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PARTICIPACIÓN FUNCIONAL DEL GEN MADS-BOX AGL14 EN REDES TRANSCRIPCIONALES QUE REGULAN EL COMPORTAMIENTO DEL MERISTEMO AÉREO

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INDICE

ABREVIATURAS

ADN: Ácido desoxirribonucleico

UTR: Región no traducida en el extremo del mensajero (del inglés “untranslated region”)

SCF: (del inglés Skp, Cullin, F-box)

ARN: Ácido ribonucleico

EDTA: Ácido etilendiaminotetraacético

LB: Luria Bertani

HEPES: Ácido N-2-hidroxietilpiperacina-N-2- etanosulfónico

PBS: Buffer fosfato salino

DEPC: Dietil pirocarbonato

PBS: Buffer fosfato salino

tARN: Ácido ribonucleico de transferencia

GFP: Proteína fluorescente verde (del inglés Green fluorescent protein)

SSC: Amortiguador de citrato de sodio

BSA: Albumina sérica bovina

TBS: Amortiguador de Tris salino

NBT: Nitroazul de tetrazolio

BCIP: 5-bromo-4-cloro-3-indol-fosfato

pb: Pares de bases

SAM: (del inglés shoot apical meristem)

CO: Centro organizador

MI: Meristemo de inflorescencia

MF: Meristemo de flor

RAM: (del inglés Root apical meristem)

XAL2: XAANTAL2

AGL: Agamous Like

SOC1: SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1

TFL1: TERMINAL FLOWER1

WUS: WUSCHEL

RESUMEN

La plantas pueden desarrollar nuevos órganos post-embrionariamente. Esta capacidad de desarrollo depende de la actividad de los meristemos apicales, los cuales son un grupo de células indiferenciadas que establecen el nicho de células troncales. Este nicho se divide para seguir manteniendo el meristemo y para proporcionar células que se diferenciarán en los diferentes órganos.

El Meristemo Apical Aéreo (SAM) transita por diversas etapas durante el ciclo de vida de una planta. En *Arabidopsis thaliana*, este meristemo da lugar al establecimiento de la roseta, posteriormente se convierte en el meristemo de inflorescencia que produce hojas caulinares y ramificaciones en sus flancos. Finalmente, forma los meristemos florales que darán lugar a los órganos florales incluido el pistilo en donde después de la fertilización se generarán las semillas con las cuales se mantendrá y propagará la especie. En contraste con el SAM, que es un meristemo indeterminado a los largo del ciclo de vida de las angiospermas, los meristemos de hojas y de flor son meristemos determinados cuyas células troncales se agotan una vez que se desarrollan los órganos correspondientes.

La transición del estado vegetativo al estado reproductivo está fuertemente regulado y a esta etapa del desarrollo la conocemos como la “floración”. La floración es fuertemente influenciada por factores ambientales como el fotoperiodo y la temperatura aunque también es regulada por factores intrínsecos de las plantas, como por ejemplo la edad y la fisiología. Para entender la base genética que sustenta la transición a la floración, se ha identificado una intrincada red genética con base a diferentes mutantes afectadas en el tiempo de floración bajo diferentes condiciones de crecimiento. Siete vías parcialmente independientes de regulación han sido propuestas. Sin embargo, cada vez es más claro que estas vías están interconectadas y resulta difícil encasillar a cada uno de los actores moleculares identificados como específico de cada una de ellas.

Mediante el empleo de mutantes y líneas de sobreexpresión para *XAANTAL2 / AGAMOUS-LIKE 14 (XAL2 / AGL14)* cuyo gen codifica para un factor transcripcional MADS-Box, se determinó que, este gen es necesario y suficiente para inducir la floración principalmente en fotoperiodo de día corto. Utilizando RT-PCR cuantitativo se determinó que *XAL2* forma parte de una red de regulación genética que activa a *AP1,* además se demostró mediante hibridación *in situ* que *XAL2* / *AGL14* es un gen que se expresa en los meristemos de inflorescencia y de flor. Por otra parte, la sobreexpresión de *XAL2,* así como *AGL24* y *SOC1* alteran el mantenimiento y determinación de los meristemos florales produciendo flores con características vegetativas en las cuales se encontró que *XAL2* regula directamente a *TFL1*. Finalmente con base en los resultados obtenidos en este trabajo y la evidencia reportada por otros grupos de investigación fue posible la modelación del sistema de regulación genético que establece los meristemos vegetativos, de inflorescencia y el de flor así como la modelación del paisaje epigenético que permitió mostrar el destino de las células así como la transición en estos meristemos en las plantas silvestres y en las líneas de sobre-expresión de *XAL2*.

ABSTRACT

The ability of plants to develop new organs post-embryonically, depends on the activity of apical meristems which are a group of undifferentiated cells that establish what is known as stem cell niche along plants life. This cell niche, is continuously dividing to maintain the meristem and provide cells which will differentiate into different organs.

The Shoot Apical Meristem (SAM) goes through various stages during the life cycle of a plant. In *Arabidopsis thaliana*, this meristem favors the establishment of the rosette, which later transits to the inflorescence meristem to produce cauline leaves and branches in their flanks and later on it forms the floral meristems that will lead to the floral organs. After ovules mature inside the carpels, double fertilization occur to form the seeds that will maintain and propagate the species.

The SAM transitions are highly regulated. In this work we particularly focus in the vegetative to reproductive transition or flowering and the maintenance of the flower meristem. This stage is strongly influenced by environmental factors such as light photoperiod and temperature and is also affected by the plant physiology and aging.. Seven independent pathways have been proposed based on several mutants that have been identified under different growing conditions. However, it is clear that these pathways are interconnected and that it is difficult to define each molecular actor as specific to each pathway. Therefore, an intricate genetic network is regulating flowering transition.

In this work, we demonstrate that *XAANTAL2* / *AGAMOUS LIKE 14* (*XAL2* / *AGL14*) a MADS-box transcription factor is necessary and sufficient to induce flowering, particularly under short-day growing condition. By RT-qPCR it was demonstrated that *XAL2* belongs to the gene regulatory network that induce *AP1*. and by using *in situ* hibridization it was shown that *XAL2* expression was detected in inflorescence and flowers meristems. On

the other hand, overexpression of *XAL2, AGL24 and SOC1* are able to induce *TFL1*, explaining at least partially the vegetative traits in these lines. Finally, with our results and other evidence previously reported by other groups it was possible to propose a gene regulatory network and epigenetic landscape models to understand normal and altered cell fate and transitions of the vegetative, inflorescence and floral meristems in Arabidopsis wild-type plants and the overexpression lines.

1. INTRODUCCION.

1.1 . Desarrollo Vegetal.

Las plantas son organismos que crecen y se reproducen en el sitio de germinación de la semilla y su desarrollo es plástico, es decir, tienen que ajustarse constantemente a las condiciones ambientales que experimentan debido a su condición sésil (Ausin et al., 2005). Por esta peculiaridad, las plantas son excelentes modelos que permiten estudiar mecanismos de integración de señales ambientales.

Una de las diferencias más grandes entre animales y plantas, es que en los primeros los patrones de desarrollo se determinan esencialmente antes del nacimiento y todos los órganode un individuo adulto se conforman durante el desarrollo embrionario (Capron et al., 2009), con excepción de algunos animales, como por ejemplo aquellos que sufren metamorfosis. En contraste, las plantas pueden desarrollar nuevos órganos después del desarrollo embrionario (Walbot y Evans, 2003). Esta capacidad de desarrollo depende de las actividades de los meristemos apicales, los cuales son un conjunto de células indiferenciadas (pluripotenciales) que se dividen para seguir manteniendo el meristemo y para proporcionar células que posteriormente se diferenciarán en los órganos (Kaufmann et al., 2010). De esta manera, las células meristemáticas generan dos células: una de ellas conservará la característica de mantenerse indiferenciada (cómo la célula madre) y la otra seguirá proliferando hasta que entra en un proceso de diferenciación. Durante la diferenciación, las células de un linaje atraviesan por cambios, dando como resultado que la célula adquiera la morfología y las funciones de un tipo celular específico y diferente al resto de tipos celulares del organismo (Long y Barton, 1998).

1.2. Meristemos apicales en las plantas.

Durante la embriogénesis las plantas angiospermas generan dos tipos de meristemos: el de la raíz (RAM por sus siglas en inglés Root Apical Meristem) y el aéreo (SAM,

Shoot Apical Meristem), los cuales originan diversos tipos celulares y órganos a través del ciclo de vida de las plantas (Doerner, 2003; Figura 1).

El RAM produce las células que formarán todos los tejidos de la raíz, como la raíz primaria, las raíces laterales y los pelos. Este órgano tiene diversas funciones en la planta ya que le sirve de sostén, la abastece de nutrientes y agua indispensables para el crecimiento, etc. En general, el crecimiento y desarrollo de la raíz ocurre por debajo de suelo (Petricka et al., 2012). En cambio, el meristemo aéreo es el responsable de la arquitectura aérea de la planta, la cual está a la vista. Este meristemo es el encargado de producir hojas, ramas, tallos, flores, meristemos axilares etc. (Barton y Poething, 1993; Barton, 2010; Bowman y Eshed, 2000). Por lo tanto, la función y comportamiento celular de los meristemos es fundamental para el crecimiento y desarrollo de las plantas, de tal manera que señales tanto intrínsecas como externas a lo largo del ciclo de vida de las plantas modulan el desarrollo de los meristemos lo que repercute indudablemente en la arquitectura general de la planta (Medford et al., 1992).

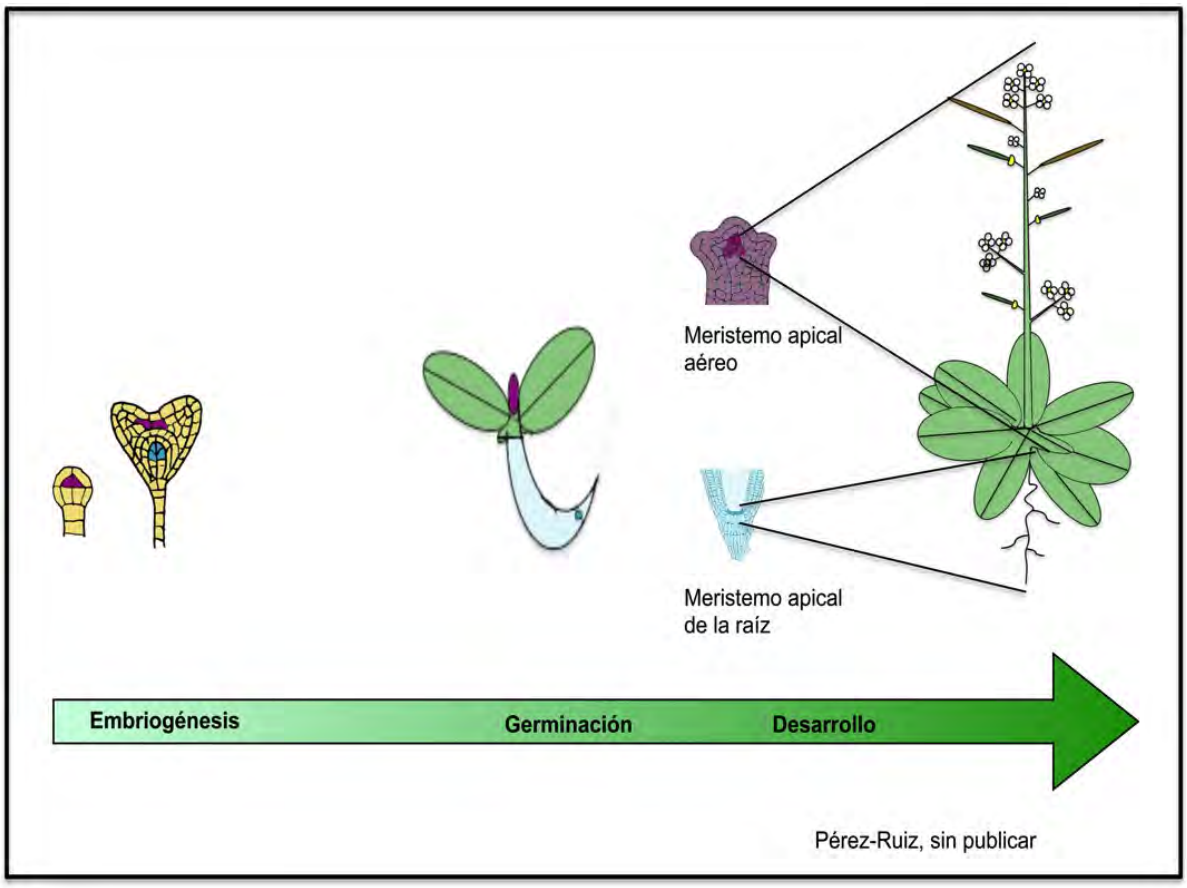


Figura 1. A partir de los meristemos apicales se desarrollan todos los órganos de las plantas a lo largo de su ciclo de vida. Dos meristemos apicales se observan desde la embriogénesis. El color púrpura representa el conjunto de células (SAM) que darán origen a toda la arquitectura aérea de la planta, mientras que el color azul representa el RAM que dará origen al sistema radicular. La flecha representa el establecimiento de los meristemos desde la embriogénesis hasta la etapa adulta de la planta.

1.3. El meristemo apical aéreo.

El meristemo apical aéreo de las angiospermas consiste en un pequeño domo celular compuesto de diferentes regiones o capas celulares. Para fines de este trabajo nos centraremos en las transiciones del meristemo apical aéreo, el cual tiene un arreglo celular que está bien estudiado. Con base en análisis citológicos se han propuesto dos principales formas de organización del meristemo apical aéreo (Carles y Fletcher, 2003), la más evidente es por capas, las cuales han sido nombradas como L1, L2 y L3 (Figura 2). La túnica esta formada por las capas L1 y L2. La capa L1 es la responsable de generar la epidermis mientras que capa L2 genera células del mesófilo y células germinales, por último la capa L3 es la responsable de generar los tejidos más internos, como el tejido vascular. Las células que se encuentran en la capa L3 conforman lo que

se conoce como el *corpus* y las células que la conforman se dividen en todas las direcciones, a diferencia de las células de las capas L1 y L2 que se dividen solamente de manera anticlinal (Clark, 1997; Carles y Fletcher, 2003; Sablowski, 2007).

Sobrelapada a la organización anterior (capas L1, L2 y L3), existe otra por medio de zonas. La zona central se encuentra en el centro del meristemo y resguarda al nicho de células troncales que tienen baja actividad mitótica y en la parte basal se encuentra el centro organizador el cual da la identidad a las células troncales; a su alrededor se encuentra la zona periférica con una alta proliferación y a partir de la cual se originan los meristemos laterales, debajo de la zona central se localiza la zona costillar la cual ha sido poco estudiada (Figura 2; Sablowski, 2007).

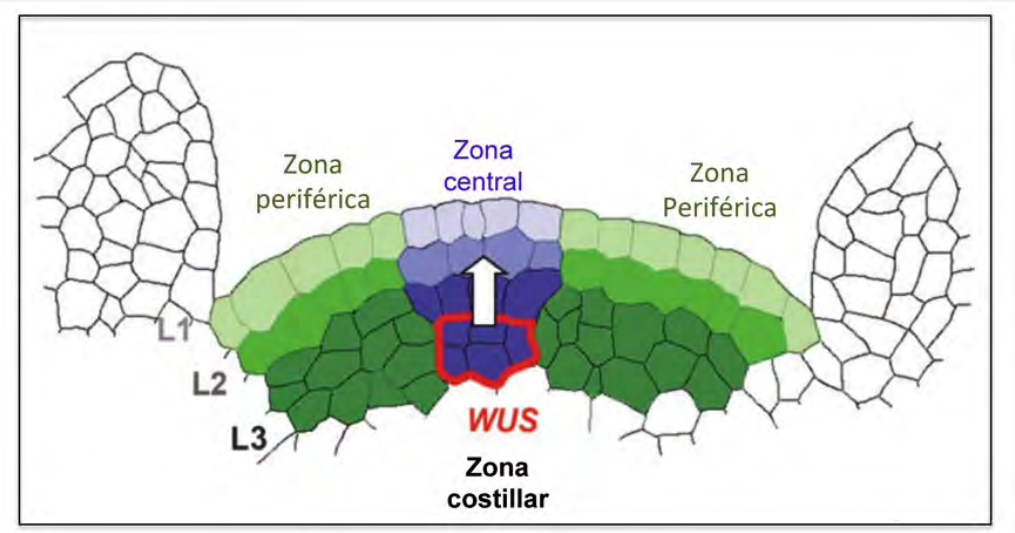


Figura 2. Distribución de las capas celulares en el meristemo apical aéreo de *A. thaliana*. Las células más externas en contacto con el ambiente forman la capa L1, la capa celular adyacente es la capa L2. Finalmente las células más internas forman la capa L3 (cada una de las capas esta marcada en color verde de diferente intensidad). Sobrelapada a la organización antes mencionada se encuentra: La zona central marcada en color púrpura, la zona periférica, marcada en verde mientras que la zona costillar lo conforman el resto de las células. El centro organizador se muestra al centro compartiendo el dominio de expresión de *WUS* delimitado en rojo. Tomado y modificado de Sablowski, 2007.

Para obtener un equilibrio en el tamaño del meristemo se requiere de genes que promuevan la proliferación y otros que lo delimiten. En el meristemo apical aéreo se ha descrito un mecanismo que regula su organización a través de un asa de regulación

entre los genes *CLAVATA3* y *WUSCHEL* (*WUS*). *WUS* codifica para una proteína homeodominio que se expresa en el Centro Organizador (CO) de la zona central del meristemo apical aéreo (Laux et al., 1996) y le da la identidad a la células troncales. *CLAVATA* 1, *CLAVATA 2* (*CLV1*, *CLV2*), *CORYNE* (*CRN) CLAVATA 3* y (*CLV3*) son requeridos para limitar el tamaño del meristemo apical aéreo, al reprimir la expresión de *WUS* vía una cadena de transducción de señales por fosforilación (Clark et al., 1993; Clark et al., 1996; Laux et al., 1996; Clark et al., 1997).

Las mutantes para los genes *CLV* presentan fenotipos de meristemos aéreos agrandados y aglomerados mientras que en la mutante *wus* se observó que terminan su crecimiento antes de tiempo y carecen de órganos florales internos debido a una aberrante división de las células del nicho de células troncales. (Clark et al., 1993; Clark et al., 1996; Laux et al., 1996; Clark et al., 1997).

CLV1 y CLV2 forman parte de una familia de receptores identificados en animales y en plantas (Jinn et al., 2000). El gen *CLV1* codifica para una proteína que consiste de 21 repetidos extracelulares ricos en leucina, un dominio transmembranal y un dominio intracelular con actividad serina/treonina y su expresión se observa fuertemente en la capa L3 de la zona central del meristemo apical aéreo (Clark et al., 1993; Clark et al., 1996; Clark et al., 1997). El gen *CLV2* codifica para un receptor que consiste de un dominio transmembranal tipo LRRs y un tallo citoplásmico de 11 aminoácidos, ambas proteínas contienen cisteínas en sus dominios extracelulares que forman homo y heterodímeros a través de puentes disulfuro. El patrón de expresión de este gen es ubicuo en comparación con los otros dos genes *CLV* (Jeong et al., 1999). El gen *CLV3* se expresa en las capas L1 y L2 de la zona central del SAM y codifica para un péptido extracelular de 96 aminoácidos que se ha sugerido es el ligando del homodímero de CLV1 y del heterodímero CLV2-CRN que actúan como sus receptores (Müller et al., 2008).

El balance entre la regulación de los genes *CLV3* y *WUS* permite la correcta formación del meristemo, se sabe que *WUS* se expresa en el CO pero su proteína migra hacia las

capas L1 y L2 en donde se une y activa al promotor de *CLV3*, esta inducción da como resultado que *CLV3* secreta un péptido inmaduro que migra de las capas L1 y L2 al CO para reprimir a *WUS* para lo cual requiere de modificaciones postraduccionales que le permiten que sea percibido por fosfatasas que son importantes para que se de la correcta interacción con los receptores CLV1 y CLV2 para desencadenar la transducción de señales para llevar a cabo la represión. (Fletcher et al., 1999; Kondo et al., 2006; Ohyama et al., 2009; Heidstra y Sabatini, 2014). Esta asa de regulación existente entre WUS-CLV3 asegura un número constante de células en el nicho de células troncales en la zona central (Schoof et al., 2000; Barton 2010; Brand et al., 2000; Schoof et al., 2000; Müller et al., 2008). Adicionalmente, también se ha observado que la hormona citoquinina juega un papel importante en la regulación WUS-CLV3, activando a *WUS* (Gordon et al., 2009; Chickarmane et al., 2012). Sin embargo, al aplicar citoquininas exógenamente no se ha observado que esto afecte el tamaño del meristemo apical aéreo en plantas silvestres, lo cual sugiere que hay un mecanismo de amortiguamiento en el que miembros de receptores quinasa de la familia *ERECTA* se han visto involucrados (Chickarmane et al., 2012; Uchida et al., 2012).

El SAM o Meristemo de Inflorescencia (MI) se encuentra indeterminado por la continúa expresión de *WUS* en el centro organizador y la capacidad de las células troncales de mantenerse indiferenciadas y proliferando por la acción de los genes KNOX y las citoquininas (Leinfried et al., 2005; Scofield et al., 2006).

1.4. Transiciones en el meristemo apical aéreo de *Arabidopsis thaliana.*

El meristemo apical aéreo de *A. thaliana* pasa por diversas etapas durante el ciclo de vida de la planta. Una vez que la semilla germina, el meristemo apical aéreo produce sólo hojas dando origen a la roseta (su tamaño y forma, depende hasta cierto punto de las condiciones ambientales). Cuando la planta alcanza la madurez, el meristemo apical aéreo se convierte en el meristemo de inflorescencia, el cual genera un escapo que produce ramas y hojas caulinares en sus flancos y dará origen a los meristemos de flor

que se diferenciarán en los órganos florales (Figura 3; Sablowski, 2007; Huijser and Schmid, 2011).

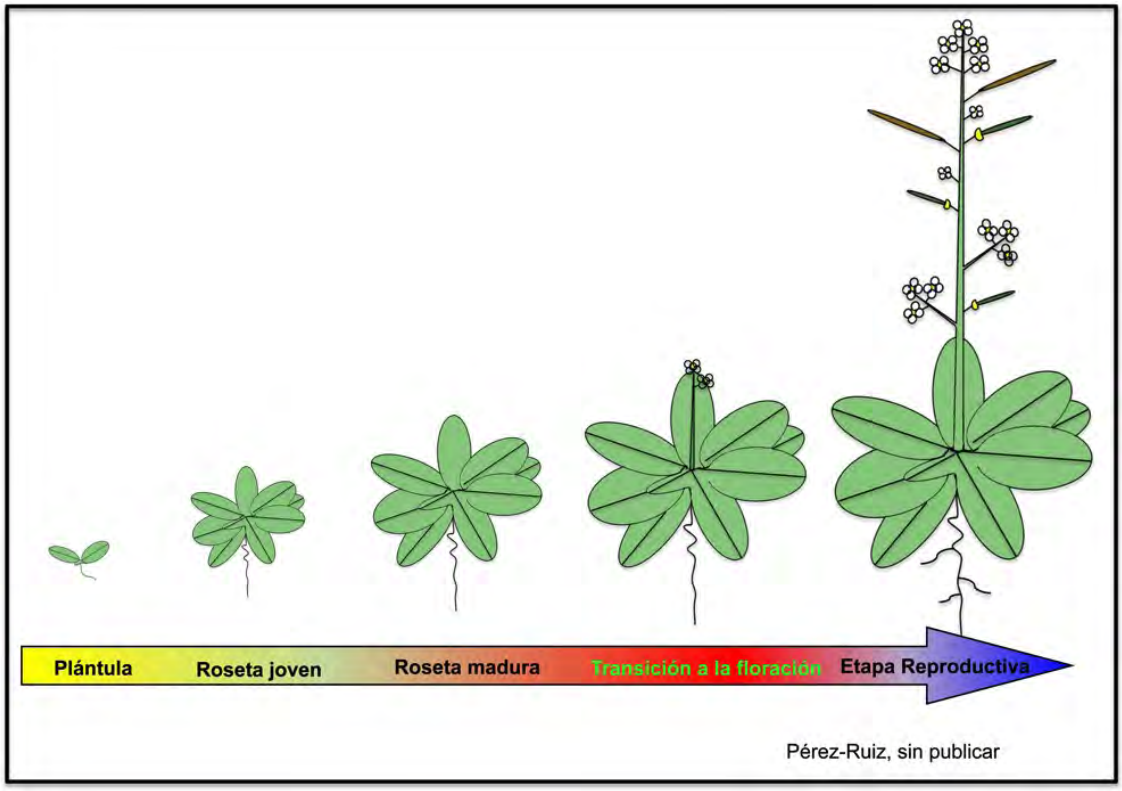


Figura 3 Etapas de transición del meristemo aéreo en *A. thaliana* a lo largo del ciclo de vida. La flecha representa el desarrollo a lo largo del tiempo hasta llegar a la etapa donde se generan las flores, a la cual se le conoce como etapa reproductiva.

En *Arabidopsis thaliana* la transición de la etapa juvenil a la adulta, durante el desarrollo vegetativo se caracteriza por la aparición de caracteres morfológicos en las hojas de la roseta, por ejemplo, la aparición de tricomas en la parte abaxial y se observa un aumento en la longitud de la hoja (Willmann y Poething, 2005). Además, las plantas son capaces de responder a estímulos que inducen la floración. Cuando el meristemo vegetativo, que únicamente produce hojas de roseta, se convierte en un meristemo de inflorescencia la planta transita a la floración y se dejan de producir las primeras (Araki, 2001). Casi simultáneamente al establecimiento del meristemo de inflorescencia se forman los meristemos florales adyacentes al meristemo de inflorescencia y ambos tipos de meristemos mantienen sus identidades uno al lado del otro hasta la senescencia. Todas las transiciones del SAM son controladas por un sistema de de

regulación genética y epigenética íntimamente relacionada con las condiciones ambientales.

1.5. Transición a la floración.

Con base en el análisis de mutantes afectadas en el tiempo de transición a la floración en *A. thaliana*, se han podido identificar una multitud de genes que participan en respuesta a una o a varias condiciones ambientales por lo que se establecieron diferentes vías de señalización. Sin embargo, cabe aclarar que cada vez hay más evidencia que indica que todas estas vías están interconectadas. Hasta el momento se han descrito siete "vías" de regulación del tiempo de transición a la floración en *A. thaliana*: una vía dependiente de la duración de la luz referida como vía del **fotoperiodo**, una independiente del fotoperiodo denominada vía **autónoma**, otra dependiente de **giberelinas** y la dependiente de bajas temperaturas conocida como **vernalización** (Figura 5; Wilson et al., 1992; Michaels et al., 2000; Suárez-López et al., 2001; Mouradov et al., 2002; Sheldon et al., 2002; Yoo et al., 2005). Adicionalmente, tres vías más han sido propuestas, una que considera cambios en la temperatura ambiental de crecimiento de la planta a la cual se le ha denominado como vía **termosensora**, otra que depende del estado de desarrollo de la planta a la cual se le ha llamado vía del **envejecimiento** (Figura 5; Wang et al., 2009, Balasubramanian et al., 2006, Srikanth y Schmid, 2011) y la que es regulada por la **trehalosa** (Wahl et al., 2013; Tsai y Gazzarrini, 2014). A continuación se describen brevemente cada una de las "vías" de señalización como se ha postulado en la literatura.

1.5.1. Vía del fotoperiodo.

El fotoperiodo es percibido en las hojas, en las cuales existen señales móviles que se transmiten al meristemo apical aéreo para inducir la floración (Srikanth y Schmid, 2011). La cascada de eventos responsable de detectar la longitud del día y la subsecuente iniciación de la floración es referida como vía del fotoperiodo (Srikanth y Schimid., 2011). En esta vía, las plantas mutantes de los genes que participan en ella, presentan

un fenotipo de floración tardía bajo la condición de fotoperiodo de día largo, es decir en un fotoperiodo de 16 h de luz por 8 h de oscuridad (Koornneff et al., 1998), pero florecen al mismo tiempo que las plantas silvestres en condiciones de días cortos (8 h de luz por 16 h de oscuridad).

La respuesta al fotoperiodo presenta dos componentes, el primer componente es el sistema para la detección de la luz. En las pla□t□s□existen tres clases de fotoreceptores especializados para este fin. Las fototropinas y los criptocromos detectan la longitud de onda para la luz azul y los fitocromos detectan las longitudes de onda para luz roja y el rojo lejano (Neff et al., 2000; Franklin et al., 2005). Mediante una cascada de señalización que involucra varios genes de regulación de ciclo circadiano, estas señales se integran con ayuda del gen *CONSTANS* (*CO*; Rédei 1992; Koornneef et al., 1991; Putteril et al., 1995) y *GIGANTEA* (*GI;* Rédei 1992; Koornneef et al., 1991; Mizoguchi et al., 2005).

El segundo componente comprende la transducción de señales sincronizadas por el ciclo circadiano y se basa en un mecanismo por el cual las plantas pueden medir la duración del día (Alabadí et al., 2001; Suárez-López et al., 2001; Doyle et al., 2002, Srikanth y Schimid, 2011; Andrés y Coupland 2012). El ciclo circadiano es un mecanismo con una periodicidad de 24 h que regula un alto porcentaje de genes en *A. thaliana*, este ciclo comprende tres asas de regulación interconectadas: el asa de regulación central y las asas de regulación de la mañana y de la tarde que pueden ser tanto positivas como negativas (Imaizumi., 2010). En *A. thaliana*, la capacidad para distinguir la duración del día y la noche se basa en la coincidencia de un “reloj” interno de la planta el cual está representado por el patrón de la expresión de *CO* un gen que codifica para un factor transcripcional con dedos de zinc, el cual responde a la luz. (Putteril et al., 1995). Experimentos con construcciones que presentan una expresión inducible de *CO* mediante dexametasona demostraron que *CO* promueve la floración al inducir la transcripción de *FLOWERING LOCUS T* (*FT),* el cual codifica para una proteína globular, similar a la familia de inhibidores de RAF cinasas y proteínas de unión de fosfatidil-etanolaminas (Kobayashi et al., 1999) de igual manera que a *TWIN SISTER*

OF FT (*TSF*) en respuesta a la luz. (Samach et al., 2000; Suarez-López et al., 2001, Yamaguchi et al., 2005).

La regulación de *CO* no es únicamente a nivel transcripcional sino que también presenta regulación en la estabilidad y acumulación de su proteína (Suárez-López et al., 2001). En la regulación de *CO* interviene el ciclo circadiano de 24 h, por lo que la transcripción y la traducción de su mensajero varía dependiendo de las condiciones del fotoperiodo en la cual crecen las plantas. Durante las condiciones de fotoperiodo de día largo los niveles de expresión de CO se van incrementando durante el día presentando los niveles más altos durante la tarde, de tal manera que la traducción de la proteína de *CO* alcanza los niveles suficientes para activar a *FT* e inducir la floración. Un caso diferente se presenta en el fotoperiodo de día corto en el cual no se alcanzan los niveles adecuados de la proteína CO para inducir a *FT* debido a que en la oscuridad la proteína se degrada vía el proteosoma, dando como consecuencia un retraso en la floración (Suárez-López et al., 2001; Imaizumi, 2010).

Durante el fotoperiodo de día largo la luz promueve la interacción entre GI y una familia de ubiquitina ligasas con caja F, como FLAVIN-BINDING, KELCH REPEAT F-BOX 1 (FKF1, Nelson et al., 2000). Estas interacciones estabilizan las proteínas, permitiendo la degradación de un conjunto de genes que reprimen a CO, incluyendo numerosos factores de transcripción DOF como CYCLING DOF FACTOR (CDF) que retrasan la floración por reprimir los niveles de expresión de *CO* (Fornara et al*.,* 2009). A nivel post-transcripcional CO es degradado en la noche por la ubiquitina ligasa CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) y en la mañana por una vía activada por el fitocromo B (PHYB).

Los niveles de regulación transcripcional y post-transcripcional aseguran que CO active la transcripción del florígeno *FT* y TSF solamente durante el fotoperiodo de día largo. FT es capaz de translocarse desde las hojas al meristemo de inflorescencia en donde se une a *FLOWERING LOCUS D* (*FD*), un factor de transcripción tipo bZIP, de esta manera se forma el complejo FT/FD el cual regula a genes que promueven la floración

en el meristemo apical aéreo (Abe et al., 2005; Wigge et al., 2005). CO también activa al factor transcripcional MADS-box *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), en parte a través de *FT* (Yoo et al., 2005). Además, *SOC1* es regulado por todas las vías de floración por lo que se le ha considerado como un integrador de todas las señales (Lee et al., 2000; Samach et al., 2000; Moon et al., 2003; Lee and Lee, 2010). Aunque se desconoce hasta el momento cómo se estructuran los complejos proteicos del que forma parte y que le permite responder a las diferentes señales que conducen a la transición floral.

1.5. 2. Via de Vernalización.

A. thaliana al igual que muchas plantas que habitan en regiones con estaciones del año marcadas, atraviesa por un periodo invernal a bajas temperaturas y esta condición favorece la floración de forma acelerada (Sheldon et al., 2000). La vía de la vernalización se detectó estudiando a aquellos mutantes que eran incapaces de responder a esta condición, y que por lo tanto, seguían floreciendo como las plantas silvestres sin vernalizar (Koorneef et al., 1998). La vía de vernalización favorece que las plantas florezcan porque reprime la transcripción de *FLOWERING LOCUS C* (*FLC*) en respuesta a tiempos prolongados a bajas temperaturas (Michaels y Amasino, 2001). *FLC* codifica para un factor transcripcional tipo MADS-box, el cual funciona como un represor de la transición a la floración al unirse a las cajas CArG del promotor de *SOC1* y FT reprimiéndolos e impidiendo así su expresión (Michaels y Amasino, 1999; Michales y Amasino, 2000; Searle et al., 2006). Durante la vernalización, actúan procesos epigenéticos que reprimen la expresión de *FLC* lo que conlleva a la activación de *SOC1* y *FT* por lo tanto se induce la floración (Michaels y Amasino, 1999; Sheldon et al., 2002; Sheldon et al., 2006).

Se ha identificado que los genes *VERNALIZATION1*, *VERNALIZATION2* y *VERNALIZATION INSENSITIVE3* (*VRN1*, *VRN2* y *VIN3* respectivamente) están involucrados en la represión epigenética de *FLC* (Gendall et al., 2001; Levi et al., 2002; Sung et al., 2006).

VRN1 participa en el proceso de vernalización y codifica para una proteína que se une a ADN y su papel es mantener la represión epigenética de *FLC* (Levy et al., 2002). *VRN2* codifica para el homólogo de Suppressor of Zeste 12 (Su(z)12) identificado inicialmente en Drosophila, y ahora se sabe que está involucrado en procesos de represión epigenética en animales y plantas, formando parte del complejo represivo Polycomb 2 (PCR2) el cual confiere silenciamiento epigenético estable al represor de la floración *FLC* (Gendall et al., 2001). El complejo PCR2 cataliza la metilación de la lisina 27 de la histona H3 (H3K27me) que es una modificación asociada con represión transcripcional apagando la expresión de *FLC* (Gendall et al., 2001). *VIN3* posee un dominio tipo Plant Homeo Domain (PHD) el cual es característico de los componentes que forman parte de los complejos de remodelación de cromatina (Sanchez y Zhou, 2011).

