un mismo organismo pueden distinguirse morfológica y/o bioquímicamente (por lo cual es posible identificar a cada tipo celular con un patrón particular de activación genética, aunque todos los tipos celulares compartan el mismo genotipo), si se modela al genoma de un organismo como una red constituída por todos los genes relevantes para un proceso dado -por ejemplo, la floración- y se identifica a cada estado de activación de la red como el patrón de expresión genética de algún tipo celular en particular, entonces la dinámica de activación de la red puede interpretarse como el proceso de diferenciación celular (Kauffman 1993).

En la Figura 5 damos un ejemplo sencillo de una red regulatoria. En ella existen dos genes -A y B- cada uno de los cuales tiene un umbral de activación -Ha y Hc- respectivamente. Si un gen es activado con un valor mayor a su umbral, este gen se activa se transcribe. Las flechas representan las interacciones entre los genes, que pueden ser positivas (activaciones) o negativas (inhibiciones). En el ejemplo numérico se muestra que si iniciamos la red prendiendo al gen A, éste activa a B con un valor de 2; como 2>-1, B se activa. Luego, en virtud de la existencia de un asa de retroalimentación positiva de 1, B se queda prendido permanentemente. Esto resulta en una inhibición de A con valor de -2. Como éste valor es menor a 1, que es el umbral de A, éste último queda permanentemente apagado. En este ejemplo sencillo, el estado de la red en que A está apagado y encendido, es un estado de activación estable que se conoce como atractor de punto fijo. Estos atractores representan los estados de activación estables característicos de distintos tipos celulares. Al conjunto de estados de activación iniciales que llevan a cada uno de los atractores, les llamamos cuencas de atracción. Es cierto que las redes genéticas modelables con datos reales resultan mucho más complicadas que la sencilla red del ejemplo anterior, pero los principios para encontrar sus atractores de punto de fijo -que corresponden a los distintos tipos celulares- son los mismos.

Con la representación de los genes involucrados en la morfogénesis como redes de regulación, se abren nuevas perspectivas para el estudio de la relación genotipo-fenotipo. Como se ha hablado en otras partes de este capítulo, los genes homeóticos juegan un papel fundamental en la morfogénesis de muchas estructuras anatómicas. Por otra parte, se sabe que el origen de las familas de genes homeóticos (homeobox, MADS-box) provienen de múltiples duplicaciones genéticas. ¿Qué efecto tienen sobre la dinámica de activación genética la duplicación, o incluso la deleción, de miembros de una familia multigénica? ¿Qué efecto tienen sobre la morfogénesis esas mismas duplicaciones y deleciones?. Utilizado el formalismo de redes para responder a la primera pregunta (Wagner 1996), se ha encontrado que bajo ciertas condiciones la aparición o desaparición de grupos de genes no tiene efectos aparentes sobre la dinámica de activación. Este tipo de resultados no son obvios; las redes han sido posiblemente la herramienta más adecuada para atacarla.

En el laboratorio hemos utilizado la gran cantidad de resultados experimentales sobre la expresión de grupos de genes que intervienen en la morfogénesis floral para proponer un modelo de red que incluye a 11 de los genes de morfogénesis floral de *Arabidopsis* (Mendoza y Alvarez-Buylla 1998, Mendoza et al. 1999; ver Figura 6). Dicho modelo, que plantea un enfoque mecanístico de los procesos de activación de genes involucrados en la morfogénesis floral, constituye la parte central de un modelo más general que describe y predice la morfología de plantas mutantes, así como su respuesta morfológica a los cambios del fotoperiodo en el que crecen las plantas. Es importante hacer notar que aunque existen cerca de 60 genes con el motivo MADS, los 11 genes que se utilizan en el modelo de red son todos aquellos que tienen un efecto reconocible sobre el fenotipo y la gran mayoría de ellos son genes homeóticos.

En nuestra interpretación, la flor de *Arabidopsis* equivale a un módulo fenotípico, al cual le corresponde otro módulo de carácter genotípico: la red de 11 genes. Hemos encontrado que la implementación de esta red predice seis estados estables; de manera notable, cuatro de ellos corresponden a los cuatro estados de activación del modelo ABC. El quinto estado corresponde a células que no están competentes para diferenciarse en células de meristemos florales, que correspondería a las células antes de la floración, mientras que el sexto estado de activación no se encuentra en plantas silvestres. Estos resultados apuntan a que estos modelos dinámicos pueden constituirse en herramientas útiles para integrar las funciones de los genes del desarrollo floral y postular hipótesis acerca de su evolución. Con análisis de simulaciones, también hemos visto que hay genes cuyos cambios tienen efectos en los estados estables predichos, mientras que hay otros genes que no afectan los estados estables de la red. Como una predicción derivada de lo anterior, esperamos

que genes del primer grupo estén mas restringidos funcionalmente que los segundos y por lo tanto, suponemos que existirá mayor variación nucleotídica entre genes ortólogos aislados de diferentes especies del segundo grupo en comparación con genes del primer grupo.

Una de las consecuencias de más largo alcance de los resultados de la aplicación de los modelos dinámicos al estudio de la morfogénesis y la diferenciación celular es el hecho de cualquier gen particular no es el responsable solitario del desarrollo de una estructura u complejos. En contraste, los modelos sugieren que lo que ocurre en la naturaleza es la generación de estados de activación estables como resultado del comportamiento dinámico de redes de interacciones genéticas complejas (Goodwin et al. 1993). Pero, entonces, ¿hasta donde importan los genes y su naturaleza molecular? ¿Cuál es la importancia de los enfoques holísticos en biología? Sin duda, en los próximos años presenciaremos grandes avances experimentales, teóricos y computacionales que nos acerquen a comprender mejor los complejos sistemas biológicos y sus alteraciones. Más allá de la comprensión básica, este avance repercutirá en aplicaciones que provean con una mayor capacidad para prevenir y curar enfermedades y -esperemos- también en la prevención de la destrucción de las especies en los ambientes naturales.

Conclusión: las plantas y animales como elaboraciones naturales independientes del desarrollo ontogenético y la diferenciación celular

Como hemos visto a lo largo de este capítulo, el estudio de los mecanismos de desarrollo de las plantas con una perspectiva genético- molecular, embriológica y principalmente evolutiva nos permitirá hacer comparaciones con lo encontrado en sistemas animales. El enfoque comparativo es imprescindible para poder responder a preguntas básicas de la biología. Por ejemplo, ¿puede el desarrollo multicelular evolucionar de nuevo? ¿Cuántas veces lo ha hecho? ¿Son las bases moleculares del desarrollo en plantas y animales diferentes o comunes? ¿Cuáles aspectos del desarrollo de plantas y animales son comunes y cuáles son diferentes? (Meyerowitz 1997). Si partimos de la base de que ambos linajes se originaron de un ancestro unicelular común, pero que en el camino evolutivo a la multicelularidad en ambos linajes hubo una larga historia independiente de unicelulares y multicelulares con diferentes grados de complejidad, uno supondría que los mecanismos de regulación a nivel celular sean comunes, pero que los mecanismos de interacción entre células sean diferentes. En realidad, esto es lo que se encuentra cuando se comparan plantas y animales (Meyerowitz 2002).

La respuesta a estas preguntas revelará aspectos importantes de la lógica del desarrollo como un fenómeno biológico general e indicaría los tipos de restricciones que ha habido en la evolución de los mecanismos de desarrollo en los grandes grupos de multicelulares de la tierra, muy probablemente determinada históricamente por su origen común. Si tal lógica puede ser dilucidada con el formalismo de redes, como hemos argumentado, y al mismo tiempo los eventos evolutivos únicos pueden ser identificados con las técnicas actuales de la sistemática, la suma de este conocimiento a lo que ahora sabemos sobre el control genético de la morfogénesis con seguridad permitirá realizar una nueva síntesis evolutiva en la cual, además de comprender las fuerzas que actúan sobre la variación, entendamos cabalmente la naturaleza misma de ella y las razones de su origen.

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Pies de figura

Figura 1. Representación esquemática del modelo de Edward Lewis acerca de la regulación genética del complejo *bithorax* y sus consecuencias sobre la especificación de identidades en los segmentos de *Drosophila*. El segundo segmento torácico (T2) es considerado el "estado basal" Para cada segmento posterior a éste, un gen adicional es activado. En el último segmento, todos los genes están activos. Modificado de Gilbert (1988). Ver el texto para una explicación más extensa.

Figura 2. Evolución de los genes *Hox* en los metazoarios. La distribución de los genes *Hox* en diversos grupos de animales se muestra con cuadros organizados en columnas, que corresponden a los diferentes grupos parálogos descritos (derecha). Asimismo, se representan la clasificación de los mismos en anteriores (blanco), centrales (gris claro) y posteriores (gris oscuro). Esta distribución se correlaciona con una reconstrucción filogenética reciente (izquierda). Sobre ésta última, se han mapeado cinco eventos importantes en la historia evolutiva de la familia multigénica: (A) expansión de los genes *Hox* centrales; (B) expansión de los genes *Hox* posteriores; (C) eventos de tetraploidización genómica; (D) origen de los genes *Ubx* y *Abd-B* (caracterizados originalmente en *Drosophila*); finalmente, origen de los genes *Lox5*, *Lox2/4* y *Post1/2* (caracterizados originalmente en los anélidos). Modificado de Carroll et al. (2001).

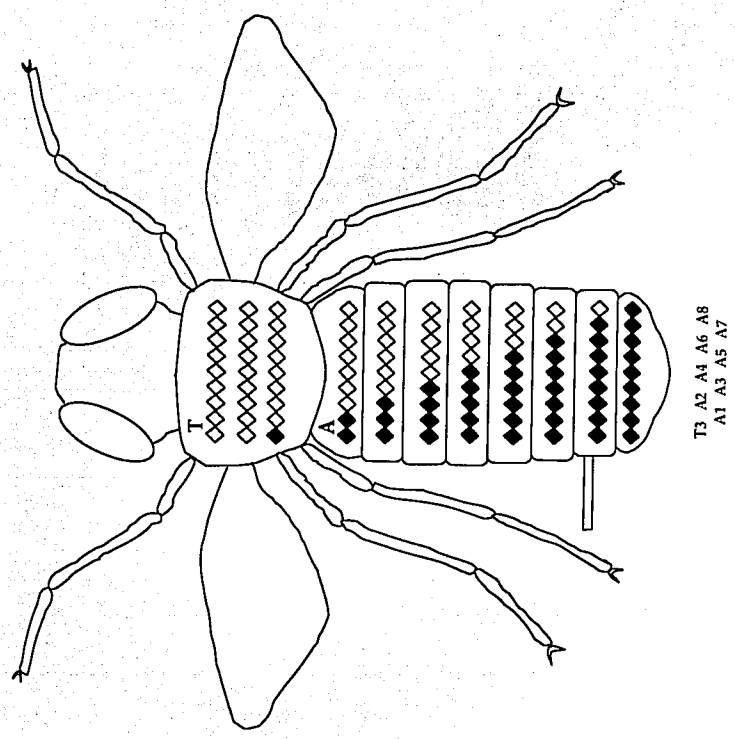
Figura 3. Flor silvestre de *Arabidopsis thaliana*, sistema modelo en genética molecular del desarrollo Se muestran de afuera hacia adentro los cuatro tipos de órganos que forman este órganos caraterístico de las angiospermas: sépalos, pétalos, estambres y carpelos.

Figura 4. Modelo ABC de desarrollo floral. Se muetra la expresión de los genes de las funciones A, B y C en el tipo silvestre y los mutantes florales que resultan cuando se desactivan o mutan una o más de éstas funciones. Ver texto para una explicación detallada.

Figura 5. Dinámica de activación de una red booleana sencialla. Los círculos representan los nodos de la red que en el caso de redes de regulación genética representan genes y las líneas representan las interacciones entre los genes: las flechas son activaciones y las líneas romas son represiones. A partir del tiempo tres se llega a un estado de activación estable o atractor de punto fijo. Los distintos atractores de una red particular se interpretan como distintos tipos celulares. Ver texto para una explicación más detallada.

Figura 6. Red de regulación genética del desarrollo floral. Ver Fig. 5 y texto para una explicación más detallada.

Fig. 1



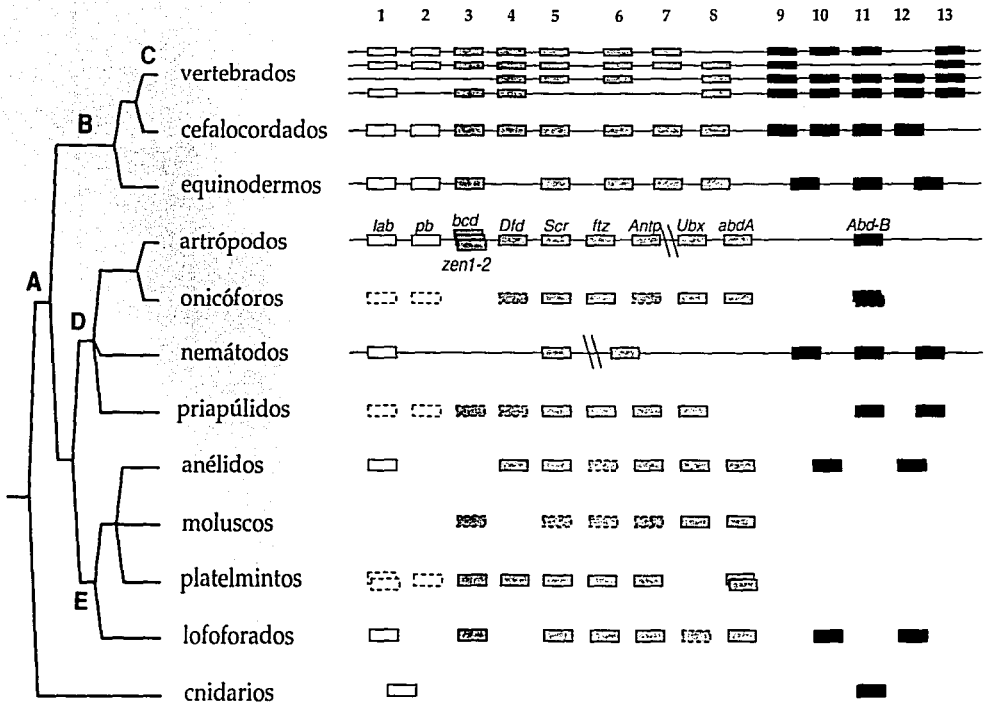


Fig. 2

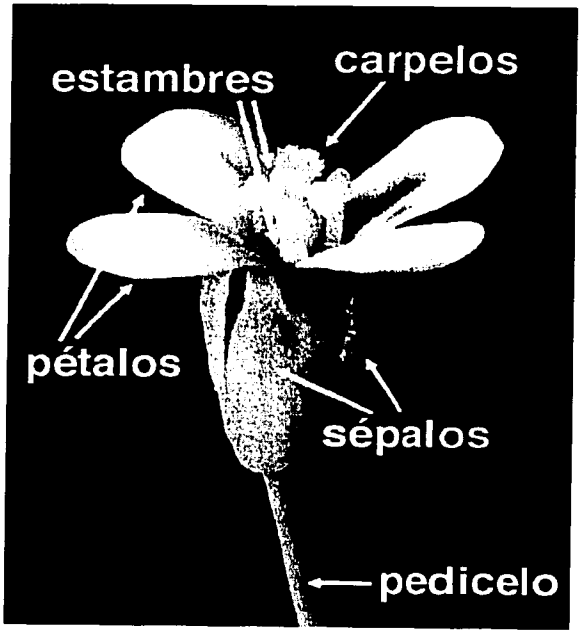


Fig. 3

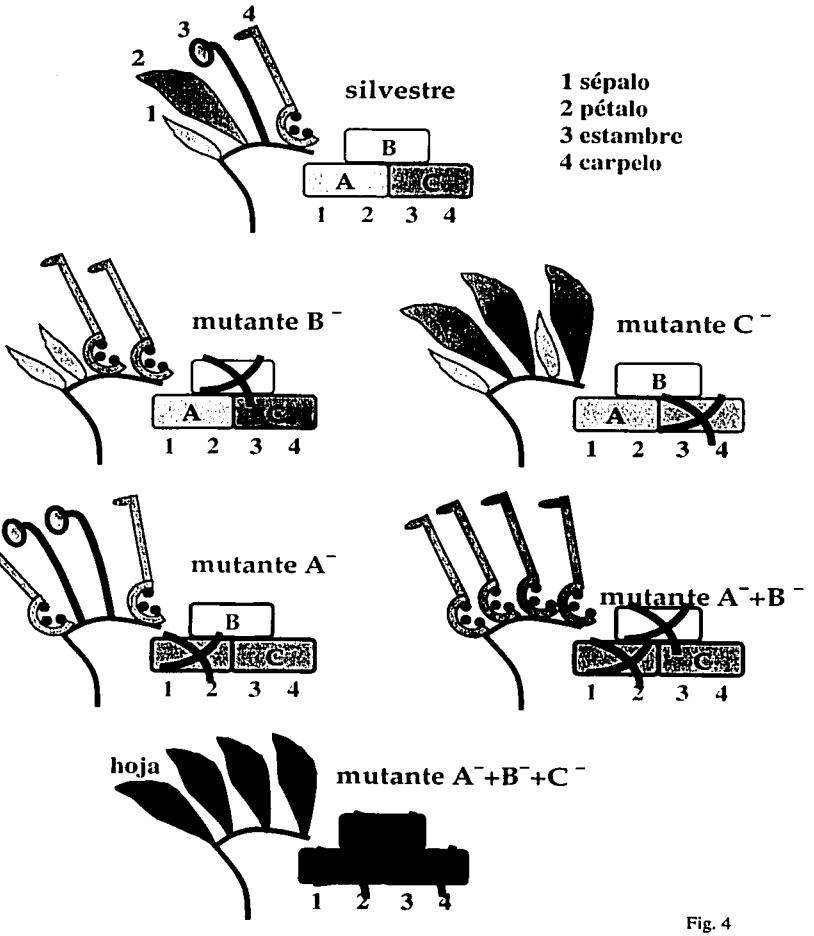


Fig. 4

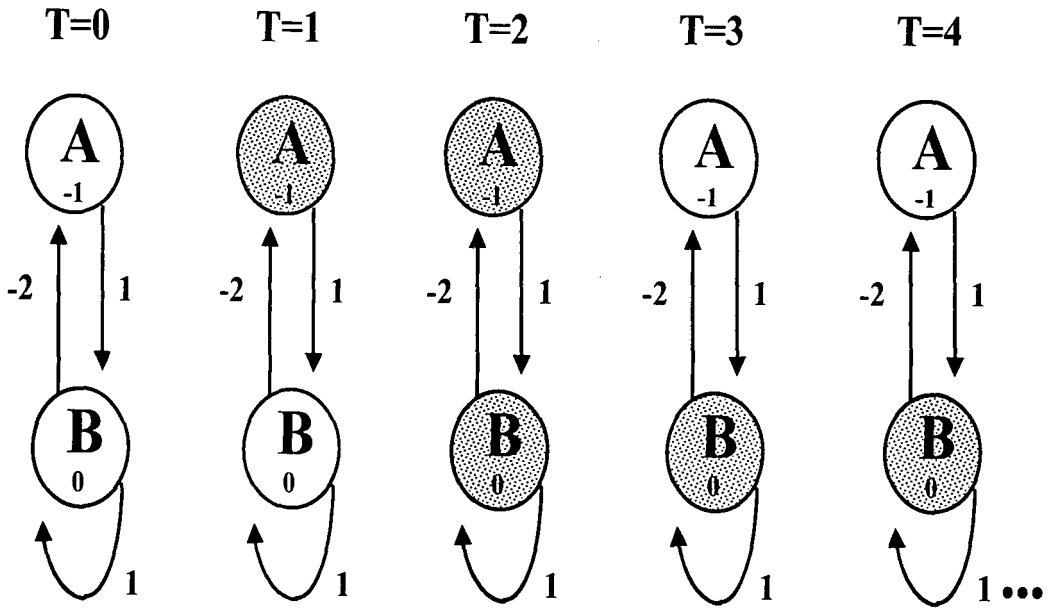


Fig. 5

punto estable
(atractor de punto fijo)

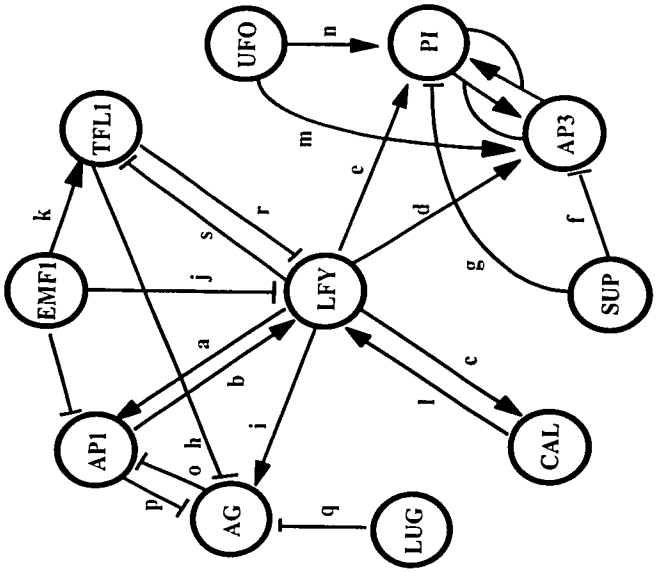


Fig. 6

Capítulos 2 y 3: generalidades

Los artículos presentados a continuación como capítulos segundo y tercero son contribuciones originales al conocimiento de (a) las relaciones filogenéticas de las triuridales mexicanas –*L. schismatica* y *Triuris brevistylis*– en el contexto de las monocotiledóneas, y (b) la variación existente en las poblaciones naturales conocidas de ambas especies. *T. brevistylis* es actualmente considerada por nuestro grupo de investigación y nuestros colegas familiarizados con este grupo taxonómico como la especie hermana de *L. schismatica*.

Con base en el análisis cladístico de una matriz morfológica trabajada durante varios años por Dennis Stevenson y M. Alejandra Gandolfo (Universidad de Cornell, EUA), asociado a análisis independientes y conjuntos basados en una matriz molecular multigénica construida por Jerrold Davis (Universidad de Cornell, EUA) y completada por el autor, hemos encontrado que (a) las triuridales *sensu* Dahlgren *et al.* (1985; Triuridaceae/Lacandoniaceae + Petrosaviaceae) incluidas en el análisis no son monofiléticas, mientras que (b) el grupo taxonómico bajo escrutinio pertenece efectivamente al Orden Pandanales, una afinidad taxonómica reportada recientemente en la literatura para nuestro taxón de estudio (Chase et al. 2000). Sin embargo, también hemos hallado soporte para afirmar que (c) la relación intrafamiliar ((Velloziaceae + Stemonaceae) (Cyclanthaceae (Pandanaceae + Triuridaceae/Lacandoniaceae))), planteada en dicho trabajo, no es correcta. En sustitución de ella, planteamos que las relaciones entre los miembros del grupo son (i) (Velloziaceae (Triuridaceae (Stemonaceae (Cyclanthaceae + Pandanaceae)))) o bien (ii) (Triuridaceae ((Stemonaceae + Velloziaceae) (Cyclanthaceae + Pandanaceae))). Es importante mencionar que el estudio presentado aquí es aún preliminar; sin embargo, si estos rearreglos alternativos se corroboraran, sería inevitable llegar a conclusiones de gran importancia para la discusión actual sobre la posibilidad de que las flores consideradas por nuestro grupo de investigación como heterotópicas sean, en realidad, pseudantios (Rudall 2002), pues ninguno de ellos apoya esta última interpretación morfológica.

Por otro lado, con base en el conocimiento de la localización geográfica de diferentes poblaciones de triuridales en la Selva Lacandona y regiones conectadas en Guatemala, así como en el análisis estructural de especímenes florales variantes, hemos concluido que (a) los morfos florales heterotopicos no son exclusivos de *L. schismatica*, sino que también se encuentran en *T. brevistylis*, incluyendo flores con estambres centrales, por lo que entonces (b) es posible que la población original de *L. schismatica* se haya originado a partir de individuos con "flores mutantes" que posteriormente pudieron reproducirse hasta constituirse en agrupaciones ecológicamente estables, tal como se observan actualmente en la naturaleza Este capítulo aparecerá publicado próximamente en la revista *International Journal of Plant Sciences*.

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Phylogenetic relationships of Triuridaceae (Liliopsida) based on cladistic analyses of molecular and morphological matrices

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Introduction

Reconstruction of the phylogenetic relationships inside the angiosperm clade has been the subject of several recent systematic studies based on morphological as well as on molecular character matrices (see, for example, Doyle et al. 1994, Nickrent and Soltis 1995, Nandi et al. 1998, Soltis et al. 1997 and 2000). A prominent trend in these studies has been to improve sampling of genes or non-coding regions for molecular matrix construction; to a lesser degree, this interest has been accompanied by the establishment of new phenotypic characters coded. Therefore, current systematic work deals with several *loci* in combination, and more morphological characters, in order to enhance the quality of inferences. This concern has been added to an increase in the number of taxa included, particularly when the traditional sampling of certain groups has not been abundant in the past, and in many cases both data generation practices have converged with the application of a whole array of methods for the estimation of statistical support for clades observed after analysis. Without disregard for important issues related to the foundation of the methods involved in some of these tasks, which have led to hot debates and unsettled discussions on the suitability and logical justification of different strategies for phylogenetic reconstruction, it would be fair to state that the scientific standards for the area have in general been benefited by the proliferation of the aforementioned methodological uses.

Within the angiosperms, the monocotyledons have proven to be an example that illustrates the justification of the previously commented trends: initial work based on the analyses of both classes of matrices -either separate or in combination- aimed to elucidate the relationships for the group have demonstrated, according to Stevenson et al. (2000), the existence of character coding problems, unstable taxa and other inconsistencies in the results produced by different analytical approaches. A clear instance of this situation is given by works on the position of *Acorus calamus*, found to be either the sister group of the monocots in maximum likelihood reconstructions based on *rbc*L matrices (see, for example, Duvall et al. 1993a and 1993b, Chase et al. 1995) or a commelinoid taxon with affinity to Typhales in cladistic analyses based on morphological matrices (Stevenson and Loconte 1995, Chase et al. 1995). So far, the solution to this (see, for example, Duvall 2000) and other related issues in monocot systematics has been based precisely on the construction of multigene molecular matrices for more numerous suites of taxa, and the refinement of morphological character state coding (see, for example, Rudall et al. 1999, Stevenson et al. 2000). To some researchers working in this field, improvements in the resolution of the reconstructions themselves derived from the implementation of these practices, has resulted in a diminished concern for the application of measures of statistical support (see, for example, Stevenson et al. 2000). For others, in contrast (see Chase et al. 2000 for an example), the outcome of the latter is considered to confer additional robustness to the inferences made.

In the present work, we report the results of the application of a standard unweighted cladistic approach to a morphological, a multigene molecular and a combined character matrices corresponding to several monocot taxa that comprise the close phylogenetic vicinity of the Family Triuridaceae, a group of mycoheterotrophic herbs that until recently had been completely unexplored from the molecular point of view, and whose taxonomic affinities have also varied across decades of research in systematic botany (compare, for example, Dahlgren et al. 1985 to Dahlgren and Bremer 1985, Cronquist 1988 and APG 1998). Besides these taxonomic assessments, background for the research conducted here consists of three reports published during the last decade. On one hand, Triuridaceae has been placed as either the sister group of Petrosaviaceae –in accordance with the classification of Dahlgren et al. (1985)- or a part of the alismatids in the morphology-based cladistic study of Stevenson and Loconte (1995) and the simultaneous analyses of Chase et al. (1995). More recently, a report in which a single sequence –that corresponding to the small subunit of the nuclear ribosomal gene complex (SSU or 18S nrDNA)- from a single triurid taxon, the genus *Sciaphila*, resulted in an association of Triuridaceae several terminals belonging to the Order Pandanales, implying the family level relationships ((Velloziaceae+Stemonaceae) (Cyclanthaceae (Pandanaceae+Triuridaceae))) (Chase et al. 2000). Although statistical support for this taxic hypothesis is higher than 70% for the entire Pandanales assemblage and for the (V+S) and (C(P+T)) clades, the association of Pandanaceae to Triuridaceae was not supported above 50%. Our results, based in the sampling of two mitochondrial *loci* (*atp*A and *cob*) and the nuclear version of the 18S ribosomal gene from two Mexican triurid taxa –the hermaphroditic *Lacandonia schismatica* and the dioecious *Triuris brevistylis*- also place Triuridaceae inside the Pandanales, but contradict their clustering with Pandanaceae, supporting instead either an association with Velloziaceae-Stemonaceae or a sister group relationship with the rest of the families included in the order. These alternative reconstructions have interesting consequences for current hypotheses on character evolution in Pandanales, particularly regarding triurid floral traits (see, for example, Rudall 2002, Vergara-Silva et al. 2002).

Materials and methods

Morphological matrices. The 103 taxa, 101 characters matrix of Stevenson and Loconte (1995) was reworked by DWS and MAG into an expanded matrix of 153 characters. A set of 33 taxa was then selected to be used in separate cladistic analyses as well as in conjunction with the molecular multigene matrix. The taxa selected for this morphological matrix are *Tofieldia* (Tofieldiaceae), *Acorus* (Acoraceae), *Gymnostachys* (Araceae), *Triglochin* (Juncaginaceae), *Japonolirion* (Petrosaviaceae), *Petrosavia* (Petrosaviaceae), *Burmannia* (Burmanniaceae), *Dioscorea* (Dioscoreaceae), *Tacca* (Taccaceae), *Trillium* (Melanthiaceae), *Smilax* (Smilacaceae), *Acanthochlamys* (Velloziaceae), *Barbaceniopsis* (Velloziaceae), *Vellozia* (Velloziaceae), *Lacandonia* (Lacandoniaceae), *Triuris* (Triuridaceae), *Sciaphila* (Triuridaceae), *Croomia* (Stemonaceae), *Stemona* (Stemonaceae), *Freycinetia* (Pandanaceae), *Pandanus* (Pandanaceae), *Cyclanthus* (Cyclanthaceae), *Sphaeradenia* (Cyclanthaceae), *Chorigyne* (Cyclanthaceae), *Carludovica* (Cyclanthaceae), *Blandfordia* (Blandfordiaceae), *Astelia* (Asteliaceae), *Hypoxis* (Hypoxidaceae), *Borya* (Boryaceae), *Ixiolirion* (Ixioliridaceae), *Tecophilaea* (Tecophilaeaceae), *Xanthorrhoea* (Xanthorrhoeaceae) and *Xeronema* (Xeronemataceae).

Field collection. Samples of inflorescences of *L. schismatica* and *T. brevistylis* were collected in four visits to the original site of discovery of the first species (Crucero Corozal, Municipio de Ocosingo, Chiapas, México; Martínez and Ramos, 1989, Coello et al. 1993) and one visit to the only locality known to date in southeastern Mexico for the latter (Segunda Ampliación de El Censo y Najá, Municipio de Ocosingo, Chiapas, México; Espinosa Matías 1991, 1994) during 1995 to 1998. The samples were separated into aerial and radical parts, stored in Eppendorf tubes, frozen inside a full LN deposit during the trips, and refrozen on arrival to the laboratory.

Extraction of genomic DNA. Aerial parts of inflorescences kept at -80°C were homogenized and subsequently treated following the CTAB extraction methods of Doyle and Doyle (1987, 1990), with some minor modifications introduced by Michael Frohlich (Natural History Museum, London). The final pellets were always resuspended in sterilized water and stored back at -80°C for further use as templates in PCR reactions.

Amplification, cloning, sequencing of products and assessment of their identities. 18S nrDNA from the two triurid species sampled in this study plus the genera *Acanthochlamys* (Velloziaceae), *Barbaceniopsis* (Velloziaceae), *Carludovica* (Cyclanthaceae), *Chorigyne* (Cyclanthaceae), *Pandanus* (Pandanaceae) and *Vellozia* (Velloziaceae), were partially amplified in two overlapping parts. Selected oligonucleotides were chosen from a collection of amplification and internal sequencing primers kindly provided by Daniel Nickrent (Southern Illinois University) and Elizabeth Zimmer (Smithsonian Institution). The 5' end was obtained using the forward primers NS1 (5'GTA GTC ATA TGC TTG TCT C3') and 18S-25EF (=23Afor; 5'TTG GTT GAT CCT GCC AGT AG3') and the reverse primer 854rev (5'ATC ATT ACT CCGATC CCG3'). On the other hand, the 3' fragment was obtained using the forward primer 366for (=18Efor; 5'GTC TGC CCT ATC AAC T3') and the reverse primers C18L (5'GAA ACC TTG TTA CGA CTT3') and 1769rev (5'CAC CTA CGG AAA CCT TGT T3'). After the preparation of separate 50 µl reaction mixes containing 1X PCR buffer +Mg (Gibco BRL), 1 mM of dNTPs (0.25 mM each; Gibco BRL), 0.5 µM of each couple of primers (two combinations for each fragment; see above), 5 µl of template at a concentration of approximately 50 ng/µl, 0.5 µl of Pfu polymerase (Stratagene) and sterilized water up to the final volume, PCR reactions were conducted in a PTC-100 Programmable Thermal Controller (MJ Research) under the following conditions: 1 cycle, 94°C 2'; 35 cycles of 94°C 20"- 52°C 30"- 72°C 3' 30"; 1 cycle 72°C 6'; 4°C final temperature. The products obtained were inspected by LMP agarose gel electrophoresis; excised bands were purified using the QIAquick Gel Extraction Kit (QIAGEN), and cloned into the pCR-Blunt vector (Zero Blunt PCR Cloning Kit, Invitrogen). Plasmid purification was attained using the QIAprep Spin Plasmid Kit (QIAGEN). Sequences were obtained by automated sequencing using M13 forward and reverse primers and ABI Prism sequencers at the Sequencing Facility of the California Institute of Technology (CalTech, Pasadena, CA). Quality of the sequences was analyzed by eye with the help of the GeneWorks software (version 2.3.1, Intelligenetics), After sequence pasting, BLAST searches at the National Center for Biotechnology Information (NCBI) web site were performed to confirm the procedence of the cloned sequences to be used in matrix construction and to rule out the possibility of working with fungal DNA-derived products. The products were considered to be of plant origin when all of the hits or at least the ones with the highest scores belonged to angiosperms in the results of the BLAST searches.

Alignment of sequences and construction of molecular and combined matrices. Sequences corresponding to homologous sequences of the 18S nrDNA from *L. schismatica, T. brevistylis, Acanthochlamys, Barbaceniopsis, Carludovica, Chorigyne, Pandanus* and *Vellozia* were aligned using the multiple alignment algorithm with default parameters available in ClustalX (software modified from ClustalV; Higgins et al. 1992) to a matrix previously constructed by JID in which the sequences corresponding to this *locus* plus the *atp*A, *cob* and *rbc*L genes had been included for 79 taxa. The automated alignment was then refined after visual inspection. Excluding the aforementioned terminals with pandanoid and non-pandanoid monocot affinity, taxa included in the matrix are *Phoenix* (Arecaceae), *Calectasia* (Dasypogonaceae), *Helmholtzia* (Philydraceae), *Callisia* (Commelinaceae), *Pontederia* (Pontederiaceae), *Canna* (Cannaceae), *Musa* (Musaceae), *Heliconia* (Heliconiaceae), *Costus* (Costaceae), *Orchidantha* (Orchidanthaceae), *Strelitzia* (Strelitziaceae), *Ananas* (Bromeliaceae), *Typha* (Typhaceae), *Sparganium* (Sparganiaceae), *Flagellaria* (Flagellariaceae), *Orectanthe*

(Xyridaceae), *Mayaca* (Mayacaceae), *Carex* (Cyperaceae), *Juncus* (Juncaceae), *Elegia* (Restionaceae), *Joinvillea* (Joinvilleaceae) and *Oryza* (Poaceae). Early branching dicotyledons included in the matrix are *Amborella* (Amborellaceae), *Nymphaea* (Nymphaeaceae), *Austrobaileya* (Austrobaileyaceae), *Illicium* (Illiciaceae), *Schisandra* (Schisandraceae), *Ceratophyllum* (Ceratophyllaceae), *Chloranthus* (Chloranthaceae), *Drymis* (Winteraceae), *Calycanthus* (Calycanthaceae), *Gyrocarpus* (Hernandiaceae), *Magnolia* (Magnoliaceae), *Annona* (Annonaceae), *Myristica* (Myristicaceae), *Aristolochia* (Aristolochiaceae), *Peperomia* (Peperomiaceae), *Piper* (Piperaceae) , *Houttuynia* (Saururaceae) and *Saururus* (Saururaceae). Additionally, seven gymnosperm taxa commonly used as outgroups (*Zamia* (Zamiaceae), *Ginkgo* (Ginkgoaceae), *Podocarpus* (Podocarpaceae), *Ephedra* (Ephedraceae), *Welwitschia* (Welwitschiaceae), *Gnetum urens* (Gnetaceae) and *G. gnemon* (Gnetaceae)) were also included in the matrix. The resulting matrix is composed of 5786 characters partioned in the following way: *atp*A, characters 1-1388; *cob*, characters 1389-2553; *rbc*L, characters 2554-3951; 18S nrDNA, characters 3952-5786. Although zones of uncertain alignment were identified, no depuration of the matrix was performed previous to the determination of informative characters.

Finally, a combined matrix was constructed, matching all taxa from the morphological matrix to the molecular one. This resulted in a matrix including the same taxa from the morphological one except *Sciaphila*, and 5939 characters.

Cladistic analyses. The matrices described above were manipulated in WinClada (Nixon 1999a) and directly submitted to NONA (Goloboff 1993) for cladistic analysis both under standard heuristic strategies and the parsimony ratchet (Nixon 1999b). A thousand heuristic searches involved 1000 subsearches with random addition sequences and up to 10 trees retained (hold/10 mult*1000); the latter were then swapped to completion (max*) using the tree bisection-reconnection (TBR) branch-swapping algorithm. For the ratchet, 1000 iterations/replication were conducted with up to 10 trees retained and 10% of the characters sampled. Bootstrapping of the entire matrices and their "mopped" versions (those including informative characters only; see the rationale for this data manipulation in Carpenter 1996) was used to estimate character support; for each of these analyses, 100 replications of a group of 10 subsearches with up to 2 trees retained were performed.

Results

Sequences. As described in Materials and Methods, the sequences obtained in this study were considered to be ready for phylogenetic analyses until their affinities with other plant sequences available in the databases were sufficiently clarified. The composed 18S nrDNAs of *L. schismatica*, *T. brevistylis*, *Acanthochlamys*, *Barbaceniopsis*, *Carludovica*, *Chorigyne*, *Pandanus* and *Vellozia* had a length of 1725-1730 nucleotides, excluding the regions corresponding to external amplification primers. Given the focus on the triurids, base composition of the 18S nrDNA sequences of *L. schismatica* and *T. brevistylis* was calculated and found to be almost exactly the same: in percentages for *L. schismatica* and *T. brevistylis*, respectively, A=25.07/25.11, C=21.56/21.65, G=27.33/26.76 and T=26.03/26.42. The high degree of similarity between the small subunit ribosomal sequences of *L. schismatica* and *T. brevistylis* reported here could be interpreted as potential support for the refusal of some authors to recognize Lacandoniaceae as a valid family inside the triurids (e. g. Maas-van de Kamer 1995). However, observations on the natural floral variation in both *L. schismatica* and *T. brevistylis*, coupled to considerations on the geographical distribution of the known populations, constitute additional evidence to validate the taxonomic status of the family (Vergara-Silva et al. 2002).

Cladistic analyses I: morphology. As in previous studies (see, for example, Stevenson and Loconte 1995), several morphological apomorphies place Triuridaceae in a close relationship to members of the Petrosaviaceae in the single most parsimonious tree found both in heuristic and parsimony ratchet analyses (L=321, CI=37, RI=60; Figure 1), although the two terminals included here which belong to the family, *Petrosavia* and *Japonolirion*, do not form a clade. The other taxon assemblage which does not form a monophyletic group are the pandans –see the position of the Velloziaceae and Stemonaceae taxa- although the genera of Pandanaceae and Cyclanthaceae appear clustered. Except for this last association and the union of Stemonaceae to a couple of dioscorid taxa –*Dioscorea* and *Smilax*- the rest of the other taxonomic groups of interest have very low bootstrap values (Figure 2).

Cladistic analyses II: molecules. The inability of our research groups to amplify an *rbc*L homolog from the triurids, coupled to success in the amplification and cloning of versions of *atp*A, *cob* and 18S nrDNA, allowed the construction of a four-gene molecular matrix (see description in Materials and Methods) in which character as well as taxon sampling was considered to be sufficiently dense to provide enough structure to the reconstructions found. The amount of informative characters (1476) in this matrix represents 25.5% of the total. The four MPTs (L=7630, CI=30, RI=49; strict consensus for the trees is shown in Figure 3) resulting from both heuristic and parsimony ratchet searches supports the findings of Chase et al. (2000), which associated the triurids to the pandans with high (>70%) bootstrap values. However, in all four trees and their consensus,

the position of the triurids corresponds to a branching event intermediate between the Velloziaceae and Stemonaceae clades (Figure 3). In any case, it should be noticed that a consideration of the same statistical measure in our analyses does not support neither the results of Chase et al. (2000) nor our MPTs, since the majority rule consensus of 300 bootstrap trees (on the "mopped" matrix) retained collapses the triurids outside the pandans in a clade that includes all the monocot taxa included except the two members of Petrosaviaceae, *Tofieldia*, *Gymnostachys*, *Acorus* and *Triglochin* (Figure 4). Inspection of individual bootstrap trees showed a large proportion of reconstructions where the triurids are associated to *Burmannia*, *Dioscorea* and *Tacca* (the dioscorids *sensu lato* included in the matrices). The same topology was obtained for the majority rule consensus of 302 trees retained from bootstrapping on the intact molecular matrix.

Cladistic analyses III: combined matrix. The amount of informative characters in the combined matrix (723) represented 12.17% of the total, due to a severe reduction in its number after the exclusion of those taxa that did not match the morphological matrix. The heuristic and parsimony ratchet simultaneous analyses conducted on it produced a single, completely resolved MPT (L=2297, CI=40, RI=49) that also places the triurids within Pandanales, but this time as the sister group of the rest of them (Figure 5). Interestingly, in this case the topology of the majority rule consensus of 229 bootstrap trees (on the "mopped" matrix) also produced this branching order (Figure 6), while the same consensus for 227 trees retained based on the 5939 characters-matrix resulted in a collapse of clades very similar to the one found for the bootstraps on the molecular matrix alone (data not shown).

Discussion

L. schismatica and *T. brevistylis* (a possible synonym of *T. hyalina*, Martínez and Gómez 1994) are a couple of mycoheterotrophic (Leake 1994) plant species that grow in restricted areas inside the Lacandon rainforest in the state of Chiapas, Mexico. The original description of the former, published in 1989, reported a small achlorophyllous saprophytic plant with hermaphroditic flowers that presented overt morphological similarities to those found among species of the Order Triuridales *sensu* Dahlgren et al. (1985), with a single major exception: instead of comprising a whorl surrounding the gynoecium (the carpels), the androecium (i. e. the stamens) was found to be placed at the center of every floral bud and adult flower analyzed. This arrangement of floral organs was not only noticed to be absent in the aforementioned Order (Maas and Rübsamen 1986), but was also considered as completely unknown in the entire universe of angiosperm species known to science (Cronquist 1988). The unexpected nature of this discovery –one that, as stated before, in the opinion of the authors of the original taxonomic description justified the creation of a new family of monocotyledons closely allied to Triuridaceae, the Lacandoniaceae (Martínez and Ramos 1989)- initially led some plant taxonomists to a position of disbelief and to suspect that the flowers of *L. schismatica* are, instead, either pseudanthia –that is, highly derived condensed inflorescences where each "organ" is in fact a flower without perianth- or "normal" bisexual flowers where stamens are projected towards the center of the reproductive structure as development advanced (J Márquez-Guzmán, personal communication). A series of detailed studies on the reproductive anatomy and embryology of the species performed subsequently (Márquez-Guzmán et al. 1989, Vázquez-Santana et al. 1998) have demonstrated that there is a continuum of the epidermal layer of cells that covers the tissue at the center of the receptacle and the stamens, thus confirming that their central position is set when the meristems are determined to a floral fate and making unlikely the veracity of the aforementioned alternative morphological interpretation. The evidences presented in the present work are not intended as a correction of the supraspecific taxonomic assignment of *L. schismatica*, although preliminar molecular evolutionary analyses of the sequences reported confirm the close relatedness of Lacandoniaceae to Triuridaceae (F Vergara-Silva, unpublished observations).

Mexican populations of the dioecious *T. brevistylis* have received relatively much less attention than the endemic *L. schismatica*. Nevertheless, it seems that the similarity in many aspects of their biologies (Espinosa Matías 1991, 1994; Espinosa-Matías et al. 2002), necessarily related to the close proximity of their habitats, must also be connected to the origin of the most salient features of the floral structures of *L. schismatica*. Recently, some of us have reported the existence of morphological variation in *T. brevistylis* flowers that consists on several different cases of organ heterotopy (Vergara-Silva et al. 2002). It is important to notice that some of the resulting bisexual flowers documented in that study have a phenotype which resembles the homeotic transformation observable in *L. schismatica* wild-type bisexual flowers. These evidences have led us to propose that *L. schismatica* arose after reproductive isolation of one or a few floral mutants occurring in *Triuris*-like populations (a suggestion independently expressed by Mabberley 1997), in which changes in the expression pattern of MADS-box genes homologous to those involved in the ABC model for the determination of floral organ identity (Coen and Meyerowitz 1991) had played a causal role.

Subsequent research over the ten years posterior to the discovery of *L. schismatica* has shown a preanthesis cleistogamic mode of fertilization (Márquez-Guzmán et al. 1993), a number of specific and unusual nuclear ultrastructural characters (Jiménez-García et al. 1992, 1998) and a previously unknown type

of female gametophyte development (Vázquez-Santana et al. 1998) all of them adding to the autapomorphic homeotic/heterotopic character of the floral structures. However, the "pseudanthial" interpretation for the flowers of *L. schismatica* has been recently revived again by Rudall (2002), this time generalized to include reproductive structures from other triurid taxa. Being one of the co-authors of Chase et al. (2000), this researcher assumes that the close relationship found for Pandanaceae and Triuridaceae in such molecular systematics study allows the interpretation of triurid "flowers"-especially the female and hermaphroditic ones- as "smaller versions of a *Pandanus* inflorescence". As a consequence, *L. schismatica* flowers should be seen instead as inflorescences where male flowers with highly reduced perianths occupy a central position and female flowers, also with reduced perianths, are distally placed. We consider that the results of our cladistic analyses, while suggesting that Triuridaceae is indeed closely related to the families comprising Pandanales as is the case in the reconstructions of Chase et al. (2000), do not support a sister group relationship with Pandanaceae, making Rudall's morphological interpretation implausible. According to exhaustive observations (see, for example, Dahlgren et al. 1985), members of Velloziaceae and Stemonaceae possess solitary flowers while, in contrast, Pandanaceae and Cyclanthaceae have inflorescences whose floral structures are greatly reduced. Therefore, we think that the two alternative hypotheses derived from our work suggest that either (a) the origin of reduced inflorescences occurred after the sequential branching of Velloziaceae, Triuridaceae and Stemonaceae (Figure 3) or (b) the flowers of Triuridaceae evolved independently of events occurring in the remaining pandans (Figures 5 and 6). Formal mappings of character evolution sequences and reconstructions of ancestral character states should be performed to establish which of these scenarios is most parsimonious.

We consider that the evidence presented here is sufficient to encourage more abundant sampling of molecular and morphological data from Triuridaceae and its allies for the refinement of taxonomic assignments and construction of a sound phylogenetic framework for the interpretation of the differing developmental-genetic hypotheses implicit in the different interpretations of the about the origin of the floral phenotypes occurring in the Mexican triurid species included in our analyses. Given the rarity and endangered status of these taxa, we also believe that our results justify more effective conservation strategies in southeastern Mexico and other areas with high indexes of botanical biodiversity inhabited by these and other species of related intrinsic importance.

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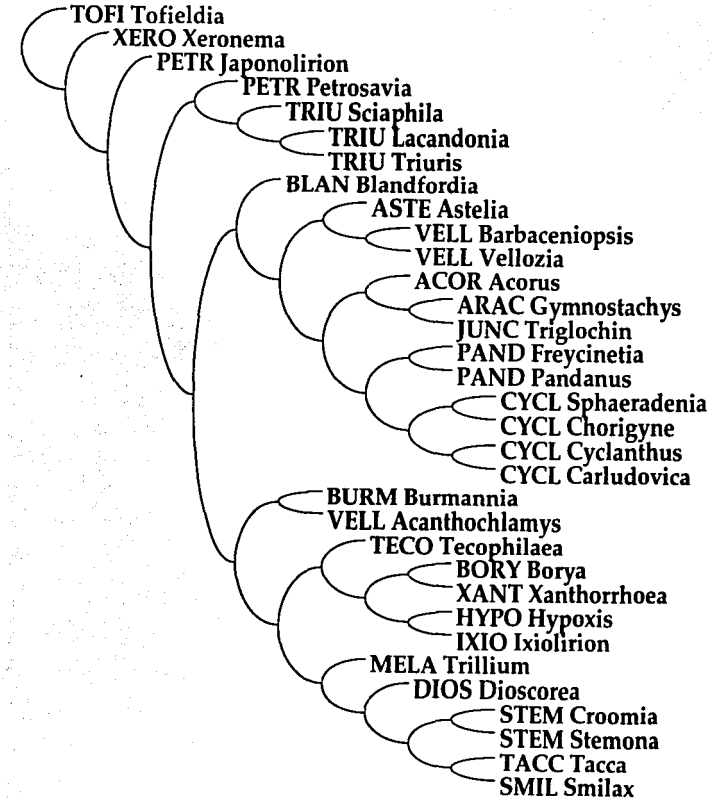
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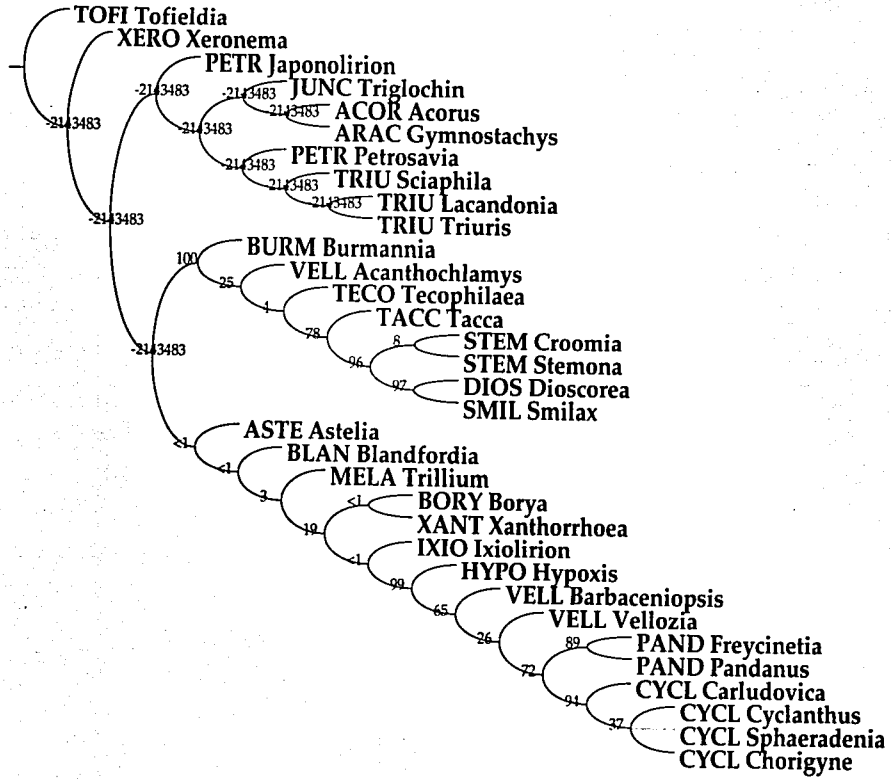
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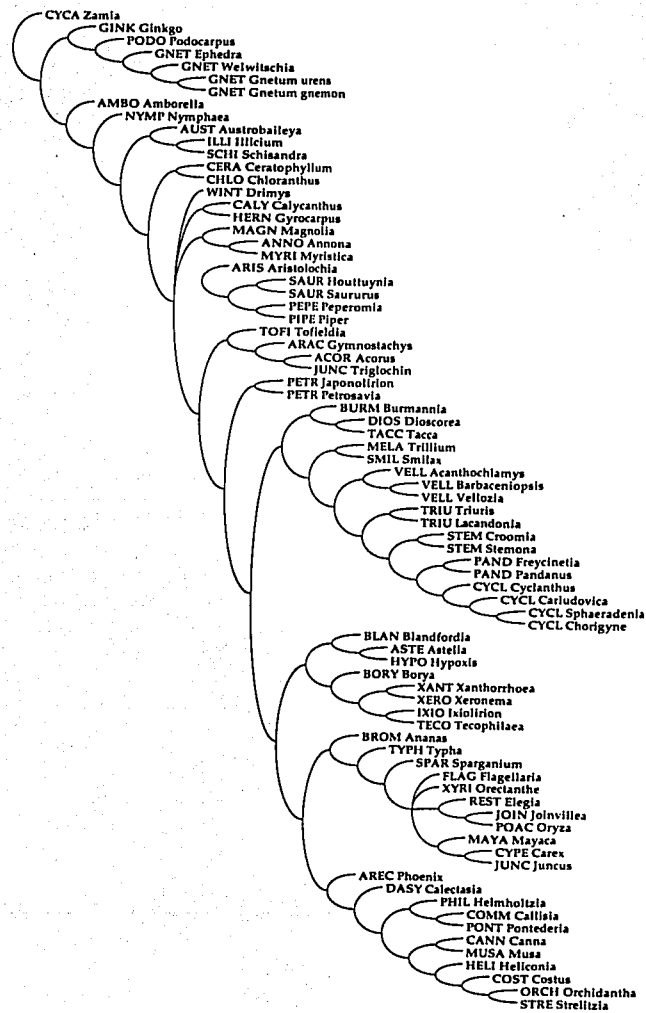
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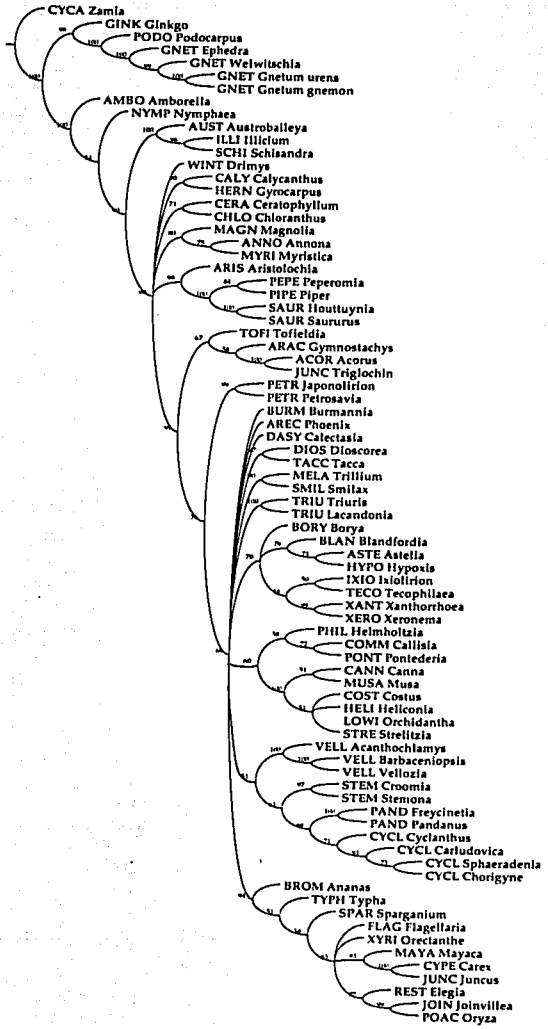
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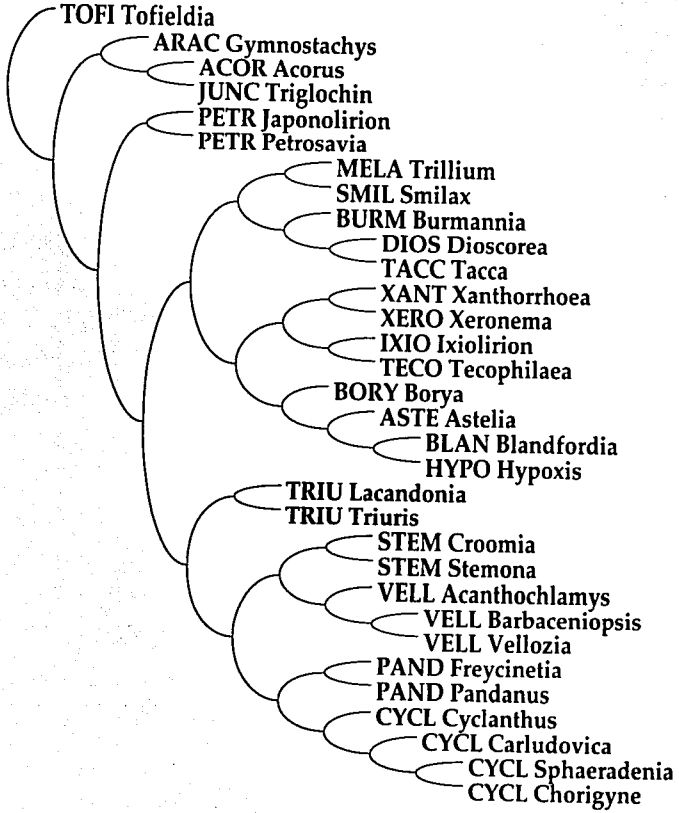
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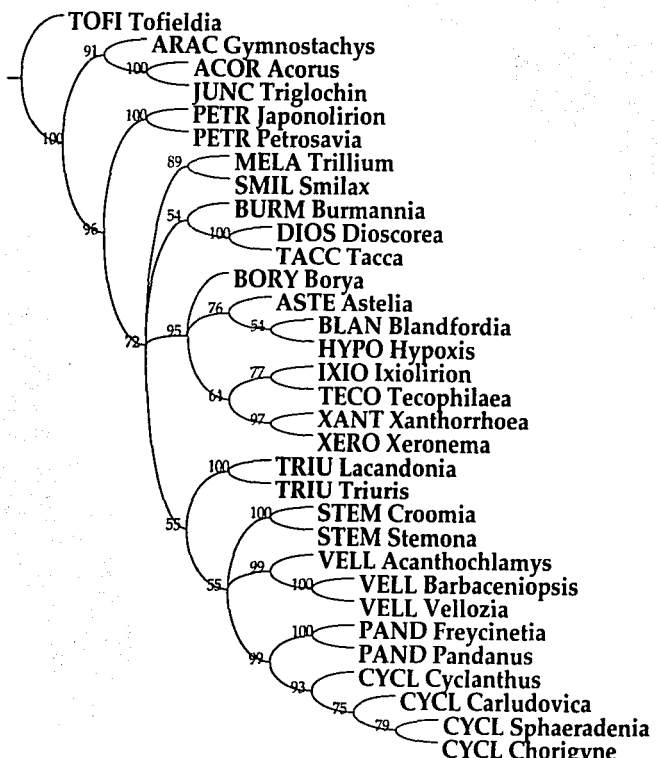
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Figure legends

Figure 1. Single most parsimonious tree (MPT) obtained from heuristic and parsimony ratchet searches with the morphological matrix (L=321, CI=37, RI=60). *L. schismatica* and the genus *Triuris* (both labeled as belonging to Triuridaceae), along with Sciaphila, cluster with Petrosavia. The genera belonging to Pandanales are not monophyletic.

Figure 2. Bootstrap support for the phylogenetic hypothesis shown in Figure 1. The grouping of the triurids with petrosaviads is not supported. While the association of Velloziaceae + Pandanaceae + Cyclanthaceae has support above 50%, Stemonaceae result clustered with *Dioscorea* and *Smilax*.

Figure 3. Strict consensus of four MPTs (L=7630, CI=30, RI=49) obtained from heuristic and parsimony ratchet analyses on the molecular matrix. Triuridaceae is clustered with the pandans, branching between Velloziaceae and Stemonaceae.

Figure 4. Majority rule tree for 300 bootstraps performed on the mopped molecular matrix. The clustering of the triurids with members of Pandanales is not supported.

Figure 5. Single MPT (L=2297, CI=40, RI=49) obtained from heuristic and parsimony ratchet analyses on the combined matrix. In this case, the placement of the triurids within Pandanales implies a sister group relationships with the rest of the members of the group.

Figure 6. Majority rule tree for 229 bootstraps performed on the mopped combined matrix. The structure of the cladograms is preserved, and the support for the monophyletic Pandanales clade (which includes the triurids) is 55%.

Inside-out flowers characteristic of *Lacandonia schismatica* (Lacandoniaceae: Triuridales) evolved at least before divergence from its putative sister taxon, *Triuris brevistylis*

Running head: Inside-out flowers in *Lacandonia schismatica* and *Triuris brevistylis*

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Abstract

Lacandonia schismatica (Lacandoniaceae: Triuridales), a mycoheterotrophic, hermaphroditic monocotyledon endemic to the Lacandon rainforest of Southeast Mexico, is the only flowering plant for which a spatial inversion (heterotopy, complete homeosis) of the reproductive floral whorls (stamens and carpels) is known to occur in natural populations. In order to investigate if this autapomorphic inside-out arrangement of the reproductive organs is fixed in natural populations, we have undertaken extensive and intensive field work spanning several years to locate new populations additional to the type locality. In parallel, we have also searched for natural variations in floral organ arrangement in *Triuris brevistylis* (Triuridaceae: Triuridales), a closely related dioecious triurid that is found in nearby areas of the Lacandon forest. We have found that a small proportion of *L. schismatica* inflorescences bear unisexual flowers of both sexes, as well as bisexual flowers with differences in the number of reproductive organs. However, in all bisexual flowers analyzed, the stamens were always central and the carpels peripheric to them, leading us to conclude that the

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Inside-out flowers characteristic of *Lacandonia schismatica* (Lacandoniaceae: Triuridales) evolved at least before its divergence from its putative sister taxon, *Triuris brevistylis*

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homeotic/heterotopic character of the latter class of flowers is fixed in nature. More importantly, we have also found that a few *T. brevistylis* individuals have bisexual flowers with altered positions of stamens and carpels. Among these, flowers with an inside-out *L. schismatica*-like floral organ arrangement were observed. We show scanning electron micrographs, histological sections and dissection scope photographs to document these findings. The information presented here implies that the developmental-genetic mechanism putatively responsible for homeotic/heterotopic transformations involving floral reproductive organs in the two triurid species studied here originated at least before these taxa diverged from each other. The case of the Mexican triurids may be an instance in which the molecular evolutionary events causally related to an autapomorphic association of morphological characters at a high taxonomic level in plants could be understood at the microevolutionary scale.

Introduction

Comparative analyses of the structure and function of floral homeotic genes is currently one of the main tools of evolutionary developmental biology studies in plants (Howell 1998, Cronk 2001) Particularly, expression patterns of MADS-box genes and members of other transcription-factor encoding gene families have been used to successfully explain differential features of homologous structures in supraspecific taxa (Theissen *et al.* 2000, Frohlich and Parker 2000). Only recently, a few studies have started to document population-level molecular variation in homeotic genes in plants, suggesting that it might have been subject to different microevolutionary forces (Barrier *et al.* 2001, Olsen *et al.* 2002). However, no study has yet established a link between morphological population-level and developmental genes structure/function variation in the field. This kind of information is needed to address the role of homeotic/heterotopic mutations in nature during the origin of morphological characters diagnostic of new species or higher taxonomic groups (Carroll 2000, Stern 2000, Haag and True 2001), and to postulate comprehensive explanations of the population-genetic events that could lead to the fixation of spontaneously occurring, *bona fide* homeotic/heterotopic phenotypes in natural populations.

The mycoheterotrophic monocot *Lacandonia schismatica* is the only flowering plant species in which flowers with central stamens surrounded by carpels have been reported (Martínez and Ramos 1989, Martínez 1994). Recently, some of us have hypothesized that alterations in the expression patterns of homologs of the ABC floral homeotic genes (Bowman *et al.* 1989, Coen 1991, Coen and Meyerowitz 1991) are causally related to the homeosis/heterotopy involving third- and fourth whorl floral organs (cited in Baum 1998, Vergara-Silva *et al.* 2000), notwithstanding that this specific transformation has not been achieved in any model experimental system. This floral feature was the key character state that justified the inclusion of *L. schismatica* in its own monotypic family, Lacandoniaceae (Martínez and Ramos 1989). Although this taxonomic assignment has been disputed (see, for example, Maas-van de Kamer 1995), there is no doubt that studies in this species would offer the opportunity to enhance our understanding of the molecular basis of the origin of unusual morphological character states, and to contribute to the clarification of the possible evolutionary role played by natural homeotic macromutations in the establishment of novel aspects of plant body plans. Clearly, a main component of these studies should focus on the phenotypic variation itself occurring in natural populations.

In this paper, we address if the homeotic/heterotopic floral transformation characteristic of *L. schismatica* is fixed in natural populations of this species, and if there is phenotypic variation linked to this floral trait within the species or in a closely related taxon, the dioecious triurid *Triuris brevistylis* (Triuridaceae: Triuridales). To pursue this objective we have conducted thorough field work to document flower morphological variation among populations of both species. This work is part of an evo-devo study on the origin of the unique floral organ arrangement found in *L. schismatica*.

We report here the existence of variants with either female or male flowers in *L. schismatica*, and the fact that all bisexual individuals examined bear inside-out flowers with central stamens. Additionally, we report that *T. brevistylis* also shows population variation in floral morphology, including some bisexual flower variants resembling the homeotic/heterotopic flowers of *L. schismatica*. We document this variation using light and scanning electron microscopy as well as histological sections. These data enables us to postulate that the mechanism underlying the evolution of inside-out flowers in *L. schismatica* evolved before its divergence from *T. brevistylis*. Given the morphological variation documented here and the population distribution of both species in the Lacandon forest, we also argue that this case may be one of the few instances in which the actual population-genetic events responsible for a large scale morphological change could eventually be traced.

Materials and methods

Species studied

L. schismatica is a mycoheterotrophic, hermaphroditic, herbaceous species with rhizomes, currently assigned to the monotypic monocotyledon family Lacandoniaceae (Martínez and Ramos 1989). This family has close affinities to Triuridaceae, a taxon comprising approximately 45 species of also mycoheterotrophic, achlorophyllous herbs distributed in tropical areas (Leake 1994, Maas-van de Kamer and Weustenfeld 1998 and references therein). In the present work, Lacandoniaceae and Triuridaceae are adscribed to the monocot Order Triuridales (sensu Dahlgren and Clifford 1982).
L. schismatica is endemic to the Lacandon rainforest in Southeastern Mexico (Martínez and Ramos 1989, Martínez 1994), and is currently considered an endangered species. The species presents scale-like leaves and fibrous roots, and has an indeterminate growth. Aerial vertical stems (3-5 cm x 0.4-0.5 mm) are simple, glabrous and bear sympodial inflorescences with ascendent pedicels subtended by bracts. Bisexual flowers in *L. schismatica* (diameter 3.4-4.1 mm) bear carpels and stamens over the receptacle, which is surrounded by 6 (4 or 5) densely papillate, equally sized sepal-like tepals with caudate apices, giving the flower the appeareance of a star (Figure 1). The apocarpic gynoecium is comprised of 60-80 free, papillate carpels with subapical glabrous style and surrounds 3 (2 or 4) stamens that are inserted around the edge of the concave center of the receptacle. Carpels are uniloculate with a single basal anatropous ovule and two integuments. The stamens are (2-)3(-4) sporangiate and sessile, with introrse longitudinal sutures but not dehiscent. Fruits are indehiscent achenes and seeds bear undifferentiated embryos and abundant endosperm. Besides the inverted position of carpels and stamens, unusual ultrastructural features related to the plastids and cytoplasm (Jiménez-García *et al.* 1998) as well as a peculiar type of megagametophyte development (Vázquez-Santana *et al.* 1998) constitute additional apomorphic character states of the species. More detailed descriptions of the species' morphology can be found in Martínez and Ramos (1989), Márquez-Guzmán *et al.* (1989 and 1993) and Vázquez-Santana *et al.* (1998).

The dioecious *T. brevistylis* is the only other triurid species reported to occur in Mexico (Martínez and Gómez 1994). Found also in isolated spots inside the Lacandon and Guatemalan rainforests, the species grows at higher altitudes (>800 m above sea level) in areas covered with evergreen rainforests and seasonal forests associated with *Quercus spp.* On the basis of its geographic distribution and the scarce number of substitutions observed in the large and small subunits of the nuclear ribosomal genes from both species (F. Vergara-Silva, E. M. Meyerowitz and E. R. Alvarez-Buylla, unpublished observations), *T. brevistylis* is currently considered by our research teams to be the sister taxon to *L. schismatica*, and also an endangered species. The embryology and aspects of the reproductive anatomy of this second triurid taxon are the subject of an accompanying study (Espinosa-Matías *et al.* 2002).

T. brevistylis shares with all other members of the genus the presence of staminate flowers with sessile anthers on an androphore (Fig. 2a). According to Maas-van de Kamer and Weustenfeld (1998) tepal structure and number of stamens, in turn, justify the current inclusion of *Triuris* in the Tribe Triuridae, along with *Lacandonia*, *Peltophyllum* and *Triuridopsis* (Maas-van de Kamer and Weustenfeld 1998). The taxonomic closeness of the two triurid genera occurring in Mexico has been confirmed by cladistic analyses of a morphological matrix of >100 characters that includes fossil taxa (M. A. Gandolfo, personal communication).

T. brevistylis plants are unbranched hyaline herbs with rhizomes. Stems are 4-11 cm, erect, and roots are up to 8 cm-long, sparsely pillose. Inflorescences are simple racemes with 1-3(-5) flowers subtended by bracts and with 3.2 mm-long pedicels. Flowers bear 3(4) equal, glabrous tepals, each one bearing a reflexed subapical appendage. Male flower (Fig. 2a) tepals with 12-15 mm caudate appendages and with a 3 x 2.5 mm widely deltoid androphore in the center of the flower that appears as a sterile subulate projection with 6(8) half-stamens inserted in small basal cavities, two of them in front of each tepal; anthers 2-sporangiate. Female flowers (Fig. 2b) are 2-5 mm diameter with a concave receptacle and glabrous triangular tepals with 3-6 mm subterminal caudas slightly ascending. Each female flower bears 120 – 600 free carpels covered with large swollen cells and centrifugal maturation, obovoid with 0.2 mm-long styles. Fruits are indehiscent achenes with 0.35 x 0.25 mm single seeds.

L. schismatica and *T. brevistylis* differ in their breeding strategies. In *L. schismatica*, an autogamic or self-pollinating system called preanthesis cleistogamy in which pollen grains germinate inside the anther and develop tubes that travel through the receptacle to the ovules (Márquez-Guzmán *et al.* 1993) is present. Hence, outcrossing is practically impossible in this species, an observation that expectedly correlates with the absence of electrophoretic variation in eight commonly used enzyme loci sampled from the type locality (Coello *et al.* 1993). Although double fertilization has not been directly observed in *T. brevistylis*, the presence of an endosperm and of pollen tube remnants suggests that sexual reproduction takes place in this species. This leads to question a natural suspicion related to the presence of is apomixis in this species, given its extreme female-biased sex ratio of 100 to 1 (Espinosa-Matías *et al.* 2002).

Information on the collection sites and initial processing of plant material

The results of yearly field work put together in this paper span a period of seven years (1995-2001), but rest heavily on previous expeditions to the Mayan rainforests started in 1984 by one of us (E. Martínez). Information on the locations found for both Mexican triurids is summarized in the map of Figure 3. GPS information for the locations was obtained with a Garmin 12X device during visits from 1998 to 2001. All collections have been performed during the rainy season in the Lacandon rainforest (July to January). Original voucher information on specimens collected in the type localities is included in Martínez and Gómez (1994). The *L. schismatica* samples appearing in Figure 4 are stored at the Institute of Ecology, UNAM. The *L. schismatica* and *T. brevistylis* materials shown in Figures 6, 7 and 8 are part of collections kept at the Faculty of Sciences, UNAM, and the Institute of Ecology, UNAM. All the materials shown were fixed *in situ* to ensure preservation.

Scanning electron microscopy

Complete inflorescences of *L. schismatica* were fixed in FAA (3.7% formaldehyde + 5% acetic acid in a 50% ethanol aequous solution), dehydrated in a graded ethanol series, critical point dried with CO_2, mounted and dissected on aluminum stubs. Samples were coated with palladium. Observations were carried out at 10KV accelerating voltage on a Cambridge 360 scanning electron microscope.

Optical microscopy and tissue sectioning

L. schismatica and *T. brevistylis* floral buds and flowers post-anthesis were fixed either in FAA or a glutaraldehyde-formaldehyde solution, dehydrated, embedded, cut, mounted and stained as in Vázquez-Santana *et al.* (1998). Histological sections were observed with an Olympus Provis AX70 microscope.

Photography

Inflorescences of *T. brevistylis* already in FAA were dehydrated and stored in 70% ethanol. Selected individuals bearing unusual arrangements of floral organs were photographed with an Olympus OM4 camera with bellows and 50 mm macro. The same procedure was used to photograph the *L. schismatica* bisexual flower shown in Figure 1.

Results

Geographical distribution of the Mexican triurids

After several years of field work, we have been able to document the distribution and habitat characteristics for the two Mexican triurids in the Lacandon rainforest (Figure 3). *L. schismatica* has been described from the type location and several other locations in the lowlands of the Lacandon forest. All the areas where this species has been found are subject to periodic water logging, since they lie in close proximity to water bodies of varying sizes. These sites are rich in endemic species (Martínez and Ramos 1989) and are aligned along the former margins of the lake that once dominated the lowlands, approximately 7000 years ago (see reconstruction in red shown in Figure 3).

T. brevistylis occupies a wider geographical area of distribution relative to *L. schismatica* (Martínez 1994, Martínez and Gómez 1994), but in contrast to the latter species, it is now found in higher lands of both Mexico and Guatemala, where temperatures are 6-8 °C lower in comparison to the lowlands (Figure 3). *T. brevistylis* is found in both evergreen and deciduous forests and it is generally associated to *Quercus spp.* However, the sites where the two Mexican triurids are now found share at least 40 herb and shrub species (list available at http://www.ibiologia.unam.mx/mexu/index.html), which are characteristic of mesophyllous forests typical of high lands.

L. schismatica floral variants

In total, more than 1000 *L. schismatica* inflorescences were analyzed under the scanning electron microscope or sectioned. Approximately 2% of the flowers observed were unisexual or had an altered number of carpels and/or stamens. Typically, central stamens of bisexual flowers in *L. schismatica* attain maturity before carpels acquire their characteristic shape at preanthesis developmental stages (Figures 4A, 4B). A diminished number of carpels (Figure 4E), as well as an increase in the number of stamens (Figure 4F) was observed in a small proportion of bisexual flowers. A separate small quantity of inflorescences bear staminate (Figures 4C and 5A) and pistillate flowers (Figures 4D and 5D). The unisexual flowers found that correspond to the former

class can bear both fertile (Figure 5B) and sterile anthers (Figure 5C), while the latter can produce viable carpels (Figure 5E). Inviability of stamens in bisexual flowers was also observed (Figures 5F and 5G). However, all hermaphroditic flowers observed had central stamens surrounded by carpels (Fig. 1). We therefore consider that this trait is fixed in the studied populations of *L. schismatica*.

T. brevistylis floral variants

T. brevistylis is considered a dioecious species (see male and female in Figures 2A and 2B, respectively) and SEM evidence show that normal male and female flowers do not have arrested organs of the opposite sex (Figure 6A-D). This has been further corroborated with sections and a detailed study of the developmental floral series of this species (B. A. Ambrose, S. Espinosa-Matías, F. Vergara-Silva and E. R. Alvarez-Buylla, in preparation). However, we found individual inflorescences that bear bisexual flowers with varying positions of stamens and carpels (Figures 7, 8, 9, 10 and 11). Pollen of normal appearance from dehiscent anthers (Figures 8D and 8F) and the cellular structure of both anthers and carpels at different stages of development seen both in scanning electron micrographs as well as in sections (Figs. 8 and 9) suggest that both male and female organs are functional in some of these flowers.

A few male-like flowers where the androphore is malformed at different degrees and with varying numbers of carpels at various positions are included in the aforementioned bisexual flowers (Figure 7 and 8C-F). Also, some individuals with female-like flowers bearing supernumerary heterotopic anthers were observed (Figure 8A-B). Of approximately 1000 flowers observed, only 1% were male and 0.5% were bisexual and falling into one of the categories of organ arrangement shown here.

Finally, *T. brevistylis* bisexual flowers with a floral arrangement that mimics that of wild-type hermaphroditic *L. schismatica* flowers were observed in two sectioned inflorescences and a single sample dissected and prepared for the SEM (Figures 10A-C and 11, respectively). In one case, carpels were apparently arrested on one side of the flower (Figure 10A) but remained functional in the other. The central anthers in this flower and in a second one (Figures 10B and 10C) bore seemingly functional pollen grains. Though collapsed, the appearance of the central stamens in the scanning electron micrograph of the inside-out *T. brevistylis* dissected floral bud resembles *L. schismatica* bisexual buds at comparable stages of development (compare Figure 11 to Figure 4A).

Discussion

Homeotic/heterotopic transformations of floral reproductive organs appeared at least before the divergence of the two Mexican triurid taxa

The finding of *L. schismatica* in 1987 and its subsequent description (Martínez and Ramos 1989) ended a period of decades during which the basic structural plan of flowers was thought to be invariably composed of a set of concentric whorls of organs in which the female one is located in the center of the meristem, without exception (Endress 1994 and 2001, Greyson 1994). *L. schismatica* flowers have a central androecium and a peripheral gynoecium (Márquez-Guzmán *et al.* 1989, Martínez and Ramos 1989). Until now, this floral arrangement had been considered an autopomorphy of this species in the context of the entire angiosperm clade. However, in this study we have shown that among the floral variants of another Mexican triurid species, *T. brevistylis*, inside-out flowers with central stamens are also found in contemporary populations.

Speculations regarding the origin of the natural floral homeotic phenotype of *L. schismatica* have already been published (Davidse and Martínez 1990; Stevens 1991). Of particular relevance to the data presented here is the hypothesis of Mabberley (1997), who postulated that *L. schismatica* could have been originated from mutations that occurred in already established members of Triuridaceae, specifically the genus *Triuris*.

We have proposed a refined hypothesis on the evolution of flower arrangement in *L. schismatica* (cited in Baum 1998, Vergara-Silva *et al.* 2000) based on the ABC model for the detemination of floral organ identity (Coen and Meyerowitz 1991). Since the ABC model predicts that stamen identity is determined by the combinatorial activities of the B- and C-function genes, while carpel identity is provided by the C-function alone, we consider that the simplest proximal explanation for the central position of the androecium in *L. schismatica* is a spatial displacement of B-function gene expression towards the center of the floral meristem. It is likely that the evolutionary changes in the expression patterns of homologs of the floral homeotic genes in *L. schismatica* are mediated by alterations in their *cis*-regulatory regions. Changes in these class of regulatory sequences, linked to modifications in the expression profiles of homeotic loci and other developmental genes, have been hypothesized to be among the main determinants of novel animal and plant morphological structures at different taxonomic levels (see, for example, Wray 1994, Carroll 1995, Baum 1998, Doebley and Lukens 1998).

It is not unconceivable, however, that other genes regulating activity of B-function homologs in *trans* could also be responsible for the postulated gene expression displacement. The morphology of some of the floral variants found in *T. brevistylis* already suggests either the involvement of other regulatory genes, besides MADS-box loci, or a more complex redeployment of ABC homolog transcription products than that implicit in our simple hypothesis. *In situ* RNA-RNA hybridization and *in situ* PCR experiments using sequence information corresponding to several MADS-box gene homologous sequences from both species are currently underway to explore these possibilities.

Dioecy seems to be the ancestral sex state among triurids

The distribution of sex states in the Mexican triurids and other members of the group is not compatible with observations in other angiosperms, in which dioecy seems to have evolved from hermaphroditism more than a hundred times (Ainsworth 2000, Weiblen *et al.* 2000). Phylogenetic analyses based on morphological matrices including fossil as well as extant taxa in the vicinity of the triurids (M. A. Gandolfo, personnal communication) currently supports the view that *Triuris* is the sister genus to *Lacandonia*. Character optimization on these reconstructions would imply that the ancestral character state for the reproductive system in Triuridaceae is dioecy and that hermaphroditism is apomorphic. The only other triurid taxon in which species with bisexual flowers occur is the genus *Sciaphila*. If correct, polarity for this character would be congruent with an additional hypothesis which states that the aforementioned molecular evolutionary events responsible for the origin of homeotic bisexual flowers in the triurid species studied here took place in populations of a dioecious ancestral species.

Microevolutionary hypotheses for the origin of *L. schismatica*

The floral variation found in contemporary natural populations of *L. schismatica* and *T. brevistylis* and their geographical distribution could lead to further speculation on the population-genetic aspects of the origin of floral homeotic/heterotopic phenotypes in these taxa. On this subject, we currently think that individuals with central stamens and surrounding carpels could have occurred in one or a few isolated populations of a *Triuris*-like ancestor, which after divergence evolved into the populations that are now recognized to constitute *L. schismatica* as a separate taxon. During the last glaciation period, the Quaternary Period in Mesoamerica (Toledo 1982 and references therein), approximately 5 million years ago, temperature at the lowlands was 6-8 °C lower than it is today in the Lacandon rainforest area. On the basis of these temperature differences and current population distribution, it is possible that the population(s) where the homeotic mutation had its origin remained isolated from their ancestors in a lowland refuge after the ice retreat. Ancestral *Triuris* populations could have concomitantly disappeared from the lowlands due to temperature increase and remained restricted to the higher lands where they are now found. The fact that the sites where the two Mexican triurids are now found share several plant species characteristic of high land-mesophyllous forests is also suggestive of this scenario. Analyses of molecular markers at the population level could be useful to test the above considerations, and to infer the role of population structure, breeding systems, drift and natural selection in the origin of *L. schismatica* from populations of *Triuris*-like ancestors.

The role of natural homeosis/heterotopy in the origin of novel morphological character states that define higher taxa

The phenomenon of homeosis is well known in plants and has been documented both before (Masters 1869, under the name of "metamorphosis") and after Bateson (1894) (see, for example, Sattler 1988, Meyerowitz *et al.* 1989 and references therein). Recently, spontaneous transformations in the identity of specific organs in flowers of several angiosperm families have been evaluated from the structural standpoint and considered as legitimate instances of homeosis (see, for example, Kirchoff 1991, Ford and Gottlieb 1992, Lehmann and Sattler 1992, 1993, 1994, 1996 and 1997, MacIntyre *et al.* 1996, Albert *et al.* 1998, Tucker 2000). In contrast with most of the previous examples, which usually involve reciprocal exchanges in the position of perianth organs (sepals, petals and tepals) and stamens, homeosis in members of the Araceae belonging mainly to the genera *Philodendron* (Barabé and Lacroix 1999 and 2000, Barabé *et al.* 2000) and *Montrichardia* (Barabé and Lacroix 2001) consist in the development of stamens and/or staminodes at places within individual flowers where carpels normally would arise in flowers that would otherwise be unisexual. These transformations, occurring in a few floral structures that are therefore bisexual and which comprose an intermediate zone in the spadix inflorescence characteristic of the genera, involve the same classes of organs interchanged in the triurid flowers with homeotic/heterotopic transformations. However, they cannot be considered cases of complete interchange of the developmental-genetic identity of the stamen and carpel whorls. In fact, homeotic transformations in bisexual flowers of *Philodendron* are always partial since they always occur in the same whorl, according to the morphological interpretation of the floral phenotypes by the authors. In accordance with them, we believe that since instances of homeosis involving the androecia and gynoecia are very rare

(see, for example, Barabé and Lacroix 2000a: 480), estimation of the homeotic floral phenotypes in the Mexican triurids as autapomorphic is still a correct cladistic assessment.
As concluded in the majority of the studies cited in the preceding paragraph, the spectrum of floral variants that we found in wild contemporary populations of both Mexican triurid taxa indicates that thorough field work that attempts to find natural variants is important to empirically support or discredit the role of homeotic/heterotopic phenotypes in the generation of plant morphological diversity. In our interpretation, the floral variants reported in this paper do not indicate that the wild-type floral phenotype in *L. schismatica* was attained through the accumulation of small morphological modifications in organ identity or through step-by-step transformations of either unisexual or hermaphroditic flowers with a regular arrangement of organs. First, the various types of bisexual *T. brevistylis* floral phenotypes reported here cannot be organized into a series of intercalary forms in which the wild-type floral phenotype in *L. schismatica* would occupy one of the extremes. Second, no intermediate arrangements of organs can be distinguished among the three different classes of floral phenotypes found in *L. schismatica*. To us, this morphological evidence indicates that development of different floral organs in the triurids behaves modularly and therefore allows the generation of discrete, non-gradual morphological variation. So far, the combinatorial nature of the floral ontogenetic specification mechanisms seemingly at work in model systems, and implicit in the ABC model, supports the possible occurrence of homeotic/heterotopic transformations in natural populations in which relevant molecular changes have taken place.

According to formal definitions of the concept of morphological innovation (see, for example, Müller and Wagner 1991), the floral organ arrangement observed in the Mexican triurids should not be included in this category of evolutionary changes, because homology at the level of organ identities is unequivocal. Instead, we consider that the discovery of these morphological apomorphies adds empirical evidence to arguments in favor of an important explanatory role for homeosis/heterotopy and related concepts –specifically, heterochrony and saltatory evolution (Sattler 1988 and 1994, Bateman and DiMichele 1994, Li and Johnston 2000, Weston 2000)- in the construction of scenarios on the evolution of morphological diversity in plants.

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Figure legends

Figure 1. Mature flower of *L. schismatica*. Three yellowish bilocular anthers, corresponding to the central stamens, can be distinguished. Scale bar = 0.5 cm.

Figure 2. Wild type *Triuris brevistylis*. (A) Male flowers with arrowheads pointing at stamens and androphores indicated (a). Note long caudate tepals. (B) Female flower with multiple carpels (c). Scale bars = 0.5 cm.