Mediante el empleo de mutantes para *VRN1*y *VRN2* se determinó que en la vernalización se puede reprimir a *FLC*, sin embargo este estado no se mantiene cuando la planta deja de estar en vernalización (Sheldon et al., 2006), estos resultados sugieren que *VRN1* y *VRN2* no están involucrados en el establecimiento del silenciamiento de *FLC* en respuesta a bajas temperaturas, pero sí participan en el mantenimiento de la represión cuando la planta se desarrolla en condiciones de temperatura normales de crecimiento. En el caso de *VIN3* su expresión se induce en vernalización y se ha propuesto que su participación es establecer el estado represivo de *FLC* (Sung y Amasino, 2004).

Por otra parte, también se ha demostrado que *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*) también llamado *TERMINAL FLOWER 2* (*TFL2*), es un homólogo de *HETEROCHROMATIN PROTEIN 1* (*HP1*) identificado en animales y levaduras, es necesario para mantener el estado reprimido de *FLC* durante la vernalización (Mylne et al., 2006; Sung et al., 2006).

FLC por otra parte es activado con ayuda del complejo PAF1, el cual fue identificado inicialmente en levadura. En *Arabidopsis* se han identificado varios genes homólogos a este complejo como por ejemplo *VERNALIZATION INDEPENDECE 4* y *VERNALIZATION INDEPENDECE 5* (*VIP4* y *VIP5*), los cuales reclutan a proteínas con

actividad de metil transferasas (Zhan y Van Nocker, 2002; Zhang et al., 2003). El estudio de los mutantes de estos complejos en *Arabidopsis* ha demostrado que en su ausencia se suprimen los niveles de *FLC*, lo que sugiere que el complejo PAF1 es indispensable para mantener los niveles de *FLC* para reprimir la floración (He et al., 2003).

Adicionalmente se ha observado que *FLC* puede generar un transcrito antisentido, el cual es diferencialmente expresado a diferentes temperaturas, este transcrito regula negativamente su propia expresión favoreciendo la transición a la floración. El transcrito antisentido más grande de *FLC* se transcribe iniciando en la región 3′ UTR donde se encuentra el poly (A), finalizando su expresión río arriba del sitio de inicio de la transcripción del mensajero en dirección sentido. Se sabe que el inicio de la transcripción del mensajero antisentido es independiente de la transcripción del mensajero sentido de *FLC*. A este transcrito antisentido se le denominó como COOLAIR (cold induced long antisense intragenic RNA) y se mostró recientemente que es la primera respuesta al tratamiento por frío y es capaz de reprimir la transcripción de la hebra sentido antes de que *VIN·3* (*VERNALIZATION INSENSITIVE 3*) genere un efecto en la represión epigenética de *FLC* (Bond et al., 2009; Swiezewski et al., 2009). Adicionalmente se ha observado que cuando las plantas son sometidas a un tratamiento por frío, *FLC* es capaz de transcribir de manera sentido el intrón 1, dando origen a un transcrito que se le ha denominado *COLDAIR* (Cold assisted intronic noncoding RNA), el cual se encontró que se asocia con el núcleo del complejo 2 represivo de Polycomb (PRC2) y se ha observado que su ausencia provoca disminución del silenciamiento epigenético llevado a cabo por el complejo remodelador de cromatinas de las proteínas Polycomb (PcG).

1.5.3. Vía dependiente de Giberelinas.

Diversas variantes del ácido giberélico han sido identificadas en plantas, hongos y bacterias, pero solamente unas cuantas presentan actividad biológica. La enzima GIBBERELLIN 20 OXIDASA (GA20ox), es la responsable de catalizar varios pasos

importantes en la síntesis de la giberelinas por llevar a cabo reacciones de oxidación en varios precursores, el efecto más fuerte de estas hormonas se presenta en condiciones no inductivas de luz, es decir en fotoperiodo de día corto (Hedden y Phillips, 2000; Mutasa-Göttgens et al., 2009). Las giberelinas son hormonas vegetales que tienen diversas funciones en el desarrollo de las plantas como son: la expansión de la hojas, elongación del tallo, germinación de las semillas, etc. Además son importantes para inducir las transiciones de fase durante el desarrollo de las plantas (Mutasa-Göttgens et al., 2009).

La ausencia de giberelinas afecta la transición a la floración en *Arabidopsis* lo cual se ha observado en mutantes incapaces de sintetizarla o en mutantes afectadas en su señalización. Plantas mutantes en el gen *GA1* el cual codifica para una ent-caureno sintasa A, que es la enzima responsable de catalizar el primer paso en la síntesis de la hormona GA, tardan más tiempo en florecer en comparación con las plantas de tipo silvestre cuando son crecidas en día corto (Wilson et al., 1992). Algo similar ocurre con plantas mutantes afectadas en la señalización de esta hormona. El receptor para giberelinas fue identificado en arroz y mediante comparaciones con el genoma de Arabidopsis se encontraron tres genes homólogos con funciones redundantes a los cuales se nombró como *GIBERRELLIN INSENSITIVE DWARF1A (GID1A)*, *GIBERRELLIN INSENSITIVE DWARF1B (GID1B)* y *GIBERRELLIN INSENSITIVE DWARF1C* (*GIB1C).* Mutantes sencillas para cada uno de estos genes fueron nombradas respectivamente como *gid1a*, *gid1b* y *gid1c* y se encontró que en las mutantes sencillas no se presentaban defectos obvios al compararlas con plantas de tipo silvestre (Griffiths et al., 2006). Sin embargo cuando se generaron mutantes múltiples, se encontró que las plantas tienen defectos como por ejemplo en la elongación del hipocótilo y en el tiempo de floración (Griffiths et al., 2006).

La estructura cristalográfica del receptor *GID1* demostró que éste contiene un sitio de unión a giberelinas y se determinó que es posible que se forme un puente de hidrógeno entre el grupo hidroxilo del carbono 3 de la molécula de la giberelina con la tirosina 31 del receptor, lo cual induce un cambio conformacional en el extremo N-terminal

favoreciendo la interacción de las proteínas DELLAS con el receptor para generar el complejo GA-GID1-DELLA (Willige et al., 2007; Murase et al., 2008; Shimada et al., 2008).

De esta manera, mientras que la ausencia de giberelinas favorece la acumulación de proteínas DELLA, las cuales a su vez reprimen respuestas a giberelinas, la formación del complejo GA-*GID1*-DELLA estimula la degradación de las proteínas DELLA, esta observación se determinó por los estudios que se llevaron a cabo en arroz en el gen *GIBERRELLIN INSENSITIVE2* (*GID2*) y en *Arabidopsis* en el gen *SLEEPY1* (*SLY1*), ambos genes codifican para proteínas de caja F, las cuales son componentes del complejo SCF (Sasaki et al., 2003; McGinnis et al., 2003).

Bajo condiciones de fotoperiodo de día corto, donde la temporalidad de luz no está participando activamente como inductora de la transición a la floración, debido a que la expresión de *FT* es baja, la floración en *Arabidopsis* depende en buena medida de las giberelinas (Wilson et al., 1992). Las plantas afectadas en los procesos antes mencionados (síntesis o señalización de GA) pueden recobrar parcialmente su tiempo de floración al suministrarse giberelinas exógenamente. Estas hormonas promueven la expresión tanto de *SOC1* como de *LFY* (Achard et al., 2004; Blázquez et al., 2000; Moon et al., 2003).

1.5.4. Vía autónoma.

Esta vía responde a señales intrínsecas del desarrollo y es independiente de las señales externas. Las plantas mutantes en la vía autónoma son aquellas en las cuales sus genes al estar mutados, provocan que las plantas presenten floración tardía en condiciones tanto de día corto como largo, sin embargo el fenotipo puede ser suprimido por vernalización (Koornneef et al., 1998). Genes de la vía autónoma regulan de forma negativa la acumulación del ARN mensajero de *FLC* cuando las plantas no han pasado por la época invernal por lo que en la ausencia de cualquiera de ellos, FLC inhibe la floración (Michaels y Amasino, 1999; Searle et al., 2006). Son varios los genes que

codifican para proteínas que participan en este proceso: *FCA, FPA*, *FLK* (*FLOWERING LATE KM MOTIF*) *FY, FVE, FLD* (*FLOWERING LOCUS D*) y *LD* (*LUMINIDEPENDENS*). *FCA*, *FPA* y *FLK* codifican para proteínas de unión a RNA, las cuales heterodimerizan con *FY* y *FVE,* que codifican para proteínas con repetidos WD-40 (Marquardt et al., 2006). Estos heterodímeros afectan post-transcripcionalmente el mensajero de *FLC* evitando que este sea traducido. Adicionalmente, FY junto con FCA participa en el procesamiento del ARN. *LD* codifica para un proteína homeodominio (Lee et al., 1994). FVE y FLD son homólogos del complejo de desacetilasas de histonas de humanos (HDAC) que también limitan la acumulación de *FLC* por participar en la remodelación de cromatina (Koorneef et al., 1991; Michaels y Amasino., 1999; Michaels y Amasino 2001; Henderson y Dean, 2004).

1.5.5 Vía termosensora.

Las plantas son organismos muy sensibles a la temperatura y pueden percibir cambios tan sutiles como 1°C (Kumar et al., 2010). Normalmente la temperatura de crecimiento en el laboratorio para *A. thaliana* se encuentran entre 20°C y 23°C, pero se ha demostrado que ligeros cambios en la temperatura de crecimiento por ejemplo a 27°C acelera la floración (Kumar et al., 2012). Se ha visto que la señal de la respuesta a los cambios pequeños de temperatura se integran por debajo del gen *CO* (Balasubramanian et al., 2006). Recientemente se ha demostrado el mecanismo por el cual se acelera la floración. Se sabe que los aumentos en la temperatura de crecimiento favorecen la transcripción del factor *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*) el cual a su vez activa al florígeno *FT* directamente al unirse a su región promotora (Kumar et al., 2012). Las señales dadas por la temperatura son mediadas a través del nucleosoma conteniendo la histona H2A.Z (Kumar et al., 2010) de esta manera al haber incrementos de temperatura, el nucleosoma libera a la región regulatoria de *FT* siendo accesible para *PIF4*, dando como resultado una unión más fuerte que favorece la transcripción de *FT*, por lo tanto un incremento del florígeno induce la floración al activar a *SOC1* (Searle et al., 2006). Por otra parte, *SHORT VEGETATIVE PHASE* (*SVP*) es un factor transcripcional tipo MADS-box que participa sensando la temperatura ambiental y

reprime a *FT* al unirse directamente a cajas CarG localizadas en su promotor (Lee et al., 2007).

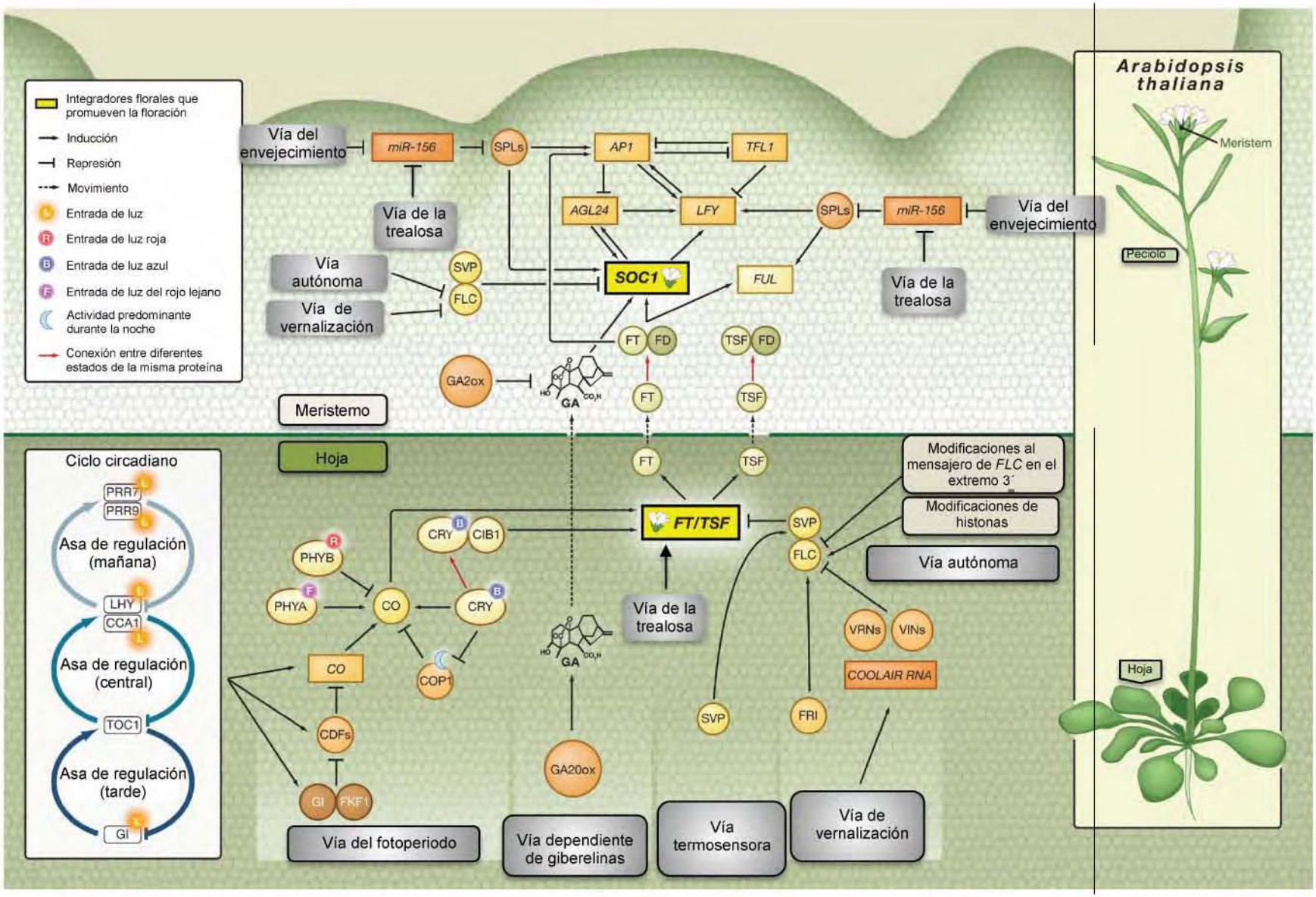
1.5.6 Vía del envejecimiento y la regulación mediada por la trehalosa.

Se ha utilizado como marcador del envejecimiento los niveles de expresión del *microRNA156 (miR156*), ya que se ha visto que estos van disminuyendo gradualmente a medida que la planta se desarrolla y envejece (Wu and Poething, 2006; Wang et al., 2009). Esta vía propone que los genes *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* 3 y 9 (*SPL3 y 9*) son los blancos del *miR156*. Cuando se analizaron plantas jóvenes en ausencia del fotoperiodo de día largo se encontraron altos niveles de miR156, pero bajos niveles de *SPL3* y *SPL9*. Con el paso del tiempo, los niveles de *miR156* disminuyen y aumentan *SPL3* y *SPL9* favoreciendo así la floración (Wang et al., 2009).. Otros genes de la familia *SPL* han sido involucrados en transiciones de fase, por ejemplo, mediante el estudio de la sobreexpresión de los genes *SLP3*, *SPL4* y *SLP5*, se observó que redundantemente integran las señales provenientes de las vías autónoma, del fotoperiodo, giberelinas y envejecimiento para favorecer la fase reproductiva (Jung et al., 2012; Porri et al., 2012; Yu et al., 2012) y que su regulación es afectada de diversas maneras en las condiciones de día corto, estos tres genes son regulados negativamente por el *miR156* dependiendo de la edad de la planta, pero son activados por *SOC1* por medio de la vía dependiente de giberelinas (Jung et al., 2011, 2012). Mientras que en día largo, *SOC1*, *FT* y *FD* regulan a *SLP3*, *SPL4* y *SLP5* en las hojas en respuesta a las señales del fotoperiodo (Jung et al., 2012). El *miR172* participa en la regulación del tiempo de floración, y se sabe que *SPL9* es su activador transcripcional el cual actúa posiblemente redundantemente con otros genes *SPL,* como *SPL10* en su regulación. (Zhao et al*.,* 2007; Wu et al., 2009). Mediante el empleo de la doble mutante *spl9 spl15* se ha encontrado que *SPL15* participa en la regulación de miR172 promoviendo su transcripción (Wu et al., 2009). Por otra parte, *miR156* regula la expresión de *miR172*, aunque esta regulación no es recíproca y se ha determinado que la expresión de *miR156* y *miR172* tiene patrones inversos, es decir cuando uno aumenta el otro disminuye (Wu et al., 2009).

El *miR172* promueve la floración por medio de un mecanismo de represión post-trancripcional de los genes *TARGET OF EAT1*, *TARGET OF EAT2* y *TARGET OF EAT3* (*TOE1*, *TOE2* y *TOE3*), *SCLAFMÜTZE* (*SMZ*) y *SCHNARCHZAPFEN* (*SNZ*), que son represores de *FT,* todos pertenecientes a la familia de genes *AP2-like* (Aukerman y Sakai 2003; Mathieu et al., 2009; Yant et al., 2009).

Recientemente se ha confirmado que el estado metabólico de las plantas, es decir si la planta cuenta con las reservas energéticas adecuadas se favorece la transición a la floración. Una de estas reservas energéticas es la trehalosa-6-fosfato (T6P) y desde hace mucho tiempo se sospechaba que la señalización mediante los azúcares estaba implicada en esta transición del desarrollo (Wahl et al., 2013; Tsai y Gazzarrini, 2014). Últimamente se descubrió que la T6P funciona en el SAM como una molécula señal que regula a genes implicados en la vía del envejecimiento inhibiendo al *miR156* que a su vez reprime a los genes *SPL3*, *SPL4* y *SPL5* mientras que en las hojas se observó que T6P favorece la floración por la vía del fotoperiodo al inducir la expresión de *FT* (Wahl et al., 2013; Tsai y Gazzarrini, 2014).

Figura 4. Integración de los diferentes componentes que participan en la transición a la floración. En *A. thaliana* la transición a la floración inicia después de pasar por un estado vegetativo. El sistema de regulación genético ha sido inferido con base en mutantes de pérdida de función y líneas de sobreexpresión. Hasta el momento, se han identificado siete vías genéticas que participan en este proceso, aquí se muestran: La vía del fotoperiodo, la vía de vernalización, la vía autónoma, la vía dependiente de giberelinas, la vía del envejecimiento, la vía termosensora y al vía en la que participa la trehalosa. Varias de estas vías convergen en genes claves a los cuales se les conoce como integradores (*SOC* y *FT*) y que además son promotores de la floración porque inducen a genes de identidad floral como *AP1* y *LFY*. Por otra parte, también hay genes que son represores de la floración, el mejor ejemplo estudiado es *FLC* (Tomado y modificado de Fornara et al., 2009).


Integradores florales que promueven la floración
Inducción
Represión
Movimiento
Entrada de luz
Entrada de luz roja
Entrada de luz azul
Entrada de luz del rojo lejano
Actividad predominante durante la noche
Conexión entre diferentes estados de la misma proteína
Vía del envejecimiento
miR-156
SPLs
AP1
TFL1
Vía de la trealosa
AGL24
LFY
SPLs
miR-156
Vía del envejecimiento
Vía autónoma
Vía de vernalización
SVP
FLC
SOC1
FUL
Vía de la trealosa
FT
FD
TSF
FD
GA2ox
GA
FT
TSF
Meristemo
Hoja
Ciclo circadiano
PRR7
PRR9
Asa de regulación (mañana)
LHY
CCA1
Asa de regulación (central)
TOC1
Asa de regulación (tarde)
GI
FT
TSF
Modificaciones al mensajero de FLC en el extremo 3´
Modificaciones de histonas
FT/TSF
SVP
FLC
Vía autónoma
CRY
CIB1
PHYB
PHYA
CO
CRY
Vía de la trealosa
VRNs
VINs
COOLAIR RNA
CO
COP1
CDFs
GI
FKF1
GA
GA20ox
SVP
FRI
Vía del fotoperiodo
Vía dependiente de giberelinas
Vía termosensora
Vía de vernalización
Arabidopsis thaliana
Meristem
Pecíolo
Hoja

1.6. Identidad del meristemo de Inflorescencia y el meristemo de flor.

Mientras que los meristemos apicales (RAM y SAM) son del tipo indeterminado, es decir continúan produciendo células hasta la senescencia, los meristemos florales son determinados y cesan la producción de nuevas células después de que se han desarrollado los órganos florales (Okamuro et al., 1996; Laudencia-Chingcuanco y Hake, 2002; Jack, 2004). Estas transiciones son reguladas genéticamente de forma que el desarrollo prosiga finalmente hasta la generación de flores sin presentar reversiones (Bäurle y Dean, 2006).

Existen genes que mantienen la identidad del meristemo de inflorescencia, tal es el caso de *TERMINAL FLOWER1* (*TFL1*) que codifica para una proteína pequeñala cual tiene homología con proteínas de unión a fosfatidiletanolamina (Bradley et al., 1997). En plantas mutantes para este gen se han observado dos características que las distinguen de las plantas de tipo silvestre, por un lado la floración es temprana y por el otro, el meristemo de inflorescencia eventualmente adquiere identidad floral lo cual provoca que se genere una flor terminal (Shannon y Meeks-Wagner, 1991; Alvarez et al., 1992). TFL1 pertenece a una familia de proteínas en las que se encuentra también FLOWERING LOCUS T (FT), sin embargo el mutante en este último gen tiene fenotipos opuestos a *tfl1* (Kobayashi et al; 1999; Hanzawa et al., 2005; Hanano y Goto, 2011; Wickland y Hanzawa 2015). Mediante herramientas moleculares se ha demostrado que *TFL1* se expresa en meristemos axilares jóvenes y en el meristemo de inflorescencia su patrón de expresión se limita a la zona central (Conti y Bradley, 2007). Sin embargo, la proteína es capaz de moverse y su rango de acción es más amplio en el meristemo de inflorescencia donde reprime a *LEAFY* (*LFY*) y *APETALA1* (*AP1*; Conti y Bradley, 2007).

Por otra parte, el meristemo de flor está especificado y le dan identidad un grupo de genes: *LFY* (Schultz y Haughn, 1991; Huala y Sussex, 1992; Blázquez et al., 1997), *AP1* (Irish y Sussex, 1990; Gustafson-Brown et al., 1994*)* y *CAULIFLOWER* (*CAL*, Kempin et al., 1995), siendo *LFY* el que induce directamente a los otros dos (Mandel et al., 1992;

Weigel et al., 1992; Parcy et al., 1998). *LFY*, es fundamental tanto para la especificación de la identidad del meristemo floral como para activar a los genes homeóticos que participan en el desarrollo de los órganos florales (Weigel and Meyerowitz, 1993; Lamb et al., 2002). *LFY* codifica para un factor transcripcional identificado hasta el momento sólo en plantas (Schultz y Haughn, 1991; Weigel et al., 1992). *LFY* se expresa en las hojas durante el estado vegetativo pero después se expresa fuertemente en los flancos del meristemo de inflorescencia, justo en las células que darán origen al meristemo de flor en una zona conocida como *anlagen* (Blazquez y Weigel, 2000). Los mutantes *lfy* producen un gran número de hojas, meristemos laterales e inflorescencias secundarias y los alelos débiles de este mutante son capaces de producir flores, pero son anormales y presentan características de inflorescencia (Weigel et al., 1992; Vijayraghavan et al., 2005).

AP1 es un factor transcripcional del tipo MADS-box cuyo mutante produce flores con características de inflorescencia (Irish y Sussex, 1990; Bowman et al., 1993). Este gen es inducido por el dímero FT-FD y adicionalmente presenta una retro-regulación positiva con *LFY*, permitiendo que este permanezca activo durante todo el desarrollo del meristemo de flor. Además, *AP1* y *LFY* son funcionalmente redundantes en el mantenimiento de la identidad de este meristemo (Huala y Sussex, 1992; Abe et al., 2005). *CAULIFLOWER* (*CAL*, Kempin et al., 1995) y *FRUITFUL* (*FUL*, Mandel y Yanofsky, 1995; Gu et al., 1998) son homólogos y funcionalmente redundantes con *AP1*, por lo que la doble mutante *ap1 cal* y la triple mutante *ful ap1 cal*, presentan fenotipos más severos que el mutante sencillo, dando como resultado inflorescencias reiterativas que dan la apariencia de una coliflor en lugar de la formación de flores (Ferrándiz et al., 2000). Cabe señalar que *FUL* normalmente se expresa en el meristemo de inflorescencia donde tiene un papel importante en la indeterminación de este meristemo y en el desarrollo del tejido vascular (Melzer et al., 2008). En presencia de AP1, *FUL* no se expresa al inicio del desarrollo del meristemo de flor, por lo que al parecer sólo puede llevar a cabo la función de AP1 en su ausencia (Blázquez et al., 2006).

Tanto los meristemos de inflorescencia como los meristemos de flor coexisten en el meristemo apical sin perder su identidad. Se ha observado que esta restricción de dominios se debe a la represión mutua entre *TFL1* y los genes de identidad floral (LFY, AP1 y *CAL*). (Figura 5; Liljegren et al., 1999; Ratcliffe et al., 1999; Moon et al., 2003; Liu et al., 2013). De esta forma, cuando *LFY* y *AP1* se expresan ectópicamente en la mutante *tfl1* se favorece la formación de flores terminales (Shannon y Mekks-Wagner, 1991; Bradley et al., 1997; Conti y Bradley, 2007). En contraste, si *LFY* y *AP1* son mutados, *TFL1* se expresa en el meristemo de flor y se indetermina (Ratcliffe et al., 1999).

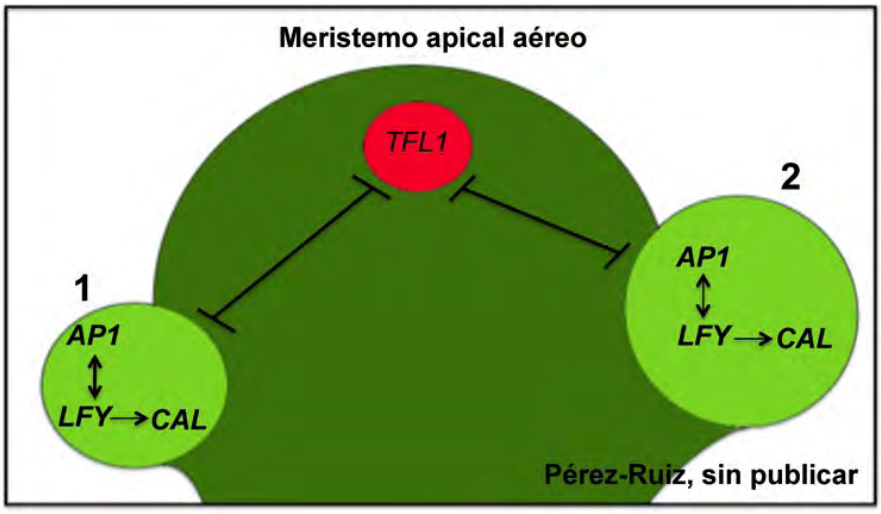


Figura 5. Represión mutua entre el meristemo de inflorescencia y el de flor. *LFY*, *AP1* y *CAL* son genes que promueven la identidad del meristemo de flor expresándose en los estados de desarrollo floral 1 y 2, mientras que *TFL1* ayuda a mantener la identidad del meristemo de inflorescencia.

1.7 Determinación del meristemo floral.

Los meristemos florales se encuentran localizados a los flancos del meristemo de inflorescencia y presentan características celulares similares a la organización que tiene el meristemo apical aéreo. La mutante *wus* produce un número cercano normal de sépalos y pétalos en los verticilos externos. Sin embargo, pierde la capacidad para generar los órganos que producen los verticilos internos y usualmente terminan en un estambre solitario. Estos fenotipos revelan que la actividad de *WUS* es necesaria para mantener cierto número de células en el meristemo floral (Schoof et al., 2000).

Adicionalmente se sabe que LFY actúa junto con WUS para inducir a *AGAMOUS* (*AG*) para formar los estambres y carpelos (Lohmann et al., 2001). A diferencia del meristemo apical aéreo la expresión de *WUS* en el meristemo floral se mantiene solamente hasta el estado de desarrollo 6 cuando AG reprime la expresión de *WUS* (Lenhard et al., 2011).

Un mecanismo molecular adicional que induce *AG*, involucra a otros genes, ahora sabemos que *REBELOTE* (*RBL*) el cual codifica para una proteína de función desconocida, *SQUINT* (*SQN*) que codifica para una ciclofilina y *ULTRAPETALA1* (*ULT1*) que codifica para un supuesto factor de transcripción (Prunet et al., 2008) actúan redundantemente para inducir la expresión de *AG* el cual a su vez reprime la expresión de *WUS*, mediante dos vías diferentes, una directa en la cual AG se une a dos cajas CArG aproximadamente 1 kb río arriba de la región codificante de *WUS*, las cuales son cruciales para su represión (Liu et al., 2011) y otra vía indirecta en la cual AG induce la activación transcripcional de *KNUCKLES* (*KNU)*, un factor transcripcional con dedos de zinc tipo C2H2 que también reprime a *WUS.* La represión de *WUS* da como consecuencia la inactividad del nicho de células troncales de la flor y por lo tanto el meristemo floral se determina (Payne et al., 2004; Sun et al., 2009) después de que se han formado los óvulos en el gineceo, así como un número fijo de órganos florales (Figura 6; Lenhard et al., 2001; Lohmann et al., 2001; Sablowski, R., 2007; Prunet et al., 2008).

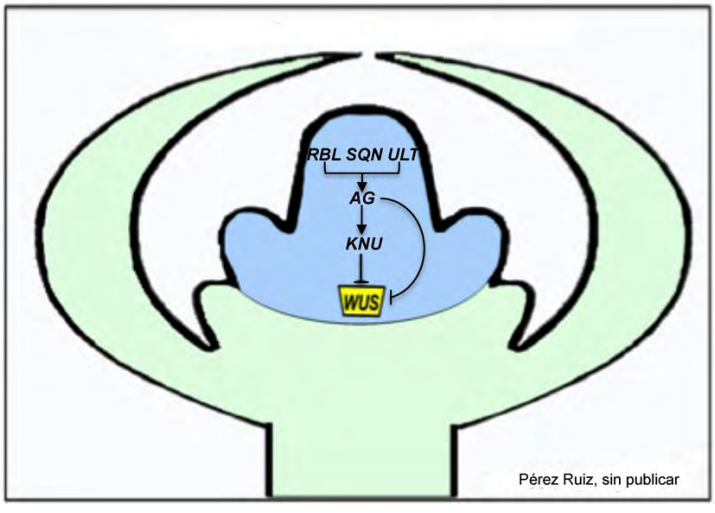


Figura 6. La represión de *WUS* en el estado 6 floral vía *AG* y *KNU* permite que el meristemo floral se determine al agotarse el nicho de células troncales.

1.8. El fenómeno de reversión floral.

La reversión floral se ha definido como el retorno o regreso a un estado de producción de hojas después de haberse desarrollado la flor, una definición más relajada de este término sería regresar a un estado temprano o anterior en el desarrollo (Tooke et al., 2005), esto implicaría una reprogramación celular. Hay dos formas de reversión: 1) Reversión del meristemos de inflorescencia: este tipo de reversión se observa cuando el desarrollo vegetativo ocurre después o es intercalado con el desarrollo de inflorescencia. 2) Reversión del meristemo floral: este tipo de reversión produce una forma de flor alterada o incompleta, con algunas partes reemplazadas por caracteres vegetativos o puede continuar la proliferación después de la formación de los órganos florales (Tooke et al., 2005).

La reversión floral en *Arabidopsis thaliana* no es común en plantas de tipo silvestre, pero se ha reportado que meristemos florales se comportan como meristemos de inflorescencia en algunos mutantes (Okamuro et al., 1996; Mizukami y Ma; 1997; Ferrándiz et al., 2000). Por ejemplo, en las plantas mutantes *ag,* la doble mutante *ap1 clv1* y la planta heterocigota lfy/*LFY* son capaces de producir primordios florales que "revierten" a meristemos de inflorescencia una vez que los verticilos florales o los órganos florales son producidos cuando éstas son crecidas en condiciones de días cortos (Clark et al., 1993; Mizukami et al., 1997; Okamuro et al., 1996).

En otro tipo de mutantes se observa la reversión cuando el meristemo de inflorescencia produce primordios que con frecuencia se comportan como meristemos de inflorescencia secundaria, esta forma de reversión es clásica en la doble mutante *ap1 cal* y de manera más severa se observa en la triple mutante *ap1 cal ful*, molecularmente se determinó en estas líneas mutantes que no tienen la expresión de *LFY* pero si presentan una expresión ectópica de *TFL1* en los meristemos laterales (Ferrándiz et al., 2000).

Para el establecimiento del meristemo floral se requiere de la expresión de genes de

LFY, *AP1* y/ *CAL* como se mencionó anteriormente, así como por la expresión de genes que regulan la transición a la floración como *AGL24*, *SOC1* y *SVP* durante los primeros dos estados del desarrollo del meristemo floral cooperando con los genes que le dan identidad. Sin embargo, se ha observado que *AGL24*, *SOC1* y *SVP* son reprimidos por AP1 y LFY a partir del estado tres del desarrollo del MF (Yu et al., 2004; Liu et al., 2007). De esta forma, los patrones de expresión indican que *AGL24* se expresa en el meristemo de inflorescencia y en la túnica en meristemos florales jóvenes de los estadios 1 y 2 (Yu et al., 2004). *SOC1* se expresa en el meristemo de inflorescencia pero casi está ausente en los estadios 1 y 2 de la flor y se vuelve a expresar ligeramente en el centro de la flor en el estadio 3, mientras que *SVP* apenas es detectable en el meristemo de inflorescencia, pero es fuertemente expresado en los estados 1 y 2 de la flor (Liu et al., 2007). Estos datos, además de las evidencias genéticas, aunadas al análisis de inmunoprecipitación de la cromatina, permitieron establecer que AGL24, SOC1 y SVP junto con AP1 reprimen la inducción de *SEPALLATA 3* (*SEP3*) en los primeros dos estados del desarrollo floral, evitando así la activación temprana de los genes homeóticos de las clases B y C (Pelaz et al., 2000; Gregis et al., 2008; Immink et al., 2009; Liu et al., 2009) y por otra parte la unión de AP1 junto con AGL24, SOC1, SVP y SEP4 reprimen la expresión ectópica de *TFL1* en el MF (Liu et al., 2013).

Sin embargo, resulta interesante que la expresión constitutiva de *AGL24*, *SOC1* y *SVP* en plantas silvestres que tienen a *LFY* y *AP1* funcionales, da como resultado flores que mantienen características de inflorescencia e indeterminación (Yu et al., 2004; Liu et al., 2007). Por ejemplo la expresión constitutiva de *AGL24* favorece la “reversión floral” al desarrollar una inflorescencia que emerge del interior del gineceo (Yu et al., 2004). En plantas 35S:*SOC1* las flores tienen pétalos verdes parecidos a sépalos, sin embargo en la planta 35S:*AGL24* 35S:*SOC1* se observa un fenotipo que produce diversas flores secundarias que emergen a partir de un mismo meristemo floral (Liu et al., 2007). En la línea 35S:*SVP* las flores son estructuras quiméricas conformadas por hojas, hojas carpeloides y estambres, pero en la línea 35S:*AGL24* 35S:*SVP* el fenotipo que se observa en la flor es más parecido a un meristemo de inflorescencia (Liu et al., 2007).Estos fenotipos se han observado también cuando se han sobre expresado los

homólogos de *AGL24* de otras especies de plantas de forma heteróloga en Arabidopsis (Masiero et al., 2004;Trevaskis et al., 2007). .Por lo tanto, toda esta información nos indica que o bien la reversión.floral se puede dar aun en presencia de AP1 en estas líneas de sobreexpresión, o los caracteres de inflorescencia que se observan no son debidos a la reprogramación del MF, que son puntos que se ahondarán en este trabajo.