Figure 3. Distribution of *L. schismatica* and *T. brevistylis* populations in the Mayan rainforests of Mexico (Lacandon rainforest) and Guatemala. The inferred shape of the large water body that existed during the last glaciation period is shown in red. San José Canyon is a geological formation through which the water body was emptied at the end of the glaciation. Locations of the documented populations of *L. schismatica* and *T. brevistylis* are labeled L1-L5 and T1-T4, respectively. Population descriptions and their GPS coordinates are the following: L1 is a small population next to Jerusalén village, at N 16° 50-51', W 91° 08.5-09'; L2 is a small population SE of Carranza Lagoon, at N 16° 48.5, W 91° 9.5'; L3 is the type location, composed by small, patchy populations found inside and in the surroundings of the triangular area formed by points [N 16° 45.37,

W 91° 00.431], [N 16° 45.398, W 91° 00.476] and [N 16° 45.356, W 91° 00.611]; L4 is a small patch SE of Corozal Crossroad, at N 16° 43-43.5', W 90° 57.5-58.5'; L5 corresponds to a small area at N 16° 22-22.5', W 91° 12-13'; T1 (N 16° 58.192-58.209', W 91° 35.218-35.250') and T2 (N 16° 57.720-57.723, W 91° 35.730-35.736') are small, well preserved patches of forest that belong to the Lacandon community at Naha; T3 corresponds to a corridor NW of the Suspiro and Ocotal Lagoons, at approximately N 16° 51-53', W 91° 24-32'; and T4 is a location east of Chixoy River, Guatemala. Scale bar = 50 km.

Figure 4. Scanning electron micrographs showing variation in sex and organ number in *L. schismatica*. (A) Dissected preanthesis floral bud, showing carpels at an early developmental stage and three fully developed anthers in the center of the flower. Scale bar = 200 μm. (B) Mature flower, showing the papillose aspect of carpel epidermal cells surrounding three dehiscent anthers. Scale bar = 200 μm. (C) Dissected unisexual floral bud, showing two stamens and no carpels. Scale bar = 200 μm. (D) Inflorescence bearing two pistillate flowers, where stamens are replaced by additional carpels. Scale bar = 2 mm. (E) Dissected floral bud showing two central stamens and a diminished number of carpels, at a developmental stage comparable to (A). Scale bar = 200 μm. (F) Mature flower with four central stamens. Scale bar = 200 μm.

Figure 5. Optical microscope sections showing unisexual flowers in *L. schismatica*. (A) Male floral bud with two central stamens. Scale bar = 500 μm. (B, C) Details of the anthers of the previous bud, showing viable and inviable pollen grains, respectively. Scale bar = 50 μm. (D, E) Two different magnifications of a unisexual female flower, showing normal cells inside carpels. Scale bars = 500 μm and 50 μm, respectively. (F, G) Two different magnifications of a bisexual flower, showing degeneration of microspore mother cells. Scale bars = 100 μm and 50 μm, respectively.

Figure 6. SEM pictures of normal male and female flowers of *T. brevistylis*. (A) Dissected young bud of a male flower with five stamen anthers (an) in which the androphore is still undeveloped. No arrested female organs are observed. Scale bar = 100 μm. (B) Mature male flower with developed androphore (a) in the center and anthers (arrowheads) displaced towards the periphery of the flower. Scale bar = 500 μm. (C) Partially dissected female flower bud with sepals removed and carpels at different stages of development (c) observed inside. No arrested anthers are observed. Scale bar = 100 μm. (D) Mature open female flower with fully developed carpels (ca). Scale bar = 500 μm.

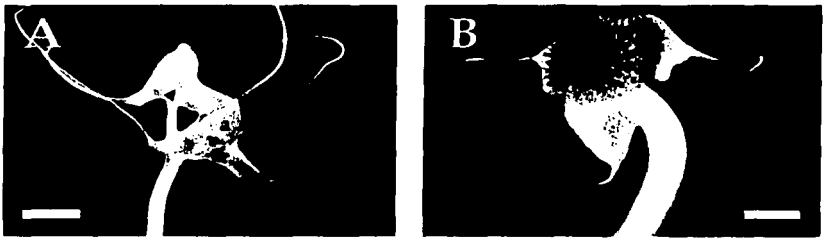
Figure 7. SEM pictures of bisexual flowers of *T. brevistylis*. (A) Male-like flower with androphore (a), stamens (arrowheads) on the right side of the flower and carpels (c) on the left one. Scale bar = 0.5 cm. (B) Enlargement of flower in A. Scale bar = 0.5 cm. (C) Female-like flower without androphore and intermingled anthers (arrowheads) and carpels (c). Scale bar = 1 cm. (D) Male-like flower with androphore (a), supernumerary stamens (arrowheads) and a few carpels (c). Scale bar = 1 cm.

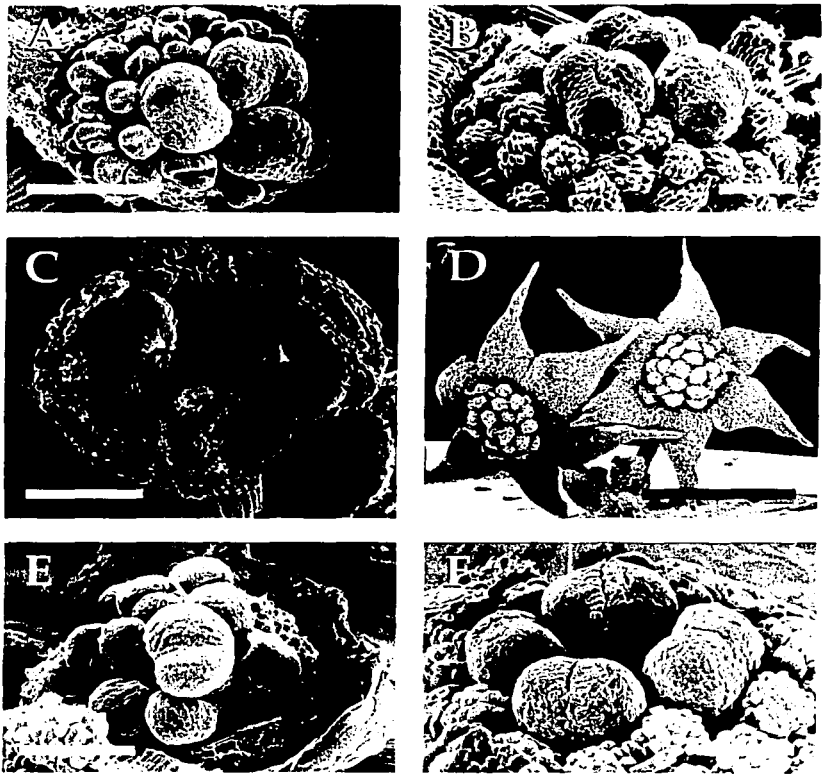
Figure 8. SEM pictures of bisexual flowers of *T. brevistylis*. (A) Female-like flower without androphore, bearing carpels (c) on the right side and anthers (arrowheads) on the left one. Scale bar = 500 μm. (B) Close-up of flower in A showing dehiscent anthers and normal-looking carpels. Scale bar = 100 μm. (C) Dissected male-like flower with androphore (a) bearing carpels (c) and one central anther (arrowhead). Scale bar = 500 μm. (D) Close-up of flower in C with anther on the right side (arrowhead) and inset showing mature pollen grains from the same anther. Scale bars = 100 μm and 5 μm (inset). (E) Male-like flower with modified androphore (a) bearing two anthers (arrowheads) and one carpel (c). Scale bar = 500 μm. (F) Close-up of flower in E with dehiscent anthers and inset showing mature pollen grain from the upper anther. Scale bars = 100 μm and 25 μm (inset).

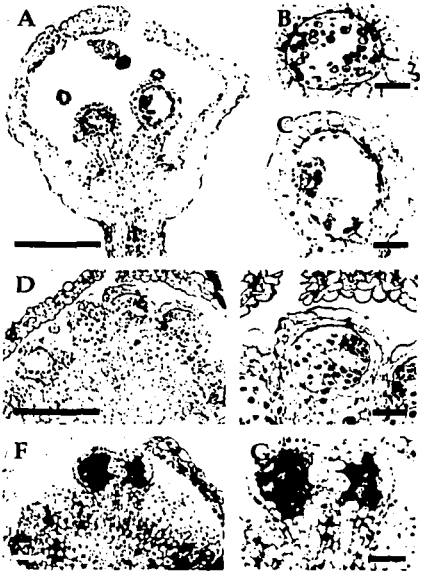
Figure 9. Sections of bisexual *T. brevistylis* variants. (A) Longitudinal section of a young flower bearing a carpel (left) and an anther (right). Scale bar = 100 μm. (B) Longitudinal section of flower with developing anther (left) and an embryo sac (right). Scale bar = 50 μm. (C) Longitudinal section of a flower bearing an anther embedded within the flower receptacle and several carpels at different developmental stages along the flower periphery. Scale bar = 100 μm. (D) Longitudinal section of a flower with an anther towards the center of the flower and a carpel towards the outer side, close to the tepal. Scale bar = 100 μm. (E) Longitudinal section of a flower with an anther surrounded by two developing carpels. Scale bar = 100 μm. (F) Longitudinal section of a flower showing one anther (arrowhead) and several carpels (c) at different stages of development. Note developed embryo sac (c). Scale bar = 100 μm. (G) Flower with many carpels and an embedded anther towards the right side (arrowhead). Scale bar = 250 μm.

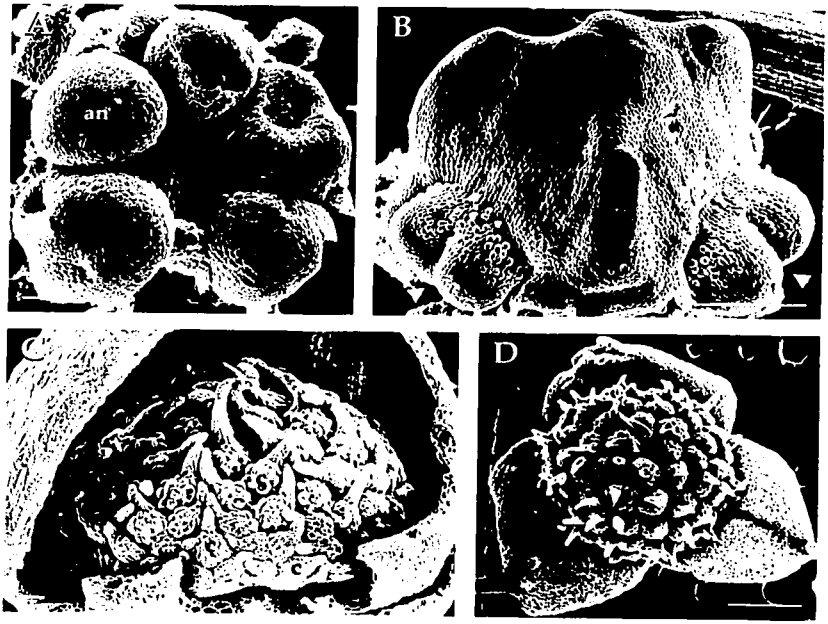
Figure 10. Sections of *T. brevistylis* bisexual flowers that mimic the arrangement of *L. schismatica* inside-out flowers. (A) Longitudinal section of an inside-out flower showing a central anther (arrowhead) and peripheral carpels. Inset shows a different serial section of the same anther (arrowhead). Scale bars = 200 μm. (B) Longitudinal section of a different inside-out flower showing a central anther with developing pollen grains inside and two peripheral carpels (c). Scale bar = 100 μm. (C) Close-up of a serial section of anther in B showing several germinating pollen grains with pollen tubes (arrowheads). Scale bar = 10 μm.

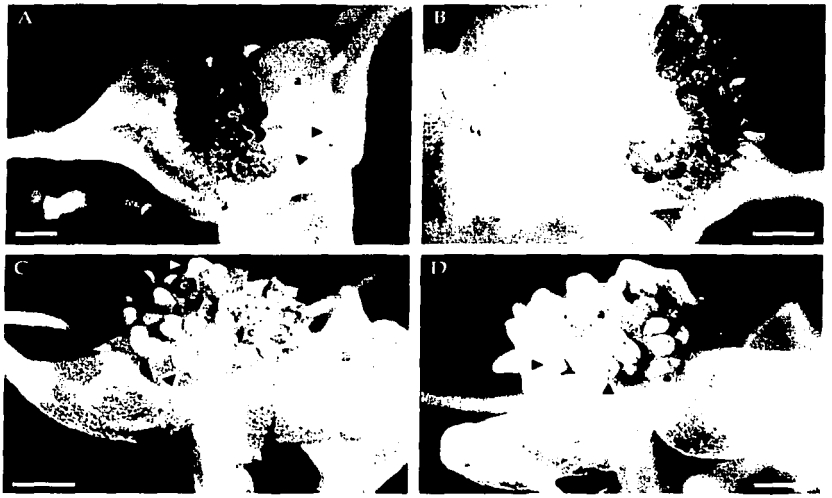
Figure 11. SEM picture of a *T. brevistylis* inside-out dissected floral bud. Three central partially collapsed anthers (an) and several carpels at different developmental stages (c) are observed. Scale bar = 100 μm.

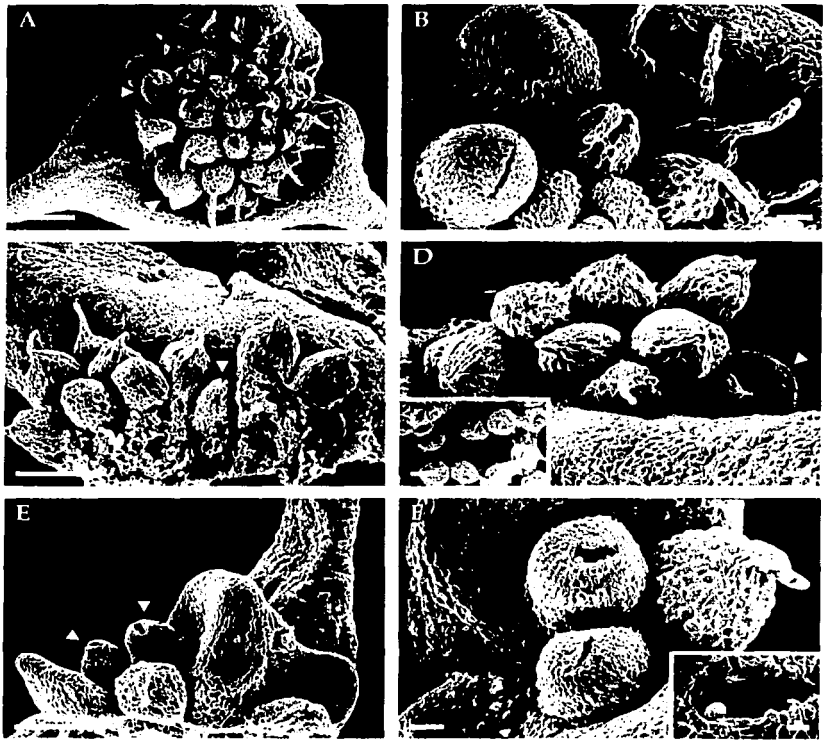


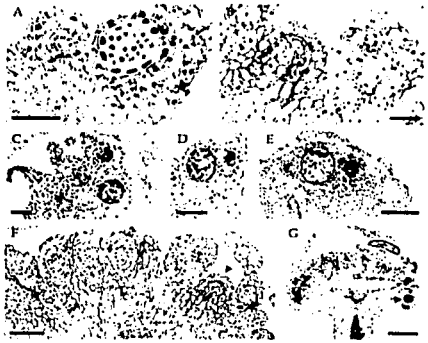












Capítulo 4: generalidades

Tal como se hizo implícito en las Generalidades de los Capítulos 2 y 3, uno de los objetivos principales del presente trabajo consistió en la búsqueda de secuencias homólogas por descendencia común (esto es, ortólogas) a los genes homeóticos florales caracterizados en los sistemas modelo. Las investigaciones sobre estos genes y su potencial importancia en la definición de los procesos ontogenéticos responsables de la variación fenotípica de las flores en las angiospermas, así como en otras estructuras morfológicas en otros grupos de plantas terrestres, ya han sido revisadas brevemente en el Capítulo 1; el artículo de revisión y mapeo filogenético incluído a continuación (publicado previamente en la revista *Journal of Phycology* 36: 803-812 (2000)) constituye una profundización en una serie de aspectos de particular relevancia para la discusión de los alcances de la evidencia genético-embriológica en la corroboración/refutación de hipótesis de homología entre órganos anatómicos en diferentes grupos de plantas. Esta revisión es particularmente adecuada como introducción a la sección adjunta de resultados experimentales ya que incluye, en el contexto de la discusión de las variaciones en los patrones de expresión de los genes homeóticos florales en las angiospermas, una mención explícita de la hipótesis mecanística más simple que puede ser postulada con base en el modelo ABC[1] para explicar la morfología floral de las flores bisexuales de *L. schismatica*. Según dicha hipótesis, planteada por E. R. Alvarez-Buylla y el autor desde 1995 (Vergara et al. 1997)[2], la homeosis/heterotopía floral observable en la mayor parte (ver Capítulo 3) de las inflorescencias que ocurren en las poblaciones naturales de esta triuridal mexicana se debe a un desplazamiento hacia el centro del meristemo floral en la expresión de los genes correspondientes a la función B –es decir, los ortólogos de los genes de *A. thaliana* bautizados como *APETALA3* (*AP3*) y *PISTILLATA* (*PI*)[3].

En virtud de los hallazgos sobre la variación floral existente tanto en *L. schismatica* como en *T. brevistylis*, discutidos en detalle en el artículo correspondiente al capítulo anterior (Vergara-Silva et al. 2002), es posible que los patrones de expresión de secuencias homólogas a los genes homeóticos florales en los diferentes morfos reproductivos de ambas especies incluya combinaciones no contempladas en la hipótesis simple mencionada arriba. Sin embargo, la dimensión microevolutiva proporcionada por la combinación de tales observaciones con la localización de las poblaciones naturales de ambos *taxa*, nos permite suponer actualmente que, en la(s) población(es) aislada(s) donde aparecieron los mutantes originales con la inversión de la posición del androceo y el gineceo, el cambio genético-molecular causalmente relacionado con dicho fenotipo floral se ajusta a una hipótesis mecanística basada en el modelo ABC.

Notas

1 El esquema abstracto para la explicación/predicción de los fenotipos florales homeóticos obtenidos mediante diferentes manipulaciones experimentales en dos importantes sistemas modelo en genética molecular de plantas, las dicotiledóneas *A. thaliana* (Brassicaceae: Magnoliopsida) y *Anthirrhinum majus* (Scrophulariaceae: Magnoliopsida), es conocido como "modelo ABC sobre la determinación de la identidad de los órganos florales". La referencia bibliográfica asociada tradicionalmente a la invención del modelo es el artículo de E. Coen y E. M. Meyerowitz publicado en *Nature* 353: 31-37 (1991). Sin embargo, es poco conocido que otros autores pertenecientes al grupo de CalTech estuvieron involucrados de manera crítica en su articulación durante al menos dos años previos a la publicación de este artículo (J. L. Bowman, comunicación personal). Los aspectos generales del modelo se discuten de modo suficientemente detallado en la sección "Los genes MADS-box y la evolución de las estructuras reproductivas de las plantas terrestres" del Capítulo 1, y en la introducción del artículo de *J Phycol*, parte del presente capítulo.

2 Durante 1995, E. R. Alvarez-Buylla y el autor –quienes previamente habían supuesto de manera independiente que un cambio en la expresión de los genes homeóticos florales podría ser responsable del fenotipo reproductivo de *L. schismatica*- se reunieron para discutir la posibilidad de realizar un proyecto de investigación dentro del cual pudiera ponerse a prueba la hipótesis mecanística correspondiente. El inicio de una colaboración con el laboratorio de E. M. Meyerowitz durante 1996, dirigida a realizar la clonación de secuencias de *L. schismatica* homólogas a los genes homeóticos florales, derivó en la asistencia al Simposio Keystone sobre "Evolución del Desarrollo en Plantas", realizado en Taos, Nuevo México, en enero de 1997 y en el cual se presentó, por primera vez en público, la hipótesis del desplazamiento de la función B. El reporte original de nuestra hipótesis puede ser confirmado en el artículo de David Baum publicado en *Current Opinion in Plant Biology* 1: 79-86 (1998).

3 La clonación de los genes *AP3* y *PI* fue lograda, respectivamente, por Thomas Jack y Koji Goto en el laboratorio de E. M. Meyerowitz en CalTech, y fue originalmente publicada en 1992 y 1994 (las citas correspondientes están incluidas en la bibliografía del Capítulo 1). En *A. thaliana*, la expresión de *AP3* se restringe a los verticilos 2 y 3, mientras que la de *PI* se extiende al verticilo 4 (Krizek y Meyerowitz 1996a). Debido a que su actividad biológica normal depende de la asociación heterodímérica de las proteínas

codificadas, la función B del modelo ABC se ubica unicamente en los verticilos donde la expresión de ambos genes –y por tanto, la presencia de las proteínas- coincide, es decir, los verticilos 2 y 3 (Krizek y Meyerowitz 1996b, Riechmann et al. 1996; ver la Figura 1B del articulo de *J Phycol*). Sin embargo, información recolectada recientemente en taxa más cercanos a las triuridales, como por ejemplo las monocotiledóneas *Sagittaria montevidensis* (Alismataceae; Kramer y Irish 2000) y *Lilium longiflorum* (Liliaceae; Tzeng y Yang 2001) indica que (a) la expresión de proteínas homólogas a AP3 y PI puede extenderse incluso al primero y cuarto verticilos florales, mientras que (b) homólogas de AP3 pueden formar homodímeros que dirigen, por sí mismos o en conjunto con las proteínas correspondientes a otras actividades dentro del modelo ABC, la diferenciación celular responsable de la determinación de la identidad de órganos como los estambres.y los carpelos. Las implicaciones de estas diferencias entre los patrones de expresión de los genes homeóticos florales en diferentes grupos taxonómicos dentro de las angiospermas son de particular interés para la interpretación de los resultados presentados más adelante en el presente capítulo.

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J. Phycol. 36, 803–812 (2000)

MINIREVIEW

MADS-BOX GENES: DEVELOPMENT AND EVOLUTION OF PLANT BODY PLANS[1]

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We review functional data on MADS-box genes, recent phylogenetic analyses of these coding regions, and their roles in the development and evolution of key morphological innovations in plants. We map the origin of important morphological structures in particular diverse stages of the life cycle in different plant clades onto organismal phylogenies, and present relevant molecular genetic aspects of development related to the MADS-box genes. We focus on reproductive structures of the sporophyte because most functional characterizations have been done of MADS-box genes involved in flower development. We discuss MADS-box evolution in flowering plants, but we also review studies in the other nonflowering vascular plants, gymnosperms (conifers and gnetales), and ferns and preliminary data from the algae. We suggest that floral (e.g. flowering time, inflorescence, and flower meristem identity) MADS-box and nonfloral plant MADS-box genes should be the focus of future comparative research. Cloning and functional analyses of MADS-box genes in bryophytes, particularly in the experimental system *Physcomitrella patens* (Hedw.) B.S.G., are needed. The ABC model of floral organ specification is an excellent general representation of an important network of genes; however, formal analytical tools are required to integrate data on complex gene interaction in comparative analyses. This and other analytical approaches to constructing gene network models will help to frame homology hypotheses in an evolutionary and developmental framework.

Key index words: **ABC model; character mapping; evolution of development; MADS-box; morphological evolution; phylogeny**

The group of evolutionarily related sequences that comprises most of the floral homeotic loci is the MADS-box multigene family (Shore and Sharrocks 1995). The acronym of the family name is derived from the initials of its first four described members (Norman et al. 1988, Jarvis et al. 1989, Sommer et al. 1990, Yanofsky et al. 1990), whereas "box" refers to a conserved sequence of approximately 180 nucleotides. In a fashion analogous to the homeobox (Gehring et al. 1994), the MADS-box encodes a DNA-binding domain that allows the products that contain it to behave as transcription factors. MADS-domain proteins have a second highly conserved segment with considerable similarity to the secondary structure of the animal cytoskeleton protein keratin (the K-domain); this segment seems to be involved in protein–protein interactions. The K-domain is connected to the MADS-box by an intermediate I region, moderately conserved among members characterized until now. Finally, the C-terminus, which encodes the putative transactivation domain, is poorly conserved among sequences (Riechmann and Meyerowitz 1997) (Fig. 1A). This is the structure of most plant MADS-box genes characterized up to now. However, recent studies have described a new group of plant MADS-box genes that lack the canonical MADS-I-K domains (MIK) structure of previously described plant genes (Alvarez-Buylla et al. 2000a). The latter are more closely related to the serum response factor (SRF)-like genes of animals than to the other plant MADS-box genes (Fig. 1A).

Molecular and genetic analyses of the mechanisms that control floral morphogenesis in *Arabidopsis thaliana* (L.) Heynh and *Antirrhinum majus* L. have provided one of the most elegant genetic models to date: the ABC model for specification of floral organ identity. In this model, the combined activities of a small number of loci are responsible for a complex phenotype through the orchestration of development (Coen and Meyerowitz 1991 and other references therein) (Fig. 1B). The main features of the model, well reviewed elsewhere (e.g. Lawton-Rauh et al. 2000), are summarized in three points. First, it comprises three partially overlapping fields of gene activity (A, B, and C; hence the name of the model) composed of genes that exclusively function in a particular domain but are not solely expressed there. Second, it defines and predicts organ identity on the basis of these combined activities. According to this idea, determination of the first floral whorl of sepals corresponds to the presence of A function alone. Similarly, petal organ specification results from the simultaneous participation of A and B functions, whereas stamens are determined from the sum of B and C functions. Finally, carpel or fourth whorl identity is specified by C function alone. A third feature of the model consists of a relationship of mutual antagonism between functions A and C, resulting in dominance of A when C is not present and vice versa.

Taking *A. thaliana* as a nomenclatural guide, the genes comprising the ABC model can be listed. All but one (*APETALA2*, an A-function gene) of the canonical ABC genes are members of the MADS-box gene fam-

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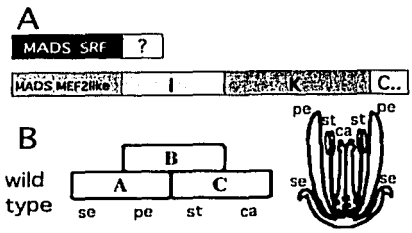

FIG. 1. (A) Schematic representation of plant MADS-domain proteins. Two types of MADS-domain proteins are found in plants: those that are more similar to the SRF-like genes from animals, called type I MADS-domain, and those more similar to the MEF2-like genes from animals, called Type II MADS-domains. Most of the latter proteins in plants have I, K, and COOH domains as shown in this figure (see text for more details). (B) The ABC combinatorial model proposes that the 4 different floral organs are determined by the specific combination of 3 different functions or activities. Activity A specifies sepals, activities A and B together specify petals, activities B and C specify stamens, and activity C alone specifies carpels. Additionally, the ABC model postulates a mutual inhibition between activities A and C, such that when function A is absent, function C takes its place and vice versa. To the right is a schematic representation of *Arabidopsis thaliana* flowers, which in wild-type plants are composed from the outside to the inside of 4 sepals (se), 4 petals (pe), 6 stamens (st), and 2 carpels (ca). The A-function genes are the MADS-box gene *APETALA1* (*AP1*) and the non-MADS-box gene *APETALA2* (*AP2*). The B-function genes are the MADS-box genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*). The C-function gene is the MADS-box gene *AGAMOUS* (*AG*). Recently, mutations in the *AGAMOUS*-like genes, *AGL2*, *AGL4*, and *AGL9*, have been identified and renamed *SEPALLATA1*, *2*, and *3* (Pelaz et al. 2000). The triple mutant of these genes yields a flower with sepals in all 4 whorls, but single and double mutants of these genes do not alter the flower phenotype. Hence, these genes are functionally redundant and necessary for the B and C function genes (see text for further details).

ily. *APETALA1* is the remaining A-function gene. The B-function genes are *APETALA3* and *PISTILLATA*, and *AGAMOUS* is the C-function gene. All MADS-box genes mentioned here are depicted in Figure 2. Similar molecular genetic studies in *A. majus* have demonstrated the functional conservation at the genetic and developmental levels between the ABC MADS-box genes of *A. thaliana* and *A. majus* (Irish and Yamamoto 1995). This conservation is also seen in other model systems—plants like maize or petunia—and, as mentioned below, the molecular conservation extends at least to the entire seed plant clade.

Recent experiments with *A. thaliana* document an alternative supernumerary activity that is an interesting addition to the ABC functions (Pelaz et al. 2000). Pelaz and collaborators found that 3 members of the MADS-box gene family related to *AGAMOUS-LIKE* 2 (*AGL2*), the genes *SEPALLATA1* (previously known as *AGL2*), *SEPALLATA2* (*AGL4*), and *SEPALLATA3* (*AGL9*) act

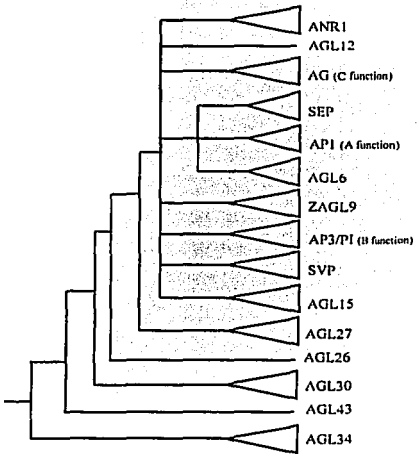

FIG. 2. Schematic representation of phylogenetic relationships among plant MADS-box genes. Groups of genes are represented by triangles and are named after *Arabidopsis thaliana* representatives except for ZAGL9 group. ANR1 group contains the *A. thaliana* genes *ANR1*, *AGL21*, *AGL17*, and *AGL16* as well as genes from *Antirrhinum*, *Medicago*, and *Gnetum*. AG group contains the *A. thaliana* genes *AGAMOUS*, *SHATTERPROOF1*, *SHATTERPROOF2* as well as genes from *Antirrhinum*, *Petunia*, *Nicotiana*, *Lycopersicon*, *Zea*, and *Oryza*. SEP group contains the *A. thaliana* genes *SEPALLATA1*, *SEPALLATA2*, *SEPALLATA3*, and *AGL3* as well as genes from *Petunia*, *Lycopersicon*, and *Pinus*. AP1 group contains the *A. thaliana* genes *APETALA1*, *CAULIFLOWER*, and *FRUITFULL* as well as genes from *Antirrhinum*, *Silene*, and *Zea*. AGL6 group contains the *A. thaliana* genes *AGL6* and *AGL13* as well as genes from *Zea*, *Pinus*, and *Picea*. ZAGL9 group contains only genes from *Zea* and *Arabidopsis*. AP3/PI group contains the *A. thaliana* genes *APETALA3* and *PISTILLATA* as well as genes from *Antirrhinum*, *Petunia*, *Medicago*, *Nicotiana*, *Syringa*, *Lycopersicon*, *Solanum*, *Silene*, *Argemoxiphuxym*, *Papaver*, *Brassica*, *Dicentra*, *Caltha*, *Ranunculus*, *Delphinium*, *Michelia*, *Liriodendron*, *Peperomia*, *Piper*, *Zea*, *Oryza*, *Gnetum*, and *Ceratopteris*. SVP group contains the *A. thaliana* genes *SHORT VEGETATIVE PHASE* and *AGL24* as well as genes from *Gnetum*. AGL15 group contains the *A. thaliana* genes *AGL15* and *AGL18* as well as genes from *Zea* and *Ceratopteris*. AGL27 group contains the *A. thaliana* genes *AGL27*, *AGL31*, and *FLOWERING LOCUS F* as well as genes from *Gnetum* and *Ceratopteris*. AGL30 group contains the *A. thaliana* genes *AGL23*, *AGL28*, *AGL29*, *AGL39*, *AGL40* as well as genes from *Gnetum*. AGL34 group contains the *A. thaliana* genes *AGL30*, *AGL33*, *AGL34*, *AGL35*, *AGL36*, *AGL37*, *AGL38*, *AGL41*, and *AGL43* as well as a gene from *Ceratopteris*. This figure is based on the combined results of two maximum parsimony analyses. Nodes with less than 50% bootstrap support have been collapsed. Clades whose members' spatiotemporal expression patterns are in agreement with ABC model functions are shown. (Modified from Alvarez-Buylla et al. [2000b] and L. Martinez-Castilla, F. Vergara-Silva and E. R. Alvarez-Buylla, UNAM [unpublished data]).

redundantly in the three inner whorls of the flowers to determine petal, stamen, and carpel identity. Mutations in these genes do not affect the mRNA expression patterns of the ABC MADS-box genes, but they interfere with B- and C-functions at the protein–protein interaction level. The proteins encoded by these genes form heterodimers with PI, AP3, and AG. These three genes could therefore be considered to constitute a D-function. This function is, however, not active in 2 but rather in 3 adjacent whorls. Because the combined gene effect of the *SEPALLATA* genes is also not defined at the transcriptional but at the protein level, this interesting function has not been widely accepted as an additional activity in the ABC model.

Functional analyses based on loss-of-function and gain-of-function phenotypes have been done for other MADS-box genes. These studies show that genes of this family are involved in diverse aspects of plant ontogeny in addition to flower development. Several MADS-box genes have been shown to be important in determining the identity and time of formation of inflorescence and floral meristems. *APETALA1, CAULIFLOWER,* and *FRUITFULL* redundantly control inflorescence architecture by altering the expression and activity of two non-MADS-box genes, *LEAFY* and *TERMINAL FLOWER,* that are involved in the transition from vegetative to reproductive development (Ferrándiz et al. 2000). *SUPPRESSION OF OVEREXPRESSION OF CONSTANS1* (*SOC1*, previously *AGL20*) is one of the targets of *CONSTANS,* which promotes flowering of *A. thaliana* in response to day length. This MADS-box gene is expressed in inflorescence meristems and then turns off in early flower meristems and comes on again later during flower development, suggesting that it might have additional roles not apparent from the single mutant (Samach et al. 2000).

Other MADS-box genes are involved in determining cell-type specification. This is the case of *SHATTERPROOF1* and *SHATTERPROOF2* that redundantly determine the proper development of the dehiscence zone of fruits (Liljegren et al. 2000). *FRUITFULL* is required for the normal pattern of cell division, expansion, and differentiation of the silique valves, for normal leaf development, and for normal inflorescence development (Gu et al. 1998). Several MADS-box genes have been shown to be involved in flowering time. *FLC* is very closely related to the *AGL27* and *AGL31* genes. They all have broad expression patterns and could share some functions, but *FLC* seems to have its own functions because the single *flc* loss-of-function mutant has a clear phenotype, suggesting that this gene is a flowering repressor (Michaels and Amasino 1999).

Several MADS-box genes are expressed predominantly or exclusively in roots, suggesting that these genes could also be involved in the morphogenesis of this plant organ. Notwithstanding the absence of mutant phenotypes that can be used to test the function of these genes in roots, cosuppression lines for one of the root genes, *ANR1*, suggests that this gene is important in controlling lateral root formation in response to local availability of nitrogen (Zhang and Forde 1998). Many other MADS-box genes have been cloned and their expression patterns characterized (Fig. 2) (Alvarez-Buylla et al. 2000b), but functional analyses based on mutant phenotypes and/or overexpression lines have not been published for them. Nonetheless, their expression patterns provide a first guide to the functional characterization of these genes and are a starting point for further studies of functional and character evolution.

PHYLOGENETIC AND MOLECULAR EVOLUTIONARY ANALYSES OF THE MADS-BOX GENE FAMILY

MADS-box genes are not restricted to plants. Phylogenetic analyses that include representative members of all eukaryotic MADS-box genes sampled suggest that the first MADS-box-containing sequence was present in the common ancestor of the three main multicellular eukaryotic groups (fungi, plants, animals). It is possible that it was present earlier in prokaryotic lineages (Mushegian and Koonin 1996) and hence in the earliest eukaryotes. In addition to flower development in plants, these roles include regulation of muscle development in mammals and insects (Martin et al. 1993, Affolter et al. 1994, Lilly et al. 1994) and arginine metabolism in yeast (Dubois and Messenguy 1991). Recent analyses show that a duplication of an ancestral MADS-box-containing sequence probably gave rise to two main lineages of MADS-box genes before animals and plants diverged. Type I MADS-box genes in plants are more similar to the animal and fungal SRF-like sequences and include a group of recently identified *A. thaliana* sequences (*AGL34* clade) (Alvarez-Buylla et al. 2000a). Type II MADS-box genes include most plant MADS-box genes previously identified and characterized and the MEF2-like genes of animals and fungi. Only plant members of this lineage encode a K-domain downstream of the MADS-domain.

Recent phylogenetic analyses of MADS-box sequences in *A. thaliana* (Alvarez-Buylla et al. 2000b) resolve 7 new MADS-box gene clades (Fig. 2). These add to those previously identified, the largely flower-specific genes that comprise the core of the ABC model (J. J. Doyle 1994, Purugganan et al. 1995, Theissen et al. 1996; see above). Among the newly identified groups, 3 monophyletic clades of genes almost exclusively expressed in roots and leaves (*ANR1* and *AGL12, AGL14*) and 2 clades of widely expressed genes (*AGL15* and *FLC*) have been resolved (Fig. 2). Additionally, there are other well-supported clades (*AGL23*-LIKE, and the plant SRF-like genes) for which no expression or functional data are yet available. Phylogenetic studies have also revealed the existence of new groups of closely related and possibly functionally redundant MADS-box sequences (Alvarez-Buylla et al. 2000b). These analyses could be useful to guide further functional characterization of these genes, as it is possible that only double, triple (see Pelaz et al. 2000), or even quadruple mutants of these closely related sequences may show phenotypes amenable to further analysis.

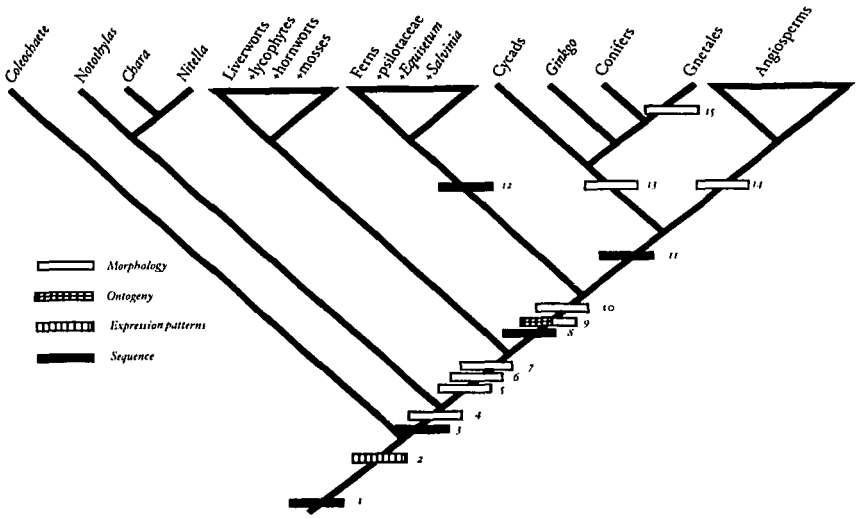

FIG. 3. Mapping of the evolutionary events leading to the establishment of the salient features of the plant body plan. Four main levels of homology defining events are depicted: appearance of protein families or subfamilies ("Sequence"), common spatiotemporal patterns of mRNA expression ("Expression patterns"), common embryological origins of plant modules and/or shifts in the relative prominence of alternating multicellular phases ("Ontogeny"), and shared morphological novelties ("Morphology"). 1, MADS-box genes; 2, Meristems; 3, MIK-type MADS-box genes; 4, Antheridia; 5, Multicellular sporophyte; 6, Sporangia; 7, Archegonia; 8, B and C classes of MIK MADS-box genes; 9, Ancestral reproductive structure of Tracheophyta (possibly, a product of B and C MADS-domain proteins acting in concert—C-class proteins would trigger the formation of either reproductive or nonreproductive structures, whereas the presence of B-class proteins would determine the sexual fate of these organs); 10, Passage from free-living or mycotrophic gametophyte to endosporic gametophyte and from dependent sporophyte to free-living sporophyte; 11, Class A of MIK MADS-box genes; 12, Non-ABC fern MADS-box genes; 13, Strobili; 14, Flowers; 15, Gnetalean "flowers." The morphological structures corresponding to 13, 14, and 15 are independent morphological elaborations based on a common genetic regulatory substrate—the ABC network. (Modified from Soltis et al. 1999.)

Arabidopsis thaliana and *A. majus* provide important models for the discovery of orthologous MADS-box sequences in a wide array of species within and outside the angiosperm clade. The successful cloning and characterization of homologous MADS-box genes in approximately 40 different plant genera have confirmed this idea. More than 30 of these taxa are angiosperms, whereas the remainder are nonflowering plants (Fig. 3). Among the latter group, *Chara* is an important taxon. Its MADS-box genes have a similar molecular structure to that of previously characterized MADS-box genes including a MADS, I, K, and COOH regions (Tanabe et al. 1999) as in Type II plant MADS-box genes. This is not surprising because phylogenetic analyses of the eukaryotic gene family suggest that both MIKC Type II and Type I (without K-box) genes should be found in green algae. This suggests that they can be used to make inferences about the molecular basis of morphological evolution in all plant groups.

All angiosperms studied to date possess members of the canonical MADS-box ABC gene subfamilies (Theissen et al. 2000). Homologous sequences of these genes have also been cloned from conifers. The search for MADS-box genes in this seed plant group started in the Norway spruce (*Picea abies* (L.) Karst., Pinaceae). In this work, 3 homologous genes were found, one of which—named *DEFICIENS-AGAMOUS-LIKE 2* (*DAL2*)—was identified as a member of the AG/PLE gene clade (Tandre et al. 1995, 1998). Additionally, the presence of B-function homologues has been reported in the same species (Sundström et al. 1999). These findings are supported by independent research on another spruce species (*P. mariana* (Mill.) B.S.P., the black spruce) (Rutledge et al.

1998) and less directly by results on *Pinus radiata* D. Don (Mouradov et al. 1998a,b).

Representative species from the Order Gnetales also possess MADS-box genes (Shindo et al. 1999, Winter et al. 1999). These studies have found genes orthologous to the ABC subfamilies and MADS-box genes that cluster elsewhere in the phylogenies (Fig. 2). Five of the 13 homologues found by Winter et al. (1999) are grouped with relatively high bootstrap support with at least one of the conifer MADS-box genes and excluded the corresponding angiosperm orthologue. Shindo et al. (1999) found that 2 of 4 genes clustered with the angiosperm AG/PLE and the DEF/GLO sequence clades, respectively (Fig. 2). These studies suggest that the ABC genes evolved before the gymnosperm/angiosperm divergence, consistent with the estimated antiquity of the main plant MADS-box gene subfamilies (Purugganan 1997). In addition, they provide conclusive evidence against the taxonomic assignment given to the Gnetales over the last 2 decades as the sister group of angiosperms (Frohlich and Meyerowitz 1997, Frohlich 1999, Winter et al. 1999, Frohlich and Parker 2000). The latter result corroborates studies of other molecular markers (Hasebe et al. 1992, Goremykin et al. 1996, Chaw et al. 1997, Bowe et al. 2000, Chaw et al. 2000).

MADS-box genes have also been cloned from the fern genera, *Ceratopteris* and *Ophioglossum*, which belong to the leptosporangiate and eusporangiate types, respectively (Münster et al. 1997, Hasebe et al. 1998), and the lycopods (Svensson et al. 2000). In contrast to the gymnosperm MADS-box genes, these sequences do not group with high bootstrap values with any of the ABC clades, suggesting that the fern and lycopod MADS-box genes characterized up to now are not orthologous to any of the seed plant ABC genes. As mentioned above, preliminary reports on MADS-box gene cloning from charophyte algae suggest that these organisms also possess MADS-box genes (Tanabe et al. 1999). Although this is not surprising, given that the MADS-box gene family has been found in all eukaryotes, algal MADS-box genes known to date have a similar status to the fern MADS-box genes in that they cluster outside the ABC gene clades.

The three A, B, and C lineages and others that are not flower specific form a monophyletic group (J. J. Doyle 1994, Purugganan et al. 1995, Alvarez-Buylla et al. 2000b). However, this basal monophyletic group is not well resolved, and the only structure that can be discerned at this level is the sister group relationship of *FLC*-like genes to the remaining sequences (Alvarez-Buylla et al. 2000a). Rapid evolution could explain the lack of resolution in the branching order of the different clades shown in Figure 2. This is an appealing ad-hoc hypothesis that should be tested with estimations of divergence times among gene clades and analyses of sequences from other species (e.g. Purugganan 1997).

The patterns of molecular evolution of the MADS-box gene family can be described by analyzing substitution rates (Nei and Gojobori 1986) of the different gene regions within and among clades. The MADS-box proper has on average the lowest overall substitution rate, and the K-box and C-terminal regions evolve at rates as high as 3 and 10 times that of the MADS-box, respectively. Analyses also suggest that the diversification of the K-box and C-terminal regions play a greater role in amino acid sequence divergence between plant MADS-box genes than the other regions and these regions appear to be under strong purifying selection. This is because their ratios of nonsynonymous to synonymous substitutions, though higher than that of the MADS-box, are still very much lower than 1, the ratio expected for sequences free of constraint (Purugganan et al. 1995). This suggests that the evolution of MADS-box floral homeotic gene lineages, which played potentially important roles in the evolution of plant form, did not occur in a neutral fashion.

Substitution rate analyses of *APETALA1*-related genes within angiosperms suggest different selection pressures for genes performing different roles in flower and inflorescence development (paralogous genes) but not for genes performing similar roles in different organisms (orthologous genes) (Lawton-Rauh et al. 1999). These conclusions are based on analyses of orthologues of the *A. thaliana* genes *AP1*, *CAL*, *FL*, and *AP3* from its sister species *A. lyrata*, the confamiliar *Brassica oleraceae* L., and the distantly related dicots *A. majus* and *Silene latifolia* Poiret. In these genes, no locus-by-lineage effects (Muse and Gaut 1997) were found, despite the considerable variation that these 5 species show in inflorescence and flower morphology. However, significant differences in the evolutionary dynamics of paralogous genes were found. For example, the *CAL* locus, which is only found in the Brassicaceae, seems to be evolving at a higher rate than the other paralogous genes, with an increase in the nonsynonymous substitution rate. Comparisons between the different domains of the genes also show differences in amino acid substitution rates, both among regions and paralogous loci.

A study in the well-known Hawaiian silversword alliance provides a very nice example of the possible coupling of MADS-box gene evolution and morphological diversification using *APETALA1* and *APETALA3* homologues. The ratios of nonsynonymous to synonymous substitutions in these genes for the Hawaiian silverswords were 3 times higher than the ratios observed for their ancestral species, the North American tarweeds (M. Barrier, M. D. Purugannan, NCSU; Robichaux, R. H., Univ. of Arizona, unpublished data). In fact, nearly 20% of the genes from the alliance members have substitution rates that are expected under adaptive selection. Additional tests have shown that the high levels of replacement substitutions are not due to an acceleration of the rate of neutral mutation in the island species (Barrier et al., unpublished data). Allozyme data suggest that structural protein evolution is not correspondingly high in the Hawaiian silverswords either (Witter and Carr 1988). Therefore, one possible hypothesis is that evolution at the studied regulatory MADS-box genes is responsible for the impressive morphological radiation observed among Hawaiian silverswords.

EVOLUTION OF PLANT MADS-BOX GENE FUNCTION

Mapping functional data of the plant MADS-box genes onto their corresponding gene trees allows the inference of different aspects about the evolutionary diversification of function within and among clades of the gene family. Previous studies had hypothesized that ancestral plant MADS-box genes were specific to vegetative structures and that selection for the evolution of specialized reproductive structures led to the high diversity of MADS-box genes approximately 450 million years ago, around the time of origin of the land plants (Purugganan 1997). However, more recent analyses made for all available data from *A. thaliana* suggest that basal genes, both in the global gene-family phylogeny and within each clade, have broader patterns of mRNA expression than more derived genes. These results suggest that the function of the plant MADS-box genes did not progress from vegetative to reproductive. Instead, it seems that differential and simultaneous gene recruitment was correlated with spatiotemporal restriction of expression pattern in both reproductive and vegetative structures as gene duplication events occurred (Alvarez-Buylla et al. 2000b) (Fig. 2). These latest results show that hypotheses related to the role of natural selection, which may explain the diversification of reproductive structures during early evolution of land plants, should also be applied to the diversification of vegetative structures.

As pointed out above, MADS-box genes that belong to different subfamilies outside the floral homeotic gene lineages have also been cloned from several angiosperm and nonangiosperm species (Fig. 2). If the occurrence of MADS-box genes in *A. thaliana* is a good guide, then homologues to several gene subfamilies have not been discovered yet in any other plant species. The analyses reviewed above will be important to interpret future findings of sequences homologous to MADS-box genes. Until now, only one monophyletic clade restricted to species other than *A. thaliana* has been found, the *ZAG1-9* clade limited to *Zea mays* L., but nothing has yet been published about these genes. Combining the expression data of MADS-box genes with the gene tree for all sequences characterized to date shows that monophyletic clades are formed and the genes within them share expression patterns of high overall similarity. However, more expression data are needed to perform formal analyses of ancestral expression patterns with gene families that include species other than *A. thaliana*.

ROLE OF MADS-BOX GENES IN PLANT BODY PLAN EVOLUTION

Data regarding the taxonomic distribution, phylogenetic relationships, expression patterns, and functional interactions of the plant MADS-box genes can be mapped onto an organismal phylogeny. Such an analysis, performed simultaneously with information on the ontogenetic and adult morphological innovations responsible for body plan diversity in the plant kingdom, is the ultimate objective of the recently emerged field of plant evolutionary developmental biology (Baum 1998). Investigating the molecular basis of morphological and developmental evolution in this way establishes a powerful approach to the problem of elucidating homology relationships among the entire spectrum of plant morphological structures. Although evolutionary and developmental character mapping exercises such as the one put forward in this work could be done for any cell, tissue, or structure type, our emphasis is on reproductive structures, because flower development has been most thoroughly studied in model systems.

Examples of the conservation of the ABC MADS-box gene regulatory network in angiosperms at the purely structural and expression pattern levels, along with mutant phenotypes, can be analyzed to corroborate or falsify homology relationship hypotheses among floral organs of species with contrasting morphologies. Flowering plants comprise more than a quarter million of species that exhibit an astounding diversity of ecological traits and interactions, as well as an apparently endless variation of floral morphological features. However, most share a stereotypical arrangement of floral organs (sepals, petals, stamens, and carpels). Perhaps the best example of the conservation of the ABC network and its use to assess homology relationships is provided by the study of Ambrose et al. (2000) on *Z. mays*. These authors established a correspondence between the highly derived floral organs of this monocot and those of the dicot model systems based on the analysis of the expression pattern of *SILKY1*, a B-function homologue and its mutants (Ambrose et al. 2000). The results of this study suggest that, despite their conspicuous differences, lodicules are modified petals and, possibly, palea and lemma are modified sepals.

Variations in the expression patterns of ABC genes can also be responsible for diversity in floral arrangements among angiosperms. For example, in the dioecious dicotyledon *Rumex acetosa* L. (sorrel; Polygonaceae), differential expression of RAP1, an AGAMOUS/PLENA (AG/PLE) homologue (Ainsworth et al. 1995), is responsible for sex determination. But the most outstanding flower morphological variation among angiosperms is the one found in the Mexican triurid, *Lacandonia schismatica* Martinez et Ramos (Martinez and Ramos 1989). This mycoheterotrophic monocot species is the only angiosperm with central stamens and carpels in the third whorl. The simplest hypothesis to explain this homeotic phenotype is to postulate a centripetal shift in the spatial domain of the B-function (Vergara et al. 1999).

In summary, comparative analyses of the ABC model have confirmed that it is a valid working hypothesis to consider the synergistic mode of floral organ determination as a synapomorphy of the angiosperms (Bowman 1997) (see the mapping of characters 9, 11, and 14 in Fig. 3). It can be concluded that the ABC model is an excellent guide for the interpretation of the molecular basis of homology among floral organs in angiosperms (Coen

and Meyerowitz 1991). Coding sequences of orthologous ABC genes are widely conserved among flowering plants, and most variations in flower arrangements can be explained in terms of changes in the expression domains of these genes, implying changes in their promoter sequences (Baum 1998).

As discussed above, studies of the occurrence of MADS-box genes successfully "went down" the species tree, resulting in the cloning and characterization of conifer MADS-box genes. Some of these genes cluster within the canonical ABC subfamilies (Fig. 2). The acceptance of an apparently natural seed plant group—implicit in the tree topology presented in Figure 3—has two inescapable consequences bearing directly on the interpretation of expression patterns of developmentally important genes in the gymnosperms, especially MADS-box genes. Given that one of the most recent estimations of the phylogenetic relationships between the Gnetales and other groups of gymnosperms actually considers them as derived conifers (Bowe et al. 2000), homology assessments among seed plant reproductive structures should be done and should be clarified first at this level. Thereafter, the common features found between conifers, Gnetales, angiosperms, and the yet to be described data in cycads and *Ginkgo* will allow the final estimation of the developmental genetic potential already present in the last common ancestor of the spermatophytes (seed plants).

Expression patterns of *DAL2*, that is, the *AG* ortholog from *Picea*, along with the phenotypes of *A. thaliana* transgenic plants overexpressing this conifer gene, suggest that this gene is the functional equivalent of the angiosperm C-function genes in reproductive organ determination (Tandre et al. 1998). In the compound female cone, this gene is exclusively expressed in the ovule-bearing scale. On the other hand, patterns of expression of B-function homologues in the same species (Sundström et al. 1999) suggest that pollen-bearing organ specification is also conserved between angiosperms and gymnosperms. Similar patterns have been found in *Gnetum*. In this gymnosperm, male reproductive structures only express these genes, whereas female reproductive axes have transcription products of both B-function and C-function homologues (Winter et al. 1999). Therefore, these authors have postulated that B-function homologues are the critical element in the developmental mechanism of sex determination in all seed plants. Clearly, this is an extraordinarily interesting conclusion because it defines a critical developmental character state of the last common ancestor of the seed plants but has yet to be tested in the cycads and *Ginkgo*.

Variations in reproductive organ molecular specification among gymnosperms would lead to possible differences in the estimation of ancestral character states in the last common ancestor of the seed plants; however, such variations are unlikely to be found. Therefore, the approach taken by Sündstrom et al. (1999), which consists of defining morphological homologues between gymnosperms and angiosperms in the most general level possible (e.g. microsporangia should be considered instead of stamens or pollen cones), seems appropriate. On the other hand, Winter et al. (1999) do not address directly homology issues between conifers and the Gnetales. In contrast, Shindo et al. (1999) suggested that the gnetalean ovule is the homologue of the conifer ovule-ovuliferous scale complex. This means that the latter structure alone is homologous to the outer envelope of the *Gnetum* ovule. Interestingly, they do so in the context of an independent derivation of this structure in each corresponding lineage from a Cordaitales-like ancestor. More work is needed to refine homology hypotheses among gymnosperm reproductive structures and between these and those of angiosperms.

Comparative evolutionary and developmental analyses in the seed plants are important because they might become the basis to elucidate the mystery of the origin of the flower, Darwin's "abominable mystery." Probably, the origin of both flowers and strobili is a consequence of ontogenetic divergence (Rieppel 1993) in the action of a progressively better defined regulatory genetic network that was already present in the last common ancestor of seed plants. According to this point of view, the first event can be conceived as the result of the constitution of a gene regulatory network that allowed the formation of structures with aggregation of sporophylls. This event most likely occurred after the divergence of the fern lineages from the seed plants. This view and the aforementioned fact that the Gnetales are part of the monophyletic gymnosperm clade, rather than a sister group to the angiosperms, justify a rejection of traditional transformational hypotheses (i.e. anthophyte and neopseudanthial) (J. A. Doyle 1994) on the origin of flowers. Neither of these hypotheses can be validated if the last common ancestor of angiosperms, conifers, and the Gnetales is actually the last common ancestor of all seed plants, because both of them assume that the angiosperm flower was transformationally derived from a particular gymnospermous reproductive morphology. The above observations are summarized in the mapping of characters 9, 11, 13, 14, and 15 in Figure 3, where we show that the establishment of the ABC MADS-box gene network is a requisite for the divergent elaboration of the characteristic reproductive structures of gymnosperms and angiosperms, strobili, and flowers, respectively. Furthermore, the morphological characters that in the past were the basis for the clustering of Gnetales and the angiosperms in the anthophyte clade actually reflect convergence on the basis of the same developmental genetic potential.

Molecular evolutionary estimations of the time of divergence among the floral homeotic gene subfamilies placed their putative ancestral sequence or sequences in the Ordovician Period 478 ± 24 million years ago (Purugannan 1997). In the species tree of Figure 3, this event falls into the node that defines the tracheophytes (ferns [gymnosperms + angiosperms]). However, as reviewed in previous sections, no estimation of the MADS-box gene genealogical relationships

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has shown robust clustering of the fern sequences with any of the ABC clades. Therefore, the reconstruction of the corresponding ancestral structures at the basal node of the vascular plant clade could not incorporate ABC gene evolution. In other words, the "developmental integration of characters" (Abouheif 1997) or "true homology" (Bolker and Raff 1996) scenarios, valid for reconstructing ancestral seed plant reproductive structures, could not be applied to reconstruct ancestral states of tracheophyte reproductive structures. According to this second scenario, the origin of fern sporangia should be correlated with the presence of MADS-box gene subfamilies that are apparently exclusive to the entire fern group (Fig. 3, mapping of character 12) (Theissen et al. 2000).

PERSPECTIVES AND CONCLUSIONS: EVOLUTION OF REGULATORY GENETIC NETWORKS

The genetic–developmental–evolutionary events analyzed above are salient episodes in the origin of diversity of body plans in extant land plants. All these events depend, in turn, on the previous appearance of molecular specification mechanisms for the ontogenetic formation of multicellular sporophytes (Fig. 3, character 5) and meristems with increasing degrees of complexity (Fig. 3, character 2). However, it is clear that such large-scale morphological innovations have involved complex regulatory gene networks in which genes from other families participate (e.g. Bowman and Eshed 2000).

Most comparative analyses have been done with floral genes. Flowering time, inflorescence, and flower meristem identity MADS-boxes should be further explored to analyze the molecular basis of inflorescence architecture evolution. However, pattern and functional data on nonfloral plant MADS-box genes will be useful for addressing questions of morphological and ontogenetic components of homology outside sporophytic reproductive structures (Figs. 2 and 3) (Alvarez-Buylla et al. 2000b). For example, genes expressed in the seed plant gametophytes (Alvarez-Buylla et al. 2000b) will likely be useful to guide the cloning and characterization of homologues in plant groups outside the tracheophytes and to clarify the molecular basis of antheridial and archegonial evolution (mapped as characters 4 and 7 in Fig. 3). Other nonfloral MADS-box genes will probably be useful for studying other aspects of body plan evolution in species with less well-characterized MADS-box gene sets. Cloning and functional analyses of MADS-box genes in bryophytes, particularly in the experimental system, *Physcomitrella patens*, will certainly shed important insights into morphological evolution in plant groups from algae, bryophytes, and tracheophytes.

The ABC model of floral organ specification is an excellent general representation of a particularly important network of genes, because members of it have been found in every angiosperm species in which they have been looked for, playing indispensable homologous roles in the determination of organ identity. However, an ultimate goal, although still far from being achieved, should be to incorporate gene network mapping into the evolutionary and developmental approach described here and to identify critical interactions at the transcriptional and postranscriptional levels responsible for morphological innovations. To this end, formal analytical tools will have to be used to integrate complex gene interaction information. Preliminary trials for flower and root genes are available (Mendoza and Alvarez-Buylla 1998, Mendoza et al. 1999). This and other analytical approaches to constructing gene network models should help to develop homology hypotheses in an evolutionary and developmental framework.

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Resultados experimentales

Los genes homeóticos florales de las triuridales mexicanas: clonación y caracterización estructural, relaciones filogenéticas y patrones de expresión

A. Clonación y caracterización estructural de cDNAs correspondientes a los genes homeóticos florales de *L. schismatica* y *T. brevistylis*

La búsqueda de secuencias homólogas a los genes homeóticos florales encaminada a la puesta a prueba de la hipótesis mecanística planteada en este capítulo para explicar el fenotipo floral de *L. schismatica* comenzó con el monitoreo de bibliotecas genómicas (genomic library screening). Estas bibliotecas fueron construidas a partir de DNA genómico extraído de inflorescencias de *L. schismatica* colectadas directamente por nuestro grupo de investigación en la localidad tipo.(Martínez y Ramos 1989, Vergara-Silva et al. 2002). Las bibliotecas se construyeron de acuerdo con las especificaciones del sistema de empaquetamiento Packagene® Lambda DNA (Promega, Madison, WI) y fueron monitoreadas de acuerdo con las técnicas convencionales descritas en Sambrook *et al.* (1989) y protocolos establecidos en el laboratorio de E. M. Meyerowitz usando fragmentos de los cDNAs correspondientes a los genes *AP3* y *PI* como sondas de hibridización marcadas radiactivamente durante los experimentos. A pesar de que las sondas empleadas carecían de la caja MADS, lo cual en principio disminuía notablemente la posibilidad de identificar clonas no deseadas, el uso de esta estrategia experimental proporcionó varios falsos positivos pero ninguna clona verdaderamente homóloga a ninguno de los genes homeóticos florales buscados. Esta estrategia experimental fue desechada a partir de 1999.

La segunda aproximación experimental consistió en la amplificación de fragmentos, mediante aplicaciones especiales de la reacción en cadena de la polimerasa (PCR), que correspondieran a los mRNAs de los genes buscados. En estos experimentos se emplearon preparaciones de RNA total proveniente de inflorescencias de *L. schismatica* y *T. brevistylis*, extraídas con el reactivo TRI® (Molecular Research Center, Cincinnati, OH) para ser usadas como templados en reacciones de amplificación de extremos de cDNAs (RACE) tanto para los extremos 3′ como los 5′ de secuencias selectas.

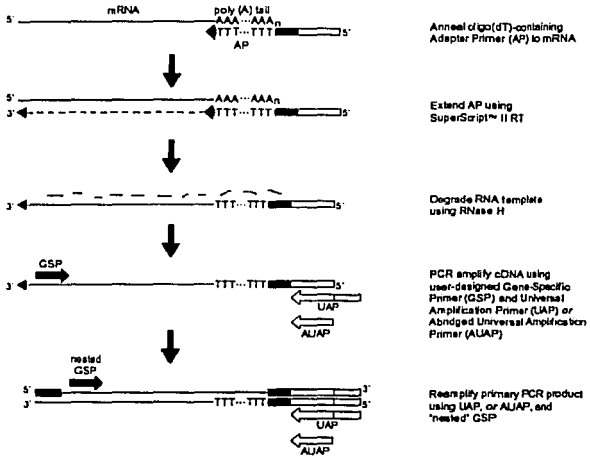

Figura 1. Resumen del procedimiento experimental correspondiente al sistema de amplificación rápida de extremos 3′ de cDNAs (3′ RACE). Para la amplificación de los fragmentos correspondientes a secuencias

homólogas a los genes *AP3* y *PI* en *L. schismatica* y *T. brevistylis*, así como fragmentos correspondientes a homólogos de *AP1* y *AG* en *L. schismatica*, se emplearon como GSPs los oligonucleótidos DEFAPzero (5′ TGA CCT ACT CCA AGC GCC 3′), DEFAP1 (5′ AGA T(CT)A AG(CA) GGA T(CA)G A(GA)A AC 3′), DEFAP2 (5′ AAC CGC CA(GA) GTG AC(CA) TA(CT) TC 3′) y PIGL1 (5′ GAG ATC AAG AGG ATC GAG AAC). Todos ellos se asocian a las correspondientes cajas MADS-box en diferentes posiciones nucleotídicas que abarcan a los codones que codifican los aminoácidos 7-23.

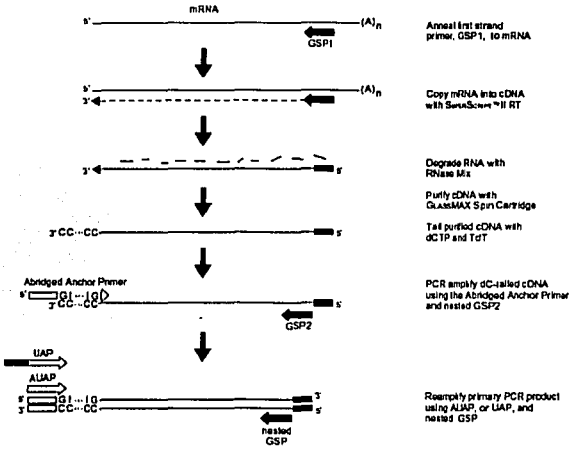

Figura 2. Resumen del procedimiento experimental correspondiente al sistema de amplificación rápida de extremos 5′ de cDNAs (5′ RACE). Para la amplificación de los fragmentos correspondientes a secuencias homólogas a los genes *AP3* y *PI* en *L. schismatica*, complementarios a los obtenidos con el protocolo de 3′ RACE, se emplearon como GSPs oligonucleótidos específicos diseñados en base a las secuencias obtenidas por 3′ RACE.

La realización de estos experimentos supuso, adicionalmente, el diseño de oligonucleótidos semiespecíficos –para los experimentos de 3′ RACE- basados en secuencias conocidas de genes homeóticos florales, así como la síntesis de oligonucleótidos específicos –para los experimentos de 5′ RACE- basados en las secuencias previamente obtenidas donde el extremo carboxilo terminal ya hubiera sido conocido. Las Figuras 1 y 2 resumen el procedimiento experimental involucrado en estos experimentos, e indican las secuencias de los oligonucleótidos usados como GSPs (gene specific primers). Los productos de las primeras amplificaciones que resultaron ser correctos fueron identificados mediante la técnica de Southern blot empleando, nuevamente, fragmentos marcados radiactivamente de los cDNAs de *AP3* y *PI* excepto sus cajas MADS; posteriormente, los fragmentos que resultaron ser positivos se identificaron por tamaño esperado al ser observados tanto en el gel de electroforesis de los productos de PCR como en las digestiones de los productos clonados. En todos los casos, los productos se clonaron dentro de vectores pGEM-T o pGEM-T Easy (Promega, Madison, WI) siguiendo las especificaciones del fabricante.

Debido a que los genes de interés fundamental para los propósitos del estudio son los ortólogos de *AP3* y *PI* en *L. schismatica*, el trabajo de obtención de secuencias completas mediante el uso conjunto de los protocolos de 3′y 5′ RACE se limitó a dichos genes. El montaje completo de estas secuencias y su alineación con el resto de las secuencias parciales de los otros homólogos de genes MADS-box obtenidos en esta especie, así como en *T. brevistylis*, fue realizado con la función de alineación tipo ClustalW incorporada en el programa MacVector™ (versión 7; Oxford Molecular/Accelrys, San Diego, CA).

En la alineación, los cDNAs ortólogos a los correspondientes a los genes *AP3* y *PI* de *L. schismatica* son denominados *LscAP3* y *LscPI*, respectivamente; los cDNAs equivalentes en *T. brevistylis*, aún incompletos por unos cuantos nucleótidos en el extremo 5′ se denominan consecuentemente *fragTbrAP3* y *fragTbrPI*. Finalmente, la alineación incluye dos fragmentos de cDNA adicionales, provenientes de *L. schismatica*, que se consideran ortólogos putativos de *API* y *AG*, pero cuya identidad no quedó demostrada de manera inequívoca después de una inspección visual de las secuencias alineadas junto con dichos genes provenientes de *A. thaliana*. Estas secuencias parciales aparecen bajo los nombres *fragputLscAG* y *fragputLscAG*, respectivamente.

B. Relaciones filogenéticas existentes entre las secuencias caracterizadas y otros genes homeóticos florales pertenecientes a otras especies de angiospermas

La pertenencia de los cDNAs encontrados a grupos específicos de genes (ver Figura 2 del artículo de *J Phycol*) dentro de la familia MADS-box fue estimada mediante el uso de las herramientas de alineación y búsqueda de similitudes de secuencia disponibles en la página web del National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), en especial la herramienta BLAST® (Basic Local Alignment Search Tool; Altschul et al. 1990). Con objeto de estimar con mayor precisión las relaciones de genealogía entre las secuencias de genes MADS-box obtenidas mediante los métodos arriba descritos y otros miembros de la familia de genes con cajas MADS, los cDNAs de las triuridales mexicanas fueron alineados a un grupo selecto de secuencias nucleotídicas provenientes de *A. thaliana* que representan la mayoría de las subfamilias identificadas en la Figura 2 de la contribución anteriormente publicada en *J Phycol* (ver alineaciones anexas). La matriz resultante fue entonces sometida a la herramienta de análisis filogenético por neighbor-joining (asociación de vecinos; Saitou y Nei 1987), un algoritmo no cladístico, verificacionista, implementado también en MacVector™ 7. De acuerdo con las simulaciones de Tateno *et al.* (1993), el método de neighbor-joining proporciona, en la menor cantidad de tiempo computacional, resultados consistentes con los de los métodos cladísticos convencionales y los obtenidos mediante algoritmos de máxima verosimilitud (maximim likelihood), especialmente cuando el porcentaje de divergencia entre las secuencias es pequeño. Adicionalmente, este método fue seleccionado para propósitos de asignación de homología a nivel se secuencias completas porque, a diferencia de las matrices analizadas con métodos cladísticos en el contexto del Capítulo 2, la matriz molecular aquí manipulada no involucra los delicados problemas de codificación de estados de caracter que se presentan al trabajar con morfología.

Como puede observarse en el árbol guía proveniente de las alineaciones múltiples, los cDNAs de las triuridales mexicanas se agrupan en las ramas esperadas, a excepción de los homólogos de *AP3* (ver árbol anexo). Sin embargo, **tanto los análisis de neighbor joining que eligieron el mejor arbol como los que involucraron pruebas de soporte estadístico por sustitución con reemplazo (bootstrap; ver reconstrucciones anexas), recuperaron, en todos los casos, la agrupación de los cDNAs de *L. schismatica* y *T. brevistylis* con el cDNA correspondiente de *A thaliana*.** Los resultados de estas reconstrucciones proporcionan fundamentos suficientes para realizar análisis con matrices más grandes compuestas por éstas y otras secuencias que puedan obtenerse en el futuro, con objeto de identificar apomorfías moleculares que eventualmente puedan ser relacionadas con características funcionales particulares para los genes homeóticos florales de las triuridales.

C. Análisis preliminar de los patrones de expresión de los genes homeóticos florales en diferentes estados de desarrollo ontogenético de los meristemos reproductivos en *L. schismatica*

A partir de las secuencias de *LscAP3* y *LscPI*, se generaron subclonas para su utilización en experimentos de transcripción *in vitro* acoplados a la realización de hibridizaciones de ácidos nucleicos *in situ*, sobre cortes de tejido de *L. schismatica* montados en laminillas para microscopía óptica. Los cortes incluyeron estados tempranos del desarrollo de los meristemos reproductivos. La realización de los cortes supuso la utilización de algunas de las muestras colectadas en el campo (ver Capítulos 2 y 3 de la presente tesis) y la conservación de las mismas en fijadores adecuados –principalmente se emplearon FAA y paraformaldehído- de acuerdo con protocolos establecidos. El protocolo específico empleado durante los experimentos es el implementado en el laboratorio de E. M. Meyerowitz.

Los resultados preliminares obtenidos para la subclona de *LscAP3* (que abarca desde el nucleótido inmediatamente posterior al final de la caja MADS hasta 124 nucleótidos antes del inicio de la cola de poliadenosina en el cDNA completo) **evidencian que el mRNA correspondiente se expresa únicamente en los estambres cuando los botones florales ya están desarrollados, pero que en estructuras más jóvenes tienen un patrón de expresión que abarca todas las células que presumiblemente están involucradas en la diferenciación del receptáculo entero** (esto es, carpelos y estambres) **y los tépalos** (ver Figura 3).

Aparentemente, en ninguno de los dos estadios mostrados en la figura parece existir expresión alguna de este mensajero en brácteas.

Debido a que hasta el momento sólo se ha encontrado un patrón de expresión confiable para el mRNA al que se complementa el producto de transcripción *in vitro* de esta subclona, la corroboración o refutación definitivas de la hipótesis genético-molecular planteada en el presente trabajo para explicar el fenotipo floral homeótico de *L. schismatica* no es posible aún. Por sí solo, sin embargo, este patrón sugiere que, al menos para uno de los genes correspondientes a la función B del modelo ABC en esta especie, sí existe un desplazamiento en el patron de expresión hacia el centro del meristemo reproductivo, al menos en ciertas etapas del desarrollo floral. En virtud de que se ha establecido que (a) en sistemas modelo como *A. thaliana*, la función B depende de la expresión simultánea de *AP3* (o su ortólogo) y de *PI* (o su ortólogo) y de la formación de heterodímeros entre las proteínas codificadas (Riechmann et al. 1996), mientras que (b) en monocotiledóneas, las proteínas codificadas por los genes de la función B pueden formar homodímeros y ser factores de transcripción activos como tales (Tzeng y Yang 2001, Winter *et al.* 2002), es posible postular que (c) aún cuando el mRNA correspondiente a *LscPI* no se tradujese en cantidades detectables -de hecho, ésta es una de las conclusiones que pueden derivarse de los resultados negativos en los experimentos de hibridización *in situ* con la subclona correspondiente- en los primordios de estambres (d) la presencia solitaria de homodímeros de LscAP3 en el centro de los meristemos florales de *L. schismatica* puede ser el mecanismo molecular responsable de la diferenciación de estambres en dicha posición, junto con la esperada expresión del ortólogo de *AG* en las mismas células en diferenciación.

A partir de numerosas investigaciones en sistemas modelo y organismos selectos (ver, por ejemplo, Carroll et al. 2001), en particular en plantas (ver, por ejemplo, Baum 1998, Doebley y Lukens 1998), actualmente existe una visión de consenso en la comunidad interesada en biología evolutiva del desarrollo según la cual las sustituciones en las regiones *cis*-regulatorias de los genes que codifican para factores de transcripción constituyen el mecanismo de evolución molecular más importante en la determinación de los patrones de evolución morfológica observados en la escala de taxa superiores. Es altamente probable que los patrones de expresión implícitos en la hipótesis mecanística de trabajo planteada en el presente capítulo dependan, por lo tanto, de cambios directos en los promotores de *LsAP3* (y tal vez también de *LsPI*), o bien en variaciones en la afinidad de asociación en las proteínas que a su vez se unen a estas regiones de DNA para activar la transcripción. Sin embargo, la participación de otros genes no contemplados en dicha hipótesis podría resultar de importancia en el futuro. Uno de los casos a considerar en este sentido es sin duda *SUPERMAN* (*SUP*, Bowman *et al.* 1992; Sakai *et al.* 1995), un gen de *A. thaliana* involucrado en la preservación de la frontera entre las células que pertenecen a los verticilos 3 y 4 (correspondientes a los estambres y carpelos, respectivamente, en las flores de dicha especie). Homólogos de *SUP*, así como elementos conservados en los correspondientes mecanismos de acción, ya han sido encontrados en el arroz, una monocotiledónea (*Oryza*: Poaceae; Nandi *et al.* 2000). La participación –o inclusive, su ausencia o bien la disrupción de su función, en términos comparativos- de un homólogo de *SUP* en la determinación de la morfología floral de las triuridales mexicanas parece ser sugerida también por la variación en los números de órganos y los arreglos de órganos en sí mismos en algunos de los morfos florales reportados en el trabajo presentado en el Capítulo 3. Un gran número de artículos publicados en los últimos años apoyan que *SUP* y homólogos del mismo gen en otras especies controlan la proliferación celular en estambres y carpelos dentro de una red compleja de interacciones genéticas que tiene, entre sus elementos componentes, a los promotores de *AP3* y *PI* (ver, por ejemplo, Kater *et al.* 2000, Sakai *et al.* 2000 y Zhao *et al.* 2001).

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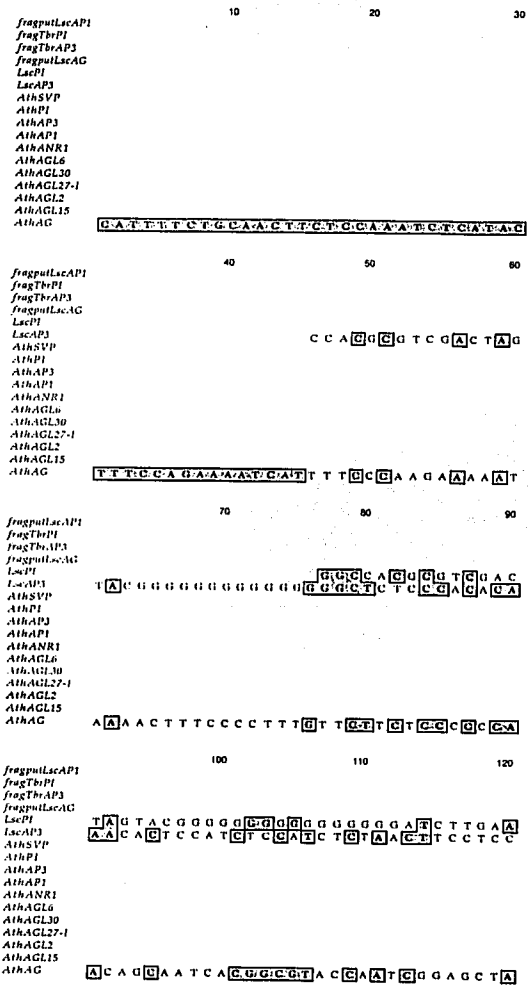

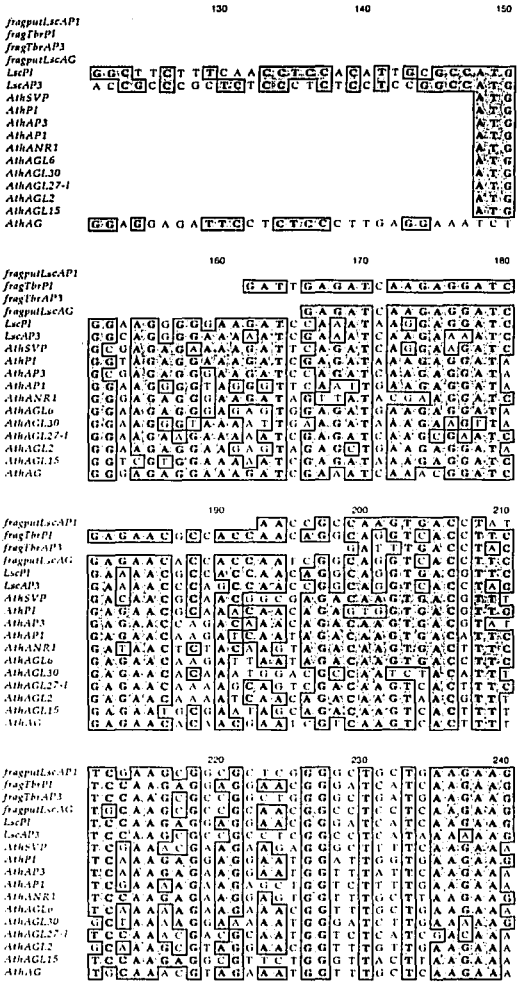

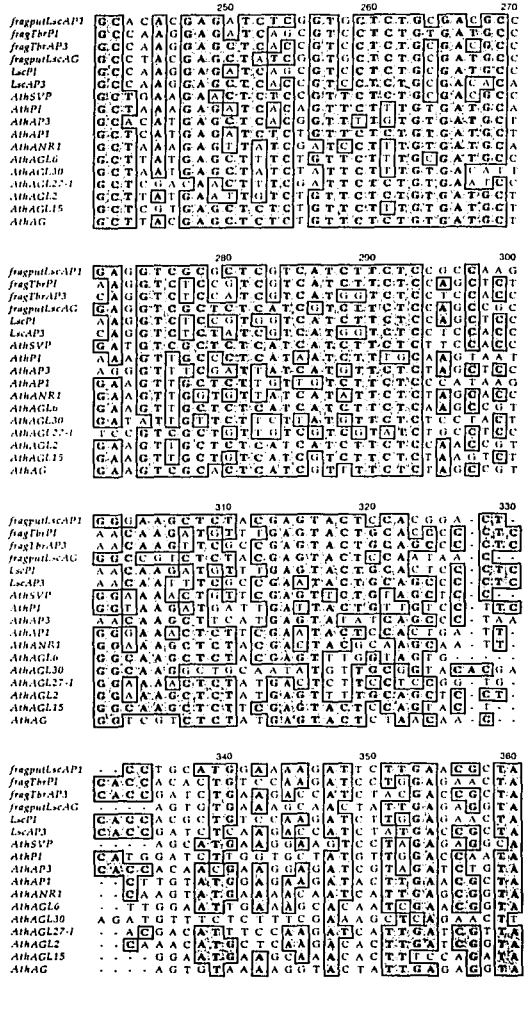
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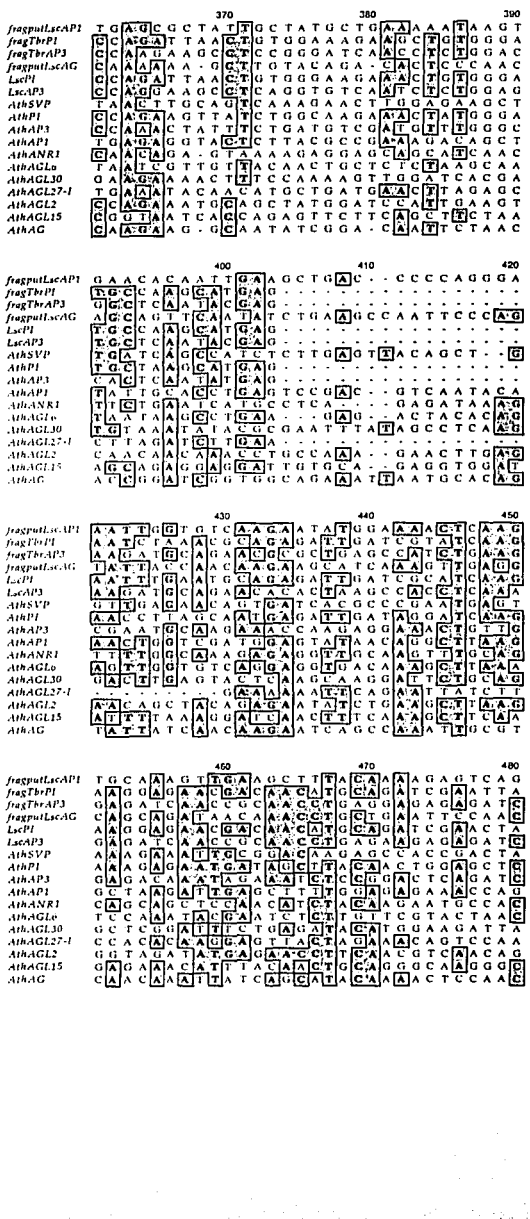
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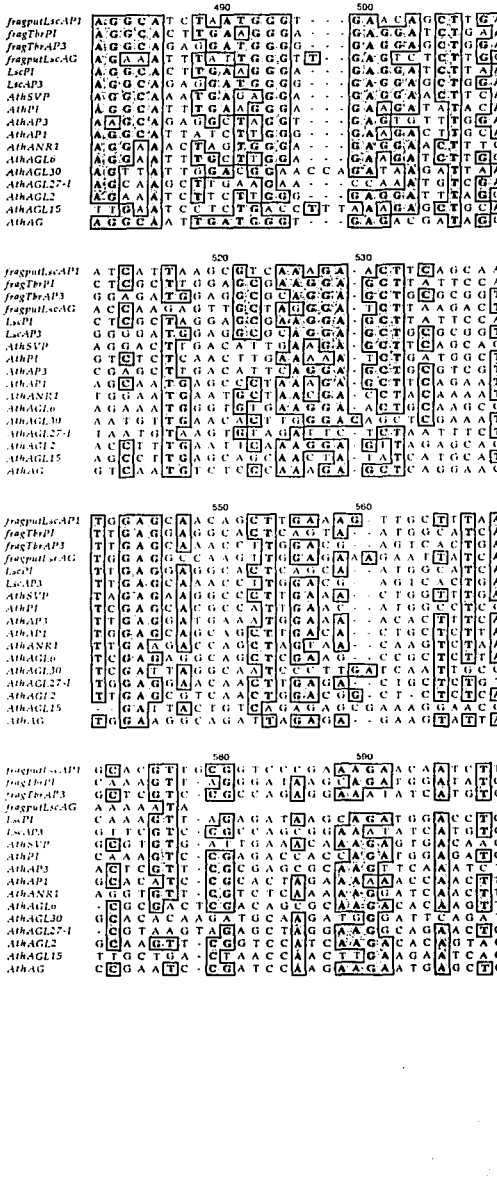
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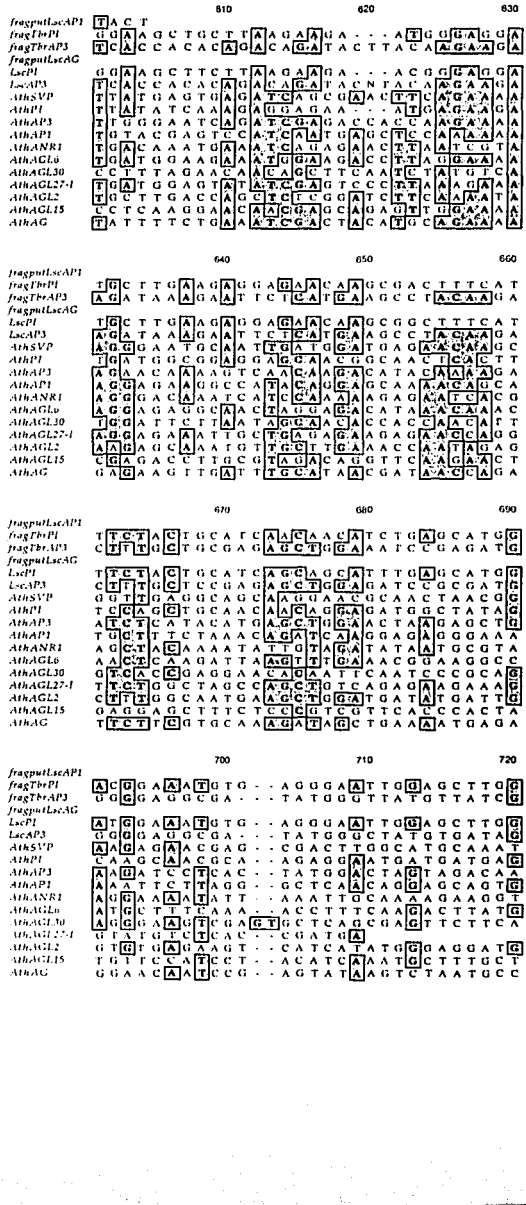
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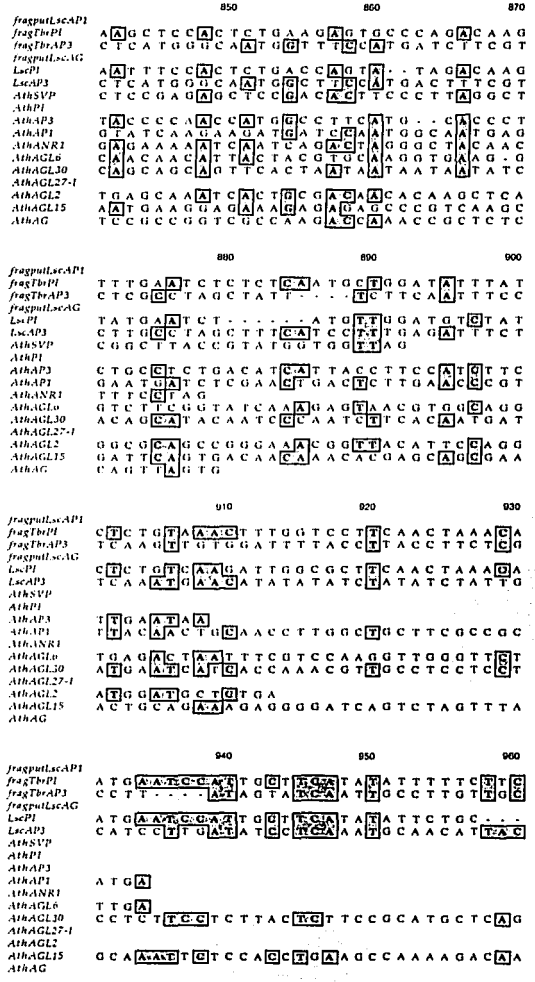