1.9. Especificación de los verticilos florales.

Una vez establecido el meristemo de flor, éste comienza a diferenciarse en los verticilos florales (Bowman et al., 1991; Coen y Meyerowitz, 1991; Mandel et al, 1992). LFY y AP1 dirigen la expresión de genes homeóticos florales que en *A. thaliana* darán origen a: cuatro sépalos, cuatro pétalos, seis estambres y dos carpelos fusionados que conforman el gineceo (Coen y Meyerowitz, 1991; Blazquez et al., 2006). La flor es una estructura muy importante en las plantas porque en ella se producirán las semillas con las cuales se propagará la especie. Su regulación genética ha sido estudiada con detalle y gracias a los análisis de dobles mutantes y perfiles de expresión de genes que participan en su desarrollo, se ha logrado proponer un modelo de regulación que establece el patrón básico de desarrollo, el cual es conocido como modelo ABC del desarrollo floral (Figura 8). A continuación se describe brevemente cada uno de los componentes de este modelo (Bowman et al., 1991; Coen y Meyerowitz, 1991).

Genes de la clase A. Esta clase de genes está representada por los genes *AP1* y *APETALA 2*. Son responsables de mantener la identidad de sépalos y en combinación con los genes de clase “B”, son responsables de establecer la identidad de pétalos. La ausencia de ellos en mutantes provocan fenotipos homeóticos en la flor, por ejemplo en *apetala 2* se generan carpelos en lugar de sépalos y estambres en lugar de pétalos (Bowman et al., 1989; Meyerowitz et al., 1989; Bowman et al., 1991).

Genes de la Clase B. Los genes que pertenecen a esta clase son *APETALA 3* y *PISTILLATA*, estos genes como se mencionó anteriormente son los responsables de generar los pétalos con la participación de los genes de clase “A” que en conjunción

con los genes de clase “C” son los responsables de establecer los estambres. Las mutantes para estos genes generan sépalos en lugar de pétalos y carpelos en lugar de estambres (Bowman et al., 1989; Bowman et al., 1991).

Genes de la Clase C. El gen que representa a esta clase es *AGAMOUS*, el cual es responsable de generar los carpelos en el centro de la flor y los estambres son generados alrededor de estos en combinación con los genes de la clase “B” como se mencionó previamente. Las mutantes para este gen provocan un cambio de identidad de estambres a pétalos y de carpelos a sépalos, además de que estas flores son indeterminadas porque AG es un represor de *WUS* como se mencionó anteriormente (Bowman et al., 1989; Yanofsky et al., 1990; Bowman et al., 1991).

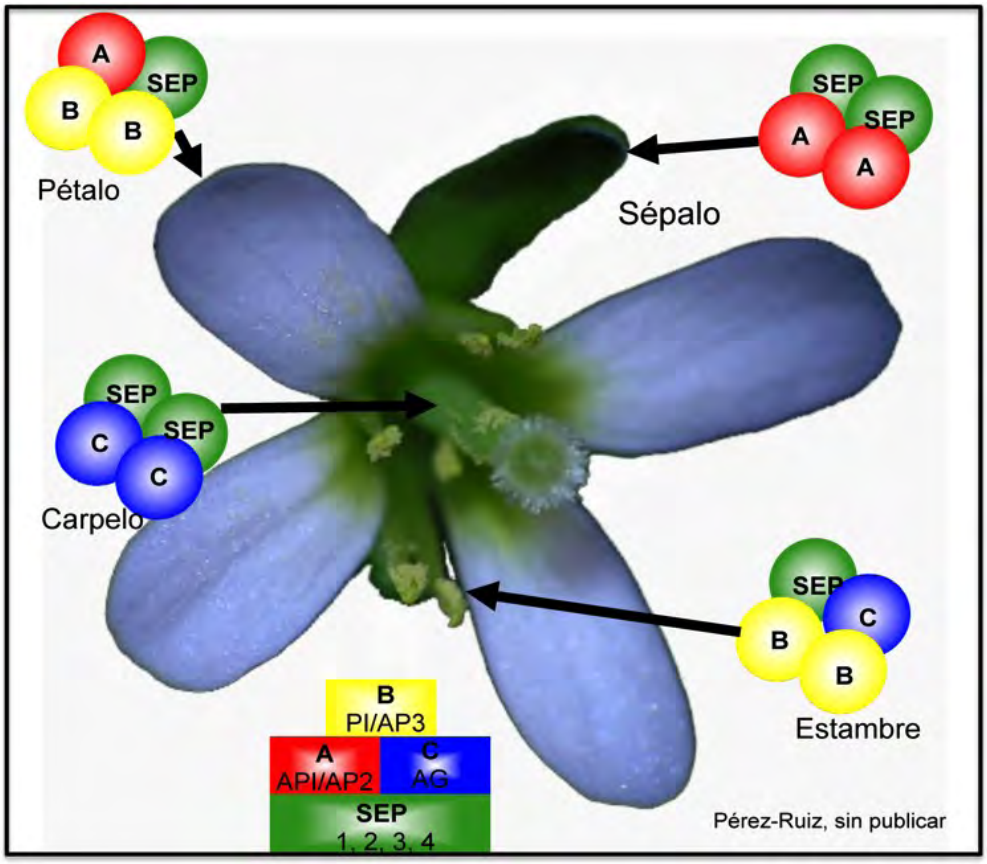


Figura 7. Modelo ABC del desarrollo de la flor. Este modelo establece que los genes de las clases A, B y C del desarrollo floral son los responsables del establecimiento de los verticilos florales que de afuera hacia adentro originarán: sépalos, pétalos, estambres y carpelos funcionados en un gineceo.

1.10. Los factores transcripcionales MADS-box y su participación en el desarrollo de las plantas.

En las secciones anteriores se puede observar que los factores de transcripción tipo MADS-box tienen un papel preponderante en la transición a la floración, el establecimiento de los meristemos florales y en la identidad de los verticilos florales, por lo que para fines de este trabajo se describen brevemente a continuación.

Los factores transcripcionales con dominio MADS constituyen una familia muy conservada en eucariontes que se caracteriza por tener un dominio de unión a ADN cuyo acrónimo MADS fue otorgado con base a las primeras letras de los genes de diversas especies en que fueron inicialmente identificados: **M**CM1 de *Saccharomyces cerevisiae*, ***A**GAMOUS* de *Arabidopsis thaliana* (Yanofsky et al., 1990), **D***EFICIENS* de *Antirrhinum majus y **S**ERUM RESPONSE FACTOR* de *Homo sapiens* (Shore y Sharrocks, 1995). En el genoma de *A. thaliana* existen más de 100 genes *AGAMOUS-LIKE* (*AGL*) y muchos de los genes de esta familia tienen un papel fundamental en la regulación de diversos procesos de desarrollo, además de los ya mencionados, como por ejemplo: el desarrollo de la raíz, mantenimiento del estado vegetativo, el desarrollo de la vaina, la gametogénesis, el desarrollo del óvulo, etc. (Figura 8; Coen y Meyerowitz, 1991; Honma y Goto, 2001; Ng y Yanofsky, 2001; Yu et al., 2002; Parenicova et al., 2003; Martínez-Castilla y Alvarez-Buylla, 2003; Tapia-López et al., 2008; Smaczniak et al., 2012, Garay-Arroyo et al., 2013).

Existen dos linajes de genes MADS-box, los del tipo I parecidos a SERUM RESPONSE FACTOR de humano y los del tipo II, homólogos a MYOCYTE ENHANCER FACTOR-2 ambos presentes en plantas y animales (Black and Olson; 1998: Chai y Tarnawski, 2002). Adicional al dominio MADS, los del linaje tipo II o MICK presentan un dominio I (intermedio) no conservado, el dominio K (Keratin-like) el cual se ha demostrado es importante para las interacciones proteína-proteína y el dominio C o carboxilo terminal, el cual es variable y puede actuar como un dominio de activación en trans que es

importante para determinar la especificidad de las interacciones entre proteínas (Purugganan et al., 1995; Riechmann y Meyerowitz 1997; Jack, 2001; Ng y Meyerowitz, 2001). El dominio MADS de estas proteínas reconoce a una secuencia concenso en el ADN de los genes blanco conocida como caja CArG ([5-CC(A/T)6-GG3´];Shore and Sharrocks, 1995).

Por estudios genéticos, ensayos de doble, triple y cuádruple híbrido en levadura así como por la inmunoprecipitación de algunos complejos, se ha propuesto que las proteínas con dominio MADS actúan como hetero y/o homodímeros que pueden conformar tetrámeros entre ellas o con otro tipo de proteínas para llevar a cabo su función transcripcional (Honma y Goto, 2001; de Folter et al., 2005).

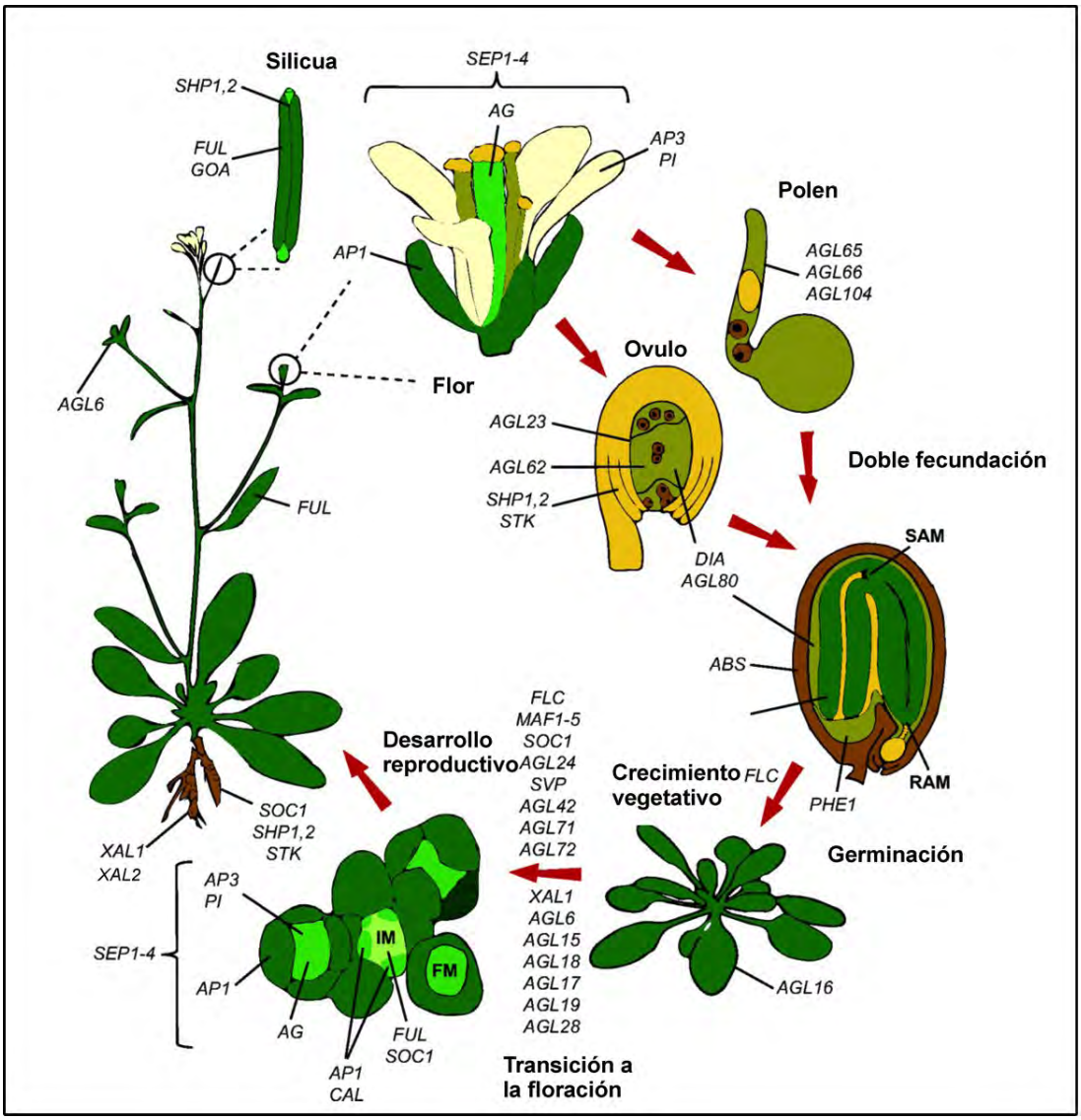


Figura 8. Participación de los genes MADS a través del ciclo de vida de *A. thaliana*. Esta crucífera pasa por diversas etapas durante su ciclo de vida y los genes MADS-box juegan papeles muy importantes en los procesos que se indican en el esquema. Una vez que la semilla germina se genera una plántula, la cual después origina una planta con una roseta joven hasta formar una roseta adulta, la cual transita hacia la floración, momento en el cual se generan los botones florales que producirán las flores, dentro de estas estructuras ocurre la doble fecundación, posteriormente las vainas elongan y permitirán el desarrollo de las semillas a partir de las cuales originarán una nueva generación de plantas (Tomado y modificado de Smaczniak et al., 2012).

1.11 Empleo de modelos para estudiar los sistemas de regulación genética

El empleo de modelos matemáticos que expliquen los sistemas de regulación genética ayuda a establecer relaciones no intuitivas en un determinado proceso en el cual

participan un conjunto de genes y/o proteínas. En plantas este tipo de modelos han sido utilizados para explicar diversos procesos como por ejemplo, el desarrollo de la flor, la Inmunidad vegetal, transporte de auxinas, desarrollo de las hojas (Alvarez-Buylla et al., 2010; Band et al., 2012; Merks et al., 2011; Muhammad et al., 2013). Para que un modelo sea creíble debe cumplir con tres características: debe ser verificable, validado y evaluado. Los modelos ayudan al avance científico de diversas maneras, por ejemplo, la experimentación es muy importante en los descubrimientos del quehacer científico, sin embargo cuando numerosos resultados son obtenidos como por medio de la secuenciación masiva, estudios de transcriptómica, proteómica, metabolómica etc. los datos y la información generada es difícil de procesar, interpretar e integrar; en este sentido los modelos pueden ayudar ordenando la información generada por la experimentación y pueden proporcionar hipótesis de trabajo la cual puede ser probada posteriormente en el laboratorio. De esta manera la combinación entre los datos obtenidos experimentalmente y las consideraciones teóricas ayudan a la generación de buenos modelos, con lo cual se pueden realizar predicciones que ayudan al avance científico.

Hace unas décadas atrás se había considerado que en biología del desarrollo un fenotipo era causado por un gen, sin embargo con el paso del tiempo esta idea se ha revolucionado y ahora sabemos que un mismo fenotipo puede ser causado por múltiples genes. En un proceso dinámico donde participan varios genes o proteínas, estas interacciones pueden ser representadas gráficamente mediante un sistema de regulación genética, el cual ilustra de manera sencilla todas las posibles interacciones de la red (Darabos et al., 2011).

Los sistemas de regulación genética son considerados en la actualidad muy importantes para estudios de frontera en las ciencias biológicas (Drabos et al., 2011). Uno de los primeros modelos dinámicos empleados es el sistema booleano al azar. En este sistema los componentes están representados por nodos y las interacciones están indicadas por uniones entre los nodos e indica cómo cada nodo ejerce una influencia con los que interacciona, por ejemplo las flechas indican activación mientras que las barras indican

represión (Alvarez-Buylla et al., 2010). En el sistema booleano las dinámicas de cada nodo se simplifican lo más posible, generalmente con valores de 0 que indican por ejemplo que los genes están apagados y 1 indican que están encendidos. (Alvarez-Buylla et al, 2010; Greil, 2012).

En biología de sistemas es necesario utilizar herramientas bioinformáticas y modelos computacionales que permitan el estudio integral de los procesos biológicos en disciplinas tan complejas como la genética, fisiología, bioquímica, etc. (Darabos et al., 2011; Gril, 2012). En el presente cada vez mas se conjuntan los datos experimentales con los análisis bioinformáticos y modelos computacionales para contar con una visión más completa de lo que ocurre en un proceso biológico.

1.12 La diferenciación celular y el paisaje epigenético.

Las células durante el desarrollo atraviesan por distintos estados celulares, algunas células permanecen indiferenciadas como las que se encuentran en el nicho de células troncales mientras que otras se especializan y se diferencian (Grafi, 2004; Takahashi, 2012). La diferenciación es el proceso que atraviesa una célula a partir de estar en un estado indiferenciado y llegar a un estado en el cual la célula adquiere características que le permiten desempeñar una función especifica alcanzando la maduración, este proceso en los seres vivos esta regulado por un intrincado sistema de regulación genético que es influenciado por factores hormonales y ambientales (Grafi, 2004; Takahashi, 2012).

Conrad Hal Waddington estableció una analogía del proceso anteriormente mencionado y en 1957 propuso el paisaje epigenético el cual es una descripción gráfica del curso por el cual atraviesa una célula en desarrollo hasta llegar a la diferenciación, este paisaje lo representó en una superficie inclinada en la cual se encuentran esferas las cuales hacen alusión a las células indiferenciadas que van rodando hasta caer dentro de sumideros o valles que representan la terminación del destino de las células porque han alcanzado la madurez. Estas células no pueden salir de los valles normalmente porque han adquirido

estados estables que dan como resultado la determinación celular (Bhattacharya et al., 2011; Takahashi, 2012). Sin embargo, Waddington también pensó que el destino celular podía modificarse y que las células podrían salir de los estados estables en lo que se encontraban y llegar a otros estados diferentes lo cual lo representó como una especie de puente entre los valles (Bhattacharya et al., 2011;Takahashi, 2012).

En biología del desarrollo los términos de: desdiferenciación, reprogramación, transdeterminación y transdesdiferenciación son utilizados para explicar la conversión de las células una vez que han adquirido identidad celular. La desdiferenciación es el proceso por el cual la célula de un estado especializado regresa a través del paisaje epigenético para convertirse en una célula inmadura hasta llegar a un estado indiferenciado. Mientras que la reprogramación o reversión, transdeterminación y transdesdiferenciación, son el proceso por el cual las células en un estado pueden pasar de un destino celular a otro sin llegar a ser una célula indiferenciada (Takahashi, K., 2012).

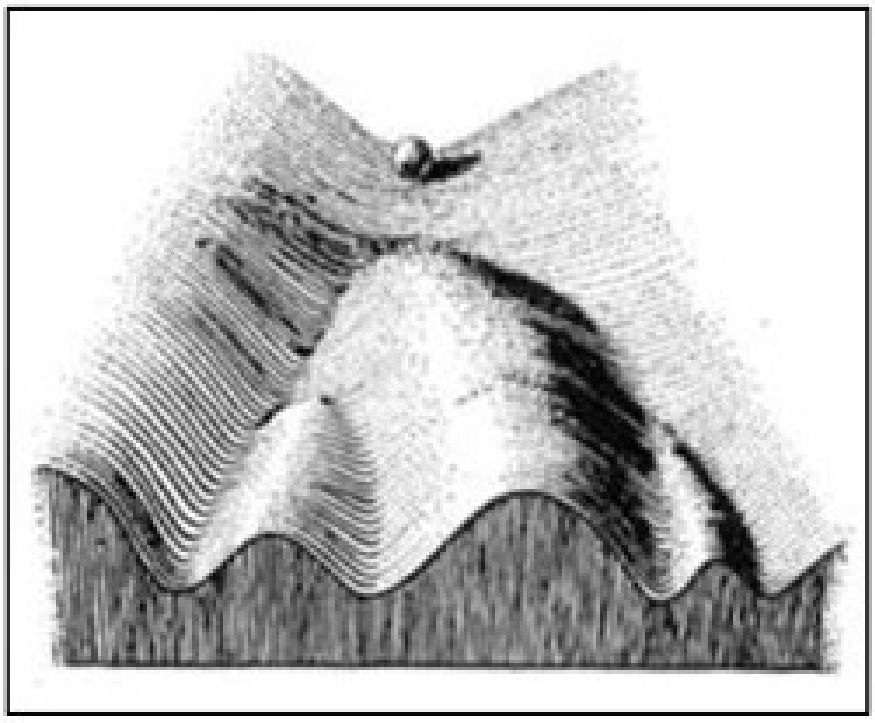


Figura 9. Paisaje epigenético de Waddington. Es una descripción gráfica para explicar como la célula en estado indiferenciado puede pasar por diversas crestas hasta llegar a un sumidero del cual normalmenteya no puede salir y en donde adquiere identidad celular (Tomado y modificado de Bhattachrya et al., 2011).

2. ANTECEDENTES.

XAL2 / *AGL14* es un factor transcripcional perteneciente a la familia de genes MADS-box el cual se encuentra dentro del clado de *SOC1*, *AGL19*, *AGL42, AGL71* y *AGL72* (Parenicovaá et al., 2003). Todos los genes de este clado con excepción de *XAL2* se ha demostrado que participan en la transición a la floración (Schönrock et al., 2006; Dorca-Fornell et al., 2011). Anteriormente otros grupos habían reportado que *XAL2* es un gen específico de raíz (Rounsley et al., 1995; Schönrock et al., 2006) en donde efectivamente tiene un papel muy importante en su desarrollo. Sin embargo, en el laboratorio de Genética Molecular, Evolución, Epigenética y Desarrollo de Plantas se encontró que una línea de expresión disminuida para *XAL2* (ahora denominada como *xal2-2* presentaba retraso en su tiempo de floración al compararse con plantas de tipo silvestre en condiciones de día largo (Villajuana-Bonequi, 2005; Garay-Arroyo et al., 2013).

Adicionalmente XAL2 / AGL14 es capaz de interaccionar con otras proteínas con dominio MADS, en ensayos de dos híbridos con: SOC1, AGL24, FUL, SHATTERPROOF 1 y 2 (SHP1 y SHP2), SEP3 y ARABIDOPSIS B SISTER-2 (ABS-2; de Folter et al., 2005). De los cuales SOC1, AGL24 y FUL son importantes en la transición a la floración (Hempel et al., 1997; Borner et al., 2000; Lee et al., 2000; Nessi et al., 2000; Liljegren et al., 2000; Michaels et al., 2003; Liu et al., 2007, Liu et al., 2008; Lee and Lee, 2010). Este trabajo sugiere que XAL2 pueda participar de forma conjunta con estas proteínas en la transición a la floración y en el mantenimiento del meristemo de flor. Con base en estos antecedentes se propone conocer con más detalle cómo *XAL2* participa en la transición a la floración y las posibles relaciones genéticas en relación a algunos otros componentes que participan en la floración.

Por lo tanto, el presente trabajo tuvo por objeto conocer cual es la participación de *XAL2* en el tiempo de floración, cuáles son sus relaciones genéticas y explicar los fenotipos de la línea de sobreexpresión.

3. HIPOTESIS.

XAL2 es parte de la red de regulación genética que participa en la transción a la floración, junto con *SOC1*, *AGL24* y *SVP*.

4. OBJETIVOS.

4.1. General

“Determinar la participación de *XAL2* durante la transición a la floración y en el mantenimiento del meristemo floral en *Arabidopsis thaliana*“.

4.2 Particulares

1.- Determinar el tiempo de floración de *xal2-1* y *xal2-2* bajo condiciones de fotoperiodo de día corto, día largo, vernalización y tratamiento con giberelinas.

2.- Analizar la regulación transcripcional de *XAL2* en la red de regulación que participa en la transición a la floración en día largo.

3.- Determinar el patrón de expresión espacio-temporal de *XAL2* en el meristemo de inflorescencia y en el meristemo floral.

4.- Documentar el fenotipo de la línea de sobreexpresión en condiciones de crecimiento de día largo y corto.

5.- Determinar si *XAL2* es capaz de inducir la expresión de *TFL1* directamente.

5. MATERIALES Y METODOS.

A continuación se describen las técnicas utilizadas para este trabajo y que no fueron detalladas en el artículo Pérez-Ruiz et al., 2015. Para complementar la información revisar la metodología del artículo.

5.1. Extracción de ADN de *Arabidopsis thaliana.*

Se tomaron 1-3 hojas de plantas creciendo en condiciones de temperatura y humedad controlados. Las hojas fueron congeladas y trituradas en N_2 líquido con ayuda de morteros de porcelana. Posteriormente se adicionaron 150 µl de un amortiguador de extracción: (6.9 M de urea, 0.31 NaCl, 0.1M Tris-HCl pH 8.0, 0.02 M EDTA pH 8.0 y 0.11 M de sarcosina). Después se adicionaron 100 µl de fenol/cloroformo (1:1). Se agitó en vortex y se centrifugó a 8049 g por 5 min. El sobrenadante se transfirió a un nuevo tubo y se adicionó 2 veces el volumen de etanol, se agitó nuevamente y se centrifugó de igual manera que el paso anterior. Se retiró el sobrenadante y se adicionaron 500 µl de etanol al 70% para lavar las sales de la pastilla, se centrifugó 1 min y se dejó secando al aire. Finalmente la pastilla se resuspendió en agua grado biología molecular y se le adicionó 1 µl RNAsa A (Invitrogen), a una concentración de 1 mg/ml.

5.2. Obtención de ARN y síntesis de ADN complementario.

De plantas de 14 días de edad crecidas en placas Petri se colectó la parte aérea y esta se trituró en morteros de porcelana previamente enfriados con N_2 líquido hasta obtener un polvo muy fino. Posteriormente el ARN fue extraído utilizando el reactivo de Trizol (Invitrogen) de acuerdo a las especificaciones del fabricante. Una vez obtenido se cuantificó y 2 µg fueron utilizados para la síntesis del ADNc utilizando la enzima Superscript II (Invitrogen), siguiendo el protocolo del fabricante.

5.3. Preparación de células competentes *Escherichia coli* DH5α, para electroporación.

A partir de una colonia aislada previamente, se inocularon 10 ml de medio Luria Broth (LB; el cual se preparó con 10 g de triptona, 5 g de extracto de levadura y 10 g de NaCl en 1 l de H_2O) se dejó creciendo toda la noche a 37ºC en agitación de 300 rpm en una incubadora (New Brunswick Scientific Co, serie 25) y este pre-cultivo sirvió para inocular 1 l de medio LB, el cultivo se creció hasta una D.O. de 0.5-0.6 a 560 nm (aproximadamente 2-4 h) en las mismas condiciones de agitación y temperatura que el pre-cultivo. Posteriormente se dejaron enfriar los matraces por 10-15 min en hielo. El cultivo fue transferido a botellas de 500 ml y fueron centrifugados en una centrífuga Beckman J2-21 utilizando un rotor de ángulo fijo por un tiempo de 10 min a una velocidad de 1400 g. La pastilla obtenida se resuspendió en 5 ml de agua enfriada a 4ºC (fria) y se añadió el mismo volumen de agua fria (por ejemplo, si se utilizó 1 l de medio, se utiliza 1 l de agua fria). Se agitó en vortex y se centrifugó nuevamente a 1400 g. Posteriormente, la pastilla se resuspendió en 5 ml de agua fria en tubos falcon y después se adicionó agua fria, la mitad del volumen inicial de medio LB que se utilizó para el cultivo (por ejemplo 500 ml si se utilizó 1 l de medio). Se centrifugó nuevamente a 2744 g. La pastilla obtenida se resuspendió nuevamente en 20 ml de glicerol al 10% frío. Se mezcló en vortex y se transfirió a tubos Corning de 50 ml, para centrifugarse 10 min a 1400 g. Se eliminó el sobrenadante y se adicionaron 2-3 ml de glicerol al 10%, se mezcló bien y se hicieron alícuotas de 40 μl que se congelaron en N_2 líquido y al finalizar se conservaron a -70°C.

5.4. Preparación de células competentes para electroporación con *Agrobacterium tumefasciens.*

Para la preparación de células competentes, se inoculó la cepa de *Agrobacterium tumefasciens* C58 en un placa conteniendo medio LB, se colocó a una temperatura de 28-30°C por dos días. Una vez observado el crecimiento en la placa se tomó una colonia bien aislada, con ayuda de un palillo estéril y se inoculó en un tubo conteniendo 3 ml de medio LB y se dejo crecer a 28-30°C por 1 día hasta observar el crecimiento de las bacterias. 100 μl del pre-cultivo fueron inoculados en 100 ml de medio LB + 0.1% glucosa, y se incubó a 28-30 °C. El cultivo fue monitoreado hasta que este alcanzó una

D.O. 600 = 0.5-0.7 e inmediatamente se incubó el matraz en hielo. Posteriormente, se colectaron las células centrifugando por 15 min a 4032 g en botellas de 250-500 ml. Después las células fueron lavadas en 10 ml de HEPES 1 mM pH 7.0. Se centrifugó nuevamente en tubos de 50 ml. Se repitió el lavado tres veces más. Se lavó la pastilla con 10 ml de glicerol al 10% y se centrifugó nuevamente. Finalmente las células se resuspendieron en un volumen de 500-700 μl con glicerol al 10% y se hicieron alícuotas de 40 μl en tubos eppendorf de 600 μl. Las alícuotas se congelaron en N_2 líquido y se almacenaron a -70 °C.

5.5. Transformación por electroporación de células de *Agrobacterium tumefasciens.*

Para este fin, se descongeló en hielo una alicuota de células previamente almacenadas a -70°C. Se pre-enfrió la celda de electroporación (BioRad). Se mezclaron 10 ng de plásmido (el que se necesitaba clonar) con 40 μl de las células y se transfirieron a la celda, formando un puente entre las dos placas de metal. Posteriormente, se utilizó un electroporador (E.C. Apparatus Corporation modelo EC100). Se ajustó el voltaje a 2800 V, se colocó la celda y se dio el choque eléctrico. Se recolectó de la celda la mayor cantidad de la mezcla, la cual se incubó en 1 ml de medio LB por 1 h a 30 °C con agitación en una incubadora (New Brunswick Scientific Co, serie 25). Después se centrifugó a 504 g x 1 min y se decantó la mayor cantidad de medio y la pastilla se resuspendió en 100 μl del mismo medio, lo cual fue plaqueado en placas conteniendo LB con el antibiótico respectivo (rifampicina y ampicilina), se incubó por dos días a 30°C. Se verificó que el plásmido se haya integrado.

5.6. Transformación de *Arabidopsis thaliana* con *Agrobacterium tumefaciens.*

Se inocularon 5 ml de medio LB complementado con 50 μl/ml con los antibióticos (kanamicina, rifampicina y ampicilina, para la cepa C58) y se incubó por 2-3 días a 300 rpm a 28°C en una incubadora (New brunswick Scientific, serie 25). Posteriormente se

escaló el cultivo a 250 ml de medio LB con los mismos antibióticos y se dejó en agitación toda la noche a 28°C. El cultivo fue colocado en botellas de 500 ml de capacidad, previamente enfriadas en hielo. Se centrifugó a 5488 g x 15 min. Una vez obtenida la pastilla, se resuspendió en medio de infiltración (2.2 g de MS, 50 g de sacarosa, 500 μl de silwet 77 y se ajustó a un volumen de 1 l). Se utilizaron plantas jóvenes (de 3-4 semanas de edad) que presentaban inflorescencias para la transformación. A estas plantas se les cortaron previamente las vainas y la mayor cantidad de flores, para dejar sólo los botones florales. Se sumergieron por inversión para que las inflorescencias quedaran dentro de la solución de infiltración durante 15-20 s, las macetas con las plantas se colocaron horizontalmente en charolas y se cubrieron con bolsas de plástico obscuro por 1 día para permitir la recuperación debido al estrés que se sometieron y se recuperaron la mayor cantidad de semillas.

5.7. Microscopía de luz y electrónica.

Para la documentación fotográfica mediante microscopía de luz de los fenotipos florales de la plantas y de las flores, se utilizó un microscopío de disección (Olympus SZ60) al cual se le montó una cámara fotográfica (Olympus C-5060) con ayuda de un adaptador. Para mayores aumentos se utilizó un microscopio óptico (Olympus BX60). En el caso del material analizado por microscopia electrónica de barrido, las muestras fueron fijadas y secadas con CO_2, después fueron cubiertas con oro y visualizadas en un microscopio electrónico (Jeol LTD) en la Facultad de Ciencias de la UNAM.

5.8. Experimentos de Hibridación *in situ.*

A continuación se describe esta técnica a partir de la obtención del tejido. Como recomendaciones generales siempre se deben de usar guantes, limpiar el área de trabajo con solución eliminadora de ARNasas y todo el material usado deberá lavarse con H_2O DEPC activo. Tener presente que trabajamos con ARN.

5.8.1 Obtención de tejido vegetal: Se sembraron 5 semillas en tierra por cada una de las 48 macetas, una vez que las plantas crecieron tanto de la línea silvestre como la de *xal2-2*, se seleccionaron escapos de 10 cm de longitud, y a las inflorescencias de estos escapos se les eliminó la mayor cantidad de botones florales con ayuda de pinzas de disección para que el meristemo de inflorescencia quedara lo más desnudo posible.

5.8.2 Fijación: Una vez que se obtuvieron las inflorescencias (al menos 30), estos fueron fijados en una solución conteniendo 300 ml de PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 a pH 11, utilizando NaOH el cual se calentó aproximadamente a 60°C en el microondas por un periodo de 30-45 s). Se adicionaron 10 gramos de paraformaldehído, se agitó muy bien para que se disolviera. Después se reajustó el pH a 7 utilizando H_2SO_4 concentrado, se esperó a que la solución se enfriará y se colocó en hielo. El fijador frío se adicionó a tubos de vidrio en donde se colocaron las inflorescencias (se pueden usar los frascos de centelleo). Las muestras sumergidas en el fijador se sometieron al vacío por dos rondas de 20 min y en cada ronda se liberó poco a poco el vacío con el fin de infiltrar el fijador. Finalmente las muestras se dejaron a 4°C por 10-12 h.

5.8.3 Hidratación: Una vez concluida la fijación se procedió al proceso de deshidratación a temperatura ambiente, para este fin se prepararon diluciones de alcohol al 60%, 70% 80%, 90%, 95% y 100% y se fueron sustituyendo cada 30 min después del proceso de fijación, se dejaron las muestras en etanol al 100% por 8-12 h en refrigeración a 4°C toda la noche (en este paso las muestras se pueden dejar el fin de semana).

5.8.4 Infiltración de Citrisolv: se prepararon soluciones en las proporciones 3:1, 1:1, 1:3 etanol:citrisolv (Fisher Brand) y se fueron cambiando cada 30 min, hasta que al final se cambió por sólo citrisolv (Fisher Brand), el cual se cambio dos-tres veces, cada 30 min a temperatura ambiente.

5.8.5 Infiltración de parafina: Después de que las muestras se dejaron en citrisolv al 100%, los viales se introdujeron en un horno a 56°C (Fisher Scientific, 650D) por aproximadamente una hora. Después se fue adicionando parafina fundida, y se dejó reposar por otra hora. Se eliminó el 50% del contenido de citrisolv-parafina (hay que tener cuidado de que no se tiren las muestras) y este cambio se realizó de 6-8 veces, para ir infiltrando poco a poco la parafina, finalmente el vial se dejó en parafina toda la noche para que se evaporara el citrisolv.