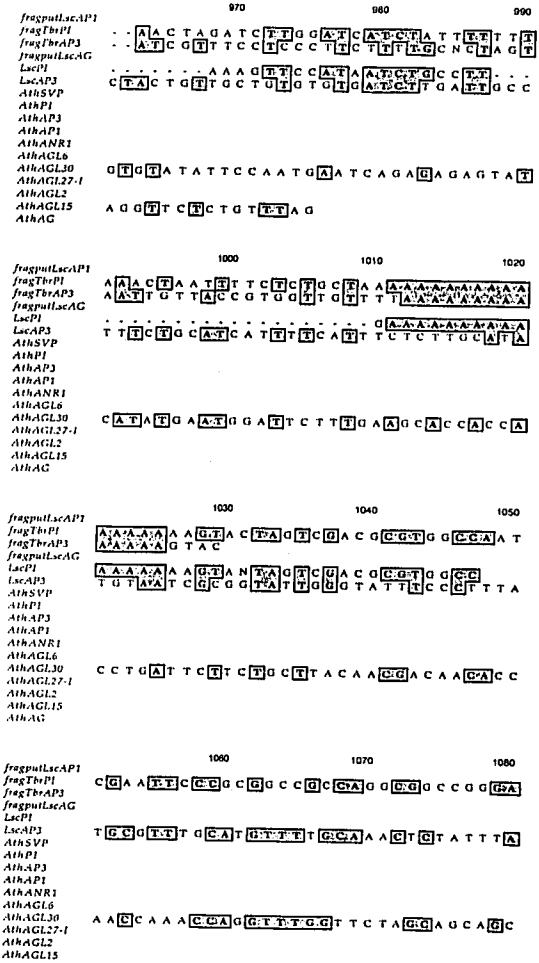

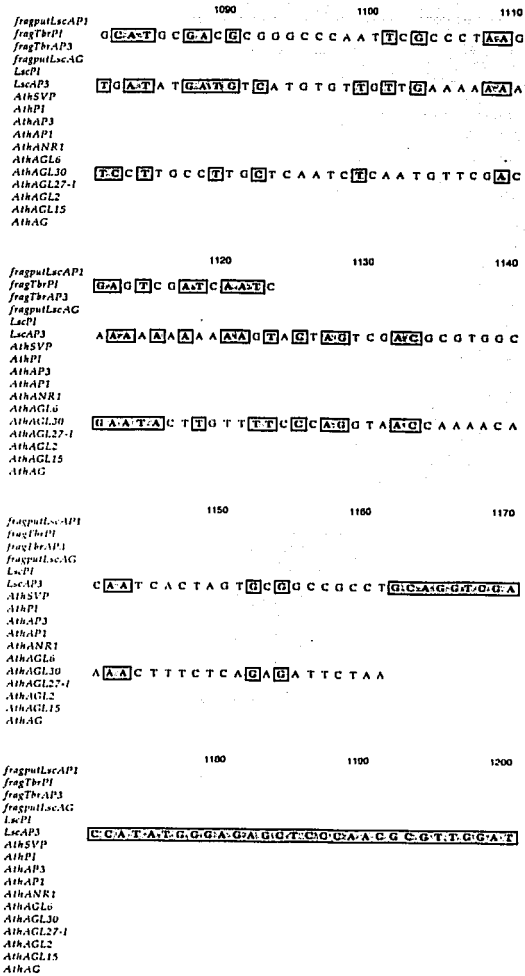
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fragTbrPI      40 GGTCACCTTCTCCAAGAGGAGGAACGGGATCATCAAGAAGGCCAAGGAGATCAGCGTCCTCTGTGATGCCAAGGTCTCCGTCGTCATCTTCTCCAGCTCT 139
fragTbrAP3      3 TTTGACCTACTCCAAGCGCCGGCTGGGGCTGATGAAGAAGGCCAAGGAGCTCACCGTCCTCTGCGACGCCCAGGTCTCCATCGTCATGGTCTCCTCCACC 102
fragputLacAG   36 GGTCACCTTCTGCAAGCGCCGCAACGGCCTCCTCAAGAAGGCCTACGAGCTATCGGTGCTCTGCGATGCCGAGGTCGCTCTCATCGTCTTCTCCAGCCGC 135
LacPI         125 GGTCACGTTCTCCAAGAGGAGGAACGGGATCATCAAGAAGGCCAAGGAGATCAGCGTCCTCTGCGATGCCAAGGTCTCCGTGGTCATCTTCTCCAGCTCC 224
LacAP3        156 GGTCACCTACTCCAAGCGCCGCCTCGGCCTCATAAAAAAGGCCAAGGAGCTCACCGTCCTCTGCGACACACAGGTCTCTATCGTCATGGTCTCCTCCACC 255
AthSVP         54 AGTGACGTTTTCGAAACGAAGAAGAGGGCTTTTCAAGAAAGCTGAAGAACTCTCCGTTCTCTGCGACGCCGATGTCGCTCTCATCATCTTCTCTTCCACC 153
AthPI          54 GGTGACGTTCTCAAAGAGGAGGAATGGATTGGTGAAGAAGGCTAAAGAGATCACAGTTCTTTGTGATGCAAAAGTTGCCCTCATAATCTTTGCAAGTAAT 153
AthAP3         54 AGTGACGTATTCAAAGAGAAGGAATGGTTTATTCAAGAAAGCACATGAGCTCACGGTTTTGTGTGATGCTAGGGTTTCGATTATCATGTTCTCTAGCTCC 153
AthAP1         54 AGTGACATTCTCGAAAAGAAGAGCTGGTCTTTTGAAGAAAGCTCATGAGATCTCTGTTCTCTGTGATGCTGAAGTTGCTCTTGTTGTCTTCTCCCATAAG 153
AthANR1        54 AGTGACTTTCTCCAAGAGAAGGAGTGGTTTGCTTAAGAAGGCTAAAGAGTTATCGATCCTTTGTGATGCAGAAGTTGGTGTTATCATATTCTCTAGCACC 153
AthAGL6        54 AGTGACCTTCTCAAAAAGAAGAAACGGTTTGCTGAAGAAAGCTTATGAGCTTTCTGTTCTTTGCGATGCCGAAGTTGCTCTCATCATCTTCTCAAGCCGT 153
AthAGL30       54 ATCTACATTTGCTAAAAGGAAAAATGGGATCTTGAAAAAGGCTAATGAGCTATCTATTCTTTGTGACATTGATATTGTTCTTCTTATGTTCTCTCCTACT 153
AthAGL27-I     54 AGTCACTTTCTCCAAACGACGCAATGGTCTCATCGACAAAGCTCGACAACTTTCGATTCTCTGTGAATCCTCCGTCGCTGTTGTCGTCGTATCTGCCTCC 153
AthAGL2        54 AGTAACGTTTTCCAAAGCGTAGGAACGGTTTGTTGAAGAAAGCTTATGAATTGTCTGTTCTCTGTGATGCTGAAGTTGCTCTCATCATCTTCTCCAACCGT 153
AthAGL15       54 AGTCACTTTTTCCAAGAGGCGTTCTGGGTTACTTAAGAAAGCTCGTGAGCTCTCTGTTCTTTGTGATGCTGAAGTTGCTGTCATCGTCTTCTCTAAGTCT 153
AthAG         201 AGTCACTTTTTGCAAACGTAGAAATGGTTTGCTCAAGAAAGCTTACGAGCTCTCTGTTCTCTGTGATGCTGAAGTCGCACTCATCGTTTTCTCTAGCCGT 300
                   ** *     ** *         ** *  *  * ** **       * *      * * ** **           *     * * * * *

fragputLacAP1 109 GGGAAGCTCTACGAGTACTCCACGGA-CT---CCTGCATGGAAAAGATTCTTGAACGCTATGAGCGCTATTGCTATGCTGAAAATAAGTGAACACAATT 204
fragTbrPI     140 AACAAGATGTTTGAGTACTGCACCCC CTCCACCACACTGTCCAAGATCCTGGAGAACTACCAGATTAACTGTGGAAAGAAGCTGTGGGATGCCAAGCAT 238
fragTbrAP3    103 AACAAGTTCCCCGAGTACTGCAGCCC-CTCCACCGATCTGAAGACCATCTACGACCGCTACCAAGAAGCCTCCGGGATCAACCTCTGGACGGCTCAATAC 201
fragputLacAG  136 GGCCGTCTCTACGAGTACTCCAATAA-C-- ---AGTGTGAAAGCAACTATTGAGAGGTACAAAAAA-GCTTGTACAGA-CACTCCCAACAGCAGTTCAA 226
LacPI         225 AACAAGATGTTTGAGTACTGCACTCC-CTCCACCACGCTGTCCAAGATCTTGGAGAACTACCAGATTAACTGTGGAAGAAACTGTGGGATGCCAAGCAT 323
LacAP3        256 AACAATTTCGCCGAATACTGCAGCCC-CTCCACCGATCTCAAGACCATCTATGACCGCTACCAGGAAGCCTCAGGTGTCAATCTCTGGAGTGCTCAATAC 354
AthSVP        154 GGAAAACTGTTCGAGTTCTGTAGCTC-C------AGCATGAAGGAAGTCCTAGAGAGGCATAACTTGCAGTCAAAGAACTTGGAGAAGCTTGATCAGCCA 246
AthPI         154 GGTAAGATGATTGATTACTGTTGTCC-TTCCATGGATCTTGGTGCTATGTTGGACCAATACCAGAAGTTATCTGGCAAGAAACTATGGGATGCTAAGCAT 252
AthAP3        154 AACAAGCTTCATGAGTATATCAGCCC-TAACACCACAACGAAGGAGATCGTAGATCTGTACCAAACTATTTCTGATGTCGATGTTTGGGCCACTCAATAT 252
AthAP1        154 GGGAAACTCTTCGAATACTCCACTGA-TT---CTTGTATGGAGAAGATACTTGAACGCTATGAGAGGTACTCTTACGCCGAAAGACAGCTTATTGCACCT 249
AthANR1       154 GGAAAGCTCTACGACTACGCAAGCAA-TT---CAAGTATGAAAACAATCATTGAGCGGTACAACAGA-GTAAAAGAGGAGCAGCATCAACTTCTGAATCA 248
AthAGL6       154 GGCAAGCTCTACGAGTTTGGTAGTG-------TTGGAATTGAAAGCACAATCGAACGGTATAATCGTTGTTACAACTGCTCTCTAAGCAATAATAAGCCT 246
AthAGL30      154 GGCAAGGCTGCAATATGTTGCGGTACACGAAGATGTTTCTCTTTCGAAAGCTCAGAACTTGAAGAAAACTTTCCAAAAGTTGGATCACGATGTAAATATA 253
AthAGL27-I    154 GGAAAACTCTATGACTCTTCCTCCGG-TG---ACGACATTTCCAAGATCATTGATCGTTATGAAATACAACATGCTGATGAACTTAGAGCCTTAGATCTT 249
AthAGL2       154 GGAAAGCTCTATGAGTTTTGCAGCTC-CT---CAAACATGCTCAAGACACTTGATCGGTACCAGAAATGCAGCTATGGATCCATTGAAGTCAACAACAAA 249
AthAGL15      154 GGCAAGCTCTTCGAGTACTCCAGTAC-T------GGAATGAAGCAAACACTTTCCAGATACGGTAATCACCAGAGTTCTTCAGCTTCTAAAGCAGAGGAG 246
AthAG         301 GGTCGTCTCTATGAGTACTCTAACAA-C------AGTGTAAAAGGTACTATTGAGAGGTACAAGAAG-GCAATATCGGA-CAATTCTAACACCGGATCGG 391
                                  *
```

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fragputLscAP1   205 GAAGCTGAC--CCCCAGGGAAATTGGTGTCAAGAATATGGAAAACTCAAGTGCAAAGTTGAAGCTTTACAAAAGAGTCAGAGGCATCTAATGGGT---GA 299
fragTbrPI       239 GAG------------------AATCTAAACGCAGAGATTGATCGTATCAAGAAGGAGAACGACAACATGCAGATCGAATTAAGGCACTTGAAGGGA---GA 318
fragTbrAP3      202 GAG------------------AAGATGCAGAACGCCCTGAGCCATCTGAAGGAGATCAACCGCAACCTGAGGAGAGAGATCAGGCAGAGGATGGGG---GA 281
fragputLscAG    227 TATCTGAAGCCAATTCCCAGTATTACCAACAAGAAGCATCAAAGTTGAGGCAGCAGATAACAAACCTGCTGAATTCCAACAGAAATTTATTGGGTT--GA 324
LscPI           324 GAG------------------AATTTGAATGCAGAGATTGATCGCATCAAGAAGGAGAACGACAACATGCAGATCGAACTAAGGCACTTGAAGGGA---GA 403
LscAP3          355 GAG------------------AAGATGCAGAACACACTAAGCCACCTCAAAGAGATCAACCGCAACCTGAGAAGAGAGATCAGGCAGAGGATGGGG---GA 434
AthSVP          247 TCTCTTGAGTTACAGCT--GGTTGAGAACAGTGATCACGCCCGAATGAGTAAAGAAATTGCGGACAAGAGCCACCGACTAAGGCAAATGAGAGGA---GA 341
AthPI           253 GAG------------------AACCTTAGCAATGAGATTGATAGGATCAAGAAAGAGAATGATAGCTTACAACTGGAGCTCAGGCATTTGAAGGGA---GA 332
AthAP3          253 GAG------------------CGAATGCAAGAAACCAAGAGGAAACTGTTGGAGACAAATAGAAATCTCCGGACTCAGATCAAGCAGAGGCTAGGT---GA 332
AthAP1          250 GAGTCCGAC--GTCAATACAAACTGGTCGATGGAGTATAACAGGCTTAAGGCTAAGATTGAGCTTTTGGAGAGAAACCAGAGGCATTATCTTGGG---GA 344
AthANR1         249 TGCCTCA----GAGATAAAGTTTTGGCAAAGAGAGGTTGCAAGTTTGCAGCAGCAGCTCCAACATCTACAAGAATGCCACAGGAAACTAGTGGGA---GA 341
AthAGL6         247 GAA---GAG--ACTACACAGAGTTGGTGTCAGGAGGTGACAAAGCTTAAATCCAAATACGAATCTCTTGTTCGTACTAACAGGAATTTGCTTGGA---GA 338
AthAGL30        254 CGCGAATTTATAGCCTCAAGGACTTGAGTACTCAAGCAAGGATTCTGCAGGCTCGGATTTCTGAGATACATGGAAGATTAAGTTATTGGACGGAACCAGA 353
AthAGL27-I      250 GAA-------------------------GAAAAAATTCAGAATTATCTTCCACACAAGGAGTTACTAGAAACAGTCCAAAGCAAGCTTGAAGAA---CC 320
AthAGL2         250 CCTGCCAAA--GAACTTGAGAACAGCTACAGAGAATATCTGAAGCTTAAGGGTAGATATGAGAACCTTCAACGTCAACAGAGAAATCTTCTTGGG---GA 344
AthAGL15        247 GATTGTGCA--GAGGTGGATATTTTAAAGGATCAACTTTCAAAGCTTCAAGAGAAACATTTACAACTGCAGGGCAAGGGCTTGAATCCTCTGACCTTTAA 344
AthAG           392 TGGCAGAAATTAATGCACAGTATTATCAACAAGAATCAGCCAAATTGCGTCAACAAATTATCAGCATACAAAACTCCAACAGGCAATTGATGGGT---GA 488

fragputLscAP1   300 ACAGCTTGA-ATCATTAAGCGTCAAAGA-ACTTCAGCAACTGGAGCAACAGCTTGAAAG-TTGCTTTAAAGCACGTTGCGGTCCCGAAAGAACAATCTTC 396
fragTbrPI       319 GGATCTGAA-CTCGCTTGGAGCGAAGGA-GCTTATTCCAATTGAGGAGGCACTCAGTA--ATGGCATCACCAAAGTT-AGGGATAAGCAGATGGATATGT 413
fragTbrAP3      282 GGAGCTGGA-GGAGATGGAGGCGCAGGA-GCTGCGCGGTCTTGAGCAAACCTTGGACG--AGTCACTGAAGCTCGTC-CGCCAGAGGAAATATCATGTGA 376
fragputLscAG    325 GTCTCTTGCCACCAAGAGTTGCTAGGGA-TCTTAAGACTCTGGAGGCCAAGTTGGAGAAAGAATTATCACAAAAATA                        400
LscPI           404 GGATCTTAA-CTCGCTAGGAGCGAAGGA-GCTTATTCCAATTGAGGAGGCACTCAGCA--ATGGCATCACCAAAGTT-AGAGATAAGCAGATGGACCTGT 498
LscAP3          435 GGAGCTGGA-GGGGATGGAGGCGCAGGA-GCTGCGCGGTCTTGAGCAAACCTTGGACG--AGTCACTGAAGTTCGTC-CGCCAGCGGAAATATCATGTGA 529
AthSVP          342 GGAACTTCA-AGGACTTGACATTGAAGA-GCTTCAGCAGCTAGAGAAGGCCCTTGAAA--CTGGTTTGACGCGTGTG-ATTGAAACAAAGAGTGACAAGA 436
AthPI           333 AGATATACA-GTCTCTCAACTTGAAAAA-TCTGATGGCTGTCGAGCACGCCATTGAAC--ATGGCCTCGACAAAGTC-CGAGACCACCAGATGGAGATCC 427
AthAP3          333 GTGTTTGGA-CGAGCTTGACATTCAGGA-GCTGCGTCGTCTTGAGGATGAAATGGAAA--ACACTTTCAAACTCGTT-CGCGAGCGCAAGTTCAAATCTC 427
AthAP1          345 AGACTTGCA-AGCAATGAGCCCTAAAGA-GCTTCAGAATCTGGAGCAGCAGCTTGACA--CTGCTCTTAAGCACATC-CGCACTAGAAAAAACCAACTTA 439
AthANR1         342 GGAACTTTC-TGGAATGAATGCTAACGA-CCTACAAAATCTTGAAGACCAGCTAGTAA--CAAGTCTAAAAGGTGTT-CGTCTCAAAAAGGATCAACTTA 436
AthAGL6         339 AGATCTTGG-AGAAATGGGTGTGAAGGA-ACTGCAAGCGCTCGAGAGGCAGCTCGAAG--CCGCTCTTAC-CGCGACTCGACAGCGCAAGACACAAGTTA 433
AthAGL30        354 TAAGATTAACAATGTTGAACACTTGGGACAGCTCGAAATTTCGATTAGGCAATCCCTTGATCAATTGCGTGCACACAAGATGCAAGATGGGATTCAGATT 453
AthAGL27-I      321 AAATGTCGA-TAATGTAAGTGTAGATTC-TCTAATTTCTCTGGAGGAACAACTTGAGA--CTGCTCTGTC-CGTAAGTAGAGCTAGGAAGGCAGAACTGA 415
AthAGL2         345 GGATTTAGG-ACCTTTGAATTCAAAGGA-GTTAGAGCAGCTTGAGCGTCAACTGGACGG-CT-CTCTCAAGCAAGTT-CGGTCCATCAAGACACAGTACA 439
AthAGL15        345 AGAGCTGCAAAGCCTTGAGCAGCAACTA-TATCATGCATT--GATTACTGTCAGAGAGCGAAAGGAACGATTGCTGA-CTAACCAACTTGAAGAATCACG 440
AthAG           489 GACGATAGG-GTCAATGTCTCCCAAAGA-GCTCAGGAACTTGGAAGGCAGATTAGAGA--GAAGTATTACCCGAATC-CGATCCAAGAAGAATGAGCTCT 583

fragputLscAP1   397 TACT                                                                                                 400
fragTbrPI       414 GGAAGCTTCTTAAGAAGA---ATGGAGGATGCTTGAAGAGGAGAACAAGCGACTTTCATTTCTACTGCATCAACAACATCTGAGCATGGACGGAAATGT 510
fragTbrAP3      377 TCACCACACAGACAGATACTTACAAGAAGAAGATAAAGAATTCTCATGAAGCCTACAAGACTTTGCTGGGAGAGCTGGAAATCCGAGATGGGGGAGGCGA 476
fragputLscAG    401                                                                                                      400
LscPI           499 GGAAGCTTCTTAAGAAGA---ACGGGAGGATGCTTGAAGAGGAGAACAAGCGGCTTTCATTTCTACTGCATCACCAGCATTTGAGCATGGATGGAAATGT 595
LscAP3          530 TCACCACACAGACAGATACNTACAAGAAGAAGATAAAGAATTCTCATGAAGCCTACAAGACTTTGCTCCGAGAGCTGGAGATCCGCGATGGGGGAGGCGA 629
AthSVP          437 TTATGAGTGAGATCAGCGAACTTCAGAAAAAGGGAATGCAATTGATGGATGAGAACAAGCGGTTGAGGCAGCAAGGAACGCAACTAACGGAAGAGAACGA 536
AthPI           428 TTATATCAAAGAGGAGAA---ATGAGAAGATGATGGCGGAGGAGCAACGGCAACTCACTTTCCAGCTGCAACAACAGGAGATGGCTATAGCAAGCAACGC 524
AthAP3          428 TTGGGAATCAGATGGAGACCACCAAGAAAAAGAACAAAAGTCAACAAGACATACAAAAGAATCTCATACATGAGCTGGAACTAAGAGCTGAAGATCCTCA 527
AthAP1          440 TGTACGAGTCCATCAATGAGCTCCAAAAAAAGGAGAAGGCCATACAGGAGCAAAACAGCATGCTTTCTAAACAGATCAAGGAGAGGGAAAAAATTCTTAG 539
AthANR1         437 TGACAAATGAAATCAGAGAACTTAATCGTAAGGGACAAATCATCCAAAAAGAGAATCACGAGCTACAAAATATTGTAGATATAATGCGTAAGGAAAATAT 536
AthAGL6         434 TGATGCAAGAAATGGAAGACCTTAGGAAAAGGAGAGGCAACTAGGAGACATAAACAAACAACTCAAGATTAAGTTTGAAACGGAAGGCCATGCTTTCAA 533
AthAGL30        454 CCTTTAGAACAACAGCTTCAATCTATGTCATGGATTCTTAATAGCAACACCACCAACATTGTCACCGAGGAACACAATTCAATCCCCGCAGAGGGAAGTCG 553
AthAGL27-I      416 TGATGGAGTATATCGAGTCCCTTAAAGAAAAGGAGAAATTGCTGAGAGAAGAGAACCAGGTTCTGGCTAGCCAGCTGTCAGAGAAGAAAGGTATGTCTCA 515
AthAGL2         440 TGCTTGACCAGCTCTCGGATCTTCAAAATAAAGAGCAAATGTTGCTTGAAACCAATAGAGCTTTGGCAATGAAGCTGGATGATATGATTGGTGTGAGAAG 539
AthAGL15        441 CCTCAAGGAACAACGAGCAGAGTTGGAAAACGAGACCTTGCGTAGACAGGTTCAAGAACTGAGGAGCTTTCTCCCGTCGTTCACCCACTATGTTCCATCC 540
AthAG           584 TATTTTCTGAAATCGACTACATGCAGAAAAGAGAAGTTGATTTGCATAACGATAACCAGATTCTTCGTGCAAAGATAGCTGAAAATGAGAGGAACAATCC 683

fragputLscAP1   401                                                                                                      400
fragTbrPI       511 G--AGGGAATTGGAGCTTGGGTATCATCACAAAGACCGCAATTACGCCTC--CCAGATTNCNGTG---TT-----TCGGGTCCAGCCGTTCCAGCCTAAC 598
fragTbrAP3      477 ---TATGGGTATGTTATCGATGAGGAGGC---TGTCACCGGT---------TATGATGGAGGTACGCTGGCG-ATGGGGAACGGAGGTAGCCCTCACAT 560
fragputLscAG    401                                                                                                      400
LscPI           596 G--AGGGAATTGGAGCTTGGGTATCATCACAAAGACCGCAATTACGCTTC--CCAGATTCCGGTGGCATT-----TCGAGTCCAGCCATTCCAGCCAAAT 686
LscAP3          630 ---TATGGGCTATGTGATAGACGACGACATCTGTNGCGCCGGCACTGCAGGATACGATGGAGGGCCACTGGCG-ATGGGGAATGGGGGAGCCCCCACAT 725
AthSVP          537 G--CGACTTGGCATGCAAATATGTAACAATGTGCATG--CACACGGTGGTGCTGAATCGGAGAACGCTGCTGT-GTACGAGGAAGGACAGTCGTCGGAGT 631
AthPI           525 A--AGAGGAATGATGATGAGAGATCATGATGGGCA---------------------GTTTGGATA---------TAGAGTGCAACCGATTCAGCCAAAT 591
AthAP3          528 C--TATGGACTAGTAGACAATGGAGGAGAT---------------------TACGACTCAGTTCT---------TGGATACCAAATCGAAGGGTCACGT 594
AthAP1          540 G--GCTCAACAGGAGCAGTGGGATCAGCAGAACCAAGGCCACAATATGCCTCCCCCTCTGCCACCGCAGCA---GCACCAAATCCAGCATCCTTACATGC 634
AthANR1         537 T--AAATTGCAAAAGAAGGTTCATGGAA--GAACAAATGTGATTGAAGGCAATTCAAGTGTAGATCCAATA---AGCA--ATGGAACCACAACATATGCA 627
AthAGL6         534 A--ACCTTTCAAGACTTATGGGCAAACTCGG--CGGCATCGGTGGCCGGGGATCCAAACAATTCTGAATTT---CCGGTAGAGCCTTCTCATCCTAATGT 626
AthAGL30        554 AGTGCTCAGCGAGTTCTTCATTCGGGAGCTATCCAGGCTACTTTGGAACAGGGAAATCTCCTGAAATGACAATTCCGGGTCAAGAAACAAGCTTTCTTGA 653
AthAGL27-I      516 C--CGATGA                                                                                            522
AthAGL2         540 T--CATCATATGGGAGGATGGGAAGGCGTTGAACAGAATGTTACCTACGGGCATCATCAAGCTCAGTCTCA---GGGACTATACCAGCCTCTTGAATGCA 634
AthAGL15        541 T--ACATCAAATGCTTTGCT--ATAGATCCAAAGAACGCTCTCATAAACCACGACAGTAAATGCAGCCTCC---AGAAC-ACCGATTCAGACACAACTTT 632
AthAG           684 G--AGTATAAGTCTAATGCCAGGAGGATCTAACTACGAGCAGCTTATGCCACCACCTCAAACGCAATCTCA---ACCG--TTTGATTCACGGAATTATTT 776

fragputLscAP1   401                                                                                                      400
fragTbrPI       599 TTGCAGGAGAATAAGTAGAGCTGTTTCTCTGTATTTAATCAAGCTCCACTCTGAAGAGTGCCCAGACAAGTTTGAATCTCTCTCAATGCTGGATATTTAT 698
fragTbrAP3      561 GTTCACATTCCGCGTTCAGCCCAGCCAGCCCAATCTCCACCTCATGGGCAATGGTTTCCATGATCTTCGTCTCGCCTAGCTATT----TCTTCAATTTCC 656
fragputLscAG    401                                                                                                      400
LscPI           687 TTGCAAGAGAATAAATAGAGTTATTTCCTTGTACTTAATCAATTTCCACTCTGACCAGTA--TAGACAAGTATGAATCT------ATGTTGGATGTCTAT 778
LscAP3          726 GTTCACGTTCCGCGTTCAGCCCAGCCAGCCCAATCTCCACCTCATGGGCAATGGCTTCCATGACTTTCGTCTTGCCTAGCTTTCATCCTTTGAGATTTCT 825
AthSVP          632 CTATTACTAACGCCGAAACTCTACCGGAGCGCCTGTTGACTCCGAGAGCTCCGACACTTCCCTTAGGCTCGGCTTACCGTATGGTGGTTAG          723
AthPI           592 CTTCAGGAAAA-GATTATGTCTTTGGTCATCGATTGA                                                                627
AthAP3          595 GCTTACCCTCTTCGTTTCCACCAGA-ACCATCACCACTATTACCCCAACCATGGCCTTCATG--CACCCTCTGCCTCTGACATCATTACCTTCCATCTTC 691
AthAP1          635 TCTCTCATCAGCCATCTCCTTTTCTCAACATGGTGGTCTGTATCAAGAAGATGATCCAATGGCAATGAGGAATGATCTCGAACTGACTCTTGAACCCGT 734
AthANR1         628 CCACCGCAACTTCAACTCATACAACTACAACCAGCTCCTAGAGAAAAATCAATCAGACTAGGGCTACAACTTTCCTAG                       705
AthAGL6         627 ATTGGATTGCAACACCGAACCCTTTTTACAAATAGGGTTTCAACAACATTACTACGTGCAAGGTGAAG-GGTCTTCGGTATCAAAGAGTAACGTGGCAGG 725
AthAGL30        654 TGAACTAAACACCGGACAGCTGAAACAGGACACAAGCTCGCAGCAGCAGTTCACTAATAATAATAATATCACAGCATACAATCCCAATCTTCACAATGAT 753
AthAGL27-I      523                                                                                                      522
AthAGL2         635 ATCCAACTCTGCAAATGGGGTATGATAATCCAGTATGCTCTGAGCAAATCACTGCGACAACACAAGCTCAGGCGCAGCCGGGAAACGGTTACATTCCAGG 734
AthAGL15        633 GCAATTAGGGTTGCCGGGAGAGGCACATGATAGAAGGACGAATGAAGGAGAAAGAGAGAGCCCCGTCAAGCGATTCAGTGACAACAAACACGAGCAGCGAA 732
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AthAG          777 CCAAGTCGCGGCATTGCAACCTAACAATCACCATTACTCATCCGCCGGTCGCCAAGACCAAACCGCTCTCCAGTTAGTG 855


fragputLscAP1  401                                                                              400
fragTbrPI      699 CTCTGTAAACTTTGGTCCTTCAACTAAACAATGAATCCATTGCTTCATATATTTTTCTTC--AACTAGATCTTGGATCATCTATTTTTTTAAACTAATTT 796
fragTbrAP3     657 TCAAGTTGTGGATTTTACCTTACCTTCTCGCGTT----ATAGTATCAATTGCCTTGTTGC--ATCGTTTCCTCCCTTCTTTTGCACTAGTAATTGTTACC 750
fragputLscAG   401                                                                              400
LscPI          779 CTCTGTCAAGATTGGCGCTTCAACTAAACAATGAATCCATTGCTTCATATATTCTGC----------AAAGTTCCATAATCTGCCTT------------ 855
LscAP3         826 TCAAATGAACATATATATCTATATCTATTGCATCCTTGATATCCTCAAATGCAACATTACCTACTGTTGCTGTGTGTGGATCTTGATTGCCTTTCTGCATC 925
AthSVP         724                                                                              721
AthPI          628                                                                              627
AthAP3         692 TTGAATAA                                                                     699
AthAP1         735 TTACAACTGCAACCTTGGCTGCTTCGCCGCATGA                                           768
AthANR1        706                                                                              705
AthAGL6        726 TGAGACTAATTTCGTCCAAGGTTGGGTTCTTTGA                                           759
AthAGL30       754 ATGAATCATCACCAAACGTTGCCTCCTCCTCCTCTTCCTCTTACTCTTCCGCATGCTCAGGTGTATATTCCAATGAATCAGAGAGAGTATCATATGAATG 853
AthAGL27-I     523                                                                              522
AthAGL2        735 ATGGATGCTCTGA                                                                747
AthAGL15       733 ACTGCAGAAAGAGGGGATCAGTCTAGTTTAGCAAATTCTCCACCTGAAGCCAAAAGACAAAGGTTCTCTGTTTAG 807
AthAG          856                                                                              855


fragputLscAP1  401                                                                              400
fragTbrPI      797 TCTCTGCTAAAAAAAAAAAAAAAAAAAAAGTACTAGTCGACGCGTGGCCAATCGAATTCCCGCGGCCGCCAGGCGGCCGGGAGCATGCGACGCGGGCCCAAT 896
fragTbrAP3     751 GTGGTTGTTTTAAAAAAAAAAAAAAGTAC                                                 779
fragputLscAG   401                                                                              400
LscPI          856 ---------GAAAAAAAAAAAAAAAAAAGTANTAGTCGACGCGTGGCC                             893
LscAP3         926 ATTTTCATTTCTCTTGCATATGTAATCGCGGTATTGGGTATTTCCCTTTATGCGTTTGCATGTTTTGCAAACTCTATTTATGATATGATGTCATGTGTTG 1025
AthSVP         724                                                                              723
AthPI          628                                                                              627
AthAP3         700                                                                              699
AthAP1         769                                                                              768
AthANR1        706                                                                              705
AthAGL6        760                                                                              759
AthAGL30       854 GATTCTTTGAAGCACCACCACCTGATTCTTCTGCTTACAACGACAACACCAACCAAACCAGGTTTGGTTCTAGCAGCAGCTCCTTGCCTTGCTCAATCTC 953
AthAGL27-I     523                                                                              522
AthAGL2        748                                                                              747
AthAGL15       808                                                                              807
AthAG          856                                                                              855


fragputLscAP1  401                                                                              400
fragTbrPI      897 TCGCCCTAAGGAGTCGATCAATC                                                       919
fragTbrAP3     780                                                                              779
fragputLscAG   401                                                                              400
LscPI          894                                                                              893
LscAP3        1026 TTGAAAAAAAAAAAAAAAAAAAAGTACTAGTCGACGCGTGGCCAATCACTAGTGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT 1125
AthSVP         724                                                                              723
AthPI          628                                                                              627
AthAP3         700                                                                              699
AthAP1         769                                                                              768
AthANR1        706                                                                              705
AthAGL6        760                                                                              759
AthAGL30       954 AATGTTTCGACGAATACTTGTTTTCCCAGGTAACCAAAACAAAACTTTCTCAGAGATTCTAA               1014
AthAGL27-I     523                                                                              522
AthAGL2        748                                                                              747
AthAGL15       808                                                                              807
AthAG          856                                                                              855


fragputLscAP1  401                                                  400
fragTbrPI      920                                                  919
fragTbrAP3     780                                                  779
fragputLscAG   401                                                  400
LscPI          894                                                  893
LscAP3        1126 GCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGNTGGG        1166
AthSVP         724                                                  723
AthPI          628                                                  627
AthAP3         700                                                  699
AthAP1         769                                                  768
AthANR1        706                                                  705
AthAGL6        760                                                  759
AthAGL30      1015                                                 1014
AthAGL27-I     523                                                  522
AthAGL2        748                                                  747
AthAGL15       808                                                  807
AthAG          856                                                  855
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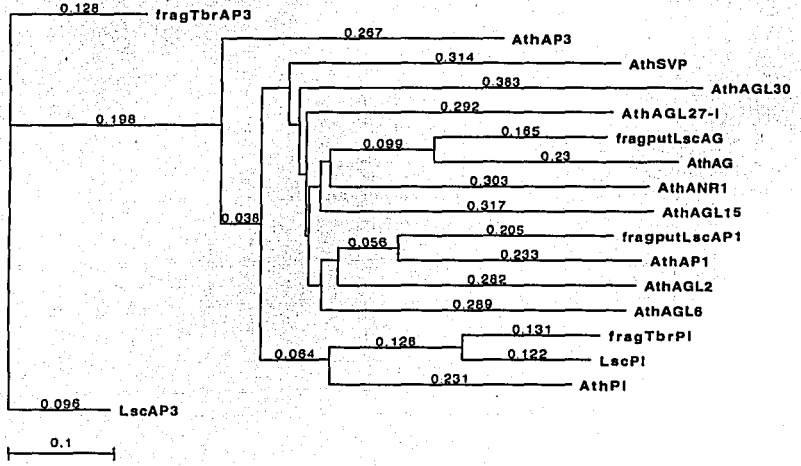
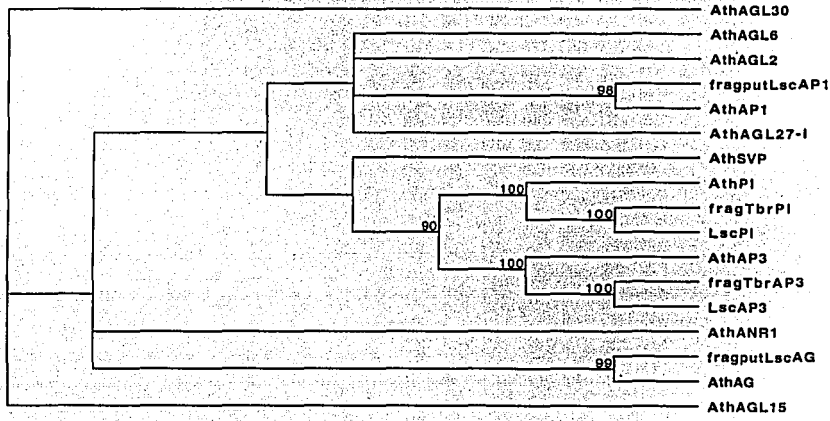
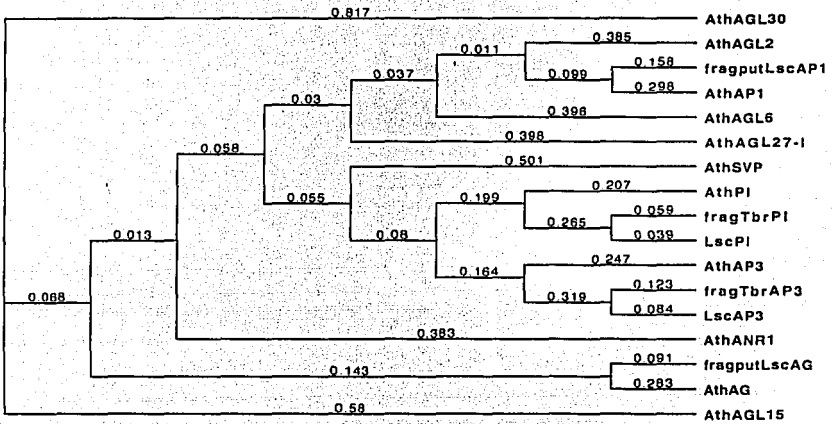
ClustalW (v1.4) Multiple Alignment Parameters:
Open Gap Penalty = 10.0; Extend Gap Penalty = 5.0; Delay Divergent = 40%
Transitions: Weighted



Method: Neighbor Joining; Bootstrap (1000 reps); tie breaking = Random
Distance: Kimura 2-parameter; Gamma correction = Off
Transition:Transversion Ratio = Estimate (Av. = 0.64); Gaps distributed proportionally



Method: Neighbor Joining; Best Tree; tie breaking = Random
Distance: Kimura 2-parameter; Gamma correction = Off
Transition:Transversion Ratio = Estimate (Av. = 0.64); Gaps distributed proportionally



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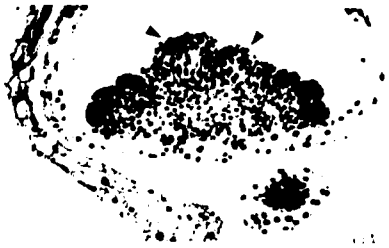

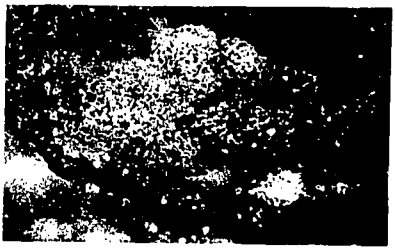

Capítulo 5: generalidades

El presente capítulo es una compilación de la información básica sobre la embriología de *T. brevistylis*, incluyendo micro- y megagametogénesis, micro- y megaesporogénesis, así como etapas más tardías del desarrollo de los gametofitos. El trabajo de botánica estructural aquí presentado fué realizado en su totalidad por Silvia Espinosa-Matías en el laboratorio de Judith Márquez-Guzmán; aparece en la presente tesis (con el permiso correspondiente) por su relación recíproca con la interpretación que el autor ha hecho de los fenotipos florales heterotópicos hallados en esta especie de triuridal (capítulo 3). El análisis de los grados de similitud entre las subunidades largas de los genes ribosomales de las dos especies de triuridales mexicanas, así como de una matriz morfológica incluyendo varios terminales miembros de Triuridaceae (datos no publicados ni incluidos en la presente tesis), sustentaron la discusión entre S. E-M. y el autor que derivó en la versión aquí presentada, actualmente en revisión para su publicación en la revista *International Journal of Plant Sciences*.

Embryology of *Triuris brevistylis* (Triuridaceae)

Running head: Embryology of *T. brevistylis*

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Abstract

Triuris brevistylis is a dioecious monocot with ocassional bisexual flowers. The male flowers have three sessile stamens. The young anther is bithecal and 4-sporangiate, as common in Triuridaceae, but the mature anther is bithecal and 2-sporangiate with an extrorse dehiscence through a longitudinal slit. The anther wall comprises four layers: an epidermis, an endothecium with U-shaped wall thickenings, a middle layer and a secretory tapetum. The microspore tetrad has an isobilateral arrangement. Mature pollen grains are oblate spheroidal, inaperturate, intectate verrucate with a thin exine and thick intine. Pollen grains are shed as monads at the 3-celled stage. In the female flowers the ovule is basal, anatropous, tenuinucellate and bitegmic. The megaspore tetrad has a linear arrangement and the embryo sac is monosporic of the Polygonum type. The first division of the zygote is transverse and the basal cell forms a suspensor. Endosperm development is of the nuclear type, after which there is a cellularization phase. Proteic bodies and non-soluble polysaccharides are reserve materials in endosperm cells. Achene maturation is asynchronous with seeds that contain embryos with approximately 14 cells at dispersal time.

Keywords: embryology, floral morphology, reproductive biology, Triuridaceae, *Triuris brevistylis*

Introduction

The order Triuridales is comprised by a single family, Triuridaceae, and has been assigned to a separate superorder on its own, Triuridiflorae (Dahlgren and Clifford 1982; Dahlgren et al. 1985).

The Triuridaceae has been subdivided into two tribes, Sciaphileae and Triurideae (Giesen 1938; Maas and Rübsamen 1986). Sciaphileae is comprised by *Sciaphila* Blume, *Soridium* Miers, *Andruris* Schlechter, *Hyalisma* Champ and *Seychellaria* Hemsley (Giesen 1938; Maas and Rübsamen 1986; Rübsamen-Weustenfeld

1991). The tribe Triurideae includes *Triuris* Miers, *Peltophyllum* Gardner (Giesen 1938; Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991), *Triuridopsis* H. Maas & Maas (Maas-van de Kamer and Maas 1994) and *Lacandonia* E. Martínez & Ramos (Maas-van de Kamer and Weustenfeld 1998). Lacandoniaceae has been proposed as a new monotypic family closely allied to the Triuridaceae (Martínez and Ramos 1989). Nevertheless, the morphological character states observed by some authors in *Lacandonia schismatica* have not been considered to justify the proposal of a new family (Rübsamen-Weustenfeld 1991; Maas-van de Kamer 1995; Maas-van de Kamer and Weustenfeld 1998).

Due to the variability in number and position of floral whorls present in *Sciaphila albescens* and *Triuris hyalina*, it has been argued that the inverted placement of the sexual organs in *Lacandonia schismatica* could be interpreted as one among other comparable floral variations. In this way, it has been suggested that alternatively to the proposals of *Lacandonia* as a separate genus, the species could be included either in *Sciaphila*, because of the shared presence of bisexual flowers, or in *Peltophyllum*, given the similarities in shape and number of tepals (Rübsamen-Weustenfeld 1991).

The Triuridaceae is a family of small, saprophytic and mycoheterotrophic plants (Maas 1988; Maas and Maas-van de Kamer 1989). These plants occur throughout the tropical and subtropical regions of the Old and New World (Maas 1979; Maas and Rübsamen 1986; Maas-van de Kamer and Weustenfeld 1998). However, it has also been recorded in cloud forests (Maas and Rübsamen 1986). Triuridaceae grow mostly on leaf mold, forest litter, rotten wood or, rarely, in termite nests (Tomlinson 1982; Maas and Rübsamen 1986).

The genus *Triuris* is comprised of *T. alata* Brade, *T. hexophthalma* Maas, and *T. hyalina* Miers. The latter has been included as synonymous of *T. brevistylis* J.D.Smith, *T. major* Poulsen and *T. mycenoides* Ule (Giesen 1938; Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991). However, Martínez (1994) analyzed samples collected by von Türckheim, (No 1384 female flower !Holotype), as well as material from both sexes obtained from extensive collections performed during five years at the Lacandon rainforest, in sites adjacent to those visited by this author with the same climatic, physiographic and floristic characteristics. According to his observation, carpellate flowers are very similar in all species. This is particularly clear in samples from herbaria. In turn, this author has argued, that male floral morphology is a useful criterion to decide on the taxonomic status of these triurid species. Focusing on the size and shape of the androphore, in the same manner as Maas and Rübsamen (1986), he concluded that *T. brevistylis* and *T. hyalina* should be considered as a separate species.

The aim of this research is to describe in detail the embryology of the female and male flowers, including pollen morphology, and seed morphology of *T. brevistylis*, in order to contribute with new embryological characters states for this species. This information could be useful in subsequent systematic studies.

Materials and methods

Plant material

Materials were collected from a population of *Triuris brevistylis* growing in the locality of Naha (16°57.716'N, 91°35.583' W and 16°57.723' N, 91°35.736' W). This site belongs to the deciduous Lacandon rainforest, located in the state of Chiapas, Southeastern Mexico. The plants were found in association with *Quercus*. Vouchers of *T. brevistylis* are deposited at the Herbarium of the Universidad Nacional Autónoma de México (MEXU): E. Martínez S. 21864 (MEXU 917742); E. Martínez S. 21920 (MEXU 917961).

Microtechniques

Floral buds, anthetic and post-anthetic pistillate, staminate and hermaphroditic flowers were collected and fixed in the field in FAA (formalin, acetic acid, 96% ethanol, water 2:1:10:7). After dehydration in a graded ethanol series, the samples were embedded in paraplast and resine J_B-4, and sectioned with an AO rotatory microtome and with a Sorvall MT2-B ultramicrotome, respectively (Johansen 1940; Ruzin 1999). Some sections of the material embedded in paraplast were stained with aniline blue for observation with a fluorescence microscope, while other sections were used to perform histochemical tests (Johansen 1940; O'Brien and McCully 1981; Ruzin 1999). Resine J_B-4 sections were stained with toluidine blue (Ruzin 1999) for observation with an Olympus BH-2 photonic microscope.

Transmission electron microscopy

At the site of collection some mature anthers were fixed in 5% glutaraldehyde + 4% paraformaldehyde + 0.1 M s-collidine buffer, pH 7.2 at 4° C, dehydrated in a graded acetone series, embedded in resine Epon 812

(Bozzola and Russell 1999) and sectioned with a Sorvall MT2-B ultramicrotome. Ultrathin sections of the pollen grains were stained with uranyl acetate followed by lead citrate and examined with an EM-10 Zeiss transmission electron microscope (TEM).

Scanning electron microscopy

Fruits, seeds and pollen grains already fixed in FAA were dehydrated in a graded ethanol series and critical point dried using liquid CO_2 in the CPD 030 Balt-Tec critical point dryer. The samples were mounted on aluminum stubs using carbon double tape and coated with approximately 200 nm of gold by means of a Denton Vacuum Desk II. Observations were carried out with a Jeol JMS-35 scanning electron microscope (SEM).

Results

Field observations

Triuris brevistylis is a dioecious, saprophytic and mycoheterotrophic plant that grows on forest litter, rotten wood or shady grounds where dead leaves accumulate. The relative humidity of the rainforest areas where *T. brevistylis* is found is amounts to approximately 90%.

Each year during field work, between September and October, most of the population was composed mainly by inflorescence axes with floral buds and female flowers at different developmental stages, including a few anthetic flowers. We found many female flowers but very few males, occurring in an approximate ratio of 100:1. During October and November, most of the female flowers were at anthesis and most of them had fruits at different developmental stages. Again, very few male flowers were present, if at all. By the end of the fruiting season, the female flowers were scarce and the few that were still present had the fruits adhered to the receptacle. The last fruits were colected on January and the male flowers were practically gone at this time. Flowers with carpels and stamens ocurring in the same receptacle were very scarce and sometimes were absolutely absent from the population.

Floral morphology

The male flowers are trimerous, with three reflexed triangular tepals in one whorl. The tepals have a long caudate apex (Fig. 1.1). The androphore is a fleshy, hyaline, central column of sterile tissue with wing-like tissue extensions at its apex. The androecium consists of three sessile anthers placed at the base of the androphore, alternating with the tepals (Fig. 1.2). Anther epidermal tissue is conspicuously papillose and the anthers dehisce extrorsely through a longitudinal slit (Fig. 1.3). The young anthers are bithecal and tetrasporangiate (Fig. 1.4), but the septum between the pollen sacs of each theca degenerates just prior to anthesis. Therefore, the pollen sacs of each thecae become connected, leading to the formation of bisporangiate mature anthers (Fig. 1.5).

The female flowers are trimerous too. The perigone has three triangular tepals, each ending in a short caudate apex. The apocarpous gynoecium has approximately 350 carpels (Fig. 1.6). The style is distal on the carpel, and along with the stigmatic region it is formed by parenchyma and epidermal cells (Fig. 1.7). The stigmatic region is not structurally differentiated (Fig. 1.8). During anthesis the hypodermal cells of the receptacle of the female flowers secrete an accumulating mucilaginous substance that contains non-soluble polysaccharides and protein. This substance disappears during later stages in the maturation of the fruits. The vascular bundles reach the floral receptacle, but they do not touch neither the carpels nor the ovules (Fig. 2.9).

Anther wall development

Male floral meristems and tepals are protected by a 2-3 layered bract. Tepals are the first floral whorl to differentiate (Fig. 2.10). The archesporial tissue is delimited by a unistratified epidermis. Primary parietal layer and sporogenous tissue are the result of periclinal divisions of archesporial tissue cells (Fig. 2.11). Subsequently, the primary parietal layer divides periclinally to originate an outer and an inner secondary parietal layers. The former differentiates into the endothecium, whereas the inner layer divides again in a periclinal manner, giving rise to the middle layer and to the secretory tapetum (Fig. 2.12). Anther wall development therefore corresponds to the monocotyledonous type, resulting in the formation of four monostratified layers: an epidermis, an endothecium, a middle layer, which disappears early and an uni- to binucleate tapetum (Fig. 2.13). At the free microspore stage, anther wall is comprised by the epidermis and the endothecium because the middle layer and tapetum become flattened and closely crushed against the endothecium (Fig. 2.14). At the 3-nucleate pollen grain stage, endothecial cell presents U-shaped wall thickenings, which are absent from the stomium (Fig. 2.15).

Microsporogenesis and microgametogenesis

During development of the anther wall, the microspore mother cells have intense meiotic activity (Fig. 2.16). These cells are surrounded by the tapetum, which by then has achieved its maximum size and amount of cytoplasmic content. At this stage, the anticlinal and internal walls of the tapetum have disappeared, allowing the formation of cytoplasmic bridges (Fig. 2.17).

Isobilateral microspore tetrads arise as a result of successive cytokinesis cycles. At this stage, the periclinal walls of the secretory tapetum become thinner and their cytoplasmic content is deposited in the microsporangial cavity (Fig. 2.18). Also, uninucleate microspores have already developed ornaments in the exine after the callose wall has degenerated (Fig. 3.19). The first mitotic division gives rise to the generative and vegetative cells (Fig. 3.20), and mitotic division of the former produces two sperm cells (Fig. 3.21). Although the pollen grains are shed as monads at the 3-celled stage through the stomium (Fig. 3.22), pre-germination stages of mature pollen grains inside the microsporangial cavity can be observed (Fig. 3.23).

Mature pollen grain morphology

Mature pollen grains are oblate spheroidal, inaperturate, 20-25 μm in diameter, and the exine is intectate verrucate (Fig. 3.24). Some pollen grains showed areas with exine detachment (Fig. 3.25). TEM photographs show a very thin exine and a thick intine that is evenly distributed all over the pollen grain (Fig.3.26).

Megasporogenesis and megagametogenesis

Female floral meristems and tepals are covered by a 2-3 layered bract (Fig. 4.27). The meristem grows asynchronously forming lobulations of tissue that will develop into unilocular carpels (Fig. 4.28). In the carpel primordia, the nucellus curves in opposite direction to the ovary wall that encloses it. The archesporial cell is conspicuous due to its large size, its denser cytoplasm and prominent nucleus. The archesporial cell acts directly as the megaspore mother cell, and shortly afterwards, integuments primordia begin to develop (Fig. 4.29). The megaspore mother cell elongates and together with the nucellus, become surrounded by outer and inner 2-layered integuments. The inner integument forms the micropyle and then degenerates, while the seed is developing (Fig. 4.30).

The megaspore mother cell divides meiotically and gives rise to a linear megaspore tetrad (Fig. 4.31). The three micropylar megaspores degenerate whereas the chalazal one remains as the functional megaspore (Fig. 4.32). The first mitotic division of the functional megaspore results in a binucleate embryo sac and the nuclei migrate to the micropylar and chalazal pole (Fig. 4.33). Two mitotic cycles occur subsequently forming a tetra-(Fig.4.34) and octonucleate coenocytic embryo sac (Fig. 4.35). At the ending of the cellularization phase, the nucellus has been reabsorbed and a 7-celled embryo sac of the Polygonum type is formed (Fig. 5.36).

Seed development

The seed coat is formed only by the outer integument. The testa is differentiated into an endotesta and an exotesta (Fig. 5.37). Endotestal cells are big, mostly at the micropylar region. Their cytoplasm has tannins and non-soluble polysaccharide inclusions. In contrast, exotestal cells are smaller than those from the endotesta, only some of them having non-soluble polysaccharide inclusions, and none having tannins (Fig. 5.38).

Endosperm development is of the nuclear type. During the first divisions, nuclei are attached to the embryo sac wall (Fig. 5.38). Afterwards, there is a cellularization phase and the endosperm consists of large cells with conspicuous nuclei and a dense cytoplasm with proteic bodies and non-soluble polysaccharides as reserve materials (Fig. 5.39), whereas cellulose and non-soluble polysaccharides were the reserve materials observed in wall thickenings of the endosperm cells. These are separate from the seed coat by the nucellus and inner integument cuticules (Fig. 5.40).

A clump of cells resulting from the division of the outer integumentary cells in the chalazal region of the ovule form a chalazal cap (Fig. 5.41). These cells dye positively with anilin blue indicating the presence of callose (Fig. 5.42).

Remains of the pollen tube occur on the micropyle region suggesting fecundation of the porogamic type. A 3-celled linear proembryo indicates that divisions at the beginning of embryogenesis are exclusively transverse (Fig. 5.43). The basal cell undergoes a transversal division and forms a 3-4 celled suspensor (Fig. 5.44). Further transverse and longitudinal divisions occur, giving rise to an undifferentiated embryo of approximately 14 cells. At this stage, the suspensor cells were not observed any more (Fig. 6.45). The seed is

brown, ovoid and the surface of the seed coat is reticulate and composed of rectangular cells. The external anticlinal walls and the cuticle are smooth (Fig. 6.46).

Fruit

The dispersion unit is an achene which develops asynchronously depending on its position on the receptacle. Achenes placed at the center of the receptacle are the first to ripe (Fig. 6. 47). Pericarpal epidermis is reticulate and deeply depressed. The style is persistent until the time of achene dispersal (Fig. 6.48).

Discussion

The available information about the reproductive aspects of the life history of Triuridaceae is compiled in the works of Tomlinson (1982), Dahlgren et al. (1985), Maas and Rübsamen (1986), Rübsamen-Weustenfeld (1991), Maas-van de Kamer (1995) and Maas-van de Kamer and Weustenfeld (1998).

As in *T. hexophthalma*, *T. hyalina* and other species of the Sciaphileae (Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991), anther wall development of the monocotyledoneous type is present in *T. brevistylis*. However, the corresponding character state in *T. alata* is unknown (Rübsamen-Weustenfeld 1991).

The fact that the septum between the pollen sacs of each theca degenerates early in floral development may be the cause of incorrect interpretations of the number of pollen sacs in the anthers of Triuridaceae, where putative bisporangiate, tetrasporangiate and even trisporangiate conditions have been reported (Tomlinson 1982; Dahlgren et al.1985; Maas and Rübsamen 1986). In *T. brevistylis*, the degeneration of the septum between the pollen sacs in each theca occurs before anthesis, and at this stage ocurring the anthers have a single cavity in each theca. The issue regarding the number of pollen sacs in each theca in anthers of pre-anthetic flowers needs to be reinvestigated in detail for other Triuridaceae, in the context of our discovery of the early degeneration of the septum between the pollen sacs of each theca.

Periplasmodial and secretory tapetum, as well as intermediate forms, were observed in Triuridaceae (Maas and Rübsamen 1986). The former is present predominantly in Sciaphileae and the second one in Triurideae (Rübsamen-Weustenfeld 1991). We have found that in *T. brevistylis*, a member of the Triurideae, the tapetum is exclusively secretory, since the intermediate forms recorded in *T. hexophthalma*, *T. hyalina* and *Andruris* cf. *andajensis* (*Sciaphila arfakiana*) (Maas and Rübsamen 1986) were not observed. Cytoplasmic bridges among tapetal cells have not been previously described for Triuridaceae, but they have been documented in *Lacandonia schismatica* (Márquez-Guzmán et al. 1993).

Isobilateral microspore tetrads occur in *T. brevistylis*, a character state also present in *S. albescens*, *S. purpurea*, *S. rubra* and *T. hexophthalma* (Rübsamen-Weustenfeld 1991), whereas the decussate microspore tetrads have been observed in *S. albescens* and *Seychellaria madagascariensis*. Observations regarding the tetrad stages for *T. alata*, *T. hyalina* and other species in the family Triuridaceae have not been reported yet (Rübsamen-Weustenfeld 1991; Maas-van de Kamer 1995).

The results from our palynological observations for *T. brevistylis* coincide with published information on *T. hyalina* (Rübsamen-Weustenfeld 1991), but differ from previous reports on prolate monosulcate pollen grains of *T. hexophthalma* and *Sciaphila* and other members of Sciaphileae (Dahlgren et al. 1985; Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991).

Pollen grains with a thin exine or with exine formed only by a few strata are known in 30 families and 13 orders of monocotyledons, among which Triuridaceae is included (Kress 1986). In this way, the presence of a very thick intine covered by irregularly sculpted thinner exine in *Seychellaria africana* and some species of *Sciaphila* has been interpreted as an aperturoid zone (Rübsamen-Weustenfeld 1991). In contrast, it is impossible to distinguish an evident aperture in *Andruris* cf. *andajensis* (*Sciaphila arfakiana*), but there is a decrease in density and ornamental degree of exine that contrasts with the highly thickened intine (Erdtman 1952; Wirz 1910). The pollen grains of *T. brevistylis* present a very thick intine all over the pollen grain and a thin exine, which detaches easily. Because of this, we suppose that the functions of protection, dispersion, harmomegathy and recognition, which usually correspond to the exine, are assumed by the intine.

Embryo sac development is monosporic and of the Polygonum type in Sciaphileae, whereas in species of Triurideae evidence allows an interpretation of embryo sac development as corresponding to the Fritillaria type (Rübsamen-Weustenfeld 1991; Maas-van de Kamer 1995). It is likely that *T. hyalina* and *T. hexophthalma* exhibit this kind of embryo sac development (Rübsamen-Weustenfeld 1991). Even though *T. brevistylis* belongs to Triurideae, this species has a linear megaspore tetrad, and according with the developmental sequence observed in the present investigation, we conclude that embryo sac development is monosporic of

the Polygonum type, as in *Sciaphila albescens* and *Soridium spruceanum* (Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991).

Mature seeds of some Triuridaceae have cell remnants from the inner integument, characterized by the presence of tannins in the chalazal region (Maas and Rübsamen 1986). A different condition is found in *T. brevistylis*, where tannins are present only in the endotesta and pericarp. Besides, the chalazal cap originates from the outer integument and only the inner integument cuticle is present.

Rübsamen-Weustenfeld (1991) recorded starch in inmature seed, which disappears continuously during seed maturation in Triurideae. Besides, this author recorded oil in the cell lumina. In contrast with those results, neither lipids nor starch were found by us at any developmental stage of the seed of *T. brevistylis*. Instead, mature seeds of this species present proteins, non-soluble polysaccharides and cellulose as major reserve materials.

Sex-allocation arguments predict male-biased sex ratios in dioecious species (Willson 1983). However, a female-biased sex ratio was observed in *T. brevistylis* (ca. 100 female:1 male). It is possible that bisexual flowers may contribute with pollen to double fertilization. Among the Triuridaceae, double fertilization has been observed only in *Soridium spruceanun* (Rübsamen-Weustenfeld 1991). In *T. brevistylis*, a double fertilization process was not directly observed but the occurrence of pollen tube remnants on the micropyle, besides an endosperm, suggest that sexual reproduction takes place. It should be noticed that until this moment we have not observed the starting point for association between mycorrhyzic fungi and the fruit/seed. However, it is most likely that this physiological relationship is of importance during germination and/or the establishment of seedlings.

Even though the species of *Triuris* have been reported as being dioecious (Giesen 1938; Jonker 1943; Dahlgren et al. 1985; Maas and Rübsamen 1986; Maas and Maas 1987; Maas 1988; Maas and Maas-vas de Kamer 1989; Rübsamen-Weustenfeld 1991; Maas-van de Kamer and Weustenfeld 1998), female flowers of *T. hyalina* from Suriname sometimes have three staminodes sunken into the receptacle alternating with perianth segments (Jonker 1943). Otherwise unisexual flowers of *T. hyalina* with many carpels and two or three stamens therefore bisexual, have also been observed (Rübsamen-Weustenfeld 1991). The production of bisexual flowers along with the occurrence of carpellate and staminate flowers has been documented in *T. brevistylis* (Martínez 1994, Vergara-Silva et al. 2002). Inflorescences bearing these flowers were found to be very scarce during our field work. Initial embryological results suggest that both sexes are functional; however, it is still necessary to perform detailed studies regarding morphological and structural aspects of bisexual flowers (e.g. arrangement and fertility of anthers and carpels, as well as ecological-populational analyses of the distribution of these. A detailed study of the reproductive anatomy of these bisexual flowers is currently in progress. In spite of the floral variation already recorded for *Seychellaria* (Hemsley 1907) *Sciaphila* (Jonker 1943), *T brevistylis* (Martínez 1994) *Sciaphila albescens* and *T. hyalina* (Rübsamen-Weustenfeld 1991; Maas-van de Kamer 1995), issues regarding the viability or functionality of carpels and anthers are not mentioned in those studies.

Dieringer and Cabrera (1994) considered that a pollen grain has germinated when the length of the cytoplasmic material coming out of the pollen grain is equal or larger than the diameter of the pollen grain. In *T. brevistylis*, the observed cytoplasmic extensions were shorter than the diameter of the pollen grain, and therefore, according to the previous definition, the pollen grain perhaps only undergoes the initial phases of germination. Nevertheless, the occurrence of pollen grains in a pre-germination phase in *T. brevistylis* is particularly significant considering the pre-anthesis cleistogamy process documented in *L. schismatica*, where pollen grains germinate inside the anthers of pre-anthetic flowers and the pollen tube grows through the floral receptacle into the carpels to fertilize the ovules (Márquez-Guzmán et al. 1993). The finding of flowers of *T. brevistylis* with stamens and carpels on the same receptacle (Vergara-Silva et al. 2002) and the presence of pollen grains in a pre-germination phase in male flowers may be related to the evolution of species with hermaphroditic flowers, through changes in the reproductive system. The question of the similarity between the floral morphology and reproductive processes in *T. brevistylis* and *L. schismatica* should be addressed in a broad evolutionary context. A detailed discussion of possible developmental-genetic and microevolutionary scenarios for the origin of *L. schismatica* from Triuris-like ancestors is given in Vergara-Silva et al. (2002).

Based on the literature regarding embryological information for *T. hexophthalma*, *T. hyalina* and *T. alata* (Maas and Rübsamen 1986; Rübsamen-Weustenfeld 1991; Maas-van de Kamer 1995), and as a result of this investigation, we suggest that the embryological features of *T. brevistylis* should be considered the best known for the genus and the family Triuridaceae.

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Figure legends

Figure 1. Floral morphology of *Triuris brevistylis*. Fig. 1.1, Male flower showing three reflexed tepals (TP) with long caudate apexs (CA). Bar = 1.8 mm. Fig. 1.2, Fleshy and hyaline androphore (AD), the arrows mark wing like tissue extension at its apex. Bar = 0.51 mm. Fig. 1.3, Anther (AN) showing extrorse dehiscence by longitudinal slit and papillose epidermis. Bar = 155 μm. Fig. 1.4, Tetrasporangiate young anther, the arrow indicate the septum. Bar = 119 μm. Fig. 1.5, Bisporangiate mature anther without septum between pollen sacs. Bar = 119 μm. Fig. 1.6, Female flower showing apocarpous gynoecium and tepals (TP) with short caudate apex (CA). Bar = 1.1 mm. Fig. 1.7, Longisection of the carpel showing style (S) terminal. Bar = 80.5 μm. Fig. 1.8, Carpels showing glabrous stigmatic zone (ST). Bar = 105 μm.

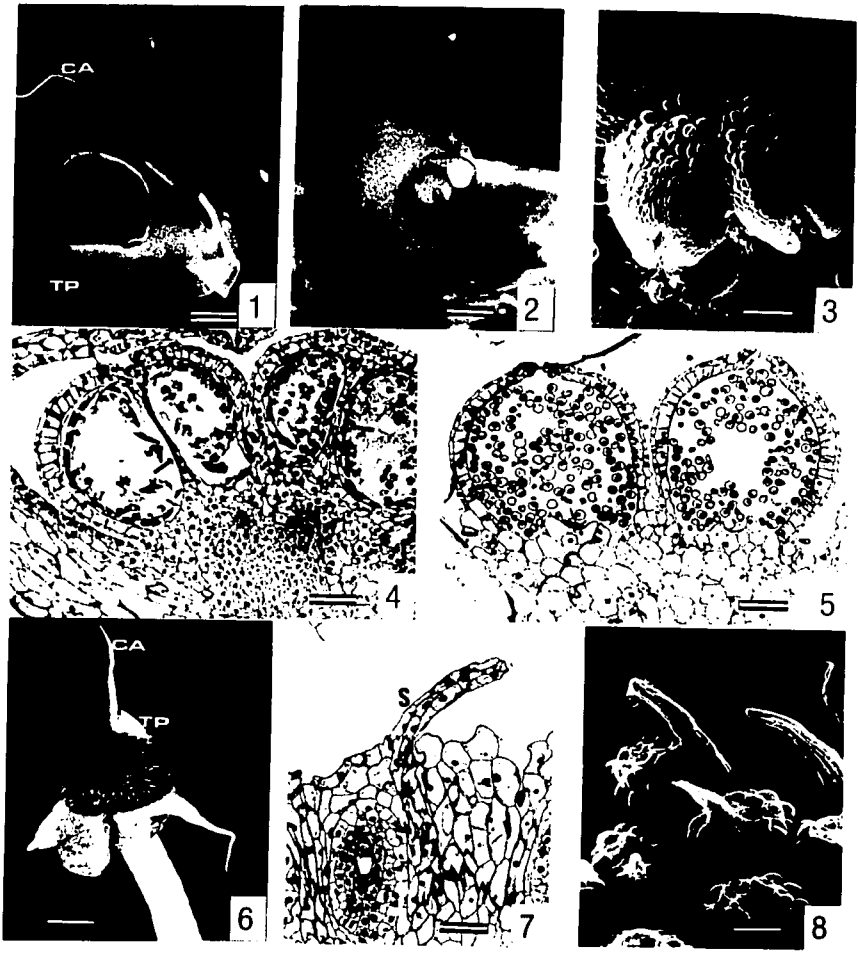
Figure 2. Light micrographs of the anther wall development and microsporogenesis. Fig. 2.9, Longisection of female flower showing vascular tissue and mucilaginous ducts (MD) in the receptacle (R). Bar = 10.2 μm. Fig. 2.10, Longisection of male floral bud showing floral meristem, tepals primordia (TP) and 2-3 layered bract (B). Bar = 53 μm. Fig. 2.11, Young anther with primary parietal (PP) layer and sporogenous tissue (SS). Bar = 20 μm. Fig. 2.12, Periclinal division of the inner secondary parietal layer (SP). Bar = 39 μm. Fig. 2.13, Young anther wall showing epidermis (EP), endothecium (ED), middle layer (M) and tapetum (T). Bar = 26 μm. Fig. 2.14, Remains of middle layer and tapetum (arrow) flattened and crushead to the endothecium (ED). Bar = 14 μm. Fig. 2.15, Mature anther wall showing epidermis and endothecium (ED) with U-shaped wall thickenings. Bar = 11 μm. Fig. 2.16, Microspore mother cells (MM) in meiosis. Bar = 48 μm. Fig. 2.17, Cytoplasmic bridges (arrow) among tapetal cells. Bar = 9 μm. Fig. 2.18, Isobilateral microspore tetrads (arrows). Bar = 26 μm.

Figure 3. Microgametogenesis and pollen grain morphology. Fig. 3. 19, Free uninucleate microspore with ornamented exine (E). Bar = 6 μm. Fig. 3.20, Pollen grain showing vegetative and generative cell. Bar = 6 μm. Fig. 3.21, 3-celled pollen grain. Bar = 9 μm. Fig. 3.22, 3-celled pollen grain shed through the stomium. Bar = 18 μm. Fig. 3.23, Pollen grain in pre-germination stage inside of the pollen sacs. Bar = 12 μm. Fig. 3.24, Oblate spheroidal, inaperturate, intectate verrugate pollen grain. Bar = 5 μm. Fig. 3.25, Partial exineless (arrow) pollen grain. Bar = 2 μm. Fig. 3.26, TEM photograph showing thin exine (E) and thick intine (I) evenly distributed all over the pollen grain. Bar = 0.5 μm.

Figure 4. Megasporogenesis and megagametogenesis. Fig. 4.27, Female floral meristem surrounded by primordia of the tepals (TP) and bract (B). Bar = 110 μm. Fig. 4.28, Receptacle (R) showing ovaries primordia. Bar = 83 μm. Fig. 4.29, Nucellus tissue and megaspore mother cell enclosed by ovary wall (OW). Bar = 41 μm. Fig. 4.30, Ovule showing elongate megaspore mother cell and outer (OI) and inner (II) 2-layered integuments. Bar = 29 μm. Fig. 4.31, Linear megaspore tetrad. Bar = 20 μm. Fig. 4.32, Functional megaspore (FM) in chalazal region, the arrows indicate megaspores degenerate. Bar = 16 μm. Fig. 4.32, Binucleate embryo sac. Bar = 20 μm. Fig. 4.34, Tetranucleate embryo sac. Bar = 20 μm. Fig. 4.35 Octanucleate embryo sac. Bar = 29 μm.

Figure 5. Embryo sac and seed morphology. Fig. 5. 36, 7-celled embryo sac showing antipodal cell (A), central cell (C) with two polar nuclei fuse and egg apparatus (EG). Bar = 24 μm. Fig. 5.37, Transection of seed showing remnants of inner integument (arrows) and endotesta (EN) with tannins and non-soluble polysacharides and exotesta (EX) with non-soluble polysacharides. Bar = 25 μm. Fig. 5.38, Free endosperm (EC) nuclei and big cells of endotesta (EN) toward micropylar region. Bar = 40 μm. Fig. 5.39, Cellular stage of endosperm (EC) the arrow indicate remnants of inner integument cuticle at the micropylar region. Bar = 61 μm. Fig. 5.40, Endosperm showing protein bodies and non-soluble polysacharides and external wall cell thick and inner integument and nucella cuticles (arrow). Bar = 9 μm. Fig. 5.41, Chalazal cap (CC) and endotesta (EN) with non-soluble polysacharides (arrow). Bar = 24 μm. Fig. 5.42, The chalazal cap (CC) showing fluorescence. Bar = 68 μm. Fig. 5.43, 3-celled linear proembryo and remnants of the pollen tube (arrow). Bar = 16 μm. Fig. 5.44, Suspensor and embryo proper. Bar = 19 μm.

Figure 6. Fig. 6.45, Undifferentiated embryo. Bar = 121 μm.Fig. 6.46, Seed. Bar = 54 μm. Fig. 6.47, Receptacle with ripe fruit in their centre. Bar = 590 μm. Fig. 6.48, Achene showing reticulate epidermis and style persistent Bar = 64 μm.



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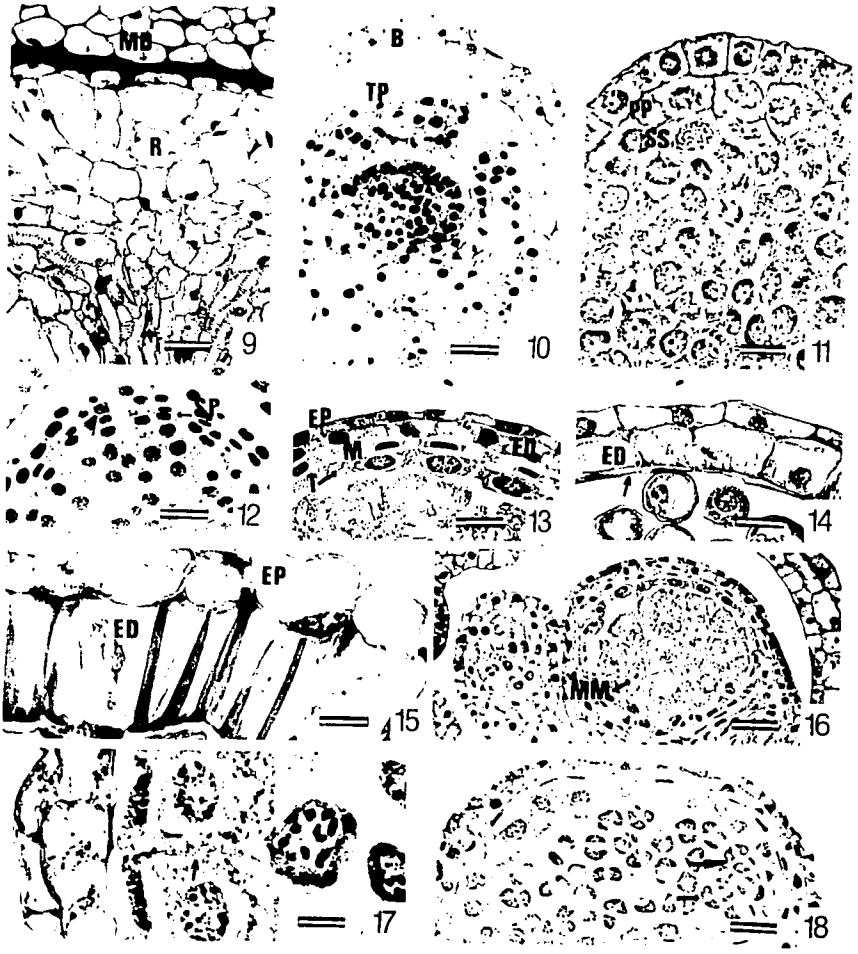

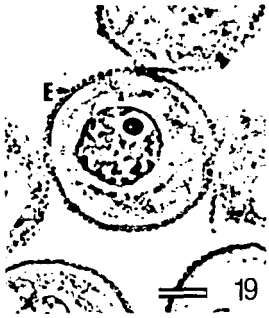

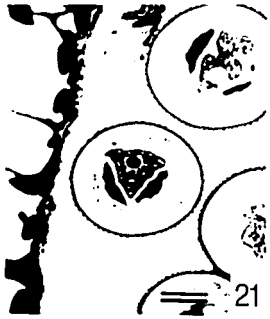

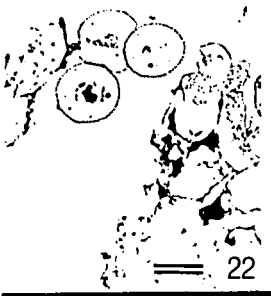

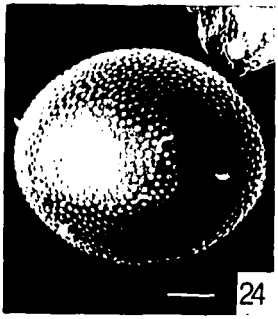

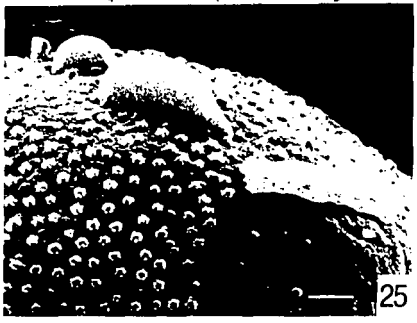

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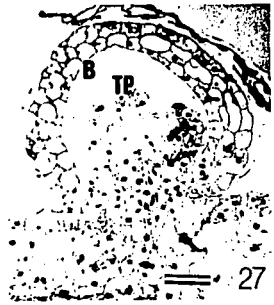

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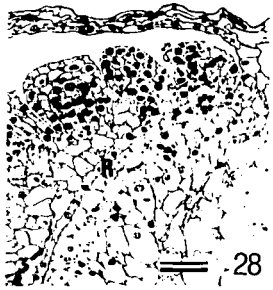

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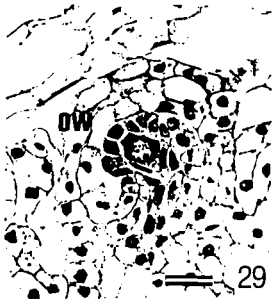

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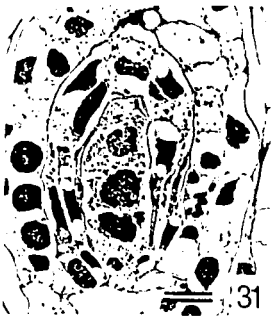