5.8.6 Preparación de muestras en bloques de parafina: Se limpiaron perfectamente los moldes de acero inoxidable con citrisolv caliente a 58°C para eliminar la parafina de muestras anteriores. Los moldes se dejaron remojando en H_2O DEPC activo por al menos 2 h, se secaron con toallas de papel hasta su uso.
Los cartuchos de soporte para las muestras también se lavaron con H_2O DEPC activo. Con ayuda de pinzas de plástico se tomaron las muestras y se colocaron en moldes calientes, se acomodaron las muestras, se fijaron permitiendo que la parafina se solidificara un poco, se colocó encima el cartucho de plástico y se adicionó parafina hasta que se cubrió la muestra, después los moldes se colocaron sobre hielo para permitir la solidificación de la parafina, transcurridos 10-20 min se separaron los cartuchos de los moldes ya que la parafina está completamente solidificada, estas muestras se guardaron a 4°C (se pueden almacenar hasta un año).

5.8.7 Cortes finos: Para este fin se sacaron del refrigerador los bloques conteniendo las muestras y se dejaron aproximadamente 1 h a temperatura ambiente para que la parafina no estuviera tan dura. Se colocaron las muestra dentro del bloque y se eliminó con navajas el exceso de parafina (formando un trapecio), se ajustó el micrótomo (Microm HM330) a un grosor del corte de 6-8 μM y se procedió a seccionar, una vez que empiezaron a salir los tejidos cortados de las muestras, estos se colocaron en toallas de papel, y se cortó todo el bloque, después las muestras se colocaron sobre portaobjetos cargados positivamente, se analizaron bajo el microscopio estereoscópico (Olympus SZ60) y se seleccionaron los mejores cortes para posteriormente guardarse a 4°C.

5.8.8 Preparación de las ribosondas: Se buscó en la base de datos del National Center for Biotechnology Information (NCBI), una región del gen *XAL2* que no hibridara cruzadamente con ningún otro gen por lo que se diseñaron los oligonucleótidos 5´-GTTTCCTCCTTCAAACTAACA-3´ y 5´-GCAACTGCTAAATTCAGTAAG-3´ que amplifican un fragmento de aproximadamente 113 pb. Posteriormente se usó ADNc para amplificar la región y se clonó en el vector P-GEM-T-Easy (Promega). Después se envió a secuenciar para verificar la presencia de la sonda dentro del plásmido. El molde para la obtención de la ribosonda fue amplificada con los oligonucleótidos SP6 y T7, el producto de PCR fue verificado y purificado usando las columnas de QIAGEN e inmediatamente se procedió a la síntesis de la ribosonda utilizando el kit de Promega con las polimerasas SP6 y T7. Se cuantificó la ribosonda con la tiras para cuantificación (Roche) y se guardaron a -70°C hasta su uso.

5.8.9 Realización del experimento: La duración de lo que propiamente es el experimento de hibridación *in situ* es de tres días.

5.8.10 Desparafinación del tejido: Las laminillas seleccionadas para el experimento se colocaron en porta laminillas, las cuales se sumergieron en citrisol V por 10 min, pasado este tiempo se cambiaron a citrisolv fresco por otros 10 min.

5.8.11 Hidratación: Las porta laminillas se colocaron en etanol al 100%, dos veces por 1 min cada vez y después se pasaron a etanol al 95%, por 30 s y posteriormente se transfirieron a soluciones de etanol al 85%, 70% 50% y 30% con 0.85% NaCl cada una, por 30 s en cada solución. Se pasaron a una solución con 0.85% NaCl por 2 min y después a una solución de PBS por 2 min.

5.8.12 Digestión con Proteinasa K: Se preparó un amortiguador que contenía 50 mM Tris-HCl pH 7.5 y 5 mM EDTA, se adicionaron 30 mg de Proteinasa K a 250 ml del amortiguador previamente calentado a 37°C y las muestras se dejaron por 25 min a 37°C. Aproximadamente cada 5 min se agitó la solución. Se colocaron los porta

laminillas en 0.2% glicina en PBS por 2 min e inmediatamente después se pasaron a PBS por otros 2 min.

5.8.13 Refijación de la muestras: Se colocaron las laminillas en solución de fijación como la descrita en punto 5.8.2, pero solamente por 2 min. Después se lavaron con PBS por 2 min. En 400 ml de una solución de 0.1 M de trietanolamina-HCl pH 8, se adicionaron 2 ml de ácido acético anhidro, el cual es inestable en agua y por tal motivo la solución de trietanolamina se mantuvo en agitación durante 10 min. Se lavó por 2 min en PBS y después en una solución all 0.85% NaCl durante 2 min.

5.8.14 Deshidratación: Los porta laminillas se colocaron en etanol 30%, 50%, 70%, 85% más 0.85% NaCl, por 30 s en cada solución. Después se pasaron a una solución con 95% etanol por 30 s y dos veces en alcohol al 100% durante 1 min en cada una. (Las laminillas se pueden guardar a 4ºC en una cámara húmeda de etanol absoluto por máximo 2 h, si no se debe proseguir inmediatamente).

5.8.15 Hibridación: Primero se llevo a cabo una pre-hibridación, se utilizaron 250 μl de solución de hibridación la cual se preparó con 4 ml de formamida, 2 ml de sulfato de dextran al 50%, 1ml de sales 10X, 100μl de tRNA a una concentración de 100 mg/ml, 200 μl de solución de Denhardts 50X y 700 μl de H_2O DEPC. Para hacer las sales al 10X, se utilizaron 6 ml de 5M NaCl, 1 ml de 1M Tris-HCl pH 6.5, 0.78 gramos de NaH_2PO_4:$2H_2O$, 0.71 gramos de Na_2HPO_4, 1 ml de 0.5M EDTA y se aforó a 10 ml con H_2O DEPC. La solución para la prehibridación se colocó entre las dos laminillas creando una especie de "sandwich" favoreciendo así su dispersión en las caras internas de las laminillas conteniendo el tejido fijado, esto con la finalidad de que todo el tejido quedara en contacto con la solución, por aproximadamente 30 min, transcurrido este tiempo las laminillas se separaron y se le adicionó la ribosonda (500 ng), la cual previamente se diluyó con 50μl de formamida al 50%, la cual se calentó por dos min a 80°C en un tubo eppendorf, e inmediatamente se colocó en hielo y se adicionó 200 μl de solución de hibridación con la cual se mezcló. Posteriormente se preparó una cámara húmeda. Para este fin se colocaron toallas de papel en un recipiente plástico y

estas fueron mojadas con formamida al 50% y 2X SSC, las laminillas fueron colocadas encima de pipetas Pasteur para impedir el contacto con las toallas de papel y evitar que se secara la solución de hibridación conteniendo la ribosonda (se hace una especie de "sandwich" entre dos laminillas, quedando en la parte interna el tejido en contacto con la solución y la ribosonda). Hay que estar seguros de que no queden burbujas de aire dentro del "sandwich" para garantizar que todo el tejido esté en contacto con la ribosonda. Finalmente el recipiente de plástico se cerró y se colocó en una incubadora a 58°C para hibridar por 10-12 h.

5.8.16 Lavados: Al siguiente día, los "sandwitches" se colocaron en un recipiente conteniendo 0.2X SSC para separar las laminillas, posteriormente se colocaron en portalaminillas con la cara que contenía el tejido viendo hacia el exterior para que los lavados se llevaran a cabo adecuadamente.
Se lavaron las laminillas por una hora a 56°C en 0.2X SSC, este lavado se realizó dos veces cambiando la solución. Posteriormente las laminillas se pasaron a un amortiguador conteniendo (0.5M NaCl, 10 mM Tris pH 7.5, 1mM EDTA) y esto se realizó dos veces por 5 min cada una. Después las laminillas se colocaron nuevamente en 0.2X SSC durante otra hora a 56°C.

5.8.17 Bloqueo: Una vez realizados los lavados se procedió a realizar el bloqueo, para esto se prepararon 50 ml al 1% del agente de bloqueo de Roche. Se incubó en agitación a 70 rpm por 45 min a temperatura ambiente. Hay que estar seguros que la solución de bloqueo está en contacto directo con las laminillas. Después se eliminó esta solución de bloqueo y se adicionó una nueva solución y se incubó por otros 20 min más. Después de eliminar la solución de bloqueo se incubaron las laminillas en 1% albumina sérica bovina, 0.3% triton X-100 diluido en TBS. Se dejó en agitación constante a temperatura ambiente por 45 min.

5.8.18 Incubación con el anticuerpo: El anticuerpo anti-DIG (Roche) fue diluido 1:1250 en 1% BSA, 0.3% Triton X-100 en TBS, se utilizaron 7.5 μl de anticuerpo en 9.3 ml de amortiguador (esta cantidad es suficiente para 40 laminillas). Se incubaron las

laminillas en otra cámara de humedad a temperatura ambiente por dos h. Se separaron las laminillas en solución conteniendo 1% BSA, 0.3% Triton X-100 en TBS (este paso se repitió 3 veces más).

Después se lavaron en un amortiguador conteniendo 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, repetir este paso por 10 min con solución fresca.

En 10 ml de solución que contenía 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, se adicionaron 8 μl de 1M de levamisol, 22 μl de NBT y 16 μl de BCIP. Se adicionaron 250 μl de esta solución por cada par de laminillas, al terminar se colocaron los sandwitches en una cámara húmeda y se dejó revelando para la obtención del color en la oscuridad, se monitoreó para que no se sobre expusieran las muestras. La reacción se detuvo con la solución: 10 mM Tris HCl pH 8 y 1 mM EDTA en donde se sumergen las laminillas. Finalmente se dejaron secando al aire y se observaron al microscopío.

5.9. Inmunoprecipitación de la cromatina.

Para este experimento se obtuvo la parte aérea de plantas de 14 días de edad. A partir de 500-1000 mg de tejido se procedió a la fijación del tejido utilizando 0.4 M sacarosa, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, formaldehído al 1%, la fijación se llevó al vacío en placas de 6 pozos utilizando una bomba de vacío (Heto Sue 300E), el tratamiento fue de 20 min. Después se adicionó glicina hasta tener una concentración final de 0.1M y se colocó nuevamente al vacío por 10 min más, al terminar este tratamiento el tejido aéreo fue lavado con agua destilada estéril al menos 4 veces y fue secado con ayuda de toallas de papel, el material se almacenó a -70°C hasta su uso.

El tejido almacenado fue molido utilizando morteros de porcelana enfriados con ayuda de nitrógeno líquido. después de obtener un polvo fino, este fue resuspendido en un amortiguador de lisis: 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Tritox X-100, 0.1% SDS, 1 mM PMSF, 10mM butirato de sodio e inhibidor de proteasas. Posteriormente el homogenizado obtenido fue sonicado 6 veces x 15 s (Sonicador Brason 150) y con intervalos de descanso de 1 min entre pulso y pulso. La muestra se incubó a 4°C en agitación durante 20 min e inmediatamente se centrifugo a 9464 g a 4°C, el sobrenadante obtenido se transfirió a un nuevo tubo y se cuantificaron las

proteínas totales mediante el reactivo de Bradford en un espectrofotómetro Beckman a 595 nm. Para cada muestra se tomaron al menos 500 μg de proteínas totales y se aforó a 1 ml con el mismo amortiguador, se separaron 100 μl y se guardaron a -70°C. Para los 900 μl restantes se adicionaron 15 μl de esperma de salmón a una concentración de 10 mg/ml y 10 μl de proteína A y se incubaron por una hora con agitación a 4°C. Por otra parte, también se prepararon tubos para bloquear los anticuerpos, a cada uno de los tubos se adicionaron 20 μl de proteína A (Santa Cruz), 500 μl de PBS 1X y 6.25 μl de anticuerpo GFP (Santa Cruz), y también se prepararon tubos que no contenían el anticuerpo, ambos tubos se incubaron por una hora a temperatura ambiente, después se centrifugaron los tubos y se eliminó el sobrenadante y se adicionaron los extractos protéicos y se dejaron en agitación a 4 °C durante toda la noche.

Al día siguiente, se realizó una centrifugación a 304 g x 2 min, se eliminó el sobrenadante evitando romper la pastilla y se procedió con los lavados, con el mismo amortiguador de lisis, una vez por 1 minuto en agitación y otra vez pero por 10 min. Posteriormente se realizó un lavado por 1 minuto en amortiguador: 50 mM Tris pH 8.0, 150 mM NaCl y 0.2% Nonidet 40. Después un lavado por 10 min en otro amortiguador: 50 mM Tris pH 8.0, 250 mM NaCl y 0.2% Nonidet 40. Dos lavados en 250 mM $LiCl_2$, 1% Triton X-100, 1 mM EDTA y 10 mM Tris pH 8.0. Finalmente, se lavó 1 vez en un amortiguador de: 10 mM Tris-HCl pH 7.5 y 1 mM EDTA por 1 min y dos veces por 10 min. Se retiró el sobrenadante y se adicionaron 300 μl de 1% SDS, 0.1 M $NaHCO_3$ con el fin de eluir los inmunocomplejos. Se adicionaron 12 μl de 5M NaCl. Para liberar el ADN se incubó a 65°C durante 6 h con ayuda de un termomixer (Eppendorf). Después se eliminó la proteína residual con ayuda de 10 μl de proteinasa K 10 mg/ml en 50 mM Tris pH 8.0 más 1 mM $CaCl_2$ y 50% Glicerol a 45 °C por toda la noche.

Finalmente, se extrajo el ADN, para este fin a 500 μl de muestra se le agregaron 250 μl de fenol-cloroformo-alcohol-isoamilico (Invitrogen) y se centrifugó a 5600 g x 5 min. El ADN se precipitó con 2.5 volumenes de etanol 1/10 de volumen. Se adicionó a cada muestra 3M acetato de sodio pH 5.2, 1 μl de glucógeno y se incubaron por una hora a -70 °C. Se lavó la pastilla 2 veces con etanol al 70%, para finalmente ser resuspendida en 20 μl de agua grado biología molecular.

6. RESULTADOS.

Los resultados de este trabajo se encuentran en el artículo**: Pérez-Ruiz R.V**, García-Ponce B., Marsch-Martínez N., Ugartechea-Chirino Y., Villajuana-Bonequi M., de Folter S., Azpeitia E., Dávila-Velderrain J., Cruz-Sánchez D., Garay- Arroyo A., de la Paz Sánchez M., Estévez-Palmas J.M., and Alvarez-Buylla E.R. (2015). *XAANTAL2* (*AGL14*) is an important component of the complex gene regulatory network that underlies Arabidopsis shoot apical meristem transitions. *Molecular Plant*. 8:796-813. El cual se presenta a continuación.

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XAANTAL2 (*AGL14*) Is an Important Component of the Complex Gene Regulatory Network that Underlies *Arabidopsis* Shoot Apical Meristem Transitions

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ABSTRACT

In *Arabidopsis thaliana*, multiple genes involved in shoot apical meristem (SAM) transitions have been characterized, but the mechanisms required for the dynamic attainment of vegetative, inflorescence, and floral meristem (VM, IM, FM) cell fates during SAM transitions are not well understood. Here we show that a MADS-box gene, *XAANTAL2* (*XAL2/AGL14*), is necessary and sufficient to induce flowering, and its regulation is important in FM maintenance and determinacy. *xal2* mutants are late flowering, particularly under short-day (SD) condition, while *XAL2* overexpressing plants are early flowering, but their flowers have vegetative traits. Interestingly, inflorescences of the latter plants have higher expression levels of *LFY*, *AP1*, and *TFL1* than wild-type plants. In addition we found that XAL2 is able to bind the *TFL1* regulatory regions. On the other hand, the basipetal carpels of the *35S::XAL2* lines lose determinacy and maintain high levels of *WUS* expression under SD condition. To provide a mechanistic explanation for the complex roles of *XAL2* in SAM transitions and the apparently paradoxical phenotypes of *XAL2* and other MADS-box (*SOC1*, *AGL24*) overexpressors, we conducted dynamic gene regulatory network (GRN) and epigenetic landscape modeling. We uncovered a GRN module that underlies VM, IM, and FM gene configurations and transition patterns in wild-type plants as well as loss and gain of function lines characterized here and previously. Our approach thus provides a novel mechanistic framework for understanding the complex basis of SAM development.

Key words: *XAL2/AGL14*, MADS-box, *TFL1*, SAM transitions, floral reversion, gene regulatory networks, epigenetic landscape modeling

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INTRODUCTION

Unraveling the molecular genetic mechanisms that underlie cell transitions and plasticity is a fundamental issue in developmental biology. Different cell states (e.g., proliferative, differentiated, transdifferentiated, or reprogrammed) are correlated to different combinations of gene activation (Sugimoto et al., 2011). Such gene configurations, and the transitions among them, emerge from complex regulatory networks (Álvarez-Buylla et al., 2010a, 2010b). Plants enable *in vivo* analyses of the molecular genetic mechanisms underlying such cell plasticity and dynamics of stem cells that remain active during their complete life cycle within meristems.

At the shoot apical meristem (SAM) the transition from a vegetative to a reproductive state is crucial, with direct fitness implications (Roux et al., 2006). Molecular genetic approaches have uncovered a complex gene regulatory network (GRN) underlying *Arabidopsis* SAM development (Srikanth and Schmid, 2011; Andrés and Coupland, 2012). Genetic screenings for mutant plants with altered bolting time under contrasting environmental conditions (Koornneef et al., 1991) have uncovered the components of flowering transition pathways in response to: photoperiod (Putterill et al., 1995; Suárez-López et al., 2001; An et al., 2004), gibberellins (gibberellic acid [GA]; Blázquez et al., 1998; Blázquez and Weigel, 2000; Porri et al., 2012), non-optimal growth temperature over 4°C (Blázquez et al., 2003; Halliday et al., 2003; Balasubramanian et al., 2006; Lee et al., 2007), vernalization (Michaels and Amasino, 1999; Sheldon et al., 2000; Michaels et al., 2003), or internal developmental cues (Koornneef et al., 1991; Simpson, 2004; Wu and Poethig, 2006).

Many of the genes that participate in floral transition are MADS-box genes (Gramzow et al., 2010). Some of them, such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), respond to more than one condition, and these have been called integrators (Blázquez and Weigel, 2000; Lee et al., 2000; Moon et al., 2003; Wang et al., 2009; Lee and Lee, 2010). Detailed functional characterization revealed that flowering transition pathways converge in the regulation of *LEAFY* (*LFY*) and *APETALA1* (*AP1*), via SOC1–AGAMOUS-LIKE 24 (AGL24) heterodimer, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL3) or FLOWERING LOCUS T–FLOWERING LOCUS D (FT–FD) complex, at the founding cells of the floral meristem (FM), thus establishing a new identity distinct from the inflorescence meristem (IM). The FM later sub-differentiates into the floral organs (Schultz and Haughn, 1991; Weigel et al., 1992; Abe et al., 2005; Yamaguchi et al., 2009).

Gene expression configurations that characterize the IM and FM identities, in addition to the floral organ primordia, have started to be recovered and explained with dynamic GRN mechanistic models, as attractors or steady states (Espinosa-Soto et al., 2004; Álvarez-Buylla et al., 2010a; van Mourik et al., 2010; Kaufmann et al., 2011; Jaeger et al., 2013). Such mechanistic explanations are still lacking for normal and altered cell-fate transitions at the SAM in wild-type plants, and for certain MADS-box overexpression lines (Yu et al., 2004; Ferrario et al., 2004; Liu et al., 2007; Fornara et al., 2008).

The coexistence and, at the same time, the clear distinction of IM and FM suggest a common underlying dynamic multi-stable mechanism. Some genes have been identified as critical markers of each of these SAM cellular identities, while others are shared among them. Distinction between IM and FM depends on the mutual repression of floral meristem identity genes, such as *LFY*, *AP1*, and *CAULIFLOWER* (*CAL*), and IM genes, particularly *TERMINAL FLOWER1* (*TFL1*), an important regulator of inflorescence development (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meeks-Wagner, 1993; Gustafson-Brown et al., 1994; Chen et al., 1997; Ohshima et al., 1997; Ratcliffe et al., 1998, 1999; Ferrándiz et al., 2000; Parcy et al., 2002). *TFL1* encodes a phosphatidylethanolamine-binding protein (PEBP) that is transcribed in the center of the IM, but the protein moves to other cells where *AP1* and *LFY* are down-regulated (Bradley et al., 1997; Conti and Bradley, 2007). *tfl1* is an early flowering mutant with a determinate inflorescence due to the ectopic expression of *LFY* and *AP1* in the IM (Shannon and Meeks-Wagner, 1991; Schultz and Haughn, 1993; Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995; Liljegren et al., 1999). Conversely, single and double mutants of *LFY* and *AP1* acquire inflorescence-like structures because of the ectopic expression of *TFL1* (Huala and Sussex, 1992; Bowman et al., 1993; Bradley et al., 1997; Ratcliffe et al., 1998, 1999; Benlloch et al., 2007).

Recent data show that the tight spatial and temporal regulation of the components of the GRN underlying the transition to flowering is also involved in FM identity and maintenance (Liu et al., 2009; Posé et al., 2012). In this sense, genes such as *SOC1*, *AGL24*, and *SHORT VEGETATIVE PHASE* (*SVP*), known to participate in the regulation of flowering transition by regulating *LFY* in the case of the first two genes (Lee et al., 2008; Liu et al., 2008), and SVP in collaboration with FLOWERING LOCUS C (FLC) by repressing *SOC1* and *FT* (Hartmann et al., 2000; Lee et al., 2007; Li et al., 2008), are also important during the first two stages of flower development (Gregis et al., 2009; Liu et al., 2009). At these stages, *SOC1*, *AGL24*, and *SVP* help to prevent the premature expression of the B and C genes (Gregis et al., 2006, 2009; Liu et al., 2009). Moreover SOC1, AGL24, SVP, and SEP4 with AP1 repress the expression of *TFL1* in the FM (Liu et al., 2013). At stage 3 of FM development, *AGL24* and *SVP* are repressed by LFY and AP1, leading to further differentiation and determinacy (Yu et al., 2004; Liu et al., 2007). Meanwhile, expression of *SOC1* and *FRUITFULL* (*FUL*, another MADS-box gene) in the IM is important to repress secondary vascular growth (Melzer et al., 2008). Therefore, *SOC1*, *AGL24*, *SVP*, and *FUL* are important in both flowering transition, and floral and inflorescence meristems identity and maintenance.

Additional evidence for the common underlying multi-stable and non-linear GRN for SAM states and transitions is the fact that several of the aforementioned MADS-domain proteins are involved in multiple SAM states and transitions (Smaczniak et al., 2012), sometimes with apparently paradoxical functions. The overexpression of some MADS-box genes, such as *AGL24* or *SOC1* and their homologs, induce early flowering by up-regulating *LFY* and *AP1* (Lee et al., 2000; Yu et al., 2002; Michaels et al., 2003; Lee et al., 2008), but at the same time produce flowers with vegetative characteristics that resemble the *ap1* mutant with elongated carpels, especially

under short-day (SD) condition (Irish and Sussex, 1990; Bowman et al., 1993; Borner et al., 2000; Ferrario et al., 2004; Masiero et al., 2004; Yu et al., 2004; Liu et al., 2007; Trevaskis et al., 2007; Fornara et al., 2008). The phenomenon known as "floral reversion" has been also described in heterozygous *lfy*, *ap1*, *ap2*, and *agamous* (*ag*) mutants, suggesting that these genes repress this process and favor FM determinacy (Battey and Lyndon, 1990; Okamuro et al., 1993, 1996, 1997). There is no explanation or mechanistic model to account for the permanence of inflorescence characteristics when *LFY* and *AP1* are prematurely expressed in the MADS-box overexpression lines.

XAANTAL2 (*XAL2*/*AGL14*) is a MADS-box gene preferentially expressed in the root (Rounsley et al., 1995; Garay-Arroyo et al., 2013). The name *XAANTAL2* was given because *xal2* mutants have short roots similar to those of *xaantal1*/*agl12* (Tapia-López et al., 2008; Garay-Arroyo et al., 2013). Here, we report that *XAL2* is also a key player in SAM cell identities and transitions. It promotes flowering and presents similar loss and gain of function phenotypes such as *AGL24* and *SOC1*. We also show that overexpression of *XAL2*, *SOC1*, and *AGL24* are able to up-regulate *TFL1*, thus explaining, at least in part, the prevalence of vegetative traits, even if *AP1* and *LFY* are prematurely expressed, supporting that *XAL2* is also important for FM maintenance. Here, we propose a dynamic GRN and epigenetic landscape (EL) models (Álvarez-Buylla et al., 2008, 2010b; Villarreal et al., 2012) that integrate our data with previous results to provide a mechanistic and dynamic framework to understanding normal and altered cell fates and transitions at the *Arabidopsis* SAM. This model thus provides a mechanistic explanation for apparently paradoxical data for other loss and gain of function phenotypes (Borner et al., 2000; Ferrario et al., 2004; Masiero et al., 2004; Yu et al., 2004; Liu et al., 2007; Trevaskis et al., 2007; Fornara et al., 2008) allowing the integration of additional components.

RESULTS

XAL2 Promotes Flowering Transition

XAL2 is a member of the TM3/SOC1 clade, belonging to the type II MADS-box genes (Álvarez-Buylla et al., 2000; Martínez-Castilla and Álvarez-Buylla, 2003; Parenicová et al., 2003; Smaczniak et al., 2012). Except for *XAL2* (Garay-Arroyo et al., 2013), all other members of this clade have been identified as activators of flowering transition (Lee et al., 2000; Moon et al., 2003; Schmid et al., 2003; Schönrock et al., 2006; Dorca-Fornell et al., 2011). Given the role of all other members of *SOC1* clade, we hypothesized that *XAL2* could also be involved in flowering and tested two *xal2* alleles under four conditions: long-day (LD) and SD photoperiods, vernalization plus LD, and GA_3 treatment plus SD. In addition, we generated double mutants using the *xal2-2* allele (which has less somatic *En*-excision rates than *xal2-1*) and *soc1-6*, *agl24-4*, and *ful-7* mutants, because SOC1, AGL24, and FUL proteins interact with XAL2 in the yeast two-hybrid system, suggesting that they form dimers (de Folter et al., 2005; van Dijk et al., 2010).

Under LD condition both *xal2* alleles (Garay-Arroyo et al., 2013) showed a subtle but significant delay in bolting time (Figure 1A and 1B and Supplemental Table 1). Under the same condition, *soc1-6* was epistatic over *xal2-2*, while *xal2-2* and *ful-7* had a slightly additive effect on bolting time compared with the parental plants. No differences were observed in the *xal2-2 agl24-4* double mutant with respect to single mutants (Figure 1B). Interestingly, the rosette leaf number (RLN) did not always coincide with the bolting time phenotype (Figure 1B and 1C). In fact, *xal2-1* and *xal2-2* alleles and *xal2-2 ful-7* have the same number of leaves as wild-type plants under LD condition, while *xal2-2 soc1-6* double mutants had fewer leaves than *soc1-6* (Figure 1B and Supplemental Table 1).

Under SD condition both *xal2* alleles are remarkably delayed compared with wild-type plants and only *xal2-2 soc1-6* plants showed an additive bolting time phenotype in comparison with both parentals (Figure 1C and Supplemental Table 1). However, *xal2-2* was epistatic over *agl24-4* and *ful-7* mutants under this condition (Figure 1C). Unexpectedly, the *xal2-2 soc1-6* RLN is lower than in both parental lines (Figure 1C). Therefore, it seems that *XAL2* effects on bolting time and rosette leaf development are partially independent. We also found that cauline leaf number is diminished in *xal2-2* only under SD condition and is epistatic over *soc1-6*, *agl24-4*, and *ful-7* (Supplemental Figure 1A).

Since GA plays a relevant role in flowering under SD, we tested the effect of this hormone in all mutants. GA application partially suppressed flowering phenotypes under SD condition in all cases except for *xal2-2 soc1-6* (Figure 1C and 1D). Interestingly, 62% of the *xal2-2 soc1-6* plants grown under SD condition were unable to flower after 117 days after sowing (DAS), and none of them flowered after GA treatment (88 DAS), thus suggesting that *XAL2* and *SOC1* additively participate in GA response during flowering transition. To explore how the impairment of GA response in *xal2-2 soc1-6* affects GA homeostasis, we assayed two GA biosynthesis genes (*GA20OX1* and *2*) and a catabolic one (*GA2OX1*; Rieu et al., 2008) at 14 DAS, when most of the flowering time genes are up-regulated under LD condition. Our results in the double mutant showed up-regulation of *GA20OX1* compared with *xal2-2* and down-regulation of *GA2OX1* compared with wild-type plants (Supplemental Figure 2A). This finding suggests a compensatory mechanism in which the plant tries to make up for reduced GA responses by producing more GA. Further analysis should be performed to clarify the role of *XAL2* in relation to *SOC1* in GA homeostasis during flowering transition.

Overall, our results for single and double mutants indicate that both *xal2* alleles have a delayed bolting time compared with wild-type plants under all conditions tested, except for vernalization treatment (Figure 1A–1D and Supplemental Table 1). To further explore the role of *XAL2* in flowering transition and to uncover possible redundancies of this gene with other related MADS-box genes, we generated several *35S::XAL2* lines and selected three of them that showed the highest levels of *XAL2* transcript accumulation (Supplemental Figure 2B) and similar phenotypes among them (see description in the following paragraphs). In Figure 1E and Supplemental Figure 1B we show that *35S::XAL2* line (9T4) has a similar early bolting time and fewer rosette and cauline leaves in comparison with wild-type plants, under both LD and SD condition. Therefore, *XAL2* is

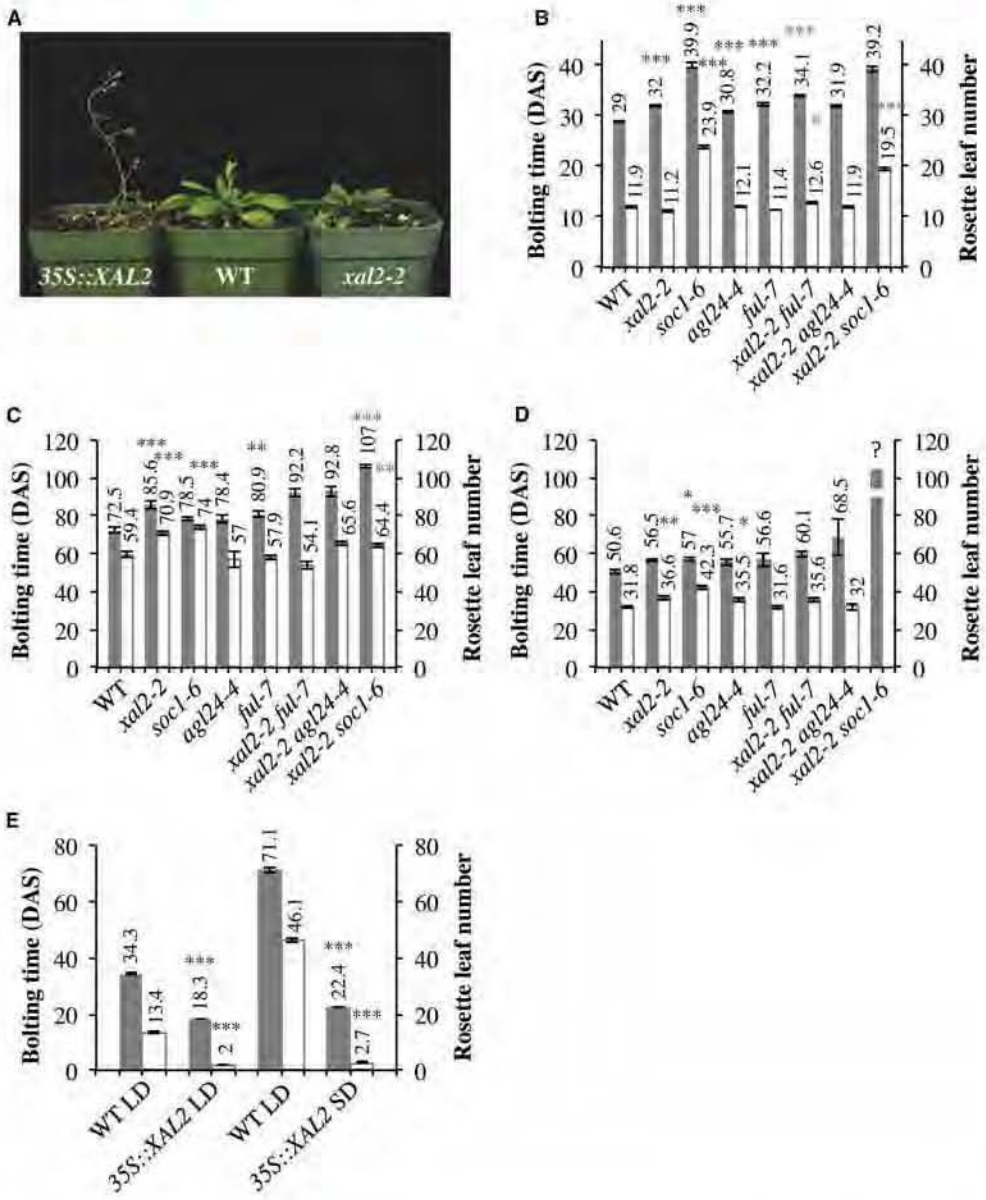

Figure 1. *XAL2* Participates in Flowering Transition.

(A) The mutant allele *xal2-2* and the overexpression line *35S::XAL2* are late and early flowering compared with wild-type (WT) plants, respectively.

(B) Flowering time of double mutant plants *xal2-2 ful-7*, *xal2-2 agl24-4*, and *xal2-2 soc1-6* compared with parental and WT plants grown under long-day (LD) condition, showing that *soc1-6* is epistatic over *xal2-2*. DAS, days after sowing.

(C) The same plants grown under short-day (SD) condition showed that the *xal2-2 soc1-6* double mutant plants have an additive effect compared with the parental and WT plants.

(D) GA_3 application mostly suppressed the late flowering phenotype of all genotypes. Note that none of the *xal2-2 soc1-6* double mutant plants flowered after 88 DAS.

(E) Overexpression of *XAL2* is sufficient to induce a similar early bolting time phenotype under LD and SD conditions.

Flowering transition was analyzed as the bolting time (gray bars) expressed in DAS and the rosette leaf number (white bars) as mean ± standard error ($n = 35–42$ plants under LD and $n = 16–23$ under SD and SD + GA). Lines with statistically significant differences compared with WT plants (black asterisks) or single mutants (red asterisks) are indicated as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ according to one-way analysis of variance (ANOVA) following Tukey's multiple comparison test.

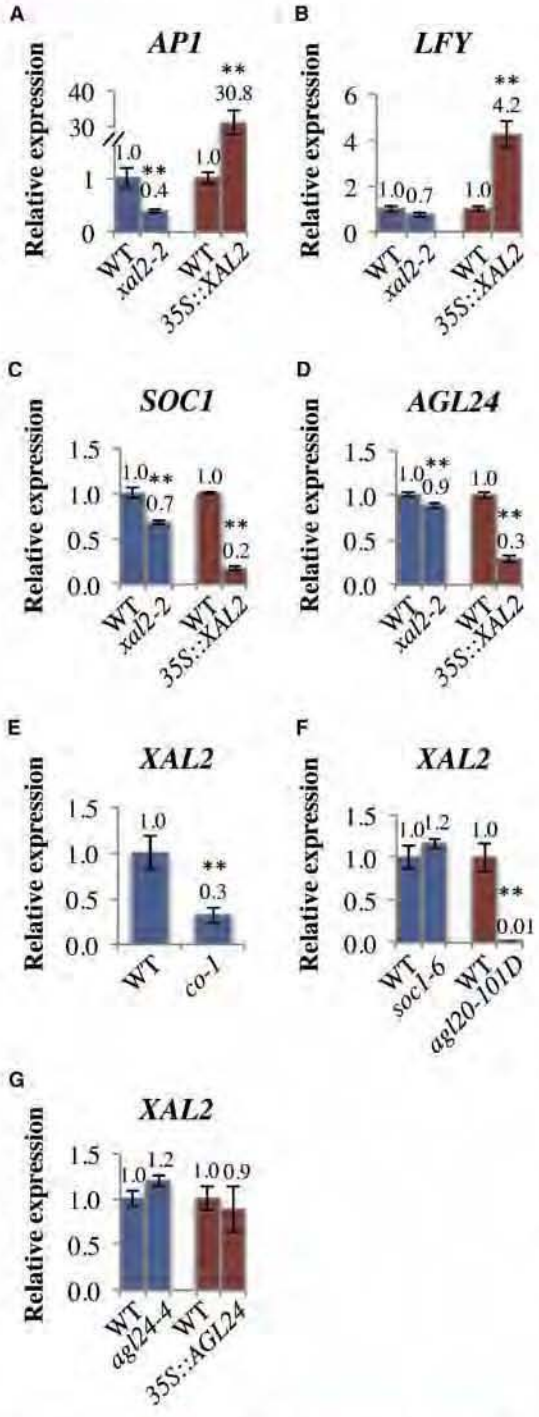

Figure 2. *XAL2* Regulation in the Flowering Gene Regulatory Network (GRN) under LD condition.

(A) XAL2 positively regulates *AP1*.

(B) *LFY* is up-regulated by XAL2 only in the overexpression line.

(C and D) *SOC1* **(C)** and *AGL24* **(D)** are down-regulated in *xal2-2*, and are repressed in the *XAL2* overexpression line.

(E) CO positively regulates *XAL2*.

(F) Overexpression of *SOC1* represses the expression of *XAL2*, but no significant difference in the latter was observed in *soc1-6* with respect to WT.

(G) AGL24 does not regulate *XAL2*.

Relative mRNA accumulation from three biological replicates were obtained from 14 DAS seedlings (blue bars) and 10 DAS plants (red bars) grown under LD condition. Data are shown as mean ± standard error. Statistical significance (**$P < 0.01$) was evaluated using the Mann–Whitney test.

sufficient to induce early flowering independently of photoperiod, and may participate in the IM to FM transition. These results confirm that *XAL2* is a key component of the GRN that controls flowering transition.

XAL2 Is Part of the GRN that Induces *AP1* during Floral Transition

We then analyzed the role of *XAL2* in the flowering transition GRN using quantitative RT–PCR. In agreement with the flowering transition phenotypes observed in Figure 1, we found that *AP1* expression was down-regulated in *xal2-2* (Figure 2A) while *LFY* did not show significant repression (Figure 2B). In contrast, both genes were up-regulated in the *XAL2* overexpression line (Figure 2A and 2B). *SOC1* and *AGL24* were also down-regulated in *xal2-2*, indicating that XAL2 positively regulates both genes. Surprisingly, *XAL2* overexpression drastically repressed *SOC1* and *AGL24* (Figure 2C and 2D). Therefore, it is possible that the early flowering phenotype observed in the *XAL2* overexpression line is due to an up-regulation of *LFY* and *AP1* and that this is partially independent of SOC1-AGL24.

XAL2 is down-regulated in *constans-1* mutant (*co-1*; Han et al., 2008), indicating that CO positively regulates *XAL2* when plants are grown under LD condition (Figure 2E). *XAL2* transcript accumulation in *soc1-6* (Wang et al., 2009) and *agl24-4* was unaffected (Figure 2F and 2G). Thus, at the transcriptional level, *XAL2* is regulated by CO and positively regulates *SOC1* and *AGL24*. Interestingly, when we analyzed *XAL2* accumulation in the *SOC1* (*agl20-101D*; Lee et al., 2000) and *AGL24* (Yu et al., 2002) overexpression lines, we found that *XAL2* was strongly repressed only in *agl20-101D* (Figure 2F and 2G). This suggests that *XAL2* and *SOC1* overexpression lines induce early flowering independently of one another.

In summary, the RT–PCR results indicate that *XAL2* is an important component of the GRN that regulates *AP1*, is under the control of CO, and participates in the up-regulation of *SOC1* and *AGL24* upon floral transition.

XAL2 Participates in Flower Meristem Maintenance and Determinacy

To address the role of *XAL2* in FM development, we analyzed its spatio-temporal expression pattern with *in situ* hybridization at different FM stages. *XAL2* expression appears very early at the flanks of the IM in the anlagens upon the transition to flowering (Figure 3A). Subsequently, *XAL2* expression levels increase in the first and second stages of the FM (Smyth et al., 1990), and

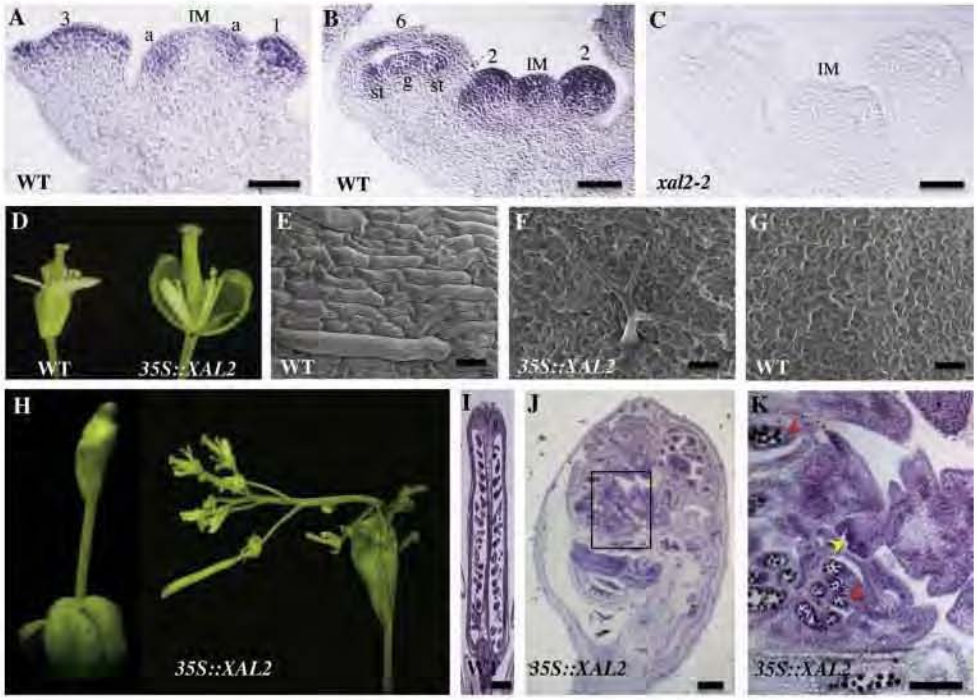

Figure 3. *XAL2* Spatial and Temporal Expression in WT SAM during the Transition to Flowering and *XAL2* Overexpression Floral Phenotypes.

(A–C) mRNA *XAL2 in situ* hybridization in WT and *xal2-2* inflorescences. **(A)** *XAL2* is detected in the anlagen (a), stage 1 of the floral meristem (FM), and L1 and L2 layers of FM stage 3. **(B)** *XAL2* accumulates at stage 2 of the FM and at the inflorescence meristem (IM). Later in FM development (stage 6), *XAL2* is also detected in the stamen (st) and gynoecium (g) primordia. **(C)** As a negative control, no signal was detected when *XAL2* antisense probe was used in the *xal2-2* mutant.

(D–G) Floral phenotype of the *35S::XAL2* compared with WT grown under LD condition. **(D)** *35S::XAL2* sepals are larger than WT sepals, and scanning electron micrographs show that the cellular identity of the *35S::XAL2* sepals **(F)** is more similar to WT leaf cells **(G)** than to WT sepal cells **(E)**, including the presence of trichomes **(F)**.

(H) Under SD condition, early arising carpels of the *35S::XAL2* plants elongate and inflorescences develop inside them.

(I–K) Longitudinal toluidine blue-stained sections confirmed that flowers at different developmental stages can be observed inside the *35S::XAL2* carpels compared with a similar stage of WT carpels based on ovule development **(I)**. **(K)** Magnification of the rectangle in **(J)** shows a FM at stage 4 of development (yellow arrowhead) and pollen grains (red arrowheads) inside the *XAL2* overexpression carpel.

Scale bars correspond to 50 μm **(A–C)**, 20 μm **(E–G)**, and 500 μm **(I–K)**.

at stage 3, *XAL2* is restricted to the L1 and L2 layers (Figure 3A and 3B). Later on, *XAL2* is expressed in the gynoecium and stamen primordia at stage 6 (Figure 3B). Interestingly, *XAL2* mRNA is also detected in the IM periphery (Figure 3B). We used *xal2-2* mutant as a negative control to rule out cross-hybridization of our probe with the closely related *AGL19* mRNA (Figure 3C).

The *XAL2* spatio-temporal expression pattern is similar to that of *AGL24* and *SVP* (Yu et al., 2004; Liu et al., 2007; Gregis et al., 2009). It has been reported that *SOC1*, in addition to the latter two genes, are important during the first two stages of FM development, but are repressed at stage 3 for proper subsequent FM differentiation (Yu et al., 2004; Gregis et al., 2006; Liu et al., 2007; Gregis et al., 2009; Liu et al., 2009). Furthermore, LFY and AP1 (particularly the latter) repress *AGL24* and *SVP* at FM stage 3 (Yu et al., 2004; Liu et al., 2007). Coincidentally, we found that *XAL2* accumulation is higher in the *ap1-15* (Ng and Yanofsky, 2001) and *ap1-1 cal-5* mutants (Ferrándiz et al., 2000) and is down-regulated in the *tfl1-2* mutant, in which the IM is converted into FM (Supplemental Figure 3A; Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). In agreement, an opposite pattern of expression for *LFY* was detected in these mutants (Supplemental Figure 3A). Therefore, AP1 and CAL probably repress *XAL2* in the FM at stage 3, as occurs with *AGL24* and *SVP*.

As already explained, *XAL2* overexpression induces early flowering with the production of very few rosette leaves (Figure 1A and 1E). It is noteworthy that cauline leaves in these lines are rounder and larger, similar to rosette leaves (Figure 5A), and flowers have leaf-like traits, such as large sepals that remain indehiscent after fertilization (Figures 3D and 5D), and sepal cells with a morphology reminiscent of wild-type leaf cells (Figure 3E–3G). These phenotypes are similar to those reported for *SOC1*, *AGL24*, and their homolog overexpression lines (Borner et al.,

2000; Michaels et al., 2003; Ferrario et al., 2004; Masiero et al., 2004; Yu et al., 2004; Liu et al., 2007; Trevaskis et al., 2007; Fornara et al., 2008). Interestingly, in the *35S::XAL2* under SD condition, early arising basipetal carpels deformed (stages 14–17 of flower development; Smyth et al., 1990; Roeder and Yanofsky, 2006) and a whole inflorescence grew from inside, bearing new fertile flowers (Figure 3H, 3J, and 3K). The flowers that develop from these indeterminate carpels attain different developmental stages, from FM (stage 4 in the picture) up to flowers with mature ovules and pollen grains (Figure 3I and 3K). These results indicate that correct spatio-temporal control of *XAL2* expression is fundamental for normal FM cell differentiation and determinacy.

In conclusion, *XAL2* overexpression accelerates the transition to flowering by terminating the vegetative phase prematurely, but at the same time the flowers produced in these lines show leaf-like traits. In addition, under non-inductive flowering conditions, overexpression of *XAL2* prevents FM determinacy, leading to what appears to be cell reprogramming with some carpel cells functioning as IM cells.

XAL2 Overexpression Positively Regulates *TFL1* and *WUS* Expression, and Directly Binds to the *TFL1* Regulatory Sequences

To unravel the molecular basis of the *XAL2* overexpression phenotypes in which this gene up-regulates *AP1* and *LFY* (Figure 2A and 2B), and at the same time yields flowers with some *ap1* mutant characteristics (Figure 3D and 3F; Irish and Sussex, 1990; Bowman et al., 1993), we hypothesized that an inflorescence identity gene capable of repressing *AP1* could be involved. Hence, we analyzed the expression of *TFL1* in *xal2-2* and the 35S::*XAL2* line, and found that it was down- and up-regulated in these lines, respectively (Figure 4A). Furthermore, to establish whether XAL2 is able to directly bind to *TFL1* regulatory sequences, we performed a chromatin immunoprecipitation (ChIP) experiment using a *35S::GFP-XAL2* line. In Figure 4B we show that three different *TFL1* regulatory regions containing CArG boxes are enriched (III, V, and VI) within the 5′ promoter and the intergenic region downstream of the 3′ stop codon of the *TFL1* gene. These results strongly support that under constitutive expression, *XAL2* directly binds to *TFL1* regulatory sequences. Since *SOC1* and *AGL24* overexpression phenotypes are similar to those of *35S:XAL2*, we further analyzed *TFL1* transcript accumulation in these two lines. *TFL1* was also up-regulated in *agl20-101D* and *35S::AGL24* (Figure 4C).

We have already shown that *AP1* is up-regulated in the *XAL2* overexpression line at 10 DAS (Figure 2A), but we wanted to be sure that the *ap1*-like phenotype was not due to its down-regulation at different developmental stages. We performed an *AP1* expression time course from 8 to 14 DAS plants, and at all time points analyzed *35S:XAL2* plants showed higher levels of *AP1* than wild-type plants (Supplemental Figure 3B). Hence, we can conclude that leaf-like traits of the *35S::XAL2* flower organs are not due to decreased levels of *AP1*. Also, *SOC1* and *AGL24* overexpressors showed up-regulation of *AP1* as expected (Figure 4D). Thus, overexpression of *XAL2*, *SOC1*, or *AGL24* is able to up-regulate both *TFL1* and *AP1* and cause early flowering, and at the same time yield flowers with leaf-like cell types.

FM cells stop proliferating after the carpels are formed in wild-type flowers (Mizukami and Ma, 1997). *WUSCHEL* (*WUS*), a key gene involved in the identity of stem cells in the SAM, is repressed in the central zone of the FM at stage 6 (Lenhard et al., 2001; Lohmann et al., 2001; Sun et al., 2009). Since we observed that in non-inductive flowering conditions FM determinacy is lost and a new inflorescence emerges from inside of early arising carpels in the *XAL2* overexpression lines (Figure 3H, 3J, and 3K), we hypothesized that *WUS* is persistently expressed in these lines. Therefore, we analyzed *WUS* and *TFL1* mRNA accumulation in these carpels compared with wild-type carpels at similar developmental stages. Indeed, we found that both genes were up-regulated in the *XAL2* overexpression line (Figure 4E and 4F), confirming that when *XAL2* is de-regulated, some of the molecular components that are important for IM and FM identity and determinacy are also altered.

To test whether the floral "reversion" phenotype of the *35S::XAL2* line was due to higher competence of *TFL1* over *AP1*, we crossed it to a *35S::AP1* plant to test whether the excess of *AP1* could counteract *TFL1* (Figure 5; Mandel and Yanofsky, 1995). As expected, both lines partially complemented each other's phenotypes in the double overexpressor line grown under SD condition, resulting in plants with smaller cauline leaves and flowers with reduced sepals compared with the *35S::XAL2* parental plant (Figure 5A, 5C, 5D, and 5F). On the other hand, the conversion of inflorescences into solitary flowers, typical of the *35S::AP1* line, disappeared (Figure 5B and 5C). Although both parental lines were early flowering, the bolting time of the *35S::AP1 35S::XAL2* line was the same as for *35S::AP1* plants, but the double overexpressor line had an intermediate number of rosette leaves with respect to both parental lines (Figure 5G). Interestingly, the double overexpressor had fewer swollen carpels compared with the *XAL2* overexpression line (Figure 5H), indicating that the indeterminacy observed in the *35S::XAL2* FM (Figure 3H, 3J, and 3K) was almost recovered when *AP1* was increased.

GRN and EL Modeling for *XAL2* Interactions under LD Condition: A Mechanistic Dynamic Explanation for *XAL2*, *SOC1*, and *AGL24* Overexpression Phenotypes

Our data uncover a complex set of interactions and roles for *XAL2* in SAM transitions. To provide an integrative, system-level, dynamic and mechanistic explanation for our results, a GRN modeling approach is required. We integrated the evidence of this work, together with previously reported information (Supplemental Table 2), to uncover a necessary and sufficient set of components and interactions (i.e., dynamic GRN module) that recover observed patterns of gene expression in vegetative meristem (VM), IM, and FM cells in wild-type plants. These patterns correspond to the expected set of steady-state gene configurations to which such wild-type GRN should converge (Figure 6 and Table 1), and can be validated if it also explains what we observe in the analyzed loss and gain of function lines.

We formalized experimental data as logical functions following previous studies (see the Methods section and Supplemental

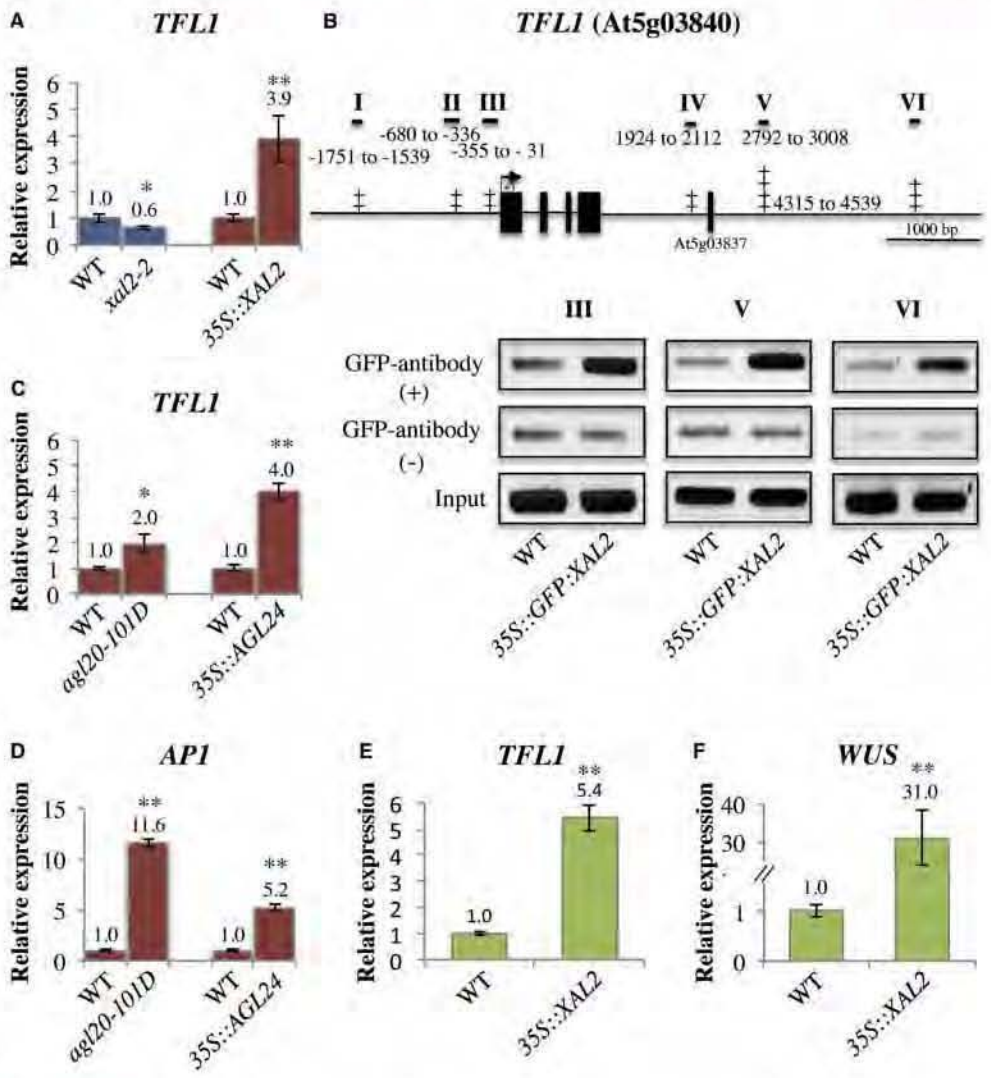

Figure 4. *XAL2* is a Positive Regulator of *TFL1*.

(A) *TFL1* relative RNA accumulation is down-regulated in *xal2-2* and up-regulated in the *XAL2* overexpression line.

(B) Chromatin Immunoprecipitation (ChIP) assay was performed to examine *in vivo* binding of XAL2 to the *TFL1* regulatory regions in the *XAL2* overexpression line. Top panel shows a schematic diagram of the *TFL1* locus, indicating in roman numerals the regions amplified by PCR after DNA immunoprecipitation. Primers flanking the CArG boxes (+) and their positions relative to the *TFL1* transcriptional start site are indicated. The bottom panel shows DNA fragments corresponding to *TFL1* III, V, and VI regions enriched in the *35S::GFP:XAL2* plants after ChIP with a GFP antibody.

(C and D) Up-regulation of *TFL1* **(C)** and *AP1* **(D)** in the *SOC1* (*agl20-101D*) and *35S::AGL24* overexpression lines.

(E and F) Higher RNA accumulation levels of both *TFL1* **(E)** and *WUS* **(F)** were detected in first arising carpels of the *35S::XAL2* plants compared with WT plants grown under SD condition.

Quantitative RT–PCR was performed with RNA extracted from 14 DAS seedlings (blue bars), 10 DAS seedlings (red bars), and carpels with similar ovule stage development (green bars). Data in **(A)** and **(C–F)** are shown as mean ± standard error. Statistical significance (*$P < 0.05$, **$P < 0.01$) was evaluated using Mann–Whitney test.

Table 3). We were able to recover a wild-type GRN model under LD condition that integrates the data presented in this work and the necessary and sufficient set of additional components and interactions from the literature, to recover the expected steady states for VM, IM, and FM for the genes considered (Figure 6C and Table 1). Loss of function lines were simulated by turning the corresponding gene to "0" during the complete simulation, while the overexpression lines were simulated by turning the corresponding gene to "2." The proposed GRN is validated because, as expected by the observed phenotypes, all single loss of function mutants qualitatively recovered the same set of steady states as wild-type GRNs, while gain of function GRN

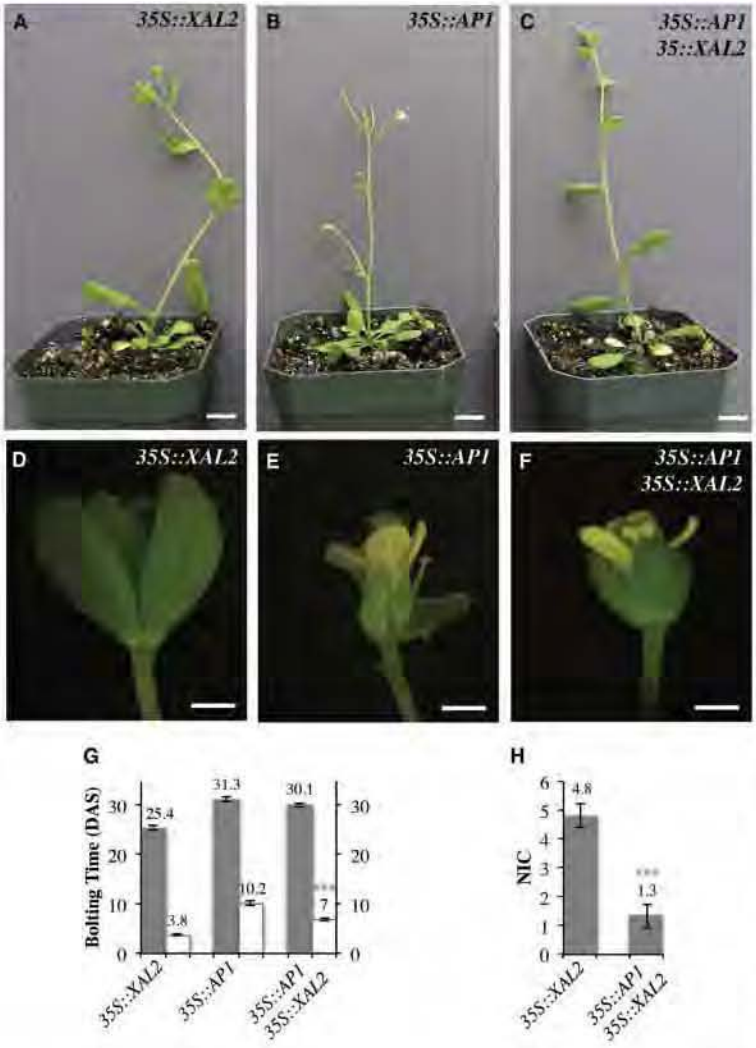

Figure 5. Complementation Analysis of the *35S::XAL2* and the *35S::AP1* Phenotypes in the Double Overexpressor Plants Grown under SD Condition.

(A and D) *XAL2* overexpression plants have large cauline leaves similar to rosette leaves **(A)** and flowers with large sepals that persist after fertilization **(D)**.

(B and E) *35S::AP1* plants show a determinate growth in which each pedicel gives rise from two to three terminal flowers **(B)**. Flowers of the *35S::AP1* are similar to those of WT **(E)**.

(C and F) Determinate growth of the *35S::AP1* line is complemented in the double overexpressor *35S::AP1 35S::XAL2* plants. On the other hand, the cauline leaves phenotype of the *35S::XAL2* is complemented to WT in this line **(A–C)**. Sepals of the double overexpressor line are partially complemented, resulting in sepals that are much smaller than the *35S::XAL2* sepals **(D and F)**. Scale bars correspond to 1 cm **(A–C)** and 2 mm **(D–F)**.

(G and H) The double overexpressor plants **(G)** have the same bolting time as the *35S::AP1* line, but have an intermediate rosette leaf number compared with parental plants. The number of indeterminate carpels (NIC) along the shoot axis of the double overexpressor **(H)** is also reduced compared with the *35S::XAL2* line. Bars correspond to standard error from average (n = 26–32 plants). Statistical significance with respect to parental plants (***P < 0.001; red asterisks) was evaluated according to one-way ANOVA following Tukey's multiple comparison test **(G)** or Mann–Whitney test **(H)**.

simulations recovered wild-type steady states, plus a new steady state with an IM/FM mixed cell identity (Figure 6C and Table 1). Indeed, experimental data has shown that *soc1*, *agl24*, and *xal2* single mutants do not modify cell identities but only flowering time, which cannot be simulated with this version of the model. On the other hand, *XAL2*, *SOC1*, or *AGL24* overexpression not only modifies flowering time, but also produces some flowers with inflorescence-like characteristics. Coincidentally, our model suggests that such flowers have some cells with a mixed IM/FM identity.

To gain further insight into how the alteration in the expression of *SOC1*, *AGL24*, and especially *XAL2* modify SAM cell transitions, we propose an EL analysis similar to that reported by Álvarez-Buylla et al. (2008) (Figure 7 and Supplemental Figure 4). Such analysis addresses whether the set of components and interactions considered in the uncovered GRN module in Figure 6B also underlies the observed temporal pattern of transition among cell types in wild-type and other lines (steady states): VM > IM > FM. Importantly, this type of model can discriminate between two hypotheses: the observed leaf-like structures in flowers of the overexpressors is due to a reversion from FM cells to IM cells, or in these lines a new type of steady state with mixed identity (IM/FM) appears during SAM development. Thus, this and the GRN modeling provide a mechanistic explanation for the apparently paradoxical phenotype of the overexpressors.

We thus performed a stochastic simulation of the proposed GRN model to propose a model for a population of cells at the SAM (see Supplemental Methods). Since VM cells are the first to attain their fate in wild-type, all cells were assumed to be in this state at initial conditions. Thus, in the vector with the proportion of cells in each GRN steady state for the dynamic stochastic equation, VM was set to 1 and the rest to 0 (Figure 7A–7D). This equation was iterated to follow the changes in the probability of reaching each one of the other steady states over time. The graph clearly shows how the trajectory for each of the steady states' probability reaches its maximum at a given time. In accordance with biological observations, the results show that the most probable sequence of cell attainment is VM > IM > FM

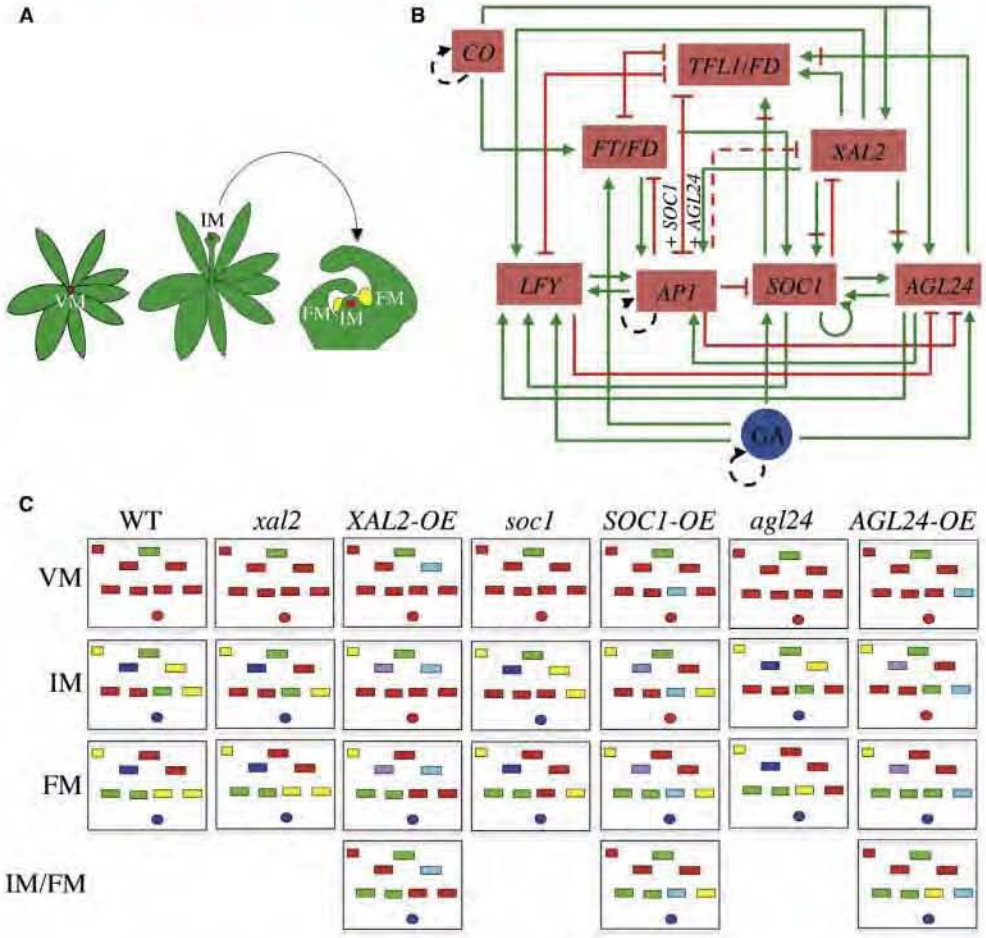

Figure 6. Model for the *XAL2* Regulatory Network Module during SAM Development and Its Steady States for the WT, Loss and Gain of Function *XAL2*, *SOC1*, and *AGL24* Lines.

(A) Schematic representation of SAM transitions from a vegetative (VM) to inflorescence (IM) and floral (FM) meristem states.

(B) GRN showing the interactions uncovered in this paper and published results (see Supplemental Table 2). Arrows (green) and bar-lines (red) indicate induction and repression, respectively. In some cases, we discovered that the sign of the interaction inferred changed depending if the loss or gain of function lines were being tested (regulation of XAL2, SOC1, and AGL24 over some of their targets). Dotted lines represent predictions of regulations that need further verification. In the case of GA and *CO*, the positive feedback loops are introduced because their upstream regulators that keep them turned on were not considered in the model proposed here. AP1 plus SOC1 or AGL24 indicate protein dimers that repress *TFL1* (Liu et al., 2013).

(C) A schematic representation of the network in **(B)** is used to represent the steady states achieved by this model under the different lines considered (columns). In each row, the steady states corresponding to the VM, IM, FM, or the novel IM/FM state recovered in the overexpressors. The components of the network are shown by squares or a circle (GA) that are turned on/off in each of the steady-state configurations being considered. The colors correspond to the activation state of the node in each case: red = 0; green = 1; yellow = 0 or 1; purple = 1 or 2; light blue = 2; and dark blue = 0, 1, or 2.

in wild-type plants (Figure 7A). In conclusion, our simulations suggest that the complex GRN that underlies the attainment of VM, IM, and FM cell identities also restricts, to a large extent, the temporal pattern of transitions among them as found for the floral organ specification GRN reported by Álvarez-Buylla et al. (2008).

Interestingly, in the case of gain of function simulations of *XAL2*, *SOC1*, and *AGL24*, the same pattern of temporal transitions as in wild-type was recovered, but in these cases the maximum probability of the mixed IM/FM identity occurs after the IM and before the FM configurations (Figure 7B–7D). This analysis also recovers all the possible transitions among the steady states (Figure 7E and 7F). The net transition rate was positive for the IM to FM direction in all the lines tested, but was lower under gain of function lines in comparison with the wild-type (Supplemental Figure 4). This means that the net probability flow preferentially follows the direction from IM to FM, both in wild-type and in each of the overexpression lines of *XAL2*, *SOC1*, and *AGL24* (Figure 7F). These results are consistent with the observed most probable temporal order of transitions in plants. Likewise, the results do not support the hypothesis of an induced, reverse

	AP1	LFY	SOC1	AGL24	XAL2	TFL1	FT	GA	CO
VM	0	0	0	0	0	1	0	*	0
IM	0	0	*	*	*	1	*	*	*
FM	1	1	*	*	*	0	*	*	*

Table 1. Observed Expression States of the Genes Considered in the Network Model in Wild-Type Plants during Different Stages of the SAM Development.

*Any possible value of the node in the network.

rate of transition from FM to IM or IM/FM cells as an explanation of the observed phenotype in the overexpressors, as both reverse transitions (FM to IM and FM to IM/FM) showed a negative net transition rate (Figure 7E). Overall, the results of the stochastic EL analysis suggest that instead of an accelerated rate of transition in the forward (IM to FM) direction, it is the novel potentiality of the IM state to now choose between two preferential (positive net transition rate) fate decisions (FM or IM/FM phenotypes) induced by gene overexpression that accounts for the observed promiscuous IM/FM state in such flowers (Figure 7F and Supplemental Figure 4).

DISCUSSION

In this work we have shown, in contrast to previous expectations (Schönrock et al., 2006; Garay-Arroyo et al., 2013), that *XAL2* is expressed in the IM and FM and is a key player in the complex GRN underlying SAM transitions (Figure 6). *XAL2* is a promoter of flowering in response to multiple signals and is also important for FM maintenance and determinacy. We propose a GRN and EL modeling approach that together provides a mechanistic dynamic framework to explain the role of *XAL2* at the SAM and the apparently paradoxical phenotypes of its overexpression. Moreover, such a modeling framework constitutes a systemic mechanistic explanation for the observed patterns of expression of multiple genes underlying VM, IM, and FM cell fates, and the observed transitions among them in wild-type *Arabidopsis*. It thus constitutes a useful framework to incorporate additional components and interactions that participate in SAM development. Finally, it provides an explanation for *AGL24*, *SOC1*, and their homolog overexpression phenotypes in *Arabidopsis* (Borner et al., 2000; Michaels et al., 2003; Ferrario et al., 2004; Masiero et al., 2004; Yu et al., 2004; Liu et al., 2007; Trevaskis et al., 2007; Fornara et al., 2008).