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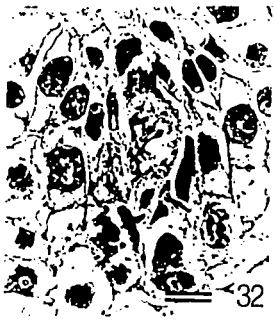
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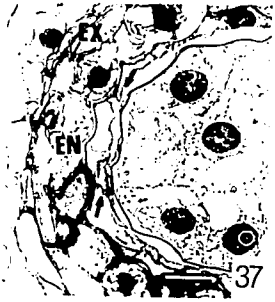

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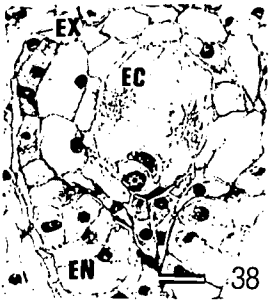

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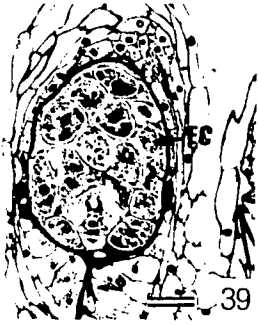

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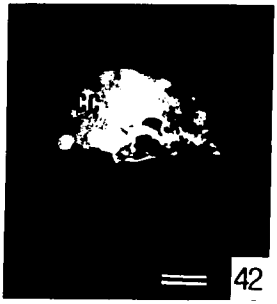
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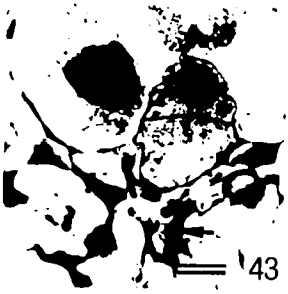
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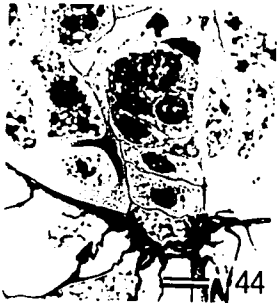
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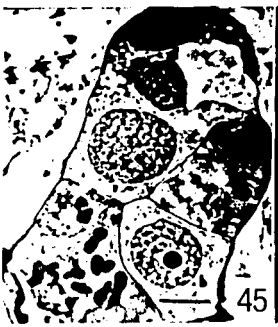
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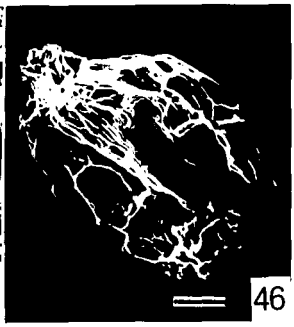
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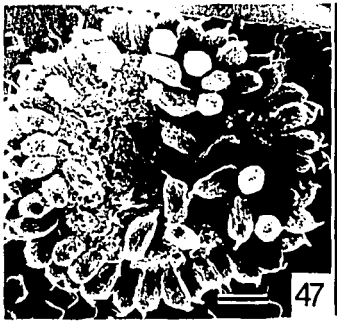
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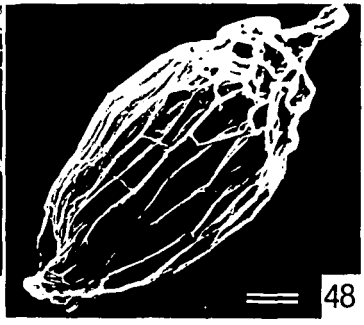
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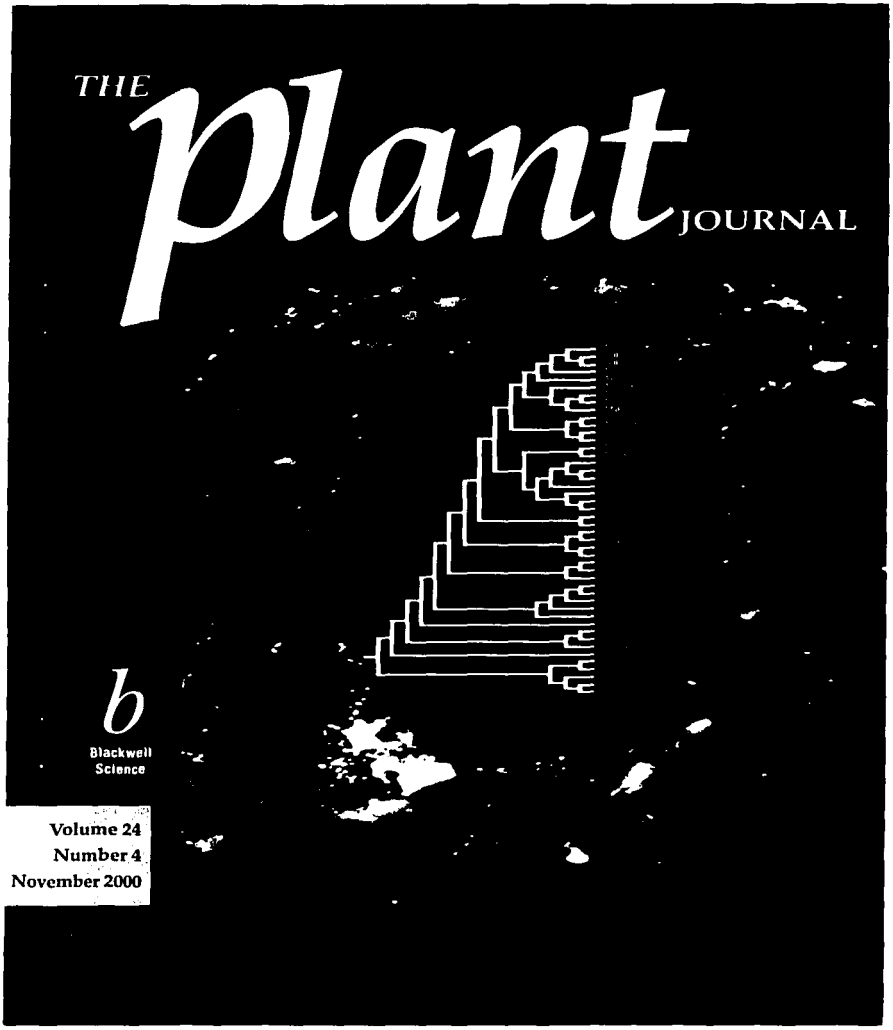

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MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes

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Summary

MADS-box genes encode transcriptional regulators involved in diverse aspects of plant development. Here we describe the cloning and mRNA spatio-temporal expression patterns of five new MADS-box genes from *Arabidopsis*: *AGL16, AGL18, AGL19, AGL27 and AGL31*. These genes will probably become important molecular tools for both evolutionary and functional analyses of vegetative structures. We mapped our data and previous expression patterns onto a new MADS-box phylogeny. These analyses suggest that the evolution of the MADS-box family has involved a rapid and simultaneous functional diversification in vegetative as well as reproductive structures. The hypothetical ancestral genes had broader expression patterns than more derived ones, which have been co-opted for putative specialized functions as suggested by their expression patterns. *AGL27* and *AGL31*, which are closely related to the recently described flowering-time gene *FLC* (previously *AGL25*), are expressed in most plant tissues. *AGL19* is specifically expressed in the outer layers of the root meristem (lateral root cap and epidermis) and in the central cylinder cells of mature roots. *AGL18*, which is most similar in sequence to the embryo-expressed *AGL15* gene, is expressed in the endosperm and in developing male and female gametophytes, suggesting a role for *AGL18* that is distinct from previously characterized MADS-box genes. Finally, *AGL16* RNA accumulates in leaf guard cells and trichomes. Our new phylogeny reveals seven new monophyletic clades of MADS-box sequences not specific to flowers, suggesting that complex regulatory networks involving several MADS-box genes, similar to those that control flower development, underlie development of vegetative structures.

Keywords: MADS-box, endosperm, guard cells, root, trichome, *Arabidopsis*.

Introduction

Transcriptional regulators play important roles in developmental pathways (Schwechheimer and Bevan, 1998), and changes in them are likely to be key molecular determinants of the morphological evolution of plants and animals (Doebley and Lukens, 1998). Phylogenies and ancestral character reconstructions of developmental regulators, such as the one presented here, provide the historical framework for studies of the evolution of developmental genetic pathways and give useful clues about the molecular basis of morphological evolution, thus linking the fields of development and evolution (Purugganan, 1998). To this end, complete phylogenies of these regulatory multigene families for model systems are fundamental for comparative analyses and interpretations of sequences from other species.

The MADS-box gene family encodes transcription factors involved in diverse biological functions in eukaryotes (Riechmann and Meyerowitz, 1997; Shore and Sharrocks, 1995). In plants these genes play central roles in flower and fruit development (Bowman *et al.*, 1999; Weigel, 1995). Other MADS-box genes are expressed in vegetative tissues, ovules and embryos, suggesting that this family of genes plays diverse roles throughout plant development (Colombo *et al.*, 1995; Ma *et al.*, 1991; Rounsley *et al.*, 1995; Zhang and Forde, 1998).

We report the isolation, chromosome mapping and mRNA *in situ* expression profiles of five new MADS-box genes from *Arabidopsis thaliana*. Our data suggest that these genes play novel regulatory roles in guard cells, trichomes, roots, pollen and endosperm, and thus may provide useful markers for further functional and evolutionary analyses. Expression data mapped onto a new MADS-box phylogeny reveal new clades that group genes with similar expression patterns, mostly in vegetative structures, and that seem to have evolved at about the same time that the major floral clades evolved (Purugganan, 1997). Mapping analyses suggest that the ancestral pattern of expression was a generalized one, and that duplications gave rise to genes with restricted spatio-temporal expression patterns probably recruited to specialized functions in either reproductive or vegetative structures. We also identify 10 new groups of potentially redundant genes that are predicted to share more than 70% amino acid identity. These results will aid future functional analyses because several closely related MADS-box genes with overlapping expression patterns encode redundant functions (Ferrándiz *et al.*, 2000; Kempin *et al.*, 1995; Liljegren *et al.*, 2000; Pelaz *et al.*, 2000). Because previous studies have found a close correlation between expression patterns of MADS-box genes and assigned functions (Gu *et al.*, 1998; Liljegren *et al.*, 2000), this study is the first step toward functional characterization of several MADS-box genes involved in vegetative development, and provides a guide to characterizing the corresponding mutant alleles.

Results and Discussion

Five new Arabidopsis *MADS-box genes*

Phylogenetic studies are most informative when all or most members of a multigene family are known. A PCR-based approach led to the identification of three new MADS-box genes: *AGL16*, *AGL18* and *AGL19*. In addition, through a yeast two-hybrid we found *AGL27* that is closely related to *AGL31*, a gene recently identified through *Arabidopsis* genome sequencing, and to *FLC* (Michaels and Amasino, 1999; Sheldon *et al.*, 1999).

AGL16, *AGL18* and *AGL19* are similar to previously characterized plant MADS-box genes. Each encodes deduced proteins of 240, 256 and 219 amino acids, respectively, with a highly conserved MADS-box at the amino-terminus, a clearly identifiable K-box region, and a divergent C-terminal region. In contrast, cDNAs for *AGL27* and *AGL31* encode deduced proteins of 192 and 178 amino acids, respectively. Two transcripts, resulting from differential splicing, were detected via RT–PCR for *AGL27* (I and II). The more abundant transcript would encode a deduced protein of 192 amino acids, and the less abundant transcript would encode a protein of 173 amino acids. *AGL27* and *AGL31* each have well defined MADS-box regions, and although their K regions are very similar to each other, they are quite different from the K regions previously described for other MIKC type MADS-box genes (Alvarez-Buylla *et al.*, 2000). As in other plant MADS-box genes, the C-terminal regions of *AGL27* and *AGL31* are also more divergent, both between themselves and with respect to other MADS-box genes.

Chromosome mapping

To discover whether any of the new MADS-box genes could be linked to known mutants, we determined the map position of each gene, shown in Figure 1 along with positions of all MADS-box sequences found in *Arabidopsis* up to now. *AGL16* maps to chromosome III between the RFLP markers 249 and g4014. *AGL18* also maps to chromosome III approximately 3.9 cM below the g2778 marker. *AGL19* maps to chromosome IV between markers g10086 and g4564a; and *AGL 27* maps to the bottom of chromosome I, approximately 2.8 cM from marker m532 and 8.6 cM from marker g17311.

Novel expression patterns of MADS-box genes

A preliminary identification of organs in which these newly isolated genes are expressed was undertaken using RNA gel-blot analyses. In addition, gene-specific probes were used in RNA *in situ* hybridization analyses to define the temporal and spatial patterns of expression of these genes.

AGL16 is expressed in trichomes and guard cells

AGL16 RNA accumulates at high levels in leaves, moderate levels in roots and stems, and at low levels in flowers and

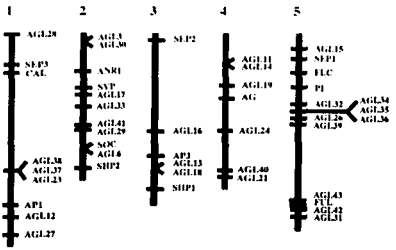

Figure 1. Map positions of *Arabidopsis* MADS-box genes.

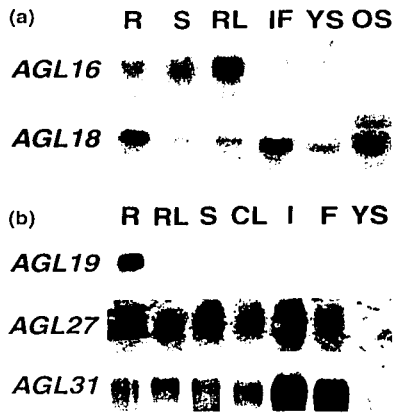

Figure 2 Expression of *AGL16*, *AGL18*, *AGL19*, *AGL27* and *AGL31* transcripts in wild type plants.
Total RNA (a) or poly(A) RNA (b) was used from roots (R), stems (S), rosette leaves (RL), cauline leaves (CL), inflorescences (I), mature flowers (F), inflorescences and mature flowers (IF), young siliques (YS), and older siliques (OS).

young siliques (Figure 2). Within rosette leaves *AGL16* is expressed in mature guard cells and trichomes found in both the abaxial and adaxial epidermis (Figure 3). Additional data suggest that *AGL16* is also expressed in epidermal cells of roots (C. Burgeff, S. Liljegren, M. Yanofsky and E.R. Alvarez-Buylla, unpublished results). Recent studies have begun to uncover the molecular mechanisms of cell-type specification in leaves and roots (reviewed by Larkin *et al.*, 1997; Schiefelbein *et al.*, 1997), including a number of genes that have been found to be expressed in both leaf and root epidermal cells. Furthermore, it has been shown that *GLABRA2* (*GL2*) is part of regulatory networks regulating both leaf and root epidermis development (Schiefelbein *et al.*, 1997). Further functional analyses should be performed to test if *AGL16* and other MADS-box genes with similar sequences and expression patterns are also part of such conserved networks (see Figure 6).

AGL16 expression was also observed in guard cells of the hypocotyl, but was not detected in guard cells of the inflorescence stem, flower pedicel or sepals (data not shown). The evolution of stomata was a key event during the early evolution of land plants, yet very little is known about the regulatory pathways underlying the development of this differentiated epidermal cell type (Larkin *et al.*, 1997). Other genes have been shown to control stomatal patterning on both the hypocotyl and root epidermis (Berger *et al.*, 1998; Lee and Schiefelbein, 1999), and it will be interesting to investigate the possible regulatory role of *AGL16* in controlling these patterning processes.

AGL18 *is expressed in pollen and endosperm*

AGL18 is expressed at highest levels in roots, flowers and siliques, and significant expression was observed in stems and leaves (Figure 2). In flowers, no expression was detected in sepals or petals. Within stamens, expression was first detected during stage 9 and was localized to the sporogenous tissue of anthers, while it was absent from filaments or anther walls. *AGL18* RNA was particularly apparent in the microspores before they separate from each other (stage 9–10, data not shown), but it was still observed at high levels within pollen grains up to stage 13 of flower development when anthers dehisce (Figure 4a). *AGL18* is the first *Arabidopsis* MADS-box gene reported to show high expression levels in pollen. Previously, pollen-specific genes have been assigned either to 'early' genes, probably encoding cytoskeletal and cell wall proteins (McCormick, 1991), or 'late' genes, expressed around the time of microspore mitosis and strongly expressed during pollen maturation. *AGL18* is expressed during both stages. Another MADS-box gene, *DEF125*, was shown to be expressed 'late' in pollen from *Antirrhinum* (Zachgo *et al.*, 1997), but this gene is more similar to the *ANR1* *Arabidopsis* root gene than to *AGL18*.

During carpel development, *AGL18* RNA is first detected in developing ovules (stage 10). Expression appears uniform early in ovule development (Figure 4b). After fertilization, *AGL18* RNA was detected only in globular structures or nodules of proliferating free nuclear endosperm that are important for embryo development (Mansfield and Briarty, 1991; Scott *et al.*, 1998). This gene was detected only up to the heart stage of embryo development, when very little nuclear endosperm remains, and it was not detected in developing embryos at any stage (Figure 4c,g; data not shown). In contrast, the closely related *AGL15* MADS-box gene is expressed in embryos, but not in the endosperm (Rounsley *et al.*, 1995).

Despite the key evolutionary and functional relevance of the endosperm, one of the key innovations of angiosperms (Friedman, 1992), little is known about the genetic circuitry controlling its development. *AGL18* represents one of the few reported molecular markers of endosperm in *Arabidopsis* (Drews *et al.*, 1998). Differential expression of *AGL18* within endosperm tissues correlates with the significant structural and developmental differences between the micropylar and the chalazal chambers in

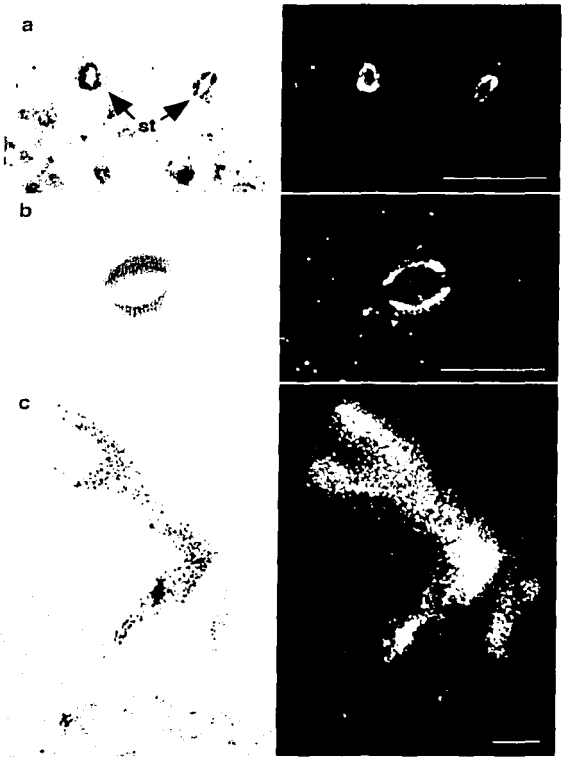

Figure 3. *AGL16* expression in leaf guard cells and trichomes.
Sections of wild-type rosette leaves were probed with *AGL16* antisense RNA, and bright-field (left) and dark-field (right) photographs of the same section are shown in panels (a–c).
(a,b) *AGL16* is expressed in stomata (st) of expanding (a) and more mature (b) leaves, along the pore-facing edge of each guard cell. (c) Stellate leaf trichome showing *AGL16* expression. Scale bars, 25 μm.

Arabidopsis and other Brassicaceae (Mansfield and Briarty, 1991).

AGL19 *is expressed in roots*

AGL19 RNA is specific to roots, and no expression was detected in leaves, stems, flowers, or siliques (Figure 2). This gene is expressed in the columella, lateral root cap and epidermal cells of the meristematic region of the primary and lateral root tips (Figure 5b–d). In the mature differentiated region of the root, *AGL19* RNA was observed in all cell types of the central cylinder from the pericycle to the inner cell types of the vascular bundles, but the endodermis, cortex and epidermis remained unlabelled (Figure 5e).

The root cap plays an important role in gravitropic sensing, and recent ablation experiments show that this tissue is important for the signaling system determining root growth rate and the suppression of lateral root formation (Tsugeki and Fedoroff, 1999). Such signaling

circuits are probably linked to transduction pathways that respond to external stimuli, as is has been proposed for the *ANR1* MADS-box gene (Zhang and Forde, 1998).

AGL27 and AGL31 are similar in sequence and expression patterns to FLC

RNA blot analyses revealed significant levels of *AGL27* and *AGL31* expression in most plant tissues, including roots, leaves, stems and flowers, and low levels of expression were detected in siliques with the *AGL31* probe (Figure 2). *In situ* data using inflorescences (data not shown) revealed that they have very similar overall expression patterns. Their mRNAs were detected in all flower organs and in early floral meristems, with the highest levels of expression observed in flower pedicels. These similar expression patterns suggest that these two closely related genes (Figure 6) probably represent functionally redundant loci.

FLC is very closely related to *AGL27* and *AGL31* (Figure 6), and they all share similar expression patterns. Therefore *FLC* could share some functions with *AGL27* and *AGL31*, but it also appears to have at least some independent roles because the single *flc* loss of function mutants have a clear phenotype of early flowering. This phenotype suggests that this gene is a repressor of flowering. Despite the generalized presence of *FLC* transcripts in all plant organs, the mutants only show alterations in flowering time (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). The overlapping expression patterns of these two genes and *FLC*, and the close sequence similarity of the three genes, suggest that *AGL27* and *AGL31* may also control the transition to flowering. These two genes could also share additional redundant functions with *FLC* that might be revealed in double or triple mutants.

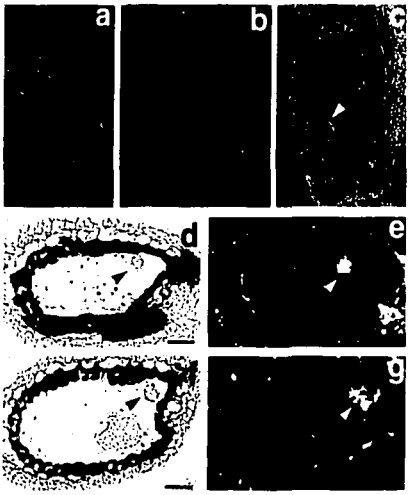

Figure 4. *AGL19* expression in wild-type flowers, ovules and developing seeds.
(a–c) Bright/dark field double exposures of *in situ* hybridizations probed with *AGL19* antisense RNA; signal in red. (a) Longitudinal section of an anther at stage 13 of flower development. (b) Longitudinal section of a carpel at stage 16 of flower development. (c) Longitudinal section of an embryo sac showing endosperm chalazal nodules. (d–g) Sections of embryo sacs were probed with *AGL19* antisense RNA, and bright field (left) and dark field (right) photographs of the same sections. Arrows point at the nodules of free-nuclear stage endosperm with strong signal. Scale bars, 50 μm.

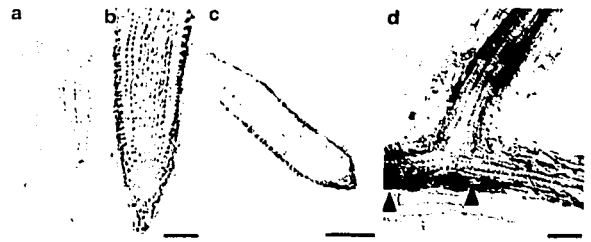

Figure 5. *AGL19* expression in wild-type roots.
(a) Longitudinal section of root meristem probed with sense *AGL19* RNA. Longitudinal sections of main (b) and lateral (c) root tips probed with antisense AGL19 RNA. Note that (c) is not a medial section and a few stained epidermal cells are seen near the tip. (d) Mature root probed with antisense *AGL19* RNA showing staining in all cell types of the central cylinder (right arrow: pericycle, phloem and parenchyma cells). Left arrow points at unstained endodermis cell. Scale bars (a,b,d) 50 μm; (c) 100 μm.

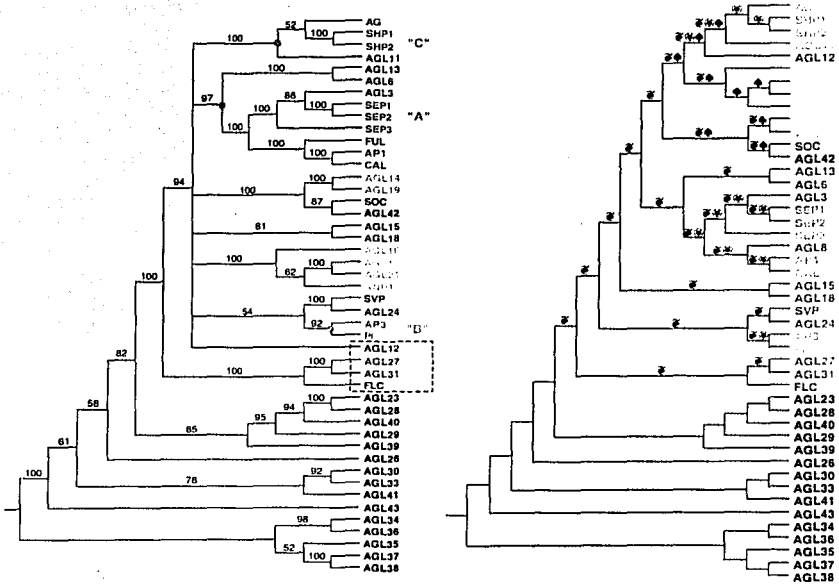

Figure 6. Phylogeny of the *Arabidopsis* MADS-box gene family.
(a) Strict consensus maximum parsimony phylogeny of the elison alignment of the MADS, I and K amino acid sequences, rooted with AGL34 clade as outgroup. Bootstrap support indicated on branches (those with <50% collapsed). Color indicates mRNA expression of genes encoding each protein as reproductive (red), vegetative (green), or both (blue). Proteins boxed in purple are those for which coiled-coils were predicted at K domain (Alvarez-Buylla *et al.*, 2000); those within dashed fine squares have conserved amino acids within the K domain but no predicted coiled-coils. ABC clades are indicated by red dots at their nodes in the tree and labeled on the right. (b) Single most parsimonious tree (CI = 0.556; RI = 0.518; RC = 0.288, length = 34345) with ancestral states of expression reconstructed under parsimony. More than one symbol indicates ambiguous reconstruction.

Phylogeny: evolution of MADS-box gene function in plants

We used MIK amino acid sequences to infer phylogenies of the newly identified MADS-box genes together with 42 previously reported MADS-box sequences (Alvarez-Buylla *et al.*, 2000 and references therein for sequence sources). The only known MADS-box sequence not included in our phylogeny was *AGL32*, as we were not able to predict its amino acid sequence reliably. We present a tree (Figure 6a) rooted with the *AGL34*-like sequences that are clear members of the Type I MADS-box lineage (Alvarez-Buylla *et al.*, 2000). It is likely that most of the different clades that define the MADS-box gene family in *Arabidopsis* are represented in this phylogeny and that the clades that include the well-characterized ABC genes are complete, while the newly uncovered clades may still lack additional members.

Sequences for which a predicted coiled-coil protein domain (K-domain) downstream of the MADS-domain was found are within the purple box outside the dash-line square (Alvarez-Buylla *et al.*, 2000). Within the dashed-square are sequences with more divergent K domains in which some of the conserved amino acids are missing, and for which the programs do not predict coiled-coils.

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These and previous analyses (Alvarez-Buylla *et al.*, 2000), which showed that predicted coiled-coils are found only in plant sequences, suggest that the K domain evolved along the plant lineage after its divergence from the animal lineage.

Our analyses (Figure 6a) confirm that genes within each monophyletic clade share similar expression patterns (Doyle, 1994; Purugganan *et al.*, 1995; Theissen *et al.*, 1996). The previously identified floral-specific clades are recovered and indicated by the letters ABC (originally typified by *AP1*, *AP3/PI* and *AG*, respectively). The ABC clades are largely flower- and fruit-specific, and none appears to be expressed in roots. However, some genes in the A clade are also expressed in leaves and stems (Purugganan, 1998; Rounsley *et al.*, 1995). Within this clade it is noteworthy that the recently characterized *SEPALLATA* (*SEP*) genes are required for the canonical B and C functions (Pelaz *et al.*, 2000). As mentioned above, B-function genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), group with *SHORT VEGETATIVE PHASE* (*SVP*, previously *AGL22*; Hartmann *et al.*, 2000) and *AGL24*. But *SVP* and *AGL24* are also expressed in inflorescence meristems, stems and leaves (C. Gustafson-Brown, C. Ferrandiz, and M. Yanofsky, unpublished results; Hartmann *et al.*, 2000).

None of the seven newly identified clades is specific to flowers or fruits (Figure 6a). The *ANR1* clade is largely root-specific, although *AGL16* is also expressed in leaves. The *SOC1* clade is also largely root-specific, with *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*, previously *AGL20*) also expressed in leaves and flowers (Samach *et al.*, 2000), while its sister gene, *AGL42*, has not yet been studied. *AGL15* and *AGL18* group together in another clade, and they are expressed in the embryo and nuclear endosperm, respectively, as well as in other plant tissues throughout the complete life cycle of *Arabidopsis*. Within the polytomy that groups all the clades described up to now, *AGL12* is the only single-gene branch. *AGL12* is also expressed at high levels in roots, and is found in a few cell types in flowers and shoots (C. Burgeff and E.R. Alvarez-Buylla, unpublished results). *FLC*, *AGL27* and *AGL31* form a sister monophyletic clade to all the groups described above (Figure 6a). All three genes have a very similar and generalized expression pattern. The newly discovered clades of MADS-box genes, whose expression is restricted to vegetative structures, suggests that complex genetic circuits also underlie development of these structures (Scheres *et al.*, 1995).

Outside the clades described above, several monophyletic groups are well resolved (Figure 6a). None of the sequences in the latter shows clear similarity beyond the MADS-domain, and cDNA clones have not been identified for any of these sequences, although an *AGL39* EST is available (Alvarez-Buylla *et al.*, 2000). Thus the expression patterns and functions of all of these sequences have yet to be determined.

Purugganan (1998) has interpreted previous similar expression and phylogenetic analyses as an indication that vegetative MADS-box functions evolved before reproductive ones. He argues that rapid early duplications and functional diversification of these genes could have been caused by selective pressures on ancestral land plant lineages to evolve more complex reproductive structures. The mapping of the expression patterns onto the gene phylogeny enables us to revisit these arguments on the functional diversification within and among gene clades in the MADS-box family.

Mapping analyses on the bootstrap (not shown) and the most parsimonious tree (Figure 6b) suggest that the ancestral pattern of expression was a generalized one, and that duplications gave rise to genes with restricted patterns of expression probably recruited to specialized functions in either reproductive or vegetative structures. In most cases the ancestral states within clades are ambiguous. However, the putative ancestral gene of the *AG–SHP* clade appears to have had an mRNA expression already restricted to reproductive structures. Similarly, the putative ancestral gene in the *AGL17–AGL21–ANR1* clade appears to have had an expression restricted to vegetative tissue, but the putative ancestor of these two clades had a generalized pattern of expression. Therefore our data suggest that the function of the plant MADS-box genes did not progress from vegetative to reproductive. Instead, it seems that differential gene recruitment was correlated with spatio-temporal restriction of expression pattern in both reproductive and vegetative structures as gene duplication events occurred.

Traditional morphological studies (Foster and Gifford, 1991) and the analyses presented here suggest that specialized vegetative structures have apparently evolved simultaneously to reproductive structures along a species tree. Furthermore, under these assumptions selectionist arguments that would explain the diversification of reproductive structures during early evolution of land plants should also be applied to the diversification of vegetative structures. On the other hand, rapid evolution could explain the lack of resolution in the branching order of the clades within the purple box in Figure 6(a). These are appealing hypotheses that should be tested with estimations of divergence times among gene clades and analyses of other species sequences (Purugganan, 1997, Purugganan, 1998).

Finally, our phylogeny reveals new groups of closely related and possibly functionally redundant MADS-box sequences, with over 70% identity at the MIK amino acid level and supported by bootstrap values >87%: *AGL13/AGL6*, *AGL14/AGL19*, *AGL17/AGL21*, *SOC/AGL42*, *SVP/AGL24*, *AGL27/AGL31/FLC*, *AGL23/AGL28/AGL40/AGL29*,

AGL30/AGL33, AGL34/AGL36 and *AGL37/AGL38*. These data should be useful to guide further functional characterization of these genes, as it is possible that only double, triple (Pelaz *et al.*, 2000) or even quadruple mutants of these closely related sequences may show phenotypes amenable to further analysis.

Given the crucial roles of MADS-box genes in plant development and the rapid pace at which new MADS-box genes from diverse plant species are being cloned, this family is becoming a promising paradigm for unravelling mysteries underlying the molecular basis of morphological evolution in plants (Kramer *et al.*, 1998; Theissen *et al.*, 2000). For example, *AGL15 and AGL18* may become useful molecular markers for studying the molecular evolution of endosperm development, and thus testing the theory that endosperm evolved from a supernumerary embryo that was transformed into nourishing tissue during early angiosperm evolution (Friedman, 1992). These two genes appear to share a more recent common ancestor with each other than with any other family member. Within the embryo sac, *AGL15* expression is restricted to the embryo, while that of *AGL18* is specific to the free nuclear endosperm. Probably both genes were expressed in embryos in the common ancestors of gymnosperms and angiosperms, and during evolution of the latter one of them (*AGL18*) was recruited to control endosperm development. This hypothesis could be tested with expression patterns of genes orthologous to *AGL15 and AGL18* in gymnosperms that constitute the sister group to angiosperms, and still have supernumerary embryos.

Experimental procedures

Cloning of new Arabidopsis *MADS-box genes*

AGL16 was cloned following Rounsley *et al.* (1995) and its 5′ coding region was derived from a genomic clone (SL76), which was identified by screening a genomic library (J. Mulligan and R. Davis, unpublished results) with a probe synthesized from a 400 bp *Eco*RI *AGL16* cDNA fragment (pSL4). *AGL18* and *AGL19* were cloned with degenerate primers based on the tomato TM8 sequence, 5′-CGGAATTCATGGG(AGCT)(CA)G(AGCT)GG(AGCT)-(AC)G(AGCT)AC-3′ and 5′-CGGGATCC(AGTC)AC(CT)TC(AGTC)-GC(GA)TC(GA)CA(AGTC)A(GA)(TGA)AT-3′. With the sequences of these novel MADS-boxes, nested oligonucleotides were designed to amplify the corresponding cDNAs by reverse transcription PCR with oligo(dT). RNA from root, from leaf and root tissues, and from whole plant tissue was used to clone *AGL19, AGL16* and *AGL18* cDNAs, respectively. Each cDNA was independently amplified, cloned, and sequenced at least twice to check for PCR-induced mutations. TAIL/PCR was used to obtain the 5′ ends (Boehringer Kit, Indianapolis, USA).

AGL27 was isolated as an interactor of AP1. The cDNA expression library was poly(T) primed (S. Pelaz *et al.*, unpublished results) and cloned into pBI771 plasmid (pPC86 plasmid from Chevray and Nathans, 1992 with minor modifications) in *Sal*I–*Not*I sites. The interactor clone was not full-length as it began 61 bases after the end of the MADS box of AGL27-I. Based on the genomic sequence that later appeared in the database, oligos were designed to isolate the entire *AGL27* cDNA using RT-PCR: YSP28.11-3′: 5′-CCAATCCGTACATTCAGACA-3′ (for RT) and AGL27-6: 5′-CCGGATCCGAAGCCATGGGAAGAAGA-3′, and AGL27-7: 5′-CCGGATCCTCAGGCTTTGAGTTTAAGG-3′. Two different full-length cDNAs were isolated: AGL27-I and AGL27-II. *AGL31* cDNA clone was deduced from the overlapping ESTs (one putative intron, by similarity with *AGL27*, present in the cDNA was removed; the ESTs are T45787 and comprise the 5′ end and H36546). cDNA sequences are in accession numbers: *AGL16* (AF312662), *AGL18* (AF312663), *AGL19* (AF312664), *AGL27-1* (AF312665), *AGL27-11* (AF312666) and *AGL31* (AF312667).

Chromosomal mapping

To map *AGL16*, a genomic clone (SL76) was used to score an *Xba*I polymorphism between the Columbia (Col) and Ler ecotypes for 36 individuals of a mapping population. *AGL18* and *AGL19* were mapped using PCR-amplified genomic clones and analyzing *Eco*RI and *Eco*RV polymorphisms, respectively, between Ler and Col for 79 individuals. To map *AGL27*, an artificial *Mae*III polymorphism between Ler and Col was created and scored for 22 individuals. The genotyping oligo designed to produce this polymorphism, 5′-CCCGGTATTTTTAATTTGTGAAATTTGTAA-3′, took advantage of two nucleotide differences between these ecotypes: AT (Col) and CA (Ler). Mapping data were analyzed with MAPMAKER MACINTOSH version 2.0 (E.I. duPont de Nemours, Wilmington, DE, USA) as described by Reiter *et al.* (1992).

RNA blot analyses

Total RNA was extracted from Landsberg *erecta* plant tissues as described by Rounsley *et al.* (1995) and used for the *AGL16, AGL18* and *AGL19* Northern blots. Poly(A)+ RNA was isolated from Columbia plant tissues using the Dynabeads Oligo(dT) kit from Dynal AS (Oslo, Norway) and used for the *AGL27* and *AGL31* RNA blots. The *AGL16* probe was synthesized from a 400 bp *Eco*RI fragment of pSL4; the *AGL18* probe from a 700 bp fragment of pAGL18–4; the *AGL19* probe from a 670 bp fragment of pAGL19-3; the *AGL27* probe from a 670 bp fragment of AGL27-I which starts at the end of the I region; and the *AGL31* probe from a 500 bp fragment of H36546 which starts at the beginning of the K-box. All probes used are gene-specific and exclude the MADS-box region. As RNA-loading controls, blots were stripped and rehybridized with *β-TUBULIN* probes (data not shown) as described by Marks *et al.* (1987).

In situ *hybridization*

Tissue fixation and sectioning were performed as described by Drews *et al.* (1991), with minor modifications. Hybridization conditions used for *AGL16* and *AGL18* were as described by Drews *et al.* (1991) and Ferrándiz *et al.* (2000) for *AGL19*. *AGL16* and *AGL18* ^{35}S-labeled antisense mRNAs were synthesized with SP6 RNA polymerase from *Bgl*I-digested pSL5 template and *Xba*I-digested pAGL18-3 template, respectively. *AGL19* digoxigenin-labeled antisense mRNA was synthesized with T7 RNA polymerase from *Bam*HI-digested pAGL19-3 template and *AGL19* sense mRNA with SP6 RNA polymerase from the same *Not*I-digested

plasmid, both according to the manufacturer's instructions (Boehringer).

Phylogenetic analyses

The sequences used and their accession numbers are given by Alvarez-Buylla *et al.* (2000). New sequences included are: *AGL41* (AC005168), *AGL42* (AB016880), and *AGL43* (AB016885). To test the robustness of the phylogeny under different alignments, we varied the gap and extension penalties to obtain 11 different alignments. These alignments were put in a large matrix that contained the 11 alignments placed one after the next. This is called an 'elison matrix'; if used to reconstruct phylogeny, the effect of ambiguous sites on the tree topology is minimized. The topology then largely depends on unambiguously aligned sites that retain their alignment across various gap penalties (Wheeler *et al.*, 1995). Phylogenetic analyses were conducted with unweighted parsimony using PAUP 4b2 (Swofford, 2000). Heuristic searching was used with 100 replicates of random addition sequences, TBR branch swapping, and no maxtrees limit, keeping all optimal trees and with gaps treated as missing data. The tree was rooted with the AGL34-like clade as outgroup, defining it as a monophyletic sister group to the ingroup. The non-parametric bootstrap (1000 replicates) was used to assess the reliability of branches. Expression patterns were assigned as reproductive, vegetative (root+leaves) or general (both reproductive and root/leaves), and the parsimony reconstruction of ancestral states method (Maddison and Maddison, 1992) was used to infer ancestral expression patterns of putative ancestral genes at each node (Figure 6b).

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Capítulo 6: generalidades

El presente artículo, correspondiente a un estudio conducido por Elena Alvarez-Buylla en el laboratorio de Martin Yanofsky (University of California -San Diego, EUA), presenta los patrones de expresión de cinco genes MADS-box en *Arabidopsis thaliana* –los genes AGL16, AGL18, AGL19, AGL27 y AGL31 en el contexto de sus relaciones filogenéticas con otros genes MADS-box provenientes del mismo genoma. La contribución del autor consistió en el mapeo de los patrones de expression sobre la filogenia; ésta manipulación resultó en la postulación de que la función de los genes MADS-box en plantas no ha progresado de atributos vegetativos a reproductivos, y en la sugerencia alternativa de eventos de reclutamiento diferencial de genes a partir de secuencias ancestrales que probablemente tenían patrones de expression generalizados.

Capítulo 7: generalidades

El artículo que concluye la presente tesis es un trabajo de filosofía de la biología, orientado a evaluar de manera crítica algunos aspectos actuales de la construcción del marco conceptual de la biología evolutiva del desarrollo ("evo-devo") en referencia a episodios históricos y hallazgos experimentales concretos en botánica, a partir de Goethe y hasta los descubrimientos contemporáneos sobre la base genético-molecular de la especificación, a lo largo de la ontogenia, de la identidad de diferentes tipos celulares, tejidos y órganos vegetales en diferentes grupos taxonómicos. El artículo problematiza alrededor de las limitaciones presentes de la versión actual de evo-devo, derivadas de su casi absoluta dependencia de investigaciones hechas en animales, al tiempo que propone, como contribución original, una nueva reflexión sobre la noción de extrapolación en biología evolutiva.

Este artículo será incluido en un número especial sobre evolución y desarrollo editado por Jason Scott Robert (Dalhousie University, Canadá) y Sahotra Sarkar (University of Texas, EUA) para la revista *Biology and Philosophy*, a publicarse en 2003.

Plants and the conceptual articulation of evolutionary developmental biology

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I. Introduction

Coming from several different standpoints, an already considerable number of biologists and a growing number of philosophers of science interested in biology are now participating in an enthusiastic discussion of the role of developmental processes in the determination of evolutionary pattern and the phenotypic aspects of biodiversity. The main themes of this discussion are not original[1], but most people concerned with the history of evolutionary biology -for professional reasons or otherwise- acknowledge that their current, renewed relevance is inevitable. On one hand, the existence of the molecular biology techniques that have led to the results that nowadays form the experimental core of evolutionary developmental biology[2] studies was not even imagined when the questions first arose; on the other, an appropriate conceptual integration between ontogeny and evolution was never fulfilled within the paradigmatic theory in the field, the Modern Synthesis (see, for example, Gilbert et al. 1996). Arguably, disagreements with the latter claim[3] and associated issues related to models of epistemological change in evolutionary biology will continue to be the main focus for philosophers and historians, regardless of the taxonomic affinity of the organisms from which the developmental-genetic data are collected (see, for example, Robert 2001, Griffiths 2002). However, since the concepts and experimental facts that give evo-devo its individuality as a scientific discourse come from the study of developmental mechanisms, it is not inconsequential that most of the current efforts to establish the boundaries and internal structure of this biological discipline rely on information pertaining to only one of the kingdoms in which ontogenetic development occurs: animals.

In this paper, I present a review of selected empirical facts and hypotheses generated in the fields of plant developmental genetics, comparative morphology and systematics, coupled to a discussion of the implications that these data could have for two issues of current interest to the community involved in evo-devo research. These issues are (a) the scope of application of the evo-devo modularity concept, and (b) the role of homeotic and other developmental genes in the determination of macroevolutionary pattern.

Consideration of these issues will lead me to postulate a scheme of relationships between developmental-genetic processes and evolutionary pattern that is in opposition to the corresponding consensus in the Modern Synthesis, and to suggest that extrapolation in evolutionary biology might not be a valid methodological principle.

II. Development occurs in plants, too, but is homoplasious with respect to animals

Evo-devo biologists appear to agree about the status of systematics or, more properly, of the branching pattern of taxa represented in phylogenies obtained after the application of objective algorithms on character matrices[4], as the indispensable framework to which aspects of ontogeny should be referred to ensure reliable inferences on the evolution of characters related to development at all levels (see, for example, Raff 2000, Arthur 2002). Phylogenetic reconstructions on matrices comprising taxa from all kingdoms consistently support a sister group-relationship between animals and fungi, with plants branching out as sister group of them, in turn (Baldauf and Palmer 1993). This taxonomic arrangement, added to the simple mapping of the presence of ontogenetic development in two of these three lineages -animals and plants[5]- and the unproblematic postulation of a unicellular ancestor for the clade that includes them all, results in a couple of alternatives for the evolution of development in its most general terms: it was either a synapomorphy for the group including the three taxa and was lost in one of them (fungi), or it was gained separately by both animals and plants.

In a couple of essays, Meyerowitz (1997, 2002) has made it possible to decide among these alternatives. He has argued that it is possible, metaphorically, to treat the evolution of development in these lineages as the result of a unique "natural experiment" that operated on common unicellular (or colonial) ancestors of unknown identity. Each of the two outcomes of the experiment -animal and plant developmental processes- seems to be, strictly speaking, independently derived: when the molecular-genetic identity of the multigenic families that are involved in the orchestration of a major proportion of the critical aspects of ontogeny -most notably, pattern formation in early precursors of complex anatomical structures- are compared, they turn out to be non-homologous. However, molecular evolutionary unrelatedness in many of these cases is accompannied by a striking similarity in "functional logic": this is the case of the transcription factor-encoding Hom/HOX metazoan homeobox genes and the MIKC MADS-box genes in spermatophytes (the seed plants)[6]. In each of these families of coding sequences, selected members behave as homeotic selector genes[7]. Following the original abstract characterization devised for them in *Drosophila*, selector loci are currently modeled as elements that act combinatorially to specify the expression of cascades of realizators[8] which in turn define cell differentiation pathways and determine organ identities in adult structures (Gerhart and Kirschner 1997, Carroll et al. 2001).

The expression patterns of angiosperm homeotic selector genes -also known as the floral homeotic loci- defined in the dicotyledon model systems *Arabidopsis thaliana* (the thale cress; simply named "Arabidopsis" in what follows) and Antirrhinum majus (the snapdragon) form the basis of the ABC model for the determination of floral organ identity (Bowman et al. 1989, Carpenter and Coen 1990, Coen and Meyerowitz 1991). The core postulation of the model states that the proper differentiation of the four organs typical of bisexual -hermaphroditic or "perfect", according to the botanical jargon- flowers is the product of the partial overlapping of three gene activities (A, B, and C) each of which is carried out by a variable number of MADS-box genes: activity A alone defines sepals, A plus B petals, B plus C stamens and C alone carpels. This simple, elegant and highly predictive model has been the main guide in evo-devo studies concerned with the evolution of reproductive structures inside the seed plant clade and in its close phylogenetic neighborhood (Theissen et al. 2000, Vergara-Silva et al. 2000, Cronk 2001)[9]. As has happened in animal evo-devo research (see, for example, Bolker and Raff 1996, Abouheif et al. 1998, Wray and Abouheif 1998) after a timely reevaluation of the contributions of Gavin de Beer (see, for example, Gilbert et al. 1996, Hall 2000, Gilbert and Bolker 2001), biologists concerned with the application of molecular-genetic information on plant development to elucidate character evolution questions are generally aware that experimental demonstration of the conservation of expression patterns of orthologous genes with a developmental role is not a definitive test of homology at higher levels in the hierarchy of structural organization (see, for example, Niklas 2000, Vergara-Silva et al. 2000).

III. In plants, differentiation and other cellular mechanisms involving the regulation of gene expression are carried out by several families of transcription factors

The ABC MADS-box genes are of inherent interest to anyone involved in the study of homeosis and related developmental-genetic phenomena, since they are genuine plant correlates of the first homeotic genes

discovered in animals. In addition to these genes, mainly expressed in tissues with a floral fate, other members of the family have non-reproductive but also specific sites of expression, like the root and the endosperm (Alvarez-Buylla et al. 2000, Burgeff et al. 2002). However, these genes are not the only group of transcription factor-encoding sequences implicated in differentiation throughout ontogeny in plants. In fact, some transcription factors involved in plant cell division processes are encoded by members of one homeobox subfamily, the KNOTTED class[10]. Experimental manipulation of these genes has not resulted in homeotic phenotypes so far, but it has been shown that they play an important role in leaf development, mainly by defining -just like animal Hom/HOX and plant MADS-box loci do- fields of cells that have unique responses to differentiation signals and therefore activate the transcription of specific sets of target genes (Pozzi et al. 1999). The patterns of expression of homologues of the maize KNOTTED1 (KN1) gene have recently been used as molecular indicators of homology in leaf form in the dicotyledon genus *Lepidium*, as well as in a discrete sampling of seed plant taxa, with the help of independently generated organismal phylogenies. An important conclusion of this study is that tests of homology based on final leaf morphology are not straightforward (Bharathan et al. 2002, see below). Among many others, additional loci with a demonstrated participation in developmental mechanisms are the YABBY genes -which are involved in the determination of the abaxial-adaxial identity of most lateral plant structures (Bowman 2000)- and CURLY LEAF (CLF), a locus which encodes a factor that helps to maintain fixed chromatin states (Goodrich et al. 1997). Interestingly, while the former group of sequences has no animal counterpart, CLF is a homolog of Enhancer of zeste, a well known member of the Polycomb group of chromatin remodellers in *Drosophila* (Meyerowitz 2002).

With the impulse provided by developmental-genetic studies, Arabidopsis is already one of the few species of eukaryotes for which a complete genomic draft is available in computer databases (The Arabidopsis Genomics Initiative 2000). According to a recent comparative analysis of quantitative and qualitative diversity of transcription factor families in this plant species and other eukaryotes for which whole genomes have been also sequenced (Riechmann et al. 2000), the number of transcriptional regulators in this plant species is 1533, which corresponds to 1.3 and 1.7 times those present in *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively. These factors are encoded by approximately 5% of the genome, and about 45% of them come from families specific to plants. Several additional estimates are interpreted by these authors to show that the regulation of transcription in plants is as complex as that in animals.

IV. Meristems, Goethe's line of intellectual descent and evo-devo -style modularity

As stated above, experimental evidence leads one to postulate that the logic behind the developmentally-relevant biochemical function of the phylogenetically diverse molecular players enumerated is similar in most animal and plant instances studied until now. This observation could provide a good ad hoc reason for the failure of textbook authors, for example, to acknowledge recent advances in the corresponding research[11]. If plant developmental genes function like their animal counterparts, sometimes very similarly, why bother to review them, also? What new things could possible come out from the analysis of the role of plant developmental processes in evolution that has not already been found or hypothesized in animals?

A. Meristems establish a qualitative difference between plant developmental processes and their animal counterparts

An implicit assumption of a major proportion of biologists, including a considerable number of researchers working on evolutionary issues, is that plants are "lower organisms". Indeed, some animal species have more -sometimes many more- organs and cell types than any known plant species do. Also, the absence in plants of emergent properties like cognition might justify that, in this zoological-versus-botanical comparison, the latter universe of processes will be forever deemed "less complex". However, as Niklas (2000) has pointed out, "the evolution of plant body plans is far more complex than that of animals because the organisms called plants are polyphyletic" (p. 413). Additionally, plant development is inextricably linked to numerous physiological processes -mainly related to signal transduction pathways triggered by external stimuli like light and temperature- that strenghten the stability of morphogenetic processes, while at the same time are at the basis of phenotypic plasticity in the face of environmental change (see, for example, Trewavas 2002)[12]. In any case, a closer look at some details of plant ontogeny and its evolutionary history could show that, in line with the developmental-genetic and genomic considerations made above, the pathway from gene networks that regulate development to adult phenotypes is hardly simpler in these taxa than in animals. Moreover, biological structure and process in plants conforms differently to some notions that are currently taken as central to the conceptual framework of evo-devo, to the point of having suggested theoretical approaches that depart from the coherency of received views.

Animal body plans are essentially complete after embryogenesis. Together with the recognition of phylotypic stages with varying degrees of flexibility, this fact has led to the postulation of the zootype (Slack

et al. 1993) and related proposals on the nature of animal body plans. An individual plant, however, continuously forms new anatomical parts throughout its life. This is possible because of the existence of meristems, structures that, in the ontology of plant biological structures, particularly from the standpoint of developmental biology, should be regarded as extremely salient. A meristem is an apparently undifferentiated tissue that provides new cells that acquire different fates (see, for example, Lyndon 1990, Howell 1998) according to their gene expression states, which in turn depend on the integration of environmental as well as internal signals. In a typical "higher plant" two primary meristems -the shoot apical meristem (SAM) and the root apical meristem (RAM)- develop during embryogenesis. In parallel, secondary meristems can be formed at specific places, starting from cell zones that previously were non-meristematic (Vroemen and the Vries 1999). Besides organs of the reproductive structures, like those comprising the floral whorls of angiosperms and gymnosperm strobili (also known as cones), each type of organ from the limited set of additional distinctive structures with this level of organization -basically the stems, the leaves and the roots- is always formed from a meristem with a specific gene expression profile (Howell 1998). The function of meristems in turn depends on the capacity that plant cells have to recapitulate development, a property called totipotency. On the basis of this property, cells from differentiated tissues like leaf mesophyll, secondary phloem, pith, cambium, petal epidermis and ovule and nucellus layers have been succesfully used in the laboratory to regenerate whole plants in culture (Lyndon 1990). Given the above, meristems provide the basis for the centenary or millenary age that individual plants of some species -for example, certain pines- can achieve. Needless to say, the biology of meristems is of potential interest to members of the biomedical community, specially to students of the molecular mechanisms underlying cancer or to those working in the now fashionable field of stem cell research (see, for example, Weigel and Jürgens 2002).

Graham et al. (2000) have recently put the evolutionary origin of meristems in perspective, along with other innovations, to assess their proper contribution to the evolution of body plans in embryophytes (the land plants). Illustrating again the use of reliable phylogenetic reconstructions, they have listed and mapped what they consider are the fundamental features that appeared during the radiation of charophycean algae -the group than includes the direct ancestors of the first land plants (see, for example, Kenrick and Crane 1997, Soltis et al. 2000)- as well as those whose origin is linked to the early stages in the evolution of the group[13]. An important observation made by these authors refers to the sexual aspects of life histories: from the beginnings of their diversification[14], land plants possess two multicellular bodies -the sporophyte and the gametophyte- which are structurally and functionally different, and that have experienced separate evolutionary processes through time. This condition, commonly known as "alternation of generations", is another important plant attribute without an animal counterpart.