XAL2 Promotes Flowering Transition

XAL2 participates in flowering transition in response to more than one signal, having a higher impact under non-inductive photoperiod conditions (Figure 1C). Flowering time is not so clearly affected in the *xal2* alleles, under all conditions tested, as is *soc1*, probably because SOC1 and AGL24 are able to directly activate *LFY* independently of *XAL2* (Lee et al., 2008; Liu et al., 2008). We proved that CO positively regulates *XAL2* and that the latter positively regulates *SOC1* and probably *AGL24* (Figure 2C–2E). Being *soc1* epistatic over *xal2* under LD condition confirms this result (Figure 1B). We also proved that under SD condition, and in response to GA, *xal2* is affected in bolting time and *xal2-2 soc1-6* has an additive effect compared with the parental plants (Figure 1C and 1D and Supplemental Table 1). These results could imply that they act independently over *LFY* and *AP1* regulation, or that they are part of the same regulatory module. We argue that *XAL2* is probably part of the same GRN in which *SOC1* participates, integrating at least some of the flowering transition pathways in response to different signals. In fact, the spatial and temporal patterns of expression of *XAL2*, and its loss and gain of function phenotypes, resemble those corresponding to *SOC1* and *AGL24* lines (Borner et al., 2000; Yu et al., 2004; Liu et al., 2007; Gregis et al., 2009), thus suggesting that *XAL2* is part of the *SOC1–AGL24* regulatory module. Moreover, XAL2 interacts with SOC1 and AGL24 according to yeast two-hybrid data (de Folter et al., 2005; Immink et al., 2009).

XAL2 Overexpression Affects FM Maintenance and Determinacy by Up-Regulating *TFL1* and *WUS*

After the flowering transition, LFY, AP1, and CAL are necessary for FM identity (Weigel et al., 1992; Bowman et al., 1993; Ferrándiz et al., 2000) by repressing the IM genes, particularly *TFL1* (Shannon and Meeks-Wagner, 1991; Schultz and Haughn, 1993; Gustafson-Brown et al., 1994; Mandel and Yanofsky, 1995; Liljegren et al., 1999). During the first and second stages of FM development, SOC1, AGL24, and SVP maintain FM identity in collaboration with AP1 by repressing *AG* and *SEP3* (Gregis et al., 2006, 2009; Liu et al., 2009). At stage 3 of flower development, LFY and AP1 repress the expression of the "flowering genes," allowing the transcription of the floral organ identity genes (Yu et al., 2004; Liu et al., 2007). LFY and WUS, among other genes, induce the expression of *AG* during this stage, which in turn represses *WUS* at stage 6, together with other proteins (Lenhard et al., 2001; Lohmann, et al., 2001; Gómez-Mena et al., 2005; Lee et al., 2006; Sun et al., 2009; Sun and Ito, 2010; Liu et al., 2011). This event drastically affects the FM stem cells, which stop proliferating (Mizukami and Ma, 1997).

These experimental data indicate that certain genes have clear effects in the FM when their expression is depleted or augmented; however, we think that FM identity, maintenance, and determinacy emerge from a complex GRN in which spatio-temporal regulations of *SOC1*, *AGL24*, *SVP*, and *XAL2* are also important. Indeed, in this study we have shown that overexpression of *XAL2* affects FM maintenance and yields phenotypes similar to those reported for the overexpression lines of *SOC1*, *AGL24*, and their homologs (Borner et al., 2000; Michaels et al., 2003; Ferrario et al., 2004; Masiero et al., 2004; Yu et al., 2004; Liu et al., 2007; Trevaskis et al., 2007; Fornara et al., 2008). More importantly, we demonstrate that overexpression of any of these genes is sufficient to induce *TFL1* expression (Figure 4A and 4C), suggesting that mis-regulation of *TFL1* underlies the "leaf-like" flower phenotype observed in the overexpression of these three MADS-box genes. In this regard, Hanano and

Goto (2011) had demonstrated that TFL1 acts as a transcriptional repressor, and the *35S::TFL1-SRDX* line phenotype reported by these authors is, in fact, very similar to the *XAL2* phenotype reported here (Figure 3).

Overexpression of *SOC1*, *AGL24*, or *XAL2* genes affects SAM transitions, causing premature flowering and *LFY/AP1* up-regulation (Figures 2, 4, and 5). At the same time, we have proved that overexpression of these MADS-box genes induces higher levels of *TFL1* mRNA accumulation compared with wild-type plants (Figure 4A and 4C). Furthermore, we have shown that XAL2 directly binds to *TFL1* regulatory sequences using the overexpression line *35S::GFP:XAL2* (Figure 4B). Interestingly, one of these binding sites (fragment V of the *TFL1* 3′ region amplified in our ChIP assay) corresponds to one of the binding sites of AP1, which has been demonstrated to be important for direct repression of *TFL1* (Kaufman et al., 2010). More recently, it was demonstrated that SOC1, AGL24, SVP, and SEP4 cooperate with AP1 in this action (Liu et al., 2013). However, it is possible that, when overexpressed, higher ratios of XAL2, SOC1, or AGL24 over AP1 are able to compete for the same binding site, affecting *TFL1* transcription in an opposite way. The partial complementation of the vegetative and indeterminacy features of the *35S::XAL2* line by crossing it with *35S::AP1* supports this hypothesis (Figure 5). If *TFL1* and, probably, other genes important for IM identity are ectopically expressed in the FM, this would explain the inflorescence characteristics of those flowers even in the presence of *AP1* which is not down-regulated (Figures 2 and 4; Supplemental Figure 3), and probably not mis-localized either, as reported for *AGL24/SVP* homolog *OsMADS47* overexpression line (Fornara et al., 2008). In this sense, the FM does not change its identity through a floral reversion process. Instead it behaves differently, probably having a mixed IM/FM identity, due to an altered behavior of the GRN (Figures 6 and 7).

Heterochronic "floral reversion" has been shown to be dependent on light and gibberellin signaling that affects a signal coming from the leaves to the SAM (Okamuro et al., 1996; Hempel et al., 2000). We now know that this signal is FT (Jaeger and Wigge, 2007; Müller-Xing et al., 2014). During flowering transition, this protein competes with TFL1 for FD, and this association up-regulates *SOC1* and *AP1* in the anlagen (Abe et al., 2005; Wigge et al., 2005; Hanano and Goto, 2011; Jaeger et al., 2013). In the overexpression lines of *XAL2*, *SOC1*, and *AGL24*, up-regulation of *TFL1* or delayed expression of *FT* under SD condition would affect such balance until endogenous FT protein attains certain levels during *Arabidopsis* inflorescence development. This would explain why the acropetal flowers show a wild-type phenotype while the early ones show IM features. This and related hypotheses could be tested by expanding the dynamic GRN and EL modeling framework proposed here.

Early Flowering and FM Phenotypes of the *XAL2* Overexpression Line under LD Condition are Reconciled Using a GRN Model and EL Analysis

We proposed GRN and EL models that provide a framework for mechanistic explanations of SAM transitions in wild-type plants, but also the complex loss and gain of function phenotypes of *XAL2* and other regulators of SAM transitions. In particular, this provides a novel framework with which to evaluate floral reversion. Floral reversion has been defined as the reappearance of vegetative traits during flower development or the loss of FM determinacy after floral organs are formed. This uncommon process in *Arabidopsis* has been attributed to reversion of the FM to the IM identity, particularly in the *lfy* and *ap1* mutants (Battey and Lyndon, 1990; Okamuro et al., 1993, 1996, 1997; Tooke et al., 2005). In contrast, based on previous data and the experimental results summarized here, we postulate an alternative explanation for the so-called floral reversion in the case of the *SOC1*, *AGL24*, and *XAL2* overexpression lines.

Our results of the deterministic GRN model suggest that a mixed meristem identity is attained as a steady state when *XAL2*, *AGL24*, or *SOC1* are overexpressed, while the same GRN yields normal configurations when the same genes are kept to "0." Indeed, based on our experimental data, in the model the IM/FM identity is the result of the positive regulation of these three MADS-box genes over *TFL1*, *LFY*, and *AP1*. When either of these genes is overexpressed, *TFL1* and *AP1* or *LFY* are activated, while at the same time the multiple feedback loops among them stabilize their expression, thus yielding the IM/FM identity (Figures 6 and 7).

The EL simulations suggest a mechanism by which *35S::SOC1*, *35S::AGL24*, or *35S::XAL2* cause a fraction of the cell population at the IM to acquire a mixed IM/FM identity (Figure 7). This could be explained by two alternative hypotheses. During normal developmental VM > IM > FM transitions, a fraction of cells may attain the new mixed identity IM/FM. Under this circumstance the establishment of the antagonistic relationship between IM and FM regulators may be weakened. On the other hand, an induced, reverse rate of transition from FM to IM or IM/FM cells could account for the results. The modeling results show that the first one is the most probable one, and the overexpressor global transition pattern is: VM > IM > IM/FM > FM (Figure 7F and Supplemental Figure 4). Therefore, for this and similar cases the term "floral reversion" should be avoided.

Loss of FM Determinacy in *XAL2* Overexpression Lines under SD Condition

Constitutive expression of *XAL2* also affects floral determinacy under SD condition. Here we showed that under this condition new inflorescences develop from the inside of the carpels of the basipetal flowers (Figure 3H, 3J, and 3K). At the molecular level, this may be explained in two different ways: either the presence of XAL2 prevents *WUS* repression or ectopic expression of this gene is sufficient to up-regulate *WUS*. We observed that *WUS* expression in the *35S::XAL2* is maintained after stage 6, enabling stem cells to remain active (Figure 3J, 3K, and 4F). At this point, we cannot know if the FM maintenance and indeterminacy phenotypes observed in the overexpression lines of *XAL2*, *SOC1*, or *AGL24* are due to a dominant negative effect or to gain of function. Interestingly, overexpression of *XAL2* or *SOC1* represses each other (Figure 2C and 2F), indicating that in these lines altered protein complexes could be formed. These hypotheses can be tested using an expanded GRN module including additional SAM genes. Furthermore, such a model could address whether FM

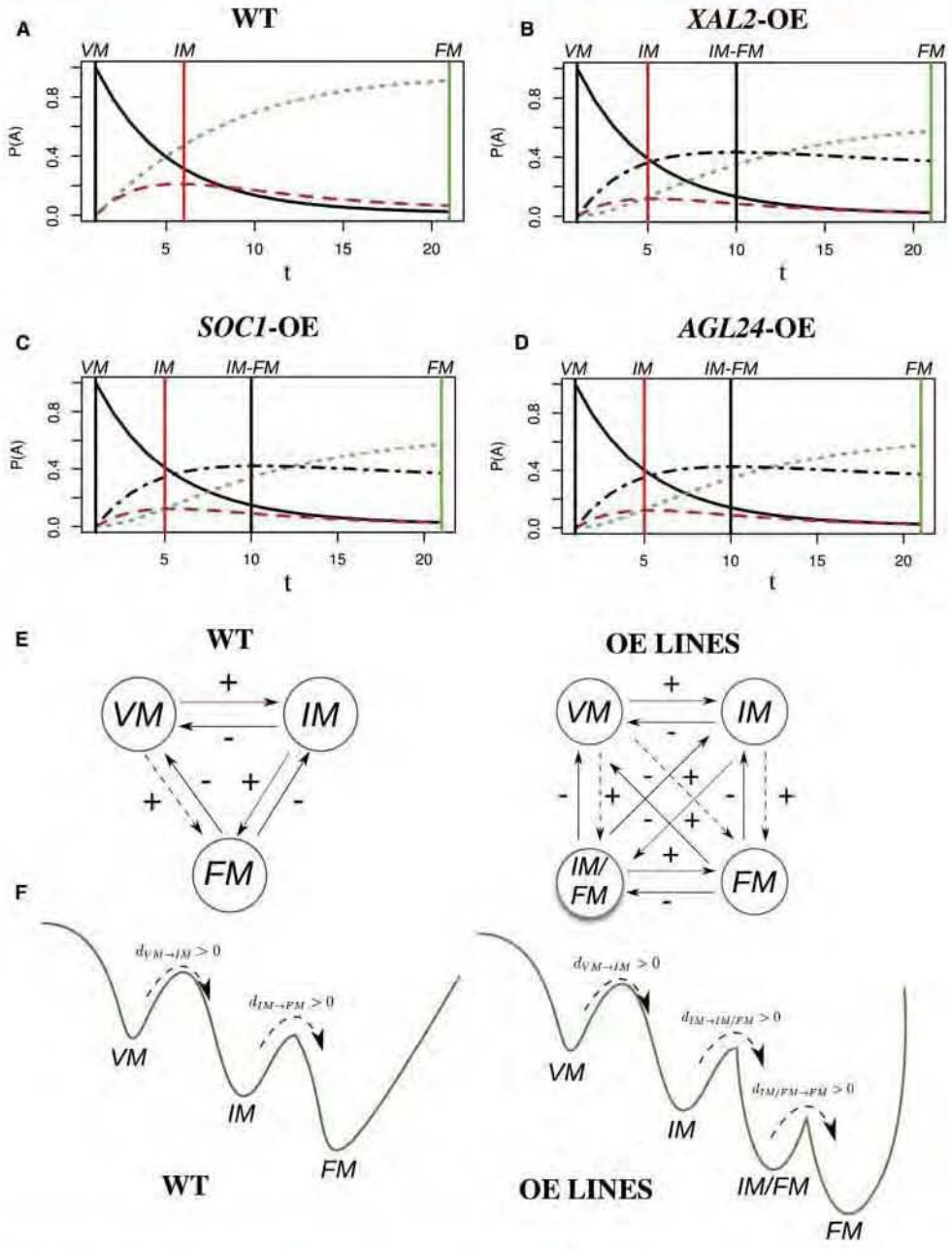

Figure 7. Epigenetic Landscape Analysis for the *XAL2* Regulatory Network Module.

(A–D) Temporal sequence of cell-fate attainment pattern under the stochastic GRN model during SAM cell-fate transitions. The maximum probability *p* of attaining each attractor, as a function of time (in iteration steps) is shown for **(A)** WT, **(B)** *XAL2* overexpression (*XAL2-OE*), **(C)** *SOC1-OE*, and **(D)** *AGL24-OE*. Vertical lines mark the time at which maximum probability of each steady state (i.e., cell fate, VM, IM, FM, or IM/FM) is attained. Note that the maximum probability for each steady state is 1. The most probable sequence of cell-fate attainment for the WT is VM, IM, FM; and for OE lines VM, IM (IM/FM), FM. The value of the error probability used in this case was $\xi = 0.05$. The same patterns were obtained with error probabilities from 0.01 to 0.1 (data not shown).

(E) Schematic representation of the possible transitions between pairs of steady states (cell fates at the SAM) for WT and OE lines. Arrows indicate the directionality of the transitions. Above each arrow a sign (+) or (–) indicates whether the calculated net transition rate between the corresponding

(legend continued on next page)

to IM transition in the indeterminate carpels, which corresponds to cell reprogramming, is favored under *XAL2* or other MADS-box overexpression.

METHODS

Plant Material and Selection of Mutant Lines

Arabidopsis thaliana wild-type and mutant plants used in this study were Col-0 with the exception of *ap1-1 cal-5* and *tfl1-2*, which are in Ler ecotype. Mutant alleles *xal2-1* and *xal2* were described previously (Garay-Arroyo et al., 2013). The *soc1-6* (SALK_138131; Wang et al., 2009), *ful-7* (SALK_033647; Wang et al., 2009), and *agl24-4* (GK674F05.03/N385337) mutant seeds were provided by the Arabidopsis Biological Resource Center or the Nottingham Arabidopsis Stock Centre, and the homozygous alleles were selected using the primers shown in Supplemental Table 4.

Plant Growth Conditions and Flowering Time Measurements

Seedlings were grown on vertical plates with 0.2× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 1% sucrose. For flowering experiments, plants were grown on soil (Metromix 200) under LD (16 h light/8 h dark) or SD (8 h light/16 h dark) condition at 22°C. For GA_3 treatment, plants were grown under SD condition for 2 weeks before they were sprayed with 100 μM GA_3 twice a week until flowering. For vernalization experiments, seeds were plated on MS medium and kept in the dark for 8 weeks at 4°C and then transferred to soil and grown under LD condition. Flowering transition was measured as bolting time (days after seed sowing required for the stem to grow to 1 cm long) and by the RLN at bolting. Inflorescences for *in situ* hybridization were collected when the stem reached 10 cm long. These comprised FM at different developmental stages.

Plasmid Constructs and Plant Selection

The *XAL2* gene was amplified from Col-0, using the XAL2g-F 5′-AGAA GAATGGTGAGGGGAAA-3′ and XAL2g-R 5′-ATGTTAGTTTGAAGGAG GAA-3′ primers. The 3603 nt DNA fragment was cloned in the pCR8/GW/TOPO-TA vector, and verified by sequencing. It was then recombined into either overexpression vectors: pGD625 (de Folter et al., 2006) or the pK7WGF2 that includes GFP (Karimi et al., 2002) carrying a kanamycin and spectinomycin/streptomycin resistance cassette, respectively. Kanamycin (50 μg/ml) resistant plants were selected on plates.

In Situ Hybridization Analysis

In situ hybridization was performed according to Tapia-López et al. (2008). *In vitro* transcription with the DIG RNA labeling Kit (Roche Molecular Biochemicals) was performed to generate the antisense *XAL2* probe using as a template the XAL2-F 5′-GTTTCCTCCTTCAAACTAACA-3′ and XAL2-R 5′-GCAACTGCTAAATTCAGTAAG-3′ amplified cDNA fragment cloned into p-GEM-T.

Quantitative Real-Time RT–PCR

Aerial tissue from three independent biological replicates (15 plants each) was used for total RNA extraction with Trizol reagent, and two independent cDNAs were reverse transcribed using Superscript II (Invitrogen). We amplified *PDF2* (AT1G13320) and *UPL7* (AT1G13320) as positive internal controls (Czechowski et al., 2005), and their stability across the compared samples was confirmed using geNorm (Vandesompele et al., 2002). Amplification efficiencies were analyzed using Real Time PCR Miner (Zhao and Fernald, 2005), and relative expression was calculated using the ΔΔCT method (Vandesompele et al., 2002). Primer sequences are presented in Supplemental Table 4.

Microscopy

An Olympus SZ60 dissecting microscope with C-5060 digital camera was used for light microscopy. Sectioned carpels were fixed in 4% paraformaldehyde, dehydrated in ethanol series, and embedded in paraffin. Sections (8 μm) were stained with toluidine blue 0.05%. For scanning electron microscopy, plant material was fixed at 4°C overnight in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde in 0.025 M phosphate buffer (pH 7.0). Samples were subsequently washed twice (30 min) in 70% ethanol in the same phosphate buffer, followed by 0.05 M phosphate buffer (pH 7.0). Samples were dehydrated gradually to ethanol 100%, and dried in liquid carbon dioxide at the critical point. Finally, samples were covered with gold using a sputter coater and observed with a scanning electron microscope.

TFL1 ChIP Assays

Wild-type and the *35S::GFP-XAL2* line were grown in MS plates under LD condition and inflorescence tissue (0.5 g) was fixed for 20 min. Chromatin was solubilized with a sonicator by three pulses of 15 s each. Immunoprecipitation was performed overnight using anti-GFP rabbit IgG fraction (A11122; Invitrogen) and protein A agarose beads (Santa Cruz). Samples were treated with proteinase K after elution followed by precipitation. Template ChIP DNA was diluted and amplified for 35–40 cycles (de Folter et al., 2007; de Folter, 2011). Primer pairs were designed in flanking regions of CArG boxes found along 2 kb upstream of the start codon, as well as 4.6 kb downstream of the *TFL1* gene (Supplemental Table 4).

GRN Model: Recovery of Gene Expression Profiles Characteristic of VM, IM, and FM Cell Types

The GRN was modeled using a discrete multi-state GRN formalism as described by Espinosa-Soto et al. (2004) and Álvarez-Buylla et al. (2010a, 2010b).

Stochastic GRN Model Implementation: EL Approach

To explore the patterns of cell-fate attainment and transition among cells, a discrete stochastic GRN dynamic model was implemented as an extension of the deterministic Boolean model described in the previous section. Stochasticity is modeled by introducing a constant probability of error for the deterministic Boolean logical functions according to:

$$x_i(t+1) = \begin{cases} f_i(t), & \text{with prob } 1-\xi \\ 1-f_i(t), & \text{with prob } \xi \end{cases}.$$

We followed Álvarez-Buylla et al. (2008). This approach yields a probability matrix that was then used to describe how the probability of being in a particular steady state changes in time by iterating the dynamic equation

$$p_x(t+1) = p_x(t)P,$$

where P is the transition probability matrix and $p_x(t)$ the distribution vector specifying the proportion of cells or the probability of a single one being in each steady state at a given time.

attractors is positive or negative. Red arrows represent the globally consistent ordering for the 3(4) attractors: the order of the attractors in which all individual transition has a positive net rate, resulting in a global probability flow across the EL as also shown in **(F)** (see Supplemental Methods).
(F) Schematic representation of the EL of the GRN modeled here. The relative barrier heights represent the hierarchy of calculated positive net probability rates, which altogether determine a consistent global ordering of the relative steady-state stabilities. According to the net probability rates, only one set of ordered transition (VM > IM > [IM/FM] > FM) produces a positive probability flow (see Supplemental Methods). As a result, a global developmental gradient in the EL is produced. Importantly this 2D representation is for illustrative purposes only and, as such, does not represent scales based on exact calculated values.

EL Exploration

To explore the EL associated with a GRN, the number, depth, width, and relative position of the GRN attractors are represented by the hills and valleys of Waddington's (EL) metaphor (Álvarez-Buylla et al., 2008). In addition to the calculation of the most probable temporal cell-fate pattern, a discrete stochastic GRN model allows calculations of the shortest and fastest pathways of cell-fate transitions, as well as possible restrictions of some cell-fate transitions that also emerge from the GRN topology and the associated EL. We calculated the mean first passage time (MFPT) between each pair of possible transitions to uncover which of these is more feasible. MFPT was estimated numerically by using the transition probabilities among steady states from a large number of samples of paths simulated as a finite Markov chain process (Wilkinson, 2011). The MFPT from one steady state (i) to another (j) corresponds to the average value of the number of steps taken to visit attractor j for the first time, given that the entire probability mass was initially localized at steady state i. The average is taken over a large number of realizations (simulations). Based on the MFPT values, a net transition rate between steady states i and j can be defined as follows: $d_{i\to j} = 1/\mathrm{MFPT}_{i\to j} - 1/\mathrm{MFPT}_{j\to i}$. This quantity effectively measures the facility by which a state transits from one state to another as a net probability flow (Zhou et al., 2014). For all stochastic modeling, robustness was assessed by comparing three different values for the error probability (0.01, 0.05, 0.1). The number of simulated samples was increased until stable results were attained. See also Supplemental Methods.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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7. DISCUSION

En este trabajo se describe el papel que tiene el gen *XAL2 / AGL14* durante el desarrollo del meristemo apical aéreo, este gen pertenece a la familia de factores transcripcionales MADS-box que llevan a cabo diversas funciones muy importantes en el desarrollo de Arabidopsis (Coen y Meyerowitz, 1991; Honma y Goto, 2001; Ng y Yanofsky, 2001; Yu et al., 2002; Parenicova et al., 2003; Martínez-Castilla y Alvarez-Buylla, 2003; Tapia-López et al., 2008; Smaczniak et al., 2012, Garay-Arroyo et al., 2013).

XAL2 pertenece al mismo clado que *AGL19*, *SOC1* / *AGL20*, *AGL42*, *AGL71* y *AGL72* y comparte un 70% de similitud con *AGL19*, el gen más cercano y un 64% de similitud con *SOC1* (Alvarez-Buylla et al., 2000; Martínez-Castilla et al., 2003; Parenicova et al., 2003Dorca-Fornell et al., 2011). Todos los miembros del clado se han caracterizado y se han descrito las funciones que llevan a cabo durante el desarrollo. Por ejemplo, *SOC1* es un gen que se ha estudiado con detalle y se sabe que participa en la transición a la floración en respuesta a señales ambientales e intrínsecas de la planta como la estado de desarrollo, etc. por lo que se le ha considerado un integrador de las diferentes vías señalización (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000; Lee y Lee, 2010).

En el caso de *AGL19* se ha observado mediante líneas mutantes para este gen, que las plantas presentan un retraso en su tiempo de floración en condiciones no inductivas de luz, después de haber sido vernalizadas, y su expresión es independiente de la represión de *FLC* por vernalización (Schönrock et al., 2006). En el caso de los genes *AGL42*, *AGL71* y *AGL72* ahora se sabe que participan de forma redundante en la transición floral al inducir genes que participan en la biosíntesis de giberelinas como *GA20OX1* y *GA20OX2* (Dorca-Fornell et al., 2011). Se ha demostrado por otros grupos de investigación que todos los genes el clado donde se ubica *XAL2* / *AGL14* participan en la transición a la floración (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000; Schönrock et al., 2006; Lee y Lee, 2010; Dorca-Fornell et al., 2011).

Mas allá del análisis de las relaciones filogenéticas para llevar a cabo este trabajo, nos centramos en el hecho de que los fenotipos descritos en la literatura de floración tardía tanto en día largo como día corto de los mutantes de *SOC1* y *AGL24*, y que sus líneas de sobre-expresión además de ser de floración temprana sus flores presentan caracteres vegetativos (Yu et al., 2004; Liu et al., 2007) fenotipos similares a los observados para los mutantes y sobre-expresión de *XAL2,* respectivamente. Además, tanto SOC1 como AGL24 y FUL interaccionan en ensayos de doble híbrido con XAL2, por lo que quisimos averiguar que tipo de relaciones genéticas y transcripcionales guardaban entre estos genes. Primeramente se hicieron los análisis floración bajo diferentes fondos genéticos.

7.1 *XAL2* es un activador de la floración.

En este trabajo se analizaron dos alelos mutantes para el gen *XAL2* / *AGL14*, utilizando diversas condiciones de crecimiento con el fin de determinar en que vía genética de transición a la floración participa este gen y se encontró que los alelos *xal2-1* y *xal2-2*, son de floración tardía en ambas condiciones del fotoperiodo (Tabla 1 material suplementario). Encontrándose que el mayor retraso en floración lo presenta *xal2-2* en el fotoperiodo de día corto aún más que *soc1-6* (Figura 1C). Mientras que la doble mutante *xal2-2 soc1-6* presentan un efecto aditivo sobre el tiempo de floración con respecto a las plantas parentales, lo que sugiere que estos genes podrían estar actuando de manera independiente en esta condición.

En el caso de día largo sucede lo contrario porque *soc1-6* es la línea que presenta el mayor retraso con 10 días a diferencia de *xal2-2* que sólo se retrasa 3 días en comparación con las planta de tipo silvestre, en el caso de la doble mutante *xal2-2 soc1-6* el tiempo de floración es idéntico al tiempo que tarda en florecer *soc1-6,* lo que sugiere que en esta condición *SOC1* es epistático sobre *XAL2* (Figura 1B). Cabe mencionar que la determinación del tiempo de floración para *xal2-2, agl24-4* y *ful-7* fue más consistente utilizando el parámetro de días en que tarda en emerger el escapo a un centímetro de la roseta ya que con el parámetro de hojas de la roseta cuando el escapo tiene un

centímetro de longitud, por ejemplo en la condición de día largo estas mutantes se comportaban como la planta de tipo silvestre. Esto hace pensar que quizás *XAL2* tiene una participación importante en el desarrollo del meristemo de las hojas o en la organogénesis de estas, probablemente participando en la regulación de algún gen implicado en su desarrollo como podrían ser los genes *BLADE ON PETIOLE1* y 2 (*BOP1 y BOP2*), que codifican para proteínas que regulan la actividad meristemática durante la morfogénesis de las hojas (Ha et al., 2004; McKim et al., 2008).

En el caso de *soc1-6* el tiempo de floración puede ser explicado mediante los dos parámetros utilizados a diferencia de *XAL2*, aunque en la literatura el parámetro más ampliamente empleado para cuantificar el tiempo de floración es mediante la cuantificación de hojas de la roseta (Borner et al., 2000; Lee et al., 2000). Los reportes existentes con respecto al comportamiento de las mutantes de *SOC1* (Borner et al., 2000; Lee et al., 2000) van en el mismo sentido que los resultados obtenidos en este trabajo, sin embargo, las determinaciones no son idénticas lo cual pueden ser debido a diversos factores como por ejemplos la diferencia de los alelos utilizados así como a cambios sutiles en las condiciones de crecimiento como la luz, temperatura, humedad, etc. en las líneas utilizadas.

Para profundizar en el entendimiento sobre la posible regulación que *XAL2* pudiera estar ejerciendo en el desarrollo de las hojas, se sugiere explorar su participación en este proceso de desarrollo mediante la generación de mutantes múltiples entre *xal2-2* y mutantes de los genes *BOP1* y *BOP2*, así como el análisis de expresión utilizando construcciones tanto transcripcionales como traduccionales de estos genes en *xal2-2*. Otra posibilidad por explorarse puede ser mediante el empleo de microarreglos.

Con respecto al estudio de las mutantes *agl24-4* y *ful-7*, se determinó que *xal2-2* es epistático sobre estos genes en la condición de día corto (Figura 1C) Mientras que en la condición de día largo, existe una diferencia sutil pero significativa en la doble mutante *xal2-2 ful-7* con respecto a sus parentales, lo que sugiere que estos genes podría actuar independientemente en esta condición. En el caso de la doble mutante *xal2-2 agl24-4*

no se encontraron diferencias significativas entre esta mutante y las parentales.

Debido a que se conoce que las giberelinas juegan un papel muy importante en la transición a la floración (Ross et al., 1997; Telfer et al., 1997) y además las mutantes para *XAL2* presentan el mayor retraso en el fotoperiodo de día corto, en el que las giberelinas tienen un papel preponderante en esta condición, se probó la hipotesis de que *XAL2* participara en esta vía genética. Para esto se aplicó la hormona exógenamente y se encontró que las plantas mutantes para *XAL2* no se recuperan completamente como las plantas control cuando se les asperjó la hormona, es decir no florecen a los 50 días como lo hace la silvestre, sino que lo hacen a 56 días (Figura 1C y D).

XAL2 podría estar afectando la vía de síntesis o de señalización de las GAs, para descartar la primera, se analizó la expresión de dos genes de síntesis *GA20OX1* y *GA20OX2* y uno de degradación de las giberelinas activas *GA2OX1*. Los resultados obtenidos sugieren que *XAL2* induce ligeramente a los genes *GA20OX1* y *GA2OX1* lo que parece contradictorio debido a que al mismo tiempo que se sintetiza la hormona también se esta degradando, sin embargo estos resultados pueden interpretarse como un mecanismo de regulación por parte de *XAL2* el cual permite mantener la homeostasis de la hormona. Para el caso de los genes *AGL42*, *AGL71* y *AGL72* se sabe que participan de forma redundante en la transición floral al inducir a los genes *GA20OX1* y *GA20OX2* favoreciendo la biosíntesis de la hormona (Dorca-Fornell et al., 2011), los cuales son expresados tanto en el desarrollo vegetativo como en el reproductivo (Rieu et al., 2008). Por otra parte la expresión de *GA20OX1* es reprimida por *SOC1*, pero en el caso del gen *GA20OX2* solamente se reprime en el fondo *soc1 agl42* ami::*agl71-72*, sugiriendo que los genes del clado de *SOC1* son importantes para el control de la expresión de los genes de síntesis de giberelinas activas (Dorca-Fornell et al., 2011).

Con los resultados obtenidos en este trabajo, no podemos concluir cual es el papel de *XAL2* sobre los genes de biosíntesis de giberelinas, cabe aclarar que el metabolismo de dichas hormonas es muy complejo. Por lo tanto será interesante la generación de

mutantes múltiples con los genes del mismo clado de *XAL2* para tratar de esclarecer cual es su papel con respecto a estas hormonas vegetales, lo cual puede determinarse por análisis cuantitativos como el PCR en tiempo real o por estudios de cromatografía de gases acoplada a espectrometría de masas para cuantificar la hormona en cada caso.

En la condición de vernalización no hay ningún efecto en el tiempo de floración de *xal2-2*, ya que las plantas se comportan de la misma manera que la plantas de tipo silvestre (Tabla 1 material suplementario). Este resultado es interesante al compararlo con *AGL19* porque es el gen hermano de *XAL2* y para el cual se ha determinado que es inducido por vernalizacion (Schönrock et al., 2006). Esta pareja de genes posiblemente sea un ejemplo más, y que puede ser interesante de estudiar para profundizar sobre el efecto de la duplicación de genes con funciones diferentes, de manera similar a lo que ocurre con *FT* y *TFL1* que presentan funciones opuestas, pues el primero es un activador y el segundo un represor de la floración que al sufrir cambios sutiles a nivel genético generan fenotipos antagónicos (Kobayashi et al., 1999; Hanzawa et al., 2005; Hanano y Goto, 2001; Wickland y Hanzawa 2015). La duplicación de genes favorece la divergencia funcional permitiendo la aparición de nuevas funciones por medio de mutaciones que afectan el patrón de expresión o la secuencia a nivel proteico, lo cual permite versatilidad y adaptación a las plantas como se ha observado con los miembros de la familia de *TFL1* (Mimida et al., 2001; Hanzawa et al., 2005). Por lo tanto, sería interesante estudiar los promotores y las proteínas con quienes forman complejos *XAL2* y *AGL19*. Por ejemplo, AGL24 y SOC1 si participan en la vía de vernalización (Yu et al., 2002; Michaels et al., 2003; Moon et al., 2003), con lo cual podemos sugerir que en esta condición de crecimiento es probable que AGL19 pueda estar formando complejos con estas proteínas para llevar cabo su función en la transición a la floración.

Por otra parte se generaron diversas líneas 35S:*XAL2* (Figura suplementaria 2B) con el fin de determinar si este gen es suficiente para inducir la transición a la floración, y efectivamente observamos que las plantas transgénicas son de floración temprana en ambas condiciones de fotoperiodo y presentan pocas hojas de la roseta al compararse con las plantas de tipo silvestre (Figura 1E).

Los datos obtenidos del tiempo de floración, tanto de los mutantes como de las líneas de expresión ectópica de *XAL2*, sugieren que *XAL2* es un inductor de la floración que presenta el efecto más severo en la condición de fotoperiodo de día corto, sugiriendo que es un componente importante del sistema de regulación genética que participa en la transición a la floración y en el que todos los genes del clado de *SOC1* participan en este proceso de desarrollo. De estos resultados es importante la de generación de mutantes múltiples para ver efectos de redundancia funcional (Borner et al., 2000; Schönrock et al., 2006; Dorca-Fornell et al., 2011).

7.2 *XAL2* es parte del sistema de regulación genética que induce a *AP1.*

Para explicar si *XAL2* es un gen que participa en el sistema de regulación genética de *AP1* y *LFY*, lo cual era muy probable debido a los fenotipos de floración observados en los alelos mutantes y las líneas de sobre-expresión de *XAL2,* que sugerían fuertemente que estos genes estuvieran reprimidos e inducidos en estas líneas, respectivamente. En este trabajo se comprobó efectivamente esta posibilidad para el caso de *AP1*, mientras que para *LFY* sólo se encontró la inducción en la línea de sobre-expresión de *XAL2*, pero no la represión en la mutante (Figura 2A y 2B). Estos resultados sugieren que *XAL2* forma parte del sistema de regulación genética que regula a *AP1*, como también lo hace *AGL24* (Grandi et al., 2012). En el caso de *LFY* se sabe que *AGL24* y *SOC1* lo inducen directamente, pero los resultados obtenidos en este trabajo sugieren que *XAL2* no participa en el sistema de regulación genética de *LFY,* o al menos no fue posible determinarlo solamente con la mutante sencilla *xal2-2* debido, posiblemente, a la redundancia funcional o a que no es una mutante nula (Lee et al., 2008; Liu et al., 2008).

Nuestros datos indican que XAL2 es uno de los reguladores positivos de *SOC1* y *AGL24* y es regulado a su vez por CO bajo condiciones de fotoperiodo de día largo. Estos datos están sustentados además por los datos genéticos arriba mencionados. Sin embargo, los datos de la línea 35S:*XAL2* muestran que reprime tanto a *SOC1* como a *AGL24*, lo que sugiere que muy posiblemente el fenotipo de floración temprana observado debido a la sobre expresión de *AP1* y *LFY* es independiente de la expresión de estos genes (Figura 2C-D). Este tipo de regulaciones contra-intuitivas sólo se pueden explicar

mediante el uso de modelos de regulación *in sílico* como el utilizado en el artículo (fig 6)

Por otra parte se encontró que *XAL2* no es regulado por *AGL24* y *SOC1* a nivel transcripcional porque, en ninguno de los fondos mutantes analizados (*agl24-4* y *soc1-6*) se encontraron diferencias significativas en los niveles de expresión de *XAL2* con respecto a la planta silvestre (Figura 2F y 2G), sin embargo sí se observó una fuerte represión de *XAL2* en la línea de sobreexpresión de *SOC1,* pero no en la de sobreexpresión de *AGL24*, lo que sugiere que tanto en la línea de sobreexpresión de *SOC1* como en la línea de sobreexpresión de *XAL2*, el fenotipo de floración temprana observado es independiente entre los dos genes, pero no ocurre lo mismo cuando se trata de *AGL24* (Figura 2D y 2G). Estos resultados a nivel transcripcional sugieren una regulación importante entre estos genes los cuales forman parte del sistema de regulación genético que induce la transición a la floración. Una posibilidad para entender mejor las relaciones de regulación trancripcional sería el análisis de la expresión de los genes en el meristemos mediante líneas reporteras en diferentes fondos genéticos.

7.3 *XAL2* se expresa en el meristemo de inflorescencia y en el meristemo de flor.

En estudios anteriores llevados a cabo por otros grupos de investigación, se propuso que *XAL2* sólo se expresaba en la raíz (Rounsley et al., 1995; Schönrock et al, 2006). En este trabajo determinamos mediante hibridación *in situ* que *XAL2* se expresa en el meristemo de inflorescencia en la zona conocida como “anlagen” y en el meristemo de flor en los estadios 1 y 2, restringiéndose en el estado tres floral a las células que darán origen a los sépalos y a las células de las capas L1 y L2, mientras que para el estado seis floral la expresión se restringe a los meristemos que darán origen al carpelo y a los estambres (Figura 3A y 3B). Estos resultados demuestran que la expresión de *XAL2* no se limita a la raíz como se pensó en un principio ya que su expresión se presenta en diversos estados del meristemo. Los resultados también sugieren que *XAL2* puede estar participando en otros procesos de desarrollo aunque esta posibilidad queda por explorarse.

Al comparar los perfiles de expresión de *XAL2* se encontró que se expresa de manera similar a *AGL24* y *SVP* que se sabe reprimen la expresión de *SEP3* para evitar que se

formen los verticilos florales en etapas tempranas del meristemo de flor (debido a que no existe quien induzca a los genes florales de la clase B y C (*AP3*, *PI* y *AG*; Gregis et al., 2008, 2009; Liu et al., 2009). Por lo tanto, es lógico pensar que *XAL2* puede tener una participación como estos genes en dar la identidad al meristemo de flor y posiblemente su represión en el estado tres floral sea regulada por *AP1*, *LFY* y *CAL*, tal como ocurre para *AGL24* y *SVP*. Es necesario e interesante determinar la expresión de *XAL2* en los fondos mutantes de estos genes y generar mutantes múltiples con ellos para comprobar o descartar su participación a este nivel.

Por otra parte, recientemente se ha demostrado que *XAL2* juega un papel importante en el desarrollo de la raíz (Garay-Arroyo et al.,2013), en donde afecta el transporte de auxinas mediante la regulación transcripcional de *PIN1;* este resultado se obtuvo mediante el análisis con las mismas mutantes que se utilizaron en este trabajo y se demostró que su ausencia genera patrones celulares alterados en el nicho de células troncales en el meristemo apical de la raíz, además de afectar el tamaño del meristemo y el crecimiento de la raíz (Garay-Arroyo et al., 2013). También en posible que en ausencia de *XAL2* puedan existir alteraciones en el meristemo aéreo de manera similar a como ocurre en la raíz, sin embargo nosotros nos fuimos capaces de ver un efecto a este nivel en *xal2-2*, posiblemente debido a redundancia funcional, por lo que una posibilidad por explorar sería generar mutantes múltiples y llevar a cabo análisis celulares para determinar si existe desarreglo.

7.4 *XAL2* regula directamente a *TFL1*.

Un fenotipo evidente en la línea 35S:*XAL2* bajo condiciones de fotoperiodo de día largo es que los sépalos en las flores presentan características vegetativas, como por ejemplo que son de tamaño grande con tricomas compuestos (Figura 3D). Además, utilizando microscopía electrónica de barrido se encontró que en las plantas que expresan ectópicamente a *XAL2*, los sépalos tienen un tipo celular que se parece mas al de las hojas de tipo silvestre (Figura 3E-3G) en vez de tener arreglos celulares de células alargadas como se observa en los sépalos de las plantas silvestres (Figura 3E). Este fenotipo se parece a las plantas mutantes *ap1* (Irish y Sussex, 1990; Bowman et al.,

1993) y sin embargo como ya se mencionó anteriormente *AP1* está sobreexpresado. Esta aparente contradicción puede conciliarse de la siguiente manera: en el caso de la mutante de *ap1-1* no hay quien reprima a *TFL1,* el gen que da identidad a las células de inflorescencia, provocando el fenotipo de características vegetativas en el meristemo de flor, mientras que en la línea *35S:XAL2* a pesar de que se encuentra *AP1* inducido, la expresión ectópica de XAL2 es capaz de inducir *TFL1*, responsable de mantener las características vegetativas lo cual explicaría en parte los fenotipos observados.

Con esta hipótesis y para tratar de entender a nivel molecular si *XAL2* es capaz de desregular a *TFL1* para permitir que se presenten las características vegetativas, se analizó la expresión de este gen en la línea de expresión ectópica de *XAL2*. En este trabajo encontramos que *TFL1* es inducido de 2 a 4 veces cuando se sobreexpresa no sólo *XAL2,* sino también *SOC1* y *AGL24* como era de esperarse (Figura 4A y 4C). Esto sugiere que la desregulación llevada a cabo por estos tres genes MADS-box puede modificar la identidad del meristemo floral y que los fenotipos observados en las tres líneas probadas puedan ser posiblemente explicados debido a la expresión ectópica de *TFL1*.

Adicionalmente se determinó que la inducción de *TFL1* por la expresión ectópica de *XAL2* es directa ya que los experimentos de immunoprecipitación de la cromatina permitieron establecer que *XAL2* es capaz de unirse directamente a tres fragmentos de la región reguladora de *TFL1* (Figura 4B), interesantemente el fragmento 5 en nuestro estudio es también blanco de la represión por AP1 (Kaufmann et al., 2010). Se ha demostrado que tanto la línea 35S:*AP1* como la línea *tfl1* presentan fenotipos similares, esto es debido a que en el caso de la línea de expresión ectópica de *AP1* se reprime a *TFL1*, dando como consecuencia que la planta se comporte como si se tratara de *tfl1* (Liljegren et al., 1999). Integrando estas evidencias a nuestros resultados, se puede sugerir un mecanismo de regulación entre *XAL2* y *AP1* sobre *TFL1,* en otras palabras mientras que la sobreexpresión del primero lo induce, la sobreexpresión de *AP1* lo reprime (Kaufmann et al., 2010), esto abre la posibilidad de que haya una competencia por estas cajas de regulación y que esto sea parte de un mecanismo de regulación fina que permita la transición hacia el meristemo de flor.

Para tratar de entender a nivel genético si *AP1* y *XAL2* son capaces de regular a *TFL1* de forma contraria, es decir mientras *AP1* lo reprime (Kaufmann et al., 2010) *XAL2* lo induce, se generó la doble línea sobreexpresora 35S:*XAL2* 35S:*AP1*, en la cual como era de esperarse se presenta una complementación parcial de ambos fenotipos (Figura 5A-C), por ejemplo el tiempo de floración en la doble sobreexpresora es intermedio al de las líneas parentales (Figura 5G) y además el número de carpelos indeterminados disminuye a 1 en comparación con la línea parental 35S:*XAL2* que generaba 5 (Figura 5H).

Cabe la posibilidad que cuando *XAL2* se expresa ectópicamente otros genes que dan identidad al meristemo de inflorescencia podrían ser desregulados adicionalmente como ocurre con *TFL1* en el meristemo de flor. Para obtener más información que ayude a comprender a nivel molecular que está ocurriendo en plantas que expresan ectópicamente a *XAL2*, sería interesante realizar análisis de microarreglos en estas líneas, y con los datos obtenidos mediante la metodología de inmunoprecipitación de la cromatina determinar si los genes afectados son regulados directamente por *XAL2*, esto permitirá entender mejor el comportamiento del desarrollo en estas líneas de expresión ectópica.

7.5 La sobreexpresión de *XAL2* afecta la determinación del meristemo de flor en condiciones de fotoperiodo de día corto.

Al crecer las plantas que sobreexpresan a *XAL2* en condiciones de fotoperiodo de día corto se observó el fenotipo de indeterminación del meristemo de flor que se ilustra en las figuras 3H, 3J y 3K el cual consiste en el desarrollo de inflorescencias dentro de carpelos. Con el fin de entender que estaba sucediendo molecularmente, se decidió analizar la expresión de *WUS* porque es el gen que mantiene el nicho de células troncales (Laux et al., 1996; Schoof et al., 2000). Cuando *WUS* esta activo existe un abastecimiento de células indiferenciadas con capacidad para formar los órganos que la planta requiere, sin embargo después del estado seis floral ocurre el agotamiento del nicho de células troncales debido a que *WUS* es apagado por la acción directa de AG, o indirectamente, al inducir a *KNU* que también lo reprime, y con lo cual el meristemo

floral se agota y la flor se determina al formar un número estable de órganos florales (Payne et al., 2004; Prunet et al., 2008; Lenhard et al., 2011). Estos antecedentes hacen suponer que en la líneas de sobreexpresión de *XAL2* se desregula a *WUS*, encontrándose que efectivamente existe una fuerte inducción de la expresión de *WUS* más allá del estado seis de desarrollo floral, en donde normalmente se encuentra apagado. Los resultados obtenidos demuestran que la expresión ectópica de *XAL2* es capaz de alterar la regulación del nicho de células troncales vía la activación de WUS ya sea por su inducción directa o por una vía indirecta reprimiendo a *KNU* u otro de sus represores (Schoof et al., 2000; Müller et al., 2008). Sería muy interesante comenzar por hacer la inmunoprecipitación de la cromatina de *WUS* utilizando la línea 35S:GFP-*XAL2*, aunque lo ideal sería generar la construcción de *XAL2* bajo su propio promotor para llevar a cabo este estudio. Por otra parte, el efecto fenotípico observado en estas líneas de sobreexpresión con los datos que obtuvimos en este trabajo no se puede explicar categóricamente si se debe a una ganancia de función o a un efecto dominante negativo. Para tratar de contestar esta pregunta propongo utilizar una versión con la proteína truncada de XAL2 por eliminaciones bajo un promotor constitutivo, esto sería un acercamiento para elucidar si el fenotipo en las plantas 35S:*XAL2* es debido a activación transcripcional o a un efecto dominante negativo.

7.6 Valoración de la reversión floral en plantas 35S:*XAL2*.

Con los datos obtenidos experimentalmente en este trabajo y mediante la incorporación de los datos de regulación para *CO*, *TFL1*, *FD*, *FT*, *LFY* y *AP1* así como de las hormonas giberelinas que otros grupos de investigación han determinado (Tabla 2, material suplementario), se realizó un modelo que estableciera las regulaciones genéticas en las que participa XAL2 y que permitiera dar una explicación mecanicista de lo fenotipos de “reversión floral” que se presentan en las líneas que sobreexpresan *XAL2*, *AGL24* y *SOC1.*

Con base en lo anterior se estableció el sistema de regulación genética que favorece el establecimiento de los meristemos: vegetativo, de inflorescencia y de flor con los mismos genes, sin embargo la configuración del sistema de regulación genética es el

que cambia para dar origen a cada uno de los meristemos, en otras palabras los mismos genes pueden estar inducidos o reprimidos dependiendo del meristemo que se trate. El modelaje permitió simular la interacción entre el grupo de genes que lo integran y se establecieron los estados estacionarios en cada uno de los meristemos en las diferentes líneas utilizadas.

Como validación de este modelo se encontró que las plantas mutantes presentan los mismos estados estacionarios que se presentan en la planta de tipo silvestre, ya que el modelo permitió establecer tres atractores o sumideros que equivalen a los tres tipos de meristemo (vegetativo, inflorescencia y flor). Asimismo, en el caso de las líneas de sobreexpresión el modelo permitió establecer un cuarto estado estacionario mixto (MI/MF) que ayuda a explicar el comportamiento del sistema de regulación genético en las plantas sobreexpresantes para *XAL2*, *SOC1* y *AGL24.*

Puede pensarse que la configuración del sistema de regulación genética que da identidad al meristemo de inflorescencia no se apaga completamente en el meristemo de flor y que en algunas células se mantenga encendido, de tal manera que el meristemo que debería ser sólo de flor se comporte como una quimera con los dos tipos de identidad celular, debido a que los dos sistemas de regulación genética se encuentran presentes generando una mezcla de las identidades de los meristemos de inflorescencia y de flor (Figura 6). Esto no ocurre en la planta silvestre en la que la configuración del meristemo de inflorescencia se apaga en su totalidad para dar lugar al establecimiento de la configuración del sistema de regulación genético que establece el meristemo floral.

La pregunta seguía siendo si como estaba descrito en la literatura esto se debía a una reversión floral es decir reprogramación de células diferenciadas de sépalo que se convirtieran en células tipo hoja o dicho en otros términos que el meristemo de flor adquiriera ciertas propiedades del meristemo de inflorescencia (Battey y Lyndon, 1990; Tooke et al., 2005). Este fenómeno en Arabidopsis sólo se ha visto en mutantes de identidad del meristemo floral y otros grupos y el nuestro (Yu et al., 2004; Liu et al., 2007;) ya habíamos demostrado que en las líneas de sobre expresión de *XAL2*, *SOC1*

y *AGL24*, los niveles de expresión de *LFY* y *AP1* también se encuentran incrementados. Por lo que recurrimos a hacer el modelamiento del paisaje epigenético de las líneas de sobreexpresión de *XAL2*, *AGL24* y *SOC1* y se pudo analizar el comportamiento de un grupo de células y no sólo de una célula como en la red de regulación genética.

En este trabajo el fenotipo de flores con rasgos vegetativos puede ser explicado de dos maneras: 1.- La primera posibilidad es que una parte de las células con características de inflorescencia adquiera características del meristemo de flor, de tal forma que el conjunto de células que deberían de comportarse sólo como meristemo de flor, se comporta como una quimera por contar con ambos tipos de meristemo. 2.- La segunda posibilidad es que una parte del meristemo de flor regrese a una etapa anterior en el desarrollo para formar el meristemo quimérico con características duales, esta posibilidad es el equivalente a una reversión o reprogramación. Sin embargo la simulación del paisaje epigenético de las líneas analizadas sugiere que la primera opción es la más posible, debido a que la probabilidad de que se establezca presenta tasa positiva a diferencia de la reversión, ya que para que esta ocurra la probabilidad es más baja debido a su tasa negativa (Figura 7). Por lo tanto, el término de reversión floral utilizado en la literatura no se acopla para el caso de la sobreexpresión de *XAL2*, *AGL24* y *SOC1* aunque no se descarta la posibilidad de que otros casos, incluso la indeterminación que observamos en los carpelos de las líneas de sobre expresión de *XAL2*, *SOC1* y *AGL24* y sus homólogos pueden ser explicados por la reversión o reprogramación de algunas células diferenciadas dentro de los carpelos. Sin embargo, se requiere de análisis celulares durante todos los estadíos de desarrollo anteriores a la emergencia de la inflorescencia para saber de donde vienen las células troncales. Así mismo, para que este tipo de modelos pueda explicar otros fenómenos como la indeterminación que se genera en la condición de día corto se deben de integrar otros actores moleculares, quizás para los cuales aún no se tengan todas las interacciones posibles así como considerar a diversos factores externos que afectan a la planta (como el fotoperiodo de día corto, temperatura etc.).

Por lo tanto es posible que en la misma línea de sobre expresión de *XAL2* durante diferentes estados de desarrollo y en respuesta a diferentes condiciones ambientales pueden estar ocurriendo diferentes procesos de (des)diferenciación, pero esto es una hipótesis que queda por probar.

8. CONCLUSIONES.

8.1. *XAL2* es necesario y suficiente para inducir la floración. Los mutantes en este gen presentan floración tardía en respuesta a las condiciones de fotoperiodo de día largo y corto, siendo en está última donde se observa un fenotipo más fuerte.

8.2. *SOC1* es epistático a *XAL2* en la condición de fotoperiodo de día largo, mientras que *XAL2 y SOC1* participan de forma aditiva en el fotoperiodo de día corto y en la respuesta a giberelinas.

8.3. XAL2 es parte de la red de regulación genética que induce a *AP1*. El análisis de expresión al comparar el mutante *xal2-2* con las plantas silvestre reveló que *XAL2* es un regulador positivo de *SOC1 y AGL24*. Sin embargo la sobre expresión de XAL2 reprime a *SOC1* y a *AGL24.* De igual forma que la sobre expresión de *SOC1* reprime a XAL2. Adicionalmente *XAL2* es inducido por *CO* en el fotoperiodo de día largo.

8.4. *XAL2* se expresa en los meristemos de inflorescencia y en el de flor, desde la formación del anlagen los estados 1 y 2 del desarrollo del meristemo de flor, restringiendo su expresión a las capa L1 y L2 en el estado 3 floral, mientras que el estado 6 floral su expresión se limita a los estambres y carpelos.

8.5. La expresión ectópica de *XAL2* induce directamente a *TFL1,* uniéndose directamente a las secuencias reguladoras de éste.

8.6. La doble línea sobreexpresora 35S:*XAL2* 35S:*AP1* complementa parcialmente los fenotipos de las líneas parentales, lo que sugiere un mecanismo de regulación de *AP1* y *XAL2* sobre *TFL1*.

8.7. La expresión ectópica de *XAL2* es capaz de mantener la expresión de *WUS* después del estadío 6 floral, sugiriendo que esta es la razón de la indeterminación del meristemo de flor en esta línea, en las condiciones de fotoperiodo de día corto.

8.8. Con base en el modelo de la red de regulación genética, y junto con la simulación del paisaje epigenético, proponemos una explicación mecanicista de los fenotipos observados de floración temprana y flores con caracteres vegetativos en las líneas de sobre expresión de *XAL2*, *SOC1* y *AGL24*, en la que las transiciones sucesivas, y una de esas transiciones, se presenta un estado adicional que resulta de la mezcla de la identidad de los dos meristemos MI/MF y no a un retorno a fases de desarrollo anteriores del MF al MI como sugiere la reversión floral.

9. PERSPECTIVAS.

9.1. Generación de mutantes múltiples entre *xal2-2* con mutantes que participan en el desarrollo de la hojas pueden dar información sobre el posible papel que *XAL2* puede estar llevando a cabo en estos órganos. Otra posibilidad para entender sobre este papel es mediante el empleo de construcciones con genes reporteros de esos genes y analizarlos bajo el fondo *xal2-2*. Finalmente microarreglos en hojas así como análisis de ChIP también pueden ser ejecutados.

9.2. Generación de mutantes múltiples de *xal2-2* con las mutantes de su clado para evitar la redundancia funcional y determinar de manera clara el efecto que pudiera tener en la regulación de las giberelinas.

9.3. Realizar mutaciones sitio dirigidas en las secuencias de *AGL14* para estudiar la divergencia funcional con *AGL19* para tratar de entender que los hace diferentes.

9.4. Obtener la triple mutante *xal2 soc1 agl24* para estudiar la participación de *XAL2* en la determinación del meristemo de flor.

9.5 Estudiar el arreglo celular del meristemo apical aéreo para determinar si *XAL2* juega un papel importante a este nivel como lo hace en la raíz, mediante construcciones con genes reporteros.

9.6. Realizar microarreglos de botones florales con potencialidad de indeterminarse con el fin de encontrar a nivel global que genes se desregulan en el fondo 35S:*XAL2* y mediante la metodología de ChIP determinar si son regulados directamente por *XAL2*.

9.7. Ampliar la capacidad de respuesta de los modelos utilizados en este trabajo mediante la integración de más actores moleculares para tener la posibilidad de explicar diversos procesos de desarrollo, porque como se ha visto los mismos genes actúan en varios procesos.

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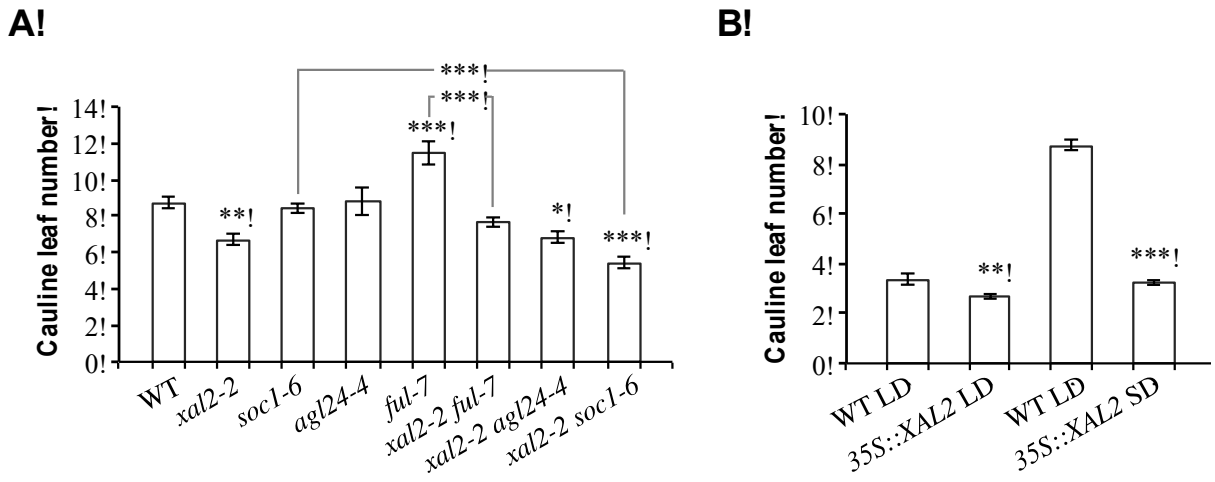
ANEXO A

Material suplementario del artículo:

Pérez-Ruiz R.V., García-Ponce B., Marsch-Martínez N., Ugartechea-Chirino Y., Villajuana-Bonequi M., de Folter S., Azpeitia E., Dávila-Velderrain J., Cruz-Sánchez D., Garay- Arroyo A., de la Paz Sánchez M., Estévez-Palmas J.M., and Alvarez-Buylla E.R. (2015). *XAANTAL2* (*AGL14*) is an important component of the complex gene regulatory network that underlies Arabidopsis shoot apical meristem transitions. *Molecular Plant*. 8:796-813.

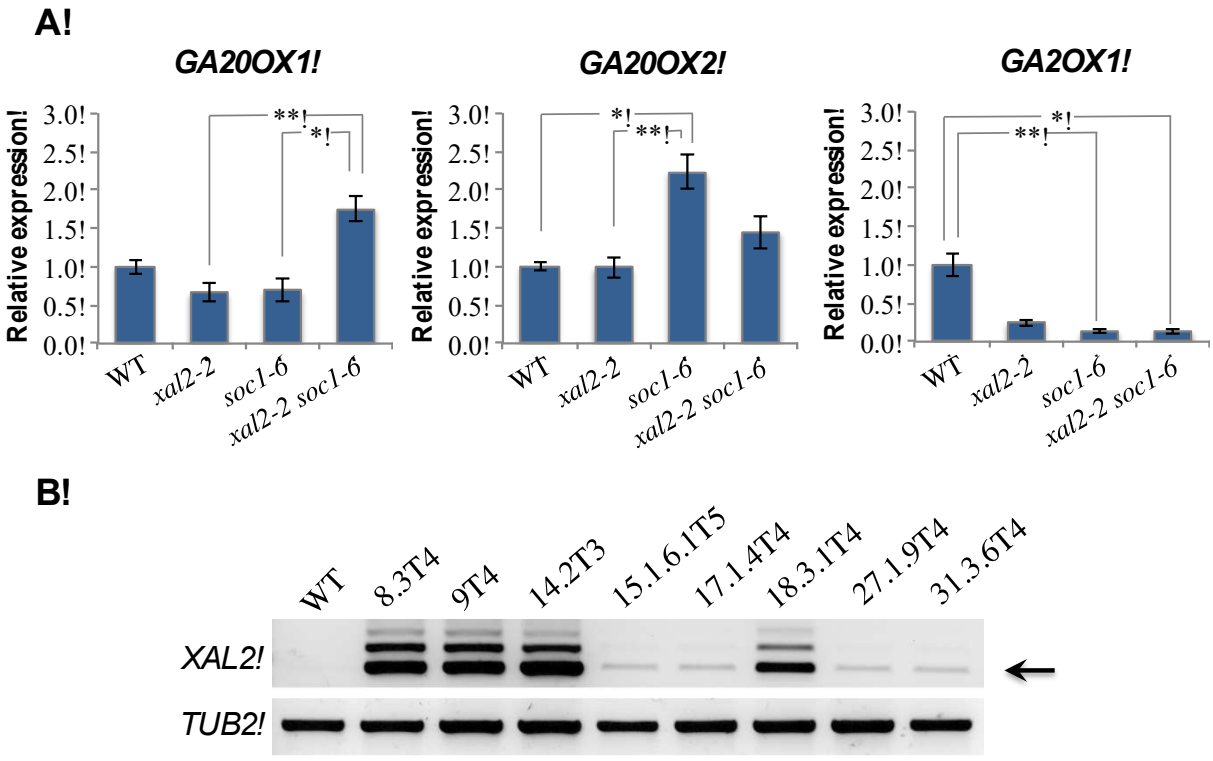


Supplemental Figure 1 Cauline leaf number is diminished in *XAL2* mutants and the overexpression line.!

(A) *xal2-2* develops fewer cauline leaves than WT plants and it is epistatic in double mutants under SD condition.!

(B) *XAL2* overpression line presents fewer cauline leaves than WT plants under both LD and SD conditions. !

Bars represent Standard Error from the average. Significant differences *p < 0.05, **p < 0.01 and ***p < 0.001 were obtained by Tukey's Multiple Comparison Test (A) or Student Test (B), respectively. !

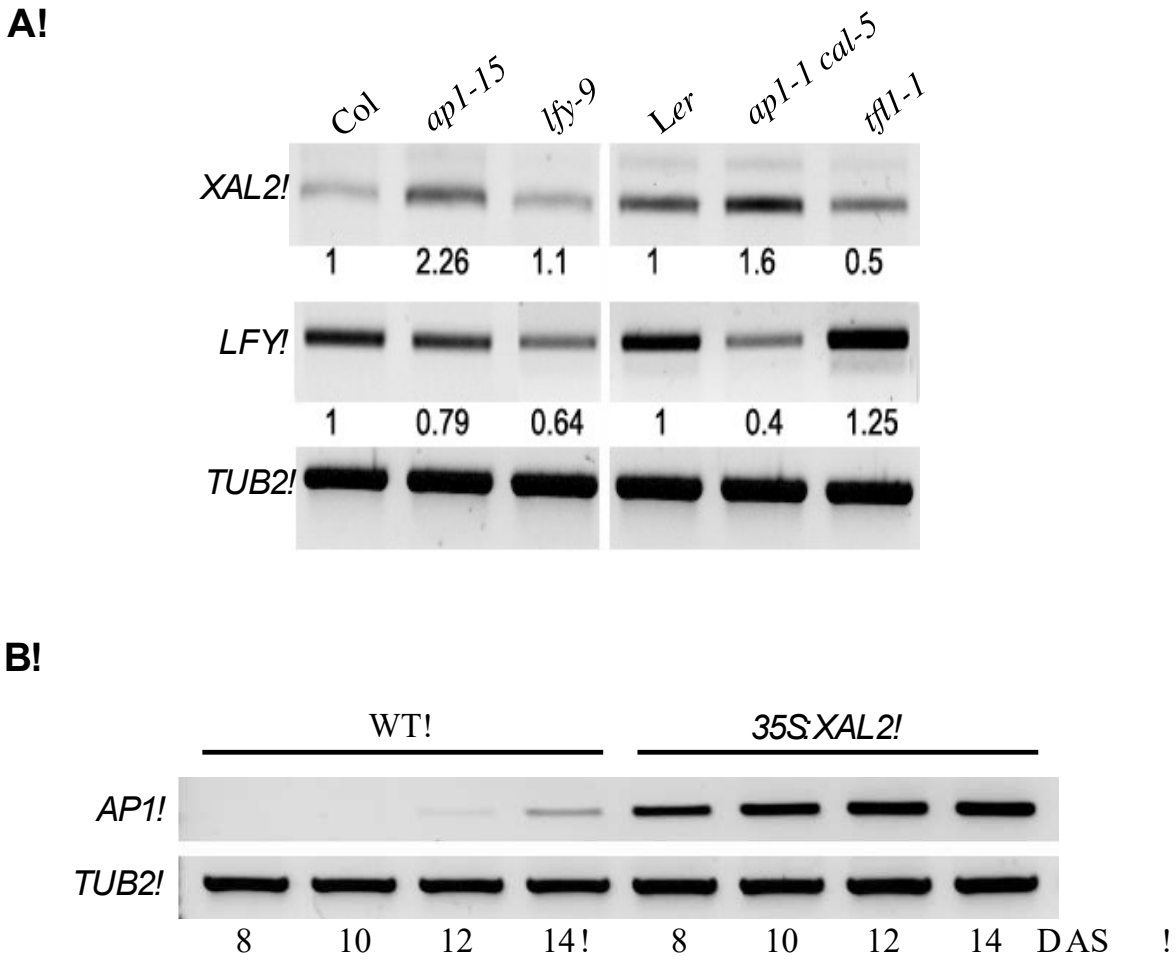


Supplemental Figure 2 Regulation of some GA metabolic genes by XAL2 and transcript accumulation of this gene in several overexpression lines. !

(A) RT-qPCR of GA biosynthetic genes *GA20OX1* and *GA20OX2*, showed that they are not regulated by XAL2, however, up-regulation of both genes is observed in the *xal2-2 soc1-6* double mutant compared to WT plants. The catabolic gene *GA2OX1* relative expression is lower in all mutants compared to WT. Bars represent Standard Error from the average. Statistical differences *p < 0.05, **p < 0.01 were obtained by Mann Whitney test. !

(B) Several independent *XAL2* overexpression lines, from which 9T4 was selected for the flowering and RT-qPCR assays presented in this work as *35S::XAL2. TUBULIN2* (*TUB2*) was used as a loading control control for this experiment.!

!

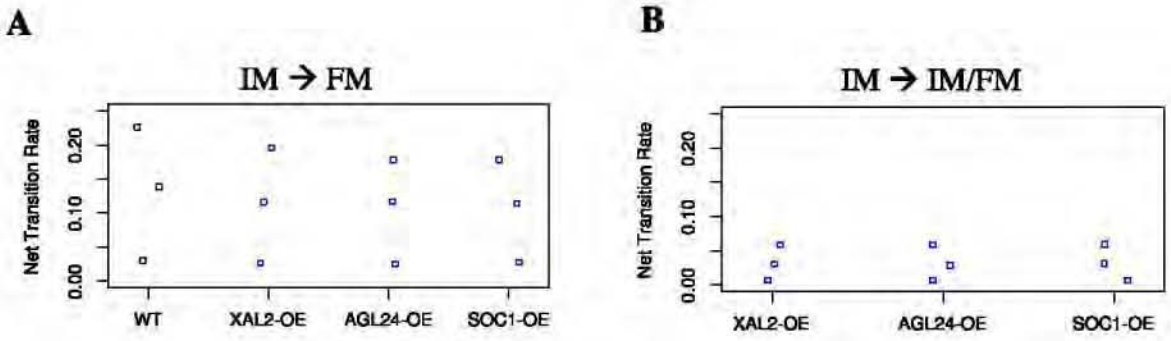


Supplemental Figure 3 *XAL2* is sufficient to induce *AP1* and the latter partially represses the former. !

(A) Comparative RT-PCR analysis show that *XAL2* is up-regulated in the *ap1-15* and *ap1-1 cal-5* plants and it is down-regulated in the *tfl1-1* mutant in which *LFY* and *AP1* are ectopically expressed in the IM. *LFY* pattern of expression is the opposite to that of *XAL2*. Numbers reflect relative mRNA accumulation compared to Columbia (Col) or Landsberg (L*er*) WT plants. !

(B) *AP1* is up-regulated in the *XAL2* overexpression line and its mRNA accumulation levels are maintained along flowering transition. Days after sowing (DAS). !

TUBULIN2 (*TUB2)* was used as a loading control.!



Supplemental Figure 4 Net transitions rates among steady states.

(A) Calculated net transition rates from IM to FM in the *XAL2- SOC1-* and *AGL24-* overexpressors (OE) compared to WT.

(B) Net transition rates from IM to IM/FM for the *XAL2- SOC1-* and *AGL24-* OE lines.

The net transition rate between IM and FM is positive in the direction IM > FM (A), and it is lower in the OE-lines. The net transition rate between IM and IM/FM states in the three OE lines (B), although positive, is an order of magnitude lower than the rate from IM to FM (A).

For each condition, each square corresponds to the value calculated taking a specific level of error probability: bottom (0.01), middle (0.05), and top (0.1) squares.

Supplemental Table 1 Comparative bolting time of *xal2-1* and *xal2-2* alleles versus wild-type (WT) plants, quantified as days after sowing (DAS) and rosette leaf number (RLN) at different growing conditions.

Plant Line	LD	LD + VER	SD	SD + GA_3
WT	36.03 ± 0.45 DAS (n=30) 14.5 ± 0.41 RLN (n=28)	25.59 ± 0.56 DAS (n=27) 7.41 ± 0.15 RLN (n=27)	70.68 ± 0.87 DAS (n=34) 52.06 ± 1.07 RLN (n=34)	46.79 ± 0.44 DAS (n=24) 25.04 ± 0.48 RLN (n=24)
xal2-1	39.43 ± 0.44*** DAS (n=30) 15.3 ± 0.26 RLN (n=30)	25.83 ± 0.61 DAS (n=30) 8.41 ± 0.34 RLN (n=29)	87.77 ± 1.24*** DAS (n=35) 61.08 ± 0.94*** RLN (n=35)	50.68 ± 0.46*** DAS (n=25) 26.8 ± 0.8 RLN (n=25)
xal2-2	40.13 ± 0.44*** DAS (n=32) 15.28 ± 0.41 RLN (n=32)	25.96 ± 0.65 DAS (n=29) 8.57 ± 0.49 RLN (n=28)		

Data is expressed as mean ± standard error. Student Test significant differences from the WT under the same growth condition at *$p < 0.001$.