The above factual considerations set the stage for a supported assessment, from a botanical point of view, of modularity. Since its introduction in contemporary discussions within evolutionary biology by Wagner (1989), the notion of modularity is a subject that has received special attention by some prominent figures in the ongoing discussion on the principles of evo-devo (see, for example, Raff 1996, Wagner 1996, Minelli 1998, Bolker 2000, Raff and Sly 2000 and Gilbert and Bolker 2001). Therefore, it has occupied a privileged position -quite justified, in my opinion- in several foundational texts commenting on the research agenda, key concepts, current issues and/or controversies in the field (Hall 2000b, Raff 2000, Wagner et al. 2000, Carroll 2001, Arthur 2002). According to the glossary in Carroll et al. (2001), the term refers to "the organization of animals into developmentally and anatomically distinct parts (and to) the organization of gene regulatory regions into discrete cis-regulatory elements". Raff and Raff (2000) have pointed out that "a view of ontogeny as composed of evolutionarily dissociable cassettes or modules with underlying identities at the cellular or gene level has become an important concept in the discussion of how development evolves" (p. 235), and Winther (2001) has elaborated a useful list of "criteria for the recognition of structural, developmental and physiological modules" (p. 119) that refer to their intra- and interconnectivity, their mechanisms of genetic specification and their variation/conservation across taxa at different taxonomic levels. Modularity, understood as a morphological and development-related property in multicellular organisms, would seem then a rather recent invention, but the modular nature of plant structure has been recognized long before.

B. Modularity in plants is more complicated than in animals: a brief account of historical events in retrospective

Many contemporary botanists talk naturally about the modular construction of plants, even when its treatment is not formally connected to developmental biology (see, for example, Niklas 1994, 1997). From what has been said before about plant life-cycles, it should be totally acceptable to postulate that a basic, ancestral kind of modularity is present in the land plant clade: the gametophyte-sporophyte distinction. In line with this, some researchers have tried to adapt -or adopt- the corresponding terms that are more commonly used in animal studies, while at the same time implying that modularity is "deeper" in plants. For example, Crane and Kenrick (1997) state that "the notions of unitary construction and strongly integrated

patterns of development may have been overemphasized and recent discussions highlight the importance of modularity and dissociation to facilitate co-option of genes, tissues and structures for new purposes (...) in plants, compartmentalized and fixed cell structure, combined with more obviously modular construction, provides a degree of inherent dissociation that makes both vegetative and reproductive parts especially susceptible to processes of co-option and modification" (p. 171, italics added). From the previous citation and many others of similar nature, it would seem that a straightforward application of the evo-devo version of the modularity concept to plants is completely justified and might (continue to) sustain productive research lines in developmental genetics.

As aptly reviewed and elaborated by Rieppel (1988), Hall (1992), Amundson (1996, 1998 and 2001), Brigandt (2001) and others -and not to be discussed here in detail- concepts like "Bauplan" and "homology", used for the description and explanation of pattern in contemporary biology, have their roots in the works of several European natural philosophers/biologists of the 18th and 19th centuries. Most of these individuals converged in deriving their speculations from the (metaphysical) idea of unity of type -a term coined by G. L. Buffon- which in turn is also a fundamental component of the structuralist (or internalist) school in comparative biology. J. W. Goethe, one of the most important structuralist thinkers during the late 18th century, had a special interest in plant form and his views have been frequently cited in association with developmental-genetic results in plant model systems (see below). The conceptions held by Goethe influenced subsequent generations of botanists, particularly in Germany, although individual workers developed divergent metaphysical and methodological stances. In her recent review of the subject, Classen-Bockhoff (2001) has identified, among others, Agnes Arber (1879-1969) and Walter Zimmermann (1892-1980) as two of the most prominent plant scientists whose works, being intellectual offsprings of Goethe's ideas, have paved the way for what now should be considered as the original contribution of botany to the understanding of the relationships between developmental-genetic process and anatomical structure -and therefore, to a contemporary conception of modularity. Interestingly, at least in the case of Arber, definitions regarding the structure and evolution of plant form provide real alternatives, because they entail a critique of the interpretation of character coding in modern comparative morphology and a dissatisfaction with the way abstractions of developmental-genetic units in the context of their causal correspondence to phenotypic traits are constructed.

Goethe was the founder of the field of "morphology" itself, conceiving it as an approach to deal "with the outer appearance of the entire organism" (Kuhn 1987). According to Classen-Bockhoff (op. cit.), two aspects of Goethe's view of plant morphology are of particular interest: (a) since the dynamic force inherent in organisms is life itself, all of them -including plants- change, transform themselves continuously, and (b) the scientific approach for the study of these changes must be an holistic one, unifying objective and subjective aspects of the cognitive process. In his best-known botanical work, Versuch die Metamorphose der Pflanzen zu erklären, Goethe first postulated that the leaf* was the primary plant organ, and that cotyledons, foliage leaves and floral organs were identical in being leaves*. The word "leaf" followed by an asterisk refers to the fact that he took this organ to be immaterial, an archetype. A series of developmental genetics studies, performed mainly in Arabidopsis, have confirmed the "leaves as archetypes" scheme for plant organ identity. This confirmation is worth noticing, though, because conceptually there is a clear opposition between the reductionistic approach that generated the results and some of Goethe's basic tenets. It has been found that a disruption in the normal function of the MADS-box genes in charge of the three canonical activities of the ABC model (see above) gives rise to flowers that, though preserving their whorled pattern and merosity, are composed only of organs with a leafy phenotype (Weigel and Meyerowitz 1994). Moreover, the general direction in the identity change of organs that Goethe hypothesized -from seed to foliage, from bracts to flowers, from carpels to fruits- could also be interpreted as supported by the recently characterized function of another subgroup of MADS-box loci -the so called SEPALLATA (SEP) genes (Pelaz et al. 2000). A mutation in each of the three members of this subgroup (SEP1, 2 and 3) resulted in flowers whose organs were transformed to a sepaloid identity, indicating that their products interact with the proteins encoded by the B and C activity genes of the ABC model. Sepals, then, would appear to be an intermediate stage in the "progressive sequential change" of structures with a foliar character towards those that occupy more central positions in the floral meristem; this in turn would make them more "derived" or "specialized" morphological structures than leaves, but less so than other floral organs (see below).

Goethe postulated the existence of an ordered series of discrete organ identities that originated from the leaf*. It is important to notice that, simultaneously, he was also introducing the complementary notion of sequential change -that is, transformation- of one anatomical part into another. To deal with this aspect, Goethe used the term "metamorphosis", and with it he was describing the process of change that the "primary unit of plant form" -that is, the leaf plus the internode placed below it and the bud located in its axil- turned into something else during growth. Taken together, both ideas constitute the basis of W. Zimmermann's conception of character transformation, a central tenet of his views on the ways in which plant form has changed over the course of evolution. For the latter author, "mutations modify determining factors relating to special characters, which then appear in the offspring in a changed form" (Classen-Bockhoff 2001: 1164); most

noticeably, he thought that phylogenetic modifications must entail changes in ontogeny. The fact that Zimmermann considered taxon phylogeny to be the result of information on these changes has led Donoghue and Kadereit (1992) to elaborate on the influence that he might have had on the conceptual development of phylogenetic systematics through Willi Hennig himself. Finally, and despite Zimmermann's fondness of linking development to phylogeny, it is important not to conflate a literal reading of character transformation -one that in principle would derive from a purely Goethian interpretation of, say, the SEP mutations- with what is actual the legacy of Zimmermann to cladistic practices. In the former instance, the "activity" of developmental genes will change in such manner that its phenotypic result would be the production of different, sequential and not necessarilly discrete (see below) states. In contrast, in the latter case character transformation (see, for example, Patterson 1982, Rieppel 1988, Carine and Scotland 1999, Brigandt 2001) only refers to the transition of one symbolic, discontinuous state to another -for example, zero to 1- over a particular branch in a phylogenetic tree which has been previously reconstructed on the basis of the identification of shared derived character states.

In what constitutes a very interesting case of intellectual divergence from a common ancestral stock of ideas, A. Arber did not endorse a categorical definition of organ types. In contrast to Zimmermann, Arber was basically a constructivist scientist: for her, "leaves", "roots", "stems" -and arguably also animal organs- should be always held as mental entities, despite their legitimate operational uses. In her "partial-shoot theory of the leaf", Arber (1950) expanded Goethe's conception of metamorphosis to specifically suggest that there is a certain degree of overlap between the identities of the shoot* (notice the asterisk) as a whole and the leaf* as its part. However, it would seem that the confirmation of Arber's theory on the basis of developmental-genetic evidence could be problematic, since from the start it is at odds with the accepted framework used for the specification of characters (in particular, organ types) in plants -a scheme that has been presupossed throughout my discussion. After identifying this, the standard methodological stance for character coding, as the "classical plant morphology (ClaM) approach" -one that looks at the root, stem and leaf as rigidly discrete units- Rutishauser and Isler (2001) have contrasted it against the "fuzzy Arberian morphology (FAM) approach". This alternative viewpoint, obviously derived from the German tradition already referred, consists in the postulation of a "continuum root-shoot model" (my italics) in which the root, stem and leaf are viewed as the products of overlapping developmental pathways. What is remarkable in this respect is that other recent developmental-genetic findings, having the same reductionistic character as the SEP MADS-box gene data discussed above have, are currently being interpreted according to generalizations that have a greater affinity to the FAM approach than to the standard framework, implying in principle that some plant morphological "modules" do not have clear-cut corresponding developmental-genetic regulatory networks. Among others, Sinha (1999) -on the basis of her work on KNOX genes and other evidences- has proposed that "the tendency to radiality and the tendency to dorsiventrality coexist (towards each other) both in shoot and leaf", while Hofer et al. (2001), with data pertaining to the pea gene UNIFOLIATA (UNI) -a homolog of LEAFY (LFY), the Arabidopsis regulator of the inflorescence/flower meristem transition- have postulated that in species with compound leaves, the "compoundedness" (branching) of these structures may be due to the expression of "shoot-specific" genes. Evidently, in both cases the interpretation of results entail that distinct groups of genes that in principle act in one categorical structure, are actually also expressed in another, and that the consequence that this overlapping pattern has on cell differentiation is an effective blurring of the phenotypic boundary between the structures themselves.

The previous paragraphs illustrate that, in plants, both "either-or" and "dynamic" conceptions of the relationship between the expression and function of developmental genes are being used by practicing scientists in their explanations on how the genome is translated into a phenotype. In animal developmental-genetic research, only recently has the item "partial homology" of developmental-genetic pathways been a matter of discussion (see Meyer 1998 and Wilkins 1998). Surprisingly (or probably not), at least in one instance the definition entertained includes the notion of overlapping interactions (see, for example, Wake 1999). Since apparently animal researchers are arriving at this conclusions so late (and without any acknowledged botanical input), it seems reasonable to conclude that theoretical pluralism in the plant sciences -exemplified, for example, by Arber's theory- has provided a wider perspective on what modularity is. From what has been presented above, though, it is difficult to assess the evolutionary significance of the partial homology scheme. For instance, would it be valid to suggests, on the basis of the Goethian interpretation of the Arabidopsis SEP mutations, that floral determination processes in angiosperms reflect an actual evolutionary acquisition of sequentially more complex developmental-genetic regulatory controls for organ identity? Clearly, more work is needed to estimate to what extent are putatively overlapping developmental pathways relevant to the discussion of the role of modularity in the generation of plant body plan diversity.

V. Natural heterotopic plant phenotypes, Rolf Sattler's "process morphology" and Modern Synthesis -style macroevolution

In congruence with the previous observations, it is probably convenient to state at this point that the role of

partial homology mechanisms as hypothetical proximal processes by which modularity of plant structures could have been related to the diversification of taxa and/or the origin of novel organs still has to be investigated in depth before leading to any definitive conclusions. Additionally, one would expect that this could take some time, because the source ideas involved are not commonly considered to belong to the conceptual foundations of the "mainstream" of experimental research. In contrast, the observation and interpretation of homeotic and related plant phenotypes in nature -stimulated in recent times, of course, by the wealth of developmental-genetic data generated in the model systems through the study of homeotic mutations- has been by far the core basis for the postulation of both general theoretical models as well as empirically testable hypotheses on the relationships between microevolution and macroevolution. Homeosis has been recently redefined in the context of heterotopy, which is currently recognized as any "positional displacement or translocation of an organ or structure" (see, for example, Li and Johnston 2000). As these authors have pointed out, the homeotic replacement or transformation of plant organs -sepals, petals, stamens, carpels and other non-floral structures- "may also be described as a displacement or translocation of an organ's development; that is, as heterotopy" (p. 75). Therefore, homeosis and heterotopy overlap, complete homeosis being equivalent to heterotopy. Under these definitions, too, homeosis is automatically placed at the opposite side of the spectrum of morphological variation when compared to the phenomena of overlapping organ identity, that in turn forms the basis of the partial homology notion.

It is interesting to notice that the analysis of heterotopy in a botanical context has also led to dissatisfaction with the accepted ways in which characters are coded in standard morphological studies (see below). On top of that, views held by a few other researchers are definitely more congruent with the postulation of saltatory modes of morphological evolution than with phyletic gradualism. As widely acknowledged (see, for example, Smocovitis 1996 and Reif et al. 2000) the postulation of an explanatory continuum that goes from the population genetic dimension to that of macroevolutionary pattern, which states that accumulation of infinitesimal morphological differences controlled by many small effect loci is the only acceptable mode of phenotypic evolution, is considered one of the main achievements of the Modern Synthesis. As such, this statement has been already incorporated almost without modification to the basic principles of the evo-devo framework (see, for example, Akam 1998, Haag and True 2001, Carroll et al. 2001). Evidently, the models and theories referred above should be of interest to anyone trying to define the structure of scientific explanations in evo-devo: confirmation of them would represent a truly profound critique to fundamental principles of the framework, in its present state.

A. Homeosis from a botanical perspective: the case of Rolf Sattler

The modern scientific documentation of natural homeotic phenotypes in plants goes back to William Bateson's *Materials for the Study of Variation*, in which he wrote that petals could be interchanged by stamens (p. 84)[15]. However, most likely in connection to the fate that embryological evidences suffered during the decades around the construction of the Modern Synthesis, the possible participation of these kinds of macromutations -sensu Dietrich (1992)- in the evolutionary process was ferociously argued against in evolutionary biology[16]. Besides the early paper by Leavitt (1909) and the review by Meyer (1966) -where dozens of angiosperm genera in which phyllody, bracteody, sepalody, staminody and carpellody occur were counted - almost nothing was published on these phenomena in plant journals. It was not until the 1980s, through the influence of the genetic investigations of Edward Lewis and subsequent workers, that hypotheses about the possible role of heterotopy and heterochronic mechanisms in general began to appear again in the botanical literature. Of course, the impact of Lewis'work on this renewed interest was arguably synergistic with the positive reception of Gould's *Ontogeny and Phylogeny*, the dispute around punctuated equilibria and, last but not least, the pervasive presence of Goldschmidt's old challenge to the Synthesis (see Dietrich 1995, 2000) in academic discussions through several decades during the 20th century.

Legitimately included in the FAM tradition (see Rutishauser and Isler 2001), Rolf Sattler is one of the most significant contemporary plant morphologists who have shown a serious interest in the ocurrence of homeosis in nature[17], as attested by his general reviews on the subject (particularly Sattler 1988) and his empirical research -most notably, his study of floral development on Calla palustris (a monocotyledon species of the family Araceae; Lehmann and Sattler 1992). As his FAM colleagues, Sattler appreciates plant developmental genetics, particularly the developmental part -probably more than any of them. Because of this, he stands apart in what represents a sort of "epistemic breakpoint" in the tradition of German plant morphologists that continues the Goethe-Arber line of intellectual descent, therefore constituting an interesting case study for historians. Sattler is an atypical structuralist: in his attempt to take further the conceptions charateristic of FAM, he has proposed that organisms are not objects that have developmental processes, but instead they are developmental processes. As advanced above, he has also suggested that morphological variation cannot be properly understood in the context of mutually exclusive categories (see Sattler 1984, 1992, 1994 and 1996). While it is by virtue of the latter standpoint that he is inserted in the Goethe-Arber tradition (Rutishauser and Isler 2001, Sattler 2001), the former conception entails the definitive

substitution of everything deemed structural in favor of what could be called a "dynamic continuum of functions" with a developmental affinity. From the previous description, it is therefore no surprise that Sattler -along with other like-minded botanists (see, for example, Hay and Mabberley 1994)- has been always unhappy not only with the ClaM approach, but also with the methodology of cladistics (see Weston 2000), especially when this one is locked into a strict methodology for morphological character coding.

While emphasizing the already mentioned drawbacks of that the "either-or" perspective on character coding could have, the alternative offered by Sattler -process morphology (see, for example, Sattler 1992 and 1994)- amounts to an "ontology of form" that in principle would appeal to any developmental biologist. The problem that the adoption of process morphology could entail, though, is clear by now: the modularity concept put forward by most evo-devo proponents is categorical and defines separate and dissociable entities, and its evolutionary interpretation (as explained in previous sections of this paper) heavily rests on the previous reconstruction of phylogenetic relationships that in turn depend on the coding of discrete characters. Now, as compellingly argued by Weston (2000), although in principle process morphology poses a problem to the logical justification of phylogenetic systematic practices, it does not "impact severely on the ability of cladistics to achieve its primary goal: to reconstruct taxic relationships" (p. 141). Instead, Sattler's contributions imply a valid criticism that is related to the transformational aspects of homology (see above). I therefore suggest that the heterodoxy reflected in the work of Sattler and other "fuzzy morphologists" could actually result in an innovation for the common practices of character coding in cladistics, in a way that does not compromise its utility for the inference of pattern: developmental-genetic modules defined by strong data could be a reliable guide in the redefinition, when needed, of morphological characters and the inclusion of new ontogenetic ones in matrices used for the reconstruction of both phylogeny and character evolution. Most importantly, it would seem that the implementation of this suggestion could give its best results if no a priori categorization related to discreteness or continuity is imposed on the data. Finally, it is tempting to predict that, if carried out carefully, such practice could contribute to the narrowing of the possible ways in which pathways composed by developmental loci could be co-opted, shuffled, duplicated and, in some cases, even "transformed" in a literal, Goethian sense through evolutionary time.

It should be noted that the above suggestion is not entirely new. Actually, arguments in pro and against the incorporation of "phylogeny-neutral principles" in systematics have contributed to an interesting, although separate, debate on tautology, redundance and circularity in evolutionary inference (see, for example, Lee 1999 and references therein). As with the partial homology proposals, it remains to be seen how much could FAM-like character coding practices, coupled to standard cladistic analyses, contribute to our understanding of the evolution of developmental processes. But as anticipated above, though, a growing body of evidence and theorizing in plant evo-devo is supporting that the generation of discontinuous, eminently heterotopic, variation could be effectively linked to major body plan transitions and morphological innovations, in contradiction to the main predictions of the gradualistic stance in evolutionary biology.

B. Current plant evo-devo models on the role of heterotopy in macroevolution are more advanced than their counterparts in animal evo-devo

Chronologically, the first significant contemporary hypotheses that connected developmental-genetic findings to the origin of (morphological characters in) plant groups of high taxonomic rank were proposed by the Russian paleobotanist S. V. Meyen, to account for the origin of gymnosperm morphological structures in general (Meyen 1984; the same year of the cloning of the first animal homeotic genes) and carpels in angiosperms (Meyen 1988). Before this work, the non-saltational models of botanists writing inside the Modern Synthesis context -among whom G. L. Stebbins is an outstanding figure (see Smocovitis 2002 and references therein)- were almost the only accepted views on plant evolution. For the first taxonomic group, Meyen postulated that three processes could have been involved in morphological evolution: "(i) homoeotic heterotopy with complete violation of the 'criterion of position' (...) (ii) postheterotopic conservation of organs, often concomitant with epimorphological reversion, and (iii) evolutionary dedifferentiation in accordance to the criterion of position (...) but with the rearrangement of several ontogenetic programs into one". Notice that the third point resembles aspects of the ontology of development promulgated by the FAM approach; Mabberley and Hay (1994) have also stated that homeotic transformations suggest "(that) disruption leads to the rearrangement of processes and destabilization of process combinations, or decanalization", a process that in turn could lead "to the expression of lost features" (p. 117). Regarding the origin of the androecium (the carpel whorl) in flowering plants, Meyen (1988) considered that it had been possible through gamoheterotopy -the transfer of characters from one sex to another- involving a microsporophyll (pollen-bearing) structure arising in the seed-bearing (female) organ, both with affinities to the structures found in the extinct seed plant group Bennettitales.

The general picture of the second Meyen hypothesis has been recently reelaborated by M. Frohlich in a highly testable and partially corroborated model on the origin of the angiosperm flower: the "mostly male

theory" (Frohlich and Parker 2000, Frohlich 2001). According to him, "developmental control of flower organization derives more from systems active in the male reproductive structures of the gymnosperm ancestor, rather than from the female, with ovules being ectopic in the original flower" (Frohlich and Parker 2000: 155); here, the direction of transfer is opposite to the one suggested in Meyen's hypothesis. Additionally, Frohlich's instance of heterotopy relies on different fossil groups -the Corystospermales and/or the pteridosperm Pteroma (Frohlich 2002)- for the reconstruction of the corresponding ancestor. The features that make the mostly male theory of flower evolutionary origins a particularly attractive model have to do mainly with its specific predictions on gene function and expression patterns, involving homologues of genes already mentioned in previous sections of this paper -namely, the LFY gene and members of the MADS-box and YABBY families (Frohlich 2001). Additionally, the model contemplates a scenario with several stages in which the potential selective value of different morphological features that appear sequentially are evaluated. In line with this, the author clearly states that these stages do not imply periodic innovation and stasis -that is, a "punctuated" tempo for the origin of structures. However, it should be observed that the ectopic expression of ovules simply does not conform to a gradualistic notion, since the identity of the organs would be preserved and no intermediates are postulated: heterotopy/homeosis are phenomena that always involve discontinuity.

Contrasting with the cautious nature of the mostly male model with regard to tempo, Bateman and DiMichele (1994) have presented a "metamodel" -a neoGoldschmidtian paradigm, in their own words- that emphatically stresses that mechanisms of saltational evolution -a term narrowly defined by them as "a genetic modification that is expressed as a profound phenotypic change across a single generation and results in a potentially independent evolutionary lineage" (p. 62) probably generated many vascular plant species and most higher taxa. As the name indicates, the proposal by these authors accepts Goldschmidt's concept of instantaneous (single-generation) speciation, while at the same time rejects his preferred causal mechanism of macromutations (a point in which everybody agrees nowadays; see Dietrich 2000 and references therein). In accordance with the existence of numerous cases of natural homeosis in several plant taxa (recall the work of Sattler, and see below), Bateman and DiMichele suggest that a considerable number of "hopeful monsters" have arisen frequently through mutations in homeotic and other developmentally relevant genes. Since the fitness of these individuals would be most likely low, the neoGoldschmidtian paradigm requires that they are temporarily released from Neodarwinian selection in locations empty of competitors in order to survive successfully. Inside the model, the period corresponding to the unfolding of the phenotypic consequences of the mutations is called the "generation phase of saltation"; concomitantly, the time during which the original mutant individuals grow into a stable population is considered an "establishment phase". In my opinion, Bateman and DiMichele's views are especially appealing for the purposes of the present discussion, because they have fearlessly embraced, in a critical manner, proposals that are unfortunately heretic for most animal researchers. At the same time, the almost complete lack of acknowledgement of these ideas by the authors currently establishing the standards of what is evo-devo simply reinforce my point on the issue.

Recently, Bateman and DiMichele (2002) have revisited their own model and tried to ammend it in places where argumentation was not strong. Among many important reformulations[18], they have suggested that fundamental elements which define a distinction between the onset of the saltation phases in plants compared to animals have to do with meristem-based modularity: explicitly, the authors make reference to the "sedentary, autotrophic lifestyle (of plants and their) reliance on a root-shoot dichotomy and (multiplicity of) localized meristems that allow open, additive growth and differentiation, offering the potential for clonal vegetative reproduction" (p. 135-136). Another noticeable improvement in their model is connected to a direct request for extensive, sharply focused field collection work, to improve our empirical knowledge of the establishment phase of saltation. Since the 1994 version, after recalling that most plant species consist of hermaphroditic individuals, Bateman and DiMichele had already suggested that when macromutations occur in these self-compatible organisms "many (...) have the potential to establish their own mutant lineages without the aid of a partner, nullifying the improbable requirement for two compatible and mutually attracted hopeful monsters that led to the ridicule of Goldschmidt's original zoological formulation of saltation (p. 75)". After several years of field work, a group of researchers working in several of the areas that converge in evo-devo have put together a series of concatenated hypotheses on the origin of the floral homeotic/heterotopic phenotype of *Lacandonia schismatica*, a Mexican mycoheterotrophic monocotyledon endemic of the Lacandon rainforest in the state of Chiapas (Vergara-Silva et al. 2002) and member of its own monotypic family, Lacandoniaceae (Martínez and Ramos 1989). Since their discovery in the 1980's, the bisexual flowers of this triurid species had been the only known case of mutual spatial inversion of the stamen and carpel whorls among the approximately 250 000 angiosperm species currently classified by plant taxonomists (Márquez-Guzmán et al. 1989 and 1993; for an independent recognition of this status see Endress 2001). In that paper, we document the existence of several classes of discontinuous floral variants in the putative sister taxon of *L. schismatica* -the dioecious *Triuris brevistylis*- some of which bear flowers that resemble those of the former species. It is likely that this and other similar instances of saltation[19] could be of special interest to animal evo-devo workers -see, for example, the concern for microevolution in evo-devo expresed by Stern (2000). Regarding the triurid heterotopic mutants, the information that we have on the exact location of the natural populations of both species makes possible a series of detailed population genetic

studies that could clarify the microevolutionary aspects of these interesting evolutionary event. In accordance with the wealth of developmental-genetic information reviewed up to now, we currently hypothesize that the described morphological change is most likely linked to a change in the expression pattern of MADS-box gene homologs and/or their regulatory regions. While awaiting for even more experimental data to accumulate, I consider fair to recognize that Bateman and DiMichele's model, together with other recent, complementary contributions to the development of more refined concepts on the role of heterotopy and developmental genes in macroevolution (see, for example, Baum and Donoghue 2002 and Kellogg 2002) put plant evo-devo ahead of its animal counterpart.

VI. Evo-devo and plants: a brief integrative perspective

In this paper I have tried to support the claim that comparative, FAM/NeoGoldschmidtian plant developmental-genetic studies have the potential to influence the "emergent conceptual framework of evo-devo" (Arthur 2002). This is to say that connections should be established with efforts by philosophy of biology-minded students of the phenomena. For example, I encounter that Bateman and DiMichele's views could be conceptually related -in a sort of "reduction"- to the idea of renewed elaboration, in each of their mutants, of developmental-genetic patways inherited from the corresponding ancestors. This kind of thinking has been compellingly exposed in the writings of O. Rieppel, particularly in his work on the connection between (the notions of) ontogeny, phylogeny and classification (Rieppel 1992). Noting that "it has been recognized that essentially similar (homologous) structures may originate through radically different ontogenetic trajectories" -which, by the way, had been de Beer's point on the nature of homology- he added that specific morphological structures are homologous to others "only in an abstract, archetypal or taxic sense" (p. 21). His example involves the bird wing with respect to other tetrapod limbs; ours could be angiosperm flowers and gymnosperm cones (see above). In summary, this kind of observations have allowed him to postulate that, for every morphological structure, there always and only exists this particular morphological structure in this particular species. In my opinion, a concept appearing in Rieppel's argumentation -ontogenetic divergence (p. 18)- could well be adopted to redefine Bateman and DiMichele's "hopeful monsters" as the evolutionary process that leads to the redeployment, in certain branches of previously reconstructed phylogenetic patterns at every taxonomic level, of developmental-genetic potentials preexistent in putative ancestors. The question of whether these potentials determine the generation of overlapping or discontinuous character states seems to be unpredictable a priori and open to empirical investigation.

Reviewing accepted facts from molecular genetics, developmental biology, morphology and systematics, as well as the more-complex-than-usually-acknowledged historical dimension of some of them, I focused on two issues that in no way exhaust the empirical observations or theoretical arguments that could be of relevance for evo-devo: modularity and the phyletic gradualism tenet on the evolution of morphological structures at different taxonomic levels. Although much lab work and conceptual integration lies ahead, i think that at least one working conclusion can be put forward for each topic.

i) It seems that the "modalities of modularity" -certainly an issue of capital importance for evo-devo- are wider than what studies in animals have suggested until now. Further studies on them would demonstrate how they have been implicated in the generation of variation. As anyone could see, this would be an enormous achievement in itself if only because it will contribute to the solution of the original Darwinian questions on the nature of evolution. So, not only should animal researchers acknowledge discoveries achieved in plants: both camps should also go deeper in their sampling of objects of study. There is currently no way to ensure that the developmental-genetic mechanisms known to date, in both animals and plants, are the only possible processes determining the potential for evolution -evolvability- existing in natural populations. In connection with this, a more vigorous interest in the possibilities of complex systems-theory and mathematical modelling derived from the work of Stuart Kauffman (see, for example, Weber 1998 and Richardson 2001) as auxilliary tools in the discovery of new properties of biological modules might be advisable.

ii) It is likely that some prominent animal evo-devo researchers will continue to be convinced that saltational views on macroevolution should be rejected, and that a sustained endorsement of the perspective created in the context of the Modern Synthesis around the dichotomy micro-macroevolution is not a bad idea (again, see Akam 1998, Haag and True 2001 and Carroll et al. 2001, but also Rieppel 2001 and Ronshaugen et al. 2002). However, it is not difficult to imagine that this position could eventually be reconsidered, too. As stated above, phylogenetic reconstruction methods and cladistics in particular can still stand criticism and their results -as attested by some of the foundational texts already cited- continue to be taken as reliable frameworks to infer character evolution at several different hierarchical levels. Then, when confronted to robust trees based on matrices that include a wide selection of taxa, particularly at high taxonomic categories, everybody should be open to consider them as strong statements suggesting either gradualism or saltation. In animals, the most recent molecular systematic evidences indicate that the traditional Hymanian hypothesis of "simple worms gradually leading to complex chordates" is not correct (see, for example, Adoutte et al. 2000

and references therein). Simultaneously, in current plant evo-devo, the longstanding transformational -sensu Modern Synthesis evolutionists- hypothesis on the origin of the angiosperm flower from the reproductive structures characteristic of a supposedly closely related group of gymnosperms, the Order Gnetales (Frohlich and Parker 2000, Theissen et al. 2000, and references therein) has been replaced by a model in which both groups of seed plants are monophyletic, sharing a last ancestor that had a developmental genetic potential (mainly constituted by a regulatory network composed of MADS-box gene homologues) acquired well before the divergence of both organismal clades (see Vergara-Silva et al. 2000 and references therein). In summary, the branching pattern of higher taxa in both the animal and plant kingdoms do not support the idea that body plans and their constituent parts arose gradually. As said before, though, it is of extreme interest to investigate in which ways could the mechanisms that determine partial homology of developmental pathways and morphological structures falsify or corroborate these views. In any case, theoretical standpoints and interpretations of data similar to the ones presented here suggest that evolutionary divergence of ontogenetic pathways whose main features had been present in hypothetical ancestors is a plausible evolutionary model to explain how the main phenotypic features of (plant and animal) body plans could have arisen through time.

VII. Concluding remark: on the nature of extrapolation in evolutionary biology

Here, I have argued that developmental genetic modules equivalent to complex gene regulatory networks (and their molecular constituent parts), specifying either continuous or discontinuous characters, could be created, shuffled or destroyed through molecular evolutionary processes and, eventually, be inherited in populations -later recognized as "ancestors"- that subsequently diversify establishing "taxa" that taxonomists have classified in categories belonging to different hierarchical levels. This description is postulated as a minimally accurate way to describe what is common in the pattern of animal and plant phenotypic evolution. However, the careful reader might have noticed that my arguments implicitly entail that an experimental result from one taxon, no matter its identity or affinity, could never be completely extrapolated to another taxon. For me, this is a direct lesson from the manner in which phylogenetic systematics has served as a reliable source of our knowledge of evolutionary pattern: pure extrapolation simply does not exist in evolutionary biology. In contrast to what seems to be the style of explanation in biomedicine and realted areas (all of them good instances of "functional biology"), in evo-devo and other "historical biology" subdisciplines, hypotheses at all hierarchical levels are put forward (or at least, should be) as a means to explain diversity, not to attempt universality or homogeneity. It is interesting, nonetheless, to notice how even debates closely related to the issues treated here could have been more readily solved if this simple idea was taken more seriously -particularly, the arguments between Rosenberg (1997) and Laubichler and Wagner (2001) come to mind. At the same time, it is likely that very valuable proposals on other subjects like, for instance, how some genome projects should be carried out (see McConkey and Goodman 1997 and McConkey et al. 2000), would have been taken more seriously if this notion had more acceptance. In summary, I conclude suggesting that, despite its apparent counterintuitive character, the practice of extrapolation in evolutionary biology should be applied with much reserve and only carried out as a guide to discover the actual, material basis of the phenotypic aspects of biodiversity.

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Notes

1 Several historians and philosophers of biology point to 20th century biologists like C. H. Waddington, Richard Goldschmidt and Gavin de Beer as prominent characters who, usually against the mainstream, were

interested in the interconnection of ontogenetic processes and the diversification of taxa; see the contributions of Hall, Gilbert and Dietrich in *American Zoologist* 40(5). The books by Hall (1992) and Schwartz (1999) deal with historical aspects of this relationship before the 20th century and around the time of constitution of the Modern Synthesis, respectively. The journal *Evolution and Development* has also recently published papers related to the subject.

2 By 1997, the term "evo-devo" had already been used as a designator of evolutionary developmental biology (see the Special News Report in Science 277 (5322)); it seems that Hall (1992) was the first author who used this combination of terms in its current sense. Despite the richness of antecedents that now are part of the discussions in the field, its foundational empirical evidence is of a molecular nature and is without question related to the cloning of the *Drosophila* homeotic genes, previously uncovered by the genetic work of Edward Lewis at CalTech.

3 The appreciation of evolutionary developmental biology as a separate "research paradigm" that challenges or competes with the Modern Synthesis is not shared by everybody in the evolutionary biology community. In 2001, for example, *The American Naturalist* published an "executive document " on evolution, science and society in which evo-devo is completely subsumed to the Synthesis, appearing as a "subdiscipline of evolutionary biology". This document has an overly adaptationist character.

4 Although Raff (2000) and Arthur (2002) -among others- converge in (correctly) considering that cladistics (that is, phylogenetic systematics) has "revolutionized phylogeny" (Raff 2000: 75) because of its "very broad applicability (and) widespread use in the analysis of molecular sequence data, including the sequences of many developmental genes"(Arthur 2002: 757), the use of other methodologies for phylogenetic reconstruction is progressively surmounting it. The discussion around the differences in the logical foundations of cladistics and other classes of reconstruction algorithms and the justification of their applicability to systematics spans decades; for a recent update on the debate, the reader is referred to Issue 3 of *Systematic Biology*, volume 50.

5 In an evolutionary sense, multicellularity is an indispensable requisite for ontogeny (Hall 1992: 131). Therefore, in taxa outside the animal and plant kingdoms, developmental processes could potentially occur. Some members of the Myxobacteria, fungal species such as *Neurospora crassa* and the still enigmatic *Dictyostelium* discoideum are able to build multicellular bodies under certain physiological conditions; since this event involves differentiation, growth and morphogenesis, it could be properly be considered developmental in nature. The complexity of gene expression in some prokaryotes also maintains the specification of different cell types and growth, although probably not morphogenesis (at least not in the usual sense, related to the formation of distinct anatomical structures). See Russo et al. (1999) for individual reviews on arguable instances of the presence of ontogenetic processes outside animals and plants.

6 Both multigenic families are composed of numerous transcription factor-encoding sequences whose DNA-binding domains are widely conserved across taxa. The name "MADS-box" actually derives from an analogy to the homeobox, where the particle "MADS" is an acronym referring to the first four characterized members of the family.

7 The term "selector gene" was coined by Spanish geneticist Antonio García-Bellido to designate the loci whose unique combinations of activities -basically, their on or off states (García-Bellido 1975)- are causal for the differentiation processes that take place in founder cells for a particular tissue or organ.

8 Also a part of García-Bellido's scheme, the realizator genes correspond to those loci "hierarchically downstream, that transform their signals into developmental operations leading to the observable morphology" (García-Bellido 1981: 194).

9 Recently, the appropriateness of the ABC model as a necessary and sufficient guide for this purpose has been critically addressed (see, for example, Egea Gutiérrez-Cortines and Davies 2000, Theissen et al. 2002). This is mainly due to the finding of additional genes -also belonging to the MADS-box family- whose protein products interact in an indispensable manner to allow the originally characterized B and C genetic activities to take place (see Section IV. B). Though it is right that the model -as it was first proposed- only partially accounts for the phenomena later observed in some angiosperm species, it has not proven false and has effectively led to the finding of valuable developmental-genetic evidence regarding the possible evolutionary events that have been at work in the specification of the identity of reproductive axes in selected gymnosperm, pteridophyte and bryophyte taxa (see, for example, Tandre et al. 1995, Münster et al. 1997, Krogan and Ashton 2000 and Svensson and Engström 2002).

10 The KNOTTED-like (KNOX) genes are included in a larger superfamily of homebox-containing sequences, the TALE ("three aminoacid loop extension") genes. In addition to the homeodomain, these genes

have other conserved motifs that could be important for protein-protein interactions. The plant KNOX genes fall in two classes (I and II), distinguished from each other by their specific conserved residues and intron positions. Class I genes are expressed mainly in meristems, while Class II members have broader patterns of expression (see Reiser et al. 2000 for a review).

11 A couple of recent outstanding books on evo-devo (Gerhart and Kirschner 1997, Carroll et al. 2001) do not cover plants; although the first states clearly that these organisms are deliberately left out and the second has a subtitle that warrants the same restriction in scope, both of them reach generalizations that in principle would apply to any taxa in which development occurs. With regard to developmental biology texts published during the last five years, only two of them have chapters devoted to plants (Wolpert et al. 1998 and Gilbert 2000).

12 Gilbert (2001) has suggested that the addition of the environment as a fundamental factor in the control of the ways in which the genotype produces a phenotype would create an "enhanced evo-devo" that should be called "ecological developmental biology" or "eco-devo". Interestingly, Gilbert does mention plants only as interactors with animals; however, in a recent comment on the subject, an animal researcher is quoted as saying that "plant people are the ones who really do eco-devo" (Dusheck 2002: 578).

13 The characters considered by Graham et al. (2000) to have evolved before the origin of apical meristematic cells and their proliferation (branching) include (i) cellulosic cell walls, (ii) multicellular bodies, (iii) cytokinetic phragmoplasts and (iv) plasmodesmata. After meristems appeared, characters involved in the establishment of the ancestral algae from which land plants arose are (v) three-dimensional tissues, (vi) assymmetric cell division, (vii) cell specialization capacity, (viii) zygote retention and (ix) placenta. Finally, characters important for the ulterior evolution of plant body plans include (x) multicellular sporophyte bodies, (xi) histogenetic apical meristems in the gametophyte body and (xii) capacity for tissue differentiation in both sporophyte and gametophyte.

14 Evidence for this assertion can be found in Remy et al. (1993) and Kenrick (1994).

15 Masters (1869) has already described examples of homeosis/heterotopy in plants under the name "metamorphosis", not to be confused with Goethe's concept under the same word.

16 Fundamental texts for the constitution of the Modern Synthesis from the 30's and 40's (especially E. Mayr and T. Dobzhansky's work; see Smocovitis 1996 and Reif et al. 2000) as well as some recent opinions (see, for example, Mayr 1997) sufficiently support the claim that evolutionary biologists working inside this research tradition rejected the participation of non-gradual modes of evolutionary change in the origin of complex organs and/or higher taxa. Traditional (evolutionary) systematics has no particular method of choice for the selection of a specific hypothesis of taxic relationships among two or more alternatives, and heavily rests in the subjectivity ("expertise") of specialists in the group under study. In general, for the evolutionists it is enough to rest on overall similarities between organisms to arrive at inductive conclusions on clustering of taxa, and their concept of transformation is a naïve notion that entails acritical acceptance of unobserved transitions between phenotypes.

17 Other contemporary botanists interested in the structural aspects of homeosis/heterotopy include Denis Barabé (Montreal Botanical Garden, Canada; see, for example, Barabé and Lacroix 2000), Shirley Tucker (University of California-Santa Barbara, USA; see, for example, Tucker 2000), Richard Bateman (Natural History Museum, London, England) and William DiMichele (National Museum of Natural History (Smithsonian), Washington, USA; see Bateman and DiMichele 2002 and references therein).

18 Bateman and DiMichele (2002) have arrived to an extraordinarily thought-provoking statement that reasonably summarizes their work: "punctuated equilibria in morphology, plus phyletic gradualism in DNA sequences, equals evolution" (p. 120). Of course, they refer here to the apparently gradual pace of mutations, even when these take place in regulatory sequences of homeotic loci or other ontogenetically critical places in the genome; however, in my opinion, punctuated equilibria could also occur there when gene shuffling or duplication, as well as other molecular evolutionary phenomena, take place. Besides this, the new version of their neoGoldschmidtian paradigm contains useful criteria for the falsification of adaptation, exaptation, transference of function and saltation itself, as well as a sound criticism to the traditional population-genetic tenets that for years made impossible a serious consideration of saltational mechanisms as explanations of macroevolutionary pattern.

19 Examples of homeotic phenotypes that are relevant to account for the origin of new taxa in orchids are also given in Bateman and DiMichele (2002). An expansion for other taxa and further elaboration of the corresponding arguments can be found in Rudall and Bateman (2002).

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