Supplemental Table 2 GRN information used to construct the model

GA self-regulation

GA active hormones increased just before flowering transition and play and important role during this process (Wilson *et al*., 1992; Putterill *et al*., 1995; Blázquez *et al*., 1997, 1998; Blázquez & Weigel, 2000; Moon *et al*., 2003; Porri *et al*., 2012). However, in our model none of the nodes of the GRN regulates GA metabolism. Thus in order to obtain different stable values of GA expression we included GA self-regulation, although is worth notice that this interaction does implicate that GA is actually positively self-regulated.

***CO* self-regulation:**

In this model CO autorregulates itself as an entrance because their known regulators are not included here.

***FT* regulators:**

CO ✎ FT (Kobayashi *et al*., 1999; Kardailsky *et al*., 1999; Wigge *et al*., 2005; Yoo *et al*., 2005; Corbesier *et al*., 2007; Jang *et al*., 2009)

GAs ✎ FT (Hisamatsu and King, 2008; Porri *et al*., 2012)

AP1 --| FT/FD (Kaufman *et al*., 2010)

TFL1/FD --| FT/FD (Jaeger *et al*., 2013)

SOC1 regulators:

GAs ✎ SOC1 (Moon *et al*., 2003; Liu *et al*., 2008)

FT ✎ SOC1 (Yoo *et al*., 2005; Searle *et al*., 2006)

AGL24 ✎ SOC1 (Liu *et al*., 2008)

SOC1 ✎SOC1 (Immink *et al*., 2012; Tao *et al*., 2012)

XAL2 ✎ SOC1 (this work)

XAL2 overexpression --| SOC1 (this work)

AP1 --| SOC1 (Liu *et al*., 2007; Gregis, *et al*., 2009; Kaufmann *et al*., 2010)

AGL24 regulators:

GAs ✎ AGL24 (Yu *et al*., 2002; Liu *et al*., 2008)

CO ✎ AGL24 (Yu *et al*., 2002)

SOC1 ✎ AGL24 (Yu *et al*., 2002; Liu *et al*., 2008)

XAL2 ✎ AGL24 (this work)

XAL2 overexpression --| SOC1 (this work)

LFY --| AGL 24 (Yu *et al*., 2004; Grandi *et al*., 2012)

AP1 --| AGL 24 (Yu *et al*., 2004; Liu *et al*., 2007; Kaufmann *et al*., 2010 S10c)

***LFY* regulators:**

GAs ✎ LFY (Blázquez *et al*., 1998; Blázquez and Weigel, 2000; Gocal *et al*., 2001; Eriksson *et al*., 2006)

SOC1 ✎ LFY (Moon *et al*., 2005; Lee *et al*., 2008; Liu *et al*., 2008)

AGL24 ✎ LFY (Yu *et al*., 2002; Lee *et al*., 2008; Grandi *et al*., 2012)

AP1 ✎ LFY (Liljegren *et al*., 1999; Ferrandiz *et al*., 2000; Kaufmann *et al*., 2010; Benlloch *et al*., 2011)

XAL2 ✎ LFY (this work)

TFL1 --| LFY (Ratcliffe *et al*., 1998, 1999)

***AP1* regulators:**

LFY ✎ AP1 **(**Parcy, *et al*.,1998; Liljegren *et al*.,1999; Wagner *et al*., 1999; William *et al*., 2004; Benlloch *et al*., 2011).

FT/FD ✎ AP1 (Abe *et al*., 2005; Wigge *et al*., 2005)

AGL24 ✎ AP1 (Grandi *et al*., 2012)

AP1 ✎ AP1. AP1 probably mantains its own expression by down-regulated TARGET OF EAT1 (TOE1), TOE3 and SCHNARCHZAPFEN (SNZ) factors that act redundantly to prevent flowering in part by directly repressing AP1 (Mathieu *et al*., PLoS Biol. 7, e1000148, 2009; Kaufmann *et al*., 2010) .

XAL2 ✎ AP1 (this work)

TFL1 --| AP1 (Gustafson-Brown, *et al*.,1994; Ratcliffe *et al*., 1998 & 1999)

***TFL1* regulators:**

XAL2 ✎ TFL1 (this work)

Overexpression of SOC1 ✎ TFL1 (this work)

Overexpression of AGL24 ✎ TFL1 (this work

AGL24 --| TFL1 (Liu *et al*., 2013)

SOC1 --| TFL1 (Liu *et al*., 2013)

LFY --| TFL1 (Ratcliffe *et al*., 1999)

AP1 --| TFL1 (Liljegren *et al*., 1999; Ferrandiz *et al*., 2000; Kaufmann *et al*., 2010; Liu *et al*., 2013)

FT/FD --| TFL1/FD (Jaeger *et al*., 2013)

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Supplemental Table 3 Network's dynamic functions.

LFY(t+1) = AP1(t) or (GA(t) > 0 or (SOC1(t) and AGL24(t))) and not TFL1(t) or (XAL2(t)=2 or SOC1(t)=2 or AGL24(t)=2) and GA(t) > 0

TFL1(t+1) = (XAL2(t) and not AGL24(t) and not SOC1(t) and not AP1(t) or not AP1(t) and (not LFY(t) or not FT(t))) or (XAL2(t)=2 or SOC1(t)=2 or AGL24(t)=2) and not FT(t)

GA(t+1) = GA(t)

FT(t+1) = CO(t) or GA(t) > 0 and not TFL1(t) and not AP1(t)

SOC1(t+1) = (not AP1(t) and XAL2(t)=1 and AGL24(t) > 0 and SOC1(t) > 0 or GA(t) > 0 or FT(t) > 0) and XAL2(t) < 2

AGL24(t+1) = ((SOC1(t) > 0 and XAL2(t)=1 and not LFY(t) and not AP1(t)) or GA(t) > 0 or CO(t)) and XAL2(t) < 2

XAL2(t+1) = not AP1(t) and CO(t) and SOC1(t) < 2

AP1(t+1) = LFY(t) or ((FT(t) > 0 or XAL2(t) >0 and AGL24(t) > 0) and not TFL1(t)) or AP1(t)

CO(t+1) = CO(t)

Supplemental Table 4 List of additional primers used in this work.

Primers for genotyping		
GK T-DNA	CCCATTTGGACGTGAATGTAGACAC	
LBb1	GCGTGGACCGCTTGCTGCAAA	
AGL24-GF	GTCTTCATGCAAGTAACATCAAC	
AGL24-GR	TCCATCGAAGTCAACTCTGCTGGATC	
SOC-GF	AAGCAGAGAGAGAAGAGACGAGTGTG	
SOC-GR	GGAGCTGGCGAATTCATAAAG	
FUL-GF	CTATGTTCGAATCCATATCTGC	
FUL-GR	TGGTGAGATGACATACTGTAG	
Primers for expression analysis		
AGL24-qF	GAGGCTTTGGAGACAGAGTCGGTGA	(Liu et al., 2008)
AGL24-qR	AGATGGAAGCCCAAGCTTCAGGGAA	(Liu et al., 2008)
AP1-qF	CATGGGTGGTCTGTATCAAGAAGAT	(Liu et al., 2008)
AP1-qR	CATGCGGCGAAGCAGCCAAGGTT	(Liu et al., 2008)
LFY-qF	ATCGCTTGTCGTCATGGCTG	(Han et al., 2008)
LFY-qR	GCAACCGCATTGTTCCGCTC	(Han et al., 2008)
LFY-F	TCATTTGCTACTCTCCGCCGCT	
LFY-R	CATTTTTCGCCACGGTCTTTCG	
SOC1-qF	AGCTGCAGAAAACGAGAAGCTCTCG	(Liu et al., 2008)
SOC1-qR	GGGCTACTCTCTTCATCACCTCTTCC	(Liu et al., 2008)

TFL1-qF	CTTCACTTTGGTGATGATAGAC	
TFL1-qR	CTTGGCAATTCATAGCTCAC	
PDF2 qF	TAACGTGGCCAAAATGATGC	Czechowski et al., 2005
PDF2 qR	GTTCTCCACAACCGCTTGGT	Czechowski et al., 2005
UPL7 qF	TTCAAATACTTGCAGCCAACCTT	Czechowski et al., 2005
UPL7 qR	CCCAAAGAGAGGTATCACAAGAGACT	Czechowski et al., 2005
GA2OX1 qF	TGAGGACGAGAGGTTGTACGA	Rieu et al., 2008
GA2OX1 qR	TCCTTTCGAATTGTTGAAGCC	Rieu et al., 2008
GA20OX1 qF	GATCCATCCTCCACTTTAGA	Rieu et al., 2008
GA20OX1 qR	GTGTATTCATGAGCGTCTGA	Rieu et al., 2008
GA20OX2 qF	ACCGAGACTATTTCCGAGGATT	Rieu et al., 2008
GA20OX2 qR	TGT TTG GCA TGG AGG ATA ATG	Rieu et al., 2008
TUB2-F	AGGACTCTCAAACTCACTACC	
TUB2-R	TCACCTTCTTCATCCGCTGTT	
WUS-qF	GCAAGCTCAGGTACTGAATGTGGTG	(Sun et al., 2009)
WUS-qR	GACCAAACAGAGGCTTTGCTCTATCG	(Sun et al., 2009)
XAL2-qF	GATAATTCACAGCAATCGAAGG	
XAL2-qR	GGTTCTCCAATTGTTGTAACTC	
XAL2-F	GTAGAAAGATATCAAAAGCGAA	
XAL2-R	GGAGGAAACTTTTTGAAGTGT	

Primers for ChIP assay

TFL1-1F	AAAGGGTTGTCTTGTTTGGAGA
TFL1-1R	CACACCCAAAGGTTTTGTCC
TFL1-2F	GCAATAATTGTATCCGGAGTTG
TFL1-2R	GCAACATTGACTGCTTCAGC
TFL1-3F	GCTGAAGCAGTCAATGTTGC
TFL1-3R	GAGACAAAGAGACGACCGAGA
TFL1-4F	AAGATTTTACTTTTTGCTACCTTGC
TFL1-4R	TGGGTCTATCATTCAGGCTGT
TFL1-5F	GCCTGTGTTGTGTTTAGCC
TFL1-5R	TGACACCAATCACCATCTCAA
TFL1-6F	TGGATTTCAATCAATCCTCTTTT
TFL1-6R	CCAATCCAAACCAAAAACTGA

Extended Methodology: Stochastic multi-cell GRN model implementation

1. **Attractor Transition Probability Approach to Explore the EL**

The Boolean GRN model was extended in order to include stochasticity in the updating rules. In this model a constant probability of error ξ is introduced for the deterministic Boolean functions:

$$x_i(t+1) = \begin{cases} f_i(t), with prob. 1-\xi \\ 1-f_i(t), with prob. \xi \end{cases}$$

thus, at each time step, each gene ``disobeys" its Boolean function with probability ξ. The obtained discrete stochastic model is then used to estimate transition probabilities between pairs of attractors. This was done by simulating a stochastic one-step transition starting from each of the possible states a large number of times (~10,000) and calculating the frequency of times the states belonging to the basin of an initial attractor were mapped into a state within the basin of a final attractor. A transition probability matrix Π is estimated. The matrix is then introduced into a discrete time Markov chain model (DTMC) obtaining a dynamic equation for the the probability distribution over the attractors P_A :

$$P_A(t+1) = \Pi P_A(t).$$

The temporal evolution of the probability distribution (see Figure 7) was simulated by iterating the previous equation. This model then follows the fate transitions of a population of cells at the SAM.

2. **Mean First Passage Time (MFPT)**

In addition to the calculation of the most probable temporal cell-fate pattern, a discrete stochastic GRN model enables the study of the ease for transitioning from one attractor to another. A transition barrier in the EL epitomizes the ease for transitioning from one attractor to another. The ease of transitions, in turn, offers a notion of relative stability. One way to formalize the ease of transitions is to calculate the mean first passage time (MFPT) between each pair of possible transitions (Zhou et al. 2014). Here, in all cases, the MFPT was estimated numerically. Using the transition probabilities among attractors, a large number of sample paths of a finite Markov chain were simulated following (Wilkinson 2011). The MFPT from attractor to attractor corresponds to the averaged value of the number of steps taken to visit attractor j for the fist time, given that the entire probability mass was initially localized at the attractor (total number of cells). The average is taken over the realizations.

A net transition rate between attractors i and j ($d_{i \to j}$) was defined based on the calculated MFPT values

ANEXO B: Capítulos en libros de las cuales soy co-autor y que fueron parte de mis actividades académicas del doctorado.

Apéndice I

El capítulo "***A MADS view of plant development and evolution***" forma parte del libro "*Topics in Animal and Plant Development: From Cell Differentiation to Morphogenesis*", 2011:181-220 ISBN: 978-81-7895-506-3. Mi participación en este capítulo fue parte de la actividad académica del semestre 2008-2 en el Programa de Doctorado en Ciencias Biomédicas. En esta capítulo se describe el papel de los factores transcripcionales MADS desde una perspectiva evolutiva y su participación en los procesos de desarrollo en plantas, uno de estos procesos es la transición a la floración el cual fue un eje de estudio en este trabajo.

Apendice II

El capítulo "**Flower Development**", forma parte del "Arabidopsis Book" 2010; 8: doi: http://dx.doi.org/10.1199/tab.0171, publicado por " The American Society of Plant Biologists". Mi participación en este capítulo fue parte de la actividad académica del semestre 2009-1 en el Posgrado en Ciencias Biomédicas. En este capítulo se presenta diferentes conceptos desde la organización estructural del meristemo apical aéreo, pasando por la especificación y determinación del meristemo de flor, muestra además una revisión detallada del desarrollo de la flor lo cual fue ilustrado mediante microscopia electrónica de barrido, esto abarco desde el meristemo apical aéreo hasta el desarrollo de la flor. Este capítulo fue muy interesante de desarrollar porque me ayudo a entender los fenotipos que se observan en las plantas *35S:XAL2* que se estudiaron en este trabajo.

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9. A MADS view of plant development and evolution

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MADS-box genes are important transcriptional regulators of plants, animals and fungi during multiple developmental processes. At least an ancestral duplication that occurred before the divergence of plants and animals gave rise to two lineages of MADS-box genes represented in these three groups of eukaryotes: Type I and Type II. The similarity of the MADS-box sequences within each lineage suggests strong functional conservation. The first and best characterized MADS-box genes in plants were those of Type II, which encode modular proteins with I, K and COOH domains in the 3' region of the MADS domain. Of these, those involved in the determination of floral organs were first characterized: the so-called ABC genes of flower development that are necessary for the specification of sepals, petals, stamens and carpels, characteristic of most angiosperms. MADS-box genes have been found to be also key integrators of signal transduction pathways in response to

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external (light and temperature) or internal (hormones) cues, to which plants respond during their transition from vegetative to reproductive growth. More recently, MADS-box genes implied in gametophytic development or in the regulation of processes of vegetative structures have been characterized. In contrast to the first studies, recent studies are suggesting that most plant MADS-box genes are expressed in multiple tissues and stages. Thus their function could be regulated at different levels (e.g., miRNA's) and/or depend upon the composition of protein complexes of MADS proteins with members of the same or other families, that are specific to different tissues and/or stages. This family will continue to be a useful system to understand the complexity of the logic of transcriptional regulation underlying developmental decisions and how these integrate multiple signal transduction pathways, as well as the relationship between molecular and morphological evolution in plants. The ease of *in vivo* studies in plant systems will likely contribute to novel insights for understanding their plastic, and at the same time, robust developmental responses. The conservation of some of the molecular components underlying such processes and the existence of generic characteristics of these will make plant studies useful for unraveling animal and fungal systems, too.

Why plants and MADS-box genes: Evolutionary context within eukaryotes and functional conservation with respect to animal genes

Plants are the source of key products and the base of the planet's ecosystem equilibrium, and it is intrinsically important to understand their development and physiology for the well being of humans. But plants also pose clear advantages in comparison to animals as research systems of the *in vivo* interplay of genetic, epigenetic and environmental factors that cause normal or aberrant morphogenesis. Plant growth and development depends largely on the cellular processes occurring in the meristems (exposed pools of undifferentiated and actively dividing cells), that contain the niches of stem cells, which remain active along the plant's whole life-cycle and adjust their response according to environmental conditions. Adult plant development, growth and form depend on equilibrium between cell proliferation and differentiation in such niches of mother cells within the meristems. Therefore, studies of the interplay of cell division and differentiation under the action of environmental factors can be readily and quantitatively studied in plant meristems *in vivo*.

Additionally, plant cellular organization is simpler than that of animals and it will be more feasible to propose computable plant models than animal ones. Such models may be used for *in silico* simulations that may help to think about the concerted action of multiple genetic and non-genetic factors during development, and the role of particular components or sets of them in cell patterning and morphogenesis *in vivo*. Finally, key molecular aspects of Gene Regulatory Networks (GRNs) and signal transduction pathways are conserved between animals and plants [1,2], thus plant research is becoming also a source of novel basic molecular and genetic knowledge that may be generally relevant for understanding development of animal, as well as plant systems, or even yield biomedical applications. Molecular genetics of plants, in particular of the experimental system *Arabidopsis thaliana*, has paved the way to key discoveries in the biomedical and biological sciences and these should now be routinely considered in the "portfolio" for the search of the basis and cure of important human diseases [3].

Recent advances in evolutionary biology have recognized that deciphering developmental processes and the mechanisms that underlie cell patterning and morphogenesis at the molecular level are necessary in order to understand morphological diversification [4]. Indeed, development determines how genetic variation is mapped into morphological or phenotypic variation [5]. MADS-box genes encode transcriptional factors that are key in plant and animal development [6,7]. Hence, phylogenetic studies of the MADS-box genes contribute to bridging the gap between molecular evolution and phenotypic evolution in a macroevolutionary scale. We uncovered two MADS lineages (Type I and II) in plant, fungi and animals [8,9], and although there is still contention on whether Type I are monophyletic or not *e.g.*, [10], our analyses suggested that at least one gene duplication occurred before the divergence of plants and animals, after which, strong functional constraints yielded widely conserved genes within most Type I and Type II MADS-box genes. Important animal genes belong to these MADS lineages [11], *e.g.* proto-oncogene, Serum Response Factor (SRF) in the Type I and Myocyte Enhancer Factors (MEF) in the Type II (see Figure 1a) together with plant MADS.

Molecular analyses of MADS-box genes in plants should, therefore, yield important insights to understanding the role of these key transcriptional regulators in animal development and human disease. This is particularly true at least concerning the role of the MADS DNA recognition domain that seems to have been conserved after plant-animal divergence within the MADS lineages.

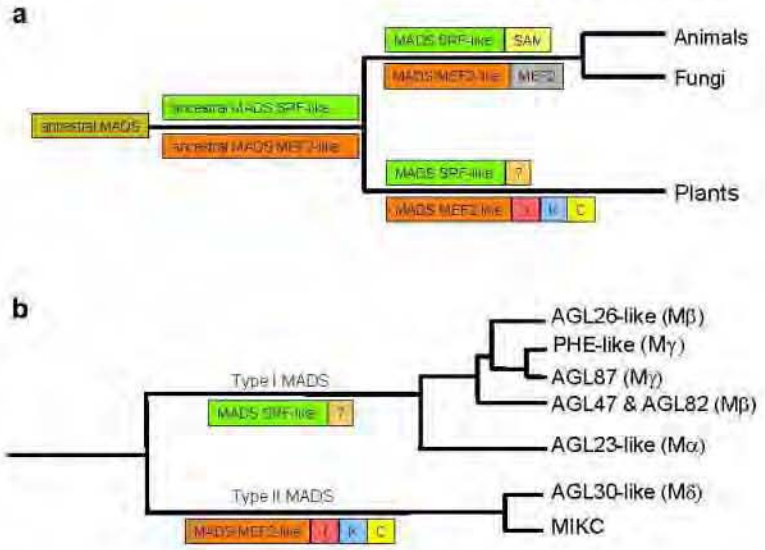

Figure 1. a) Schematic representation of the domains (boxes) of the MADS proteins from different taxa and their phylogenetic relations. At least one duplication of an ancestral MADS-box gene must have occurred before the divergence of plants and animals giving rise to the Type I (SRF-like) and the Type II (MEF2-like) lineages present in plants, animals and fungi [11,9]. After the divergence, each group acquired different accompanying domains. It has also been described that bacteria may have MADS-domain-like proteins [12], but their phylogenetic relationships with respect to those from other organisms has not been determined. b) Schematic representation of the tree classification made by Martínez-Castilla & Álvarez-Buylla (2003), [9]. In boxes are the MADS-domains of the proteins from angiosperms and in parenthesis the classification made by Parenicová & collaborators (2003) [10]. Phylogenetic relationships among Type I genes result in the separation of different clades whose monophyletic origin is still controversial [13]. According to Parenicová et al. (2003) both Mα and Mγ lineages are present in monocots and dicots, but there might be an absence of Mβ sequences in monocots. Type II proteins clearly are divided in to two clades that include the same genes in both classifications. In plants, the K-domain appears as part of the Type II lineage proteins, forming the basic MIKC structure. However, a small group of 6 genes (out of 45) do not have a clear K-domain (AGL30-like or Mδ clade). Other classifications describe variability among MIKC proteins and distinguish two groups having either a classic MIKC or a non-classic MIKC structure [14]. MADS-box genes have also been found in other terrestrial plants (i.e. gymnosperms and ferns [14,15]) as well as algae [3].

MADS-box genes

MADS-domain proteins regulate different aspects of development or cell differentiation in a variety of organisms [16,17,18]. The MADS acronym was derived from the initials of the first genes of this kind that were cloned: *MCM1* from *Saccharomyces cerevisiae* [19], *AGAMOUS* from *Arabidopsis thaliana* [20], *DEFICIENS* from *Antirrhinum majus* [21] and SRF from mammals [22].

Plant Type II MADS-box genes encoded proteins share a conserved structural organization, the so called MIKC structure including, from the amino to the carboxi-terminal part of the protein: a MADS (M) conserved DNA-binding domain, a more divergent intervening (I) region, a conserved (K) domain which may participate in protein interactions, and a divergent COOH (see below and Figure 1b) [23,24,25,26,27].

The 60 amino acid MADS-domain is at the N-terminus of the proteins. It has been shown that this domain is involved in specific binding to DNA sequences (CArG boxes) conforming the consensus sequence $CC(A/T)_6GG$ in both animals and plants [28,24,25]. This is a characterized conserved motif in the promoter of different MADS target genes [29,30,31,32,33]. However, an *Arabidopsis* genome study demonstrated that, during early flower development where MADS box proteins are fundamental, the occurrence of regulated genes with CArG boxes in the promoter was not significantly different to that of their genome distribution. Therefore, these transcription factors might either be able to recognize another element (*e.g.*, a less conserved CArG box) or they might have a limited number of target genes in this particular developmental stage [34].

The formation of dimers that are capable of DNA binding requires the I-region (60–86 aa long); this domain is at the 3' of the MADS and is a key determinant for the specificity of DNA-binding dimer formation [24,25]. After the I, a second conserved domain, the K (87–150 aa long), is postulated to form three α-helices referred to as K1, K2, and K3 that potentially form coiled-coils structural motifs, with K1 and K2 helices located entirely within the K domain, while K3 helix spans the boundary between the K and the C domains [35]. The K-domain is assumed to generate a three-dimensional structure important for protein–protein interactions [36,26]. Finally, the C-terminal domain is a length-variable amino-acid stretch that may have several functions. For example, it is thought to participate in higher-order MADS interactions [37], it is required for functional specificity [38], it may be involved in transcriptional activation [39], and can also enhance/stabilize interactions that are mediated by the K-domain [26,40].

All MADS-domain proteins studied until now bind to the CArG sequence in the DNA as dimers, either as homo- or heterodimers [29,24,25]. For example, *AG* can bind to DNA either as a homodimer or a heterodimer with *SEP1* [41]. On the contrary, *AP3* and *PI* can only bind to DNA as heterodimers in *Arabidopsis*. Moreover, It has been shown that these genes are only able to enter the nucleus as heterodimers in this species [42].

Plant MADS evolution and diversification: Duplications and natural selection

Representatives of at least two lineages of MADS-box genes are found in most plant lineages. However, while in animals MADS-domain transcription factors and the plants Type I proteins, the box that codifies for the MADS DNA-binding domain is only followed by a few 3'amino acids, in the plant Type II proteins, after the MADS-box, we can find, as shown above, three other boxes (I-, K- and C-regions; Figure 1a). Another trend of plant MADS-box genes is that there are many more types of these genes in plants than in animals. Previous studies suppor the hypothesis that the diversification of these genes could have been important during the evolution of plant form [43,44]. Indeed the more complex combinatory code underlying MADS-protein function could contribute to the robustness and plasticity of plant development.

We recovered 107 MADS sequences from the study plant, *Arabidopsis thaliana* [9]; see also [10,14]; and Figure 1b. Type II genes have been more extensively studied than Type I [10], although a few studies on the latter have started to emerge. These studies shows that although they had faster evolutionary and birth/death rates and some seem to be pseudogenes, several other Type I MADS-box genes are also functional [13]. Extensive homology search studies found that there are 64 presumed functional Type I genes, while there are 43 presumed functional Type II genes in *Arabidopsis*. Genomic studies on MADS-box genes have also started to appear on other angiosperm study systems, such as rice. These studies confirm the evolutionary tendencies of these two types of MADS-box genes (24 functional and 6 nonfunctional Type I; and 47 functional and 1 nonfunctional Type II genes in rice; [45,13]). The first MADS-box genes that were functionally characterized were those involved in cell type determination during flower development, whose loss of function yielded flower organ homeotic phenotypes [46]. It seems that alterations in the floral ABC MADS-box gene expression patterns (see below) could have contributed to the origin and structural evolution of flowers [47,48].

Floral organ identity MADS-box genes are overall conserved across angiosperms and to some extent even in gymnosperms [49,50,51,52,53,54,55,56]. These genes have been shown to be important and overall conserved in monocot and dicot flower development [57]. A recent network dynamic model has uncovered the developmental module of ABC and interacting non-ABC proteins that are necessary and sufficient for floral organ determination [58]. This model robustly converges only to gene activation profiles that correspond to those observed during early flower development in primordial cells of: the inflorescence meristem, and the sepal, petal, stamen and carpel primordia within the flower meristem. This model and its perturbation analyses have been used to propose an explanation for the robustness of the floral genetic developmental program and its observed evolutionary conservation among core eudicots (See: [58,59,60,61] for details on Network Model approaches).

At least ten Type II MADS-box genes are found in all angiosperms (including the flower ABC ones), suggesting that all of these originated before the radiation of the flowering plants and since then have been conserved. These data suggest that MADS-box genes could have been important for the origin of flowers and the establishment of the bauplan of flowers that is overall quite conserved: whorled structure with sepals, petals, stamens and carpels from the outermost to the inner of the flower [62]. This implies that further floral morphological diversification (*e.g.*, meristic structure, symmetry, color, size) among flowering plant species could have been regulated by genes of other families; or additional MADS-box genes with distinct functions yet to be characterized.

In addition to all the flower ABC organ identity MADS-box genes: *APETALA1* (*AP1*), *PISTILLATA / APETALA3* (*PI / AP3*), *AGAMOUS (AG)* and the *SEPALLATA* (*SEP1-4*); the common ancestor of all flowering plant species also had representatives of the *AGL15-*, *AGL17- AGL20,* and B-sister genes. On the other hand, the common ancestor of all seed plants (ca. 300 million years ago) already had MADS-box members of at least six families of MADS-box genes: *AGAMOUS-*, *AGL2-*, *AGL6-*, *AP3/PI*, GGM13- and TM3-like [63].

Available data suggest that the common ancestor of seed plants and ferns (400 million years-old), had at least two genes that are related to the flower MADS-box genes. But it is interesting that in ferns there were independent duplication events and 15 MADS members of different families to those of seed plants, have been identified in one fern species [63]. Recent studies in the moss (Bryophyte), *Physcomitrella patens* and in several algae have also demonstrated that the common ancestor of all land plants already had MADS-box genes [63,44].

Gene duplication is a fundamental substrate for evolution [64]. The question concerning the role of natural selection on the persistence and diversification of duplicates is therefore crucial [65]. After duplication, one duplicate may become a pseudogene, functional divergence (subfunctionalization or neofunctionalization) between the two may operate, or they may remain with overlapping functions. It is hence important to elucidate the role of evolutionary forces in molecular change and the fate of duplicates for clarifying mechanisms of genetic redundancy, as well as the link between gene family diversification and phenotypic evolution [64,65]. Indeed, the evolutionary forces that play during functional divergence of duplicates are still under debate [66,67]. However, the presence of large gene families as that of the MADS-box in plants, suggests that positive diversifying selection might have been important in preserving duplicates for longer periods of time than expected by classical stochastic neutral models [68,69,70].

Recently, we have documented the role of positive selection (PS) in the evolution of the MADS-box gene family [9,71]. We have achieved this for the complete MADS Arabidopsis family and the B-type angiosperm families addressing if positive selection has been important during MADS-box gene evolution among paralogous and orthologous genes (within the B-MADS-box gene class), respectively. Adaptive evolution in developmental regulatory loci, such as the MADS [72] is likely to act along particular lineages and at specific amino acid sites. Interestingly, our analyses have yielded statistically significant results for residues within the different domains of MADS proteins during the diversification of genes that control transition to flowering; a stage that is clearly linked to plant fitness. These findings suggest that changes in coding sequences of transcriptional regulators, rather than only alterations in their regulatory regions, may have been important during phenotypic evolution [9].

Plant form diversification

Probably the major evolutionary event during plant evolution was the relatively abrupt and extensive diversification of angiosperms shortly after they appeared in the fossil record [73,74]. This species radiation has been linked to the origin of flowers. As will be described below, a wealth of molecular genetic information is now available to understand the regulation of flower development using experimental plant species (*Arabidopsis thaliana* and *Antirrhinum majus*). However, it is from Arabidopsis that the most detailed and complete perspective has been obtained and it is going to be used as the model plant in this chapter. Nearly all of the floral organ

identity regulators belong to the Type II MADS-box genes [11,8,75,76,37,77] and available functional studies (see below) strongly suggest that these genes and their regulatory networks are key for flower development, and most probably played important roles during flower evolution, as well [47,48,5,78,79,80,81].

B genes specify petal formation, while when co-expressed with C function gene, they specify stamen formation [82,46,83]. AP3 and PI proteins interact to form a heterodimer that indirectly regulates *PI* expression and directly binds to the *AP3* promoter in a self activating regulatory loop that maintains the B function in the second and third whorls of the meristem during flower development [23,30,31]. Obligate heterodimerization of PI and AP3-like proteins of core eudicots [36,24,25] evolved from the homodimerization typical of gymnosperms, perhaps via a transitory state of facultative homo-heterodimerization [84]. Such heterodimerization evolution in B-class proteins could have been fundamental during the origin and diversification of flowers. Hence we addressed whether or not adaptive evolution was important during the origin of obligate B-protein heterodimerization, particularly following the critical AP3-PI duplication towards the base of angiosperms. Then a main duplication occurred in the AP3 lineage, leading to two AP3-like sublineages distinguished by characteristic motifs in their C-terminal region [85]: paleoAP3 lineage in basal angiosperms, monocots, magnoliids and basal eudicots (with a *paleoAP3* motif in their C-terminal) [86]; and euAP3 lineage of core eudicots B-class genes (*euAP3* C-terminal motif). These two seem to have functionally diverged [38,87]. Interestingly the expression pattern of B genes in core eudicots, which have a conserved floral plan (sepals, petals, stamens and carpels), is preserved [88,49,51], while these genes have divergent expression patterns in non-core eudictos. Coincidently, the flowers of the later do not have a well differentiated calyx and corolla [51,54].

The phylogenies of A, B, C, and *SEP1/2/4* floral MADS-box genes show a duplication close to the base of the core eudicot clade [89,90], coinciding with the moment at which the B genes split into euAP3 and paleoAP3-like lineages [89]. It has been suggested that this duplication played an important role in the origin of clearly differentiated petals in core eudicots [85,38]. Interestingly, our results strongly suggest that shortly after the duplication that led to the *AP3*-like and *PI*-like genes, functional diversification driven by PS acting on different sites within the K domain, which is key for heterodimerization, occurred along both duplicated gene lineages [71, 91].

On the other hand, our studies have suggested a possible functional divergence of *AP3* duplicates that might have been important for the

evolution of the core eudicot floral developmental genetic program. We found three major things associated with this divergence: a) an early origin for heterodimerization of B MADS-box proteins in angiosperms shortly after they diverged from gymnosperms, b) the *euAP3-TM6* duplication coincides with the origin of the core eudicots lineage, and c) a strong signal for positive selection along the *euAP3* branch lineage [71]. The B genes constitute a clear example in which duplicates evolved towards completely different functions.

A second possibility is that each duplicate may evolve a subset of all the functions originally performed by the ancestral gene (called subfunctionalization; [69]); the MADS-box genes that are expressed in carpel and stamen primordia are an example of this. While in *Arabidopsis* there is a single such gene (*AG*) important for the development of both organs, in rice and maize there are two such genes, each expressed in a single whorl (*e.g.*, [57,92]; see review of MADS subfunctionalization in: [93]). A third possibility is that one of the duplicates evolves a new function. Fewer examples of this have been documented and an outstanding one concerns the MADS-box gene that underlies *Physalis* encapsulating fruit structure [94]. The molecular basis of the novel or sub- functions of MADS-box duplicated genes has been documented in very few cases still.

After duplication, redundant genes with overlapping function could also remain for some time as is the case of some MADS-box genes [95,77]. In fact, purifying selection could keep redundant genes that may guard against deleterious mutations [96], or positive selection could favor duplicated copies if there is dose-dependence. *AP1* and *CAULIFLOWER* (*CAL*) are two recently duplicated MADS-box genes that in *Arabidopsis* underlie flower meristem identity, as well as sepal and petal identity [97,98]. While *AP1* can substitute *CAL* for both functions and single *cal1* loss of function mutants lack any visible phenotype, *CAL* cannot substitute for *AP1* functions and hence loss of function mutants of the former have clear phenotypes in flower determinancy and sepal/petal identity. The enhanced phenotype of the double mutant suggests that these genes overlap in meristem identity determinancy [81].

By expressing all the possible chimaeric proteins that result from combining the cDNA boxes that encode the four domains typical of plant Type II MADS-box genes (MADS, I, K and COOH) from *AP1* and *CAL*, under the *AP1* promoter, on an *ap1-1* loss of function background, we were able to map the unique and redundant functions of these two genes. Interestingly, the K and COOH domains, that are important for the formation of high-order multimers characteristic of MADS transcriptional

complexes, seem to be key for the unique and indispensable functions of *API* [81].

We have exemplified with some studies that MADS-box genes and plant evolution are clearly interconnected. Thus the study of these genes function in a comparative framework is likely to continue providing important clues to understanding development and phenotypical evolution beyond flowers.

Role of different MADS-box gene lineages in plant development

Plant MADS-box gene function during transition to flowering [99] and flower patterning [100] has been extensively studied ([101]; and updated reviews in sections below). The first characterizations stressed MADS-box gene role during specific stages of development as organ- [20,76], cell- [102] or meristem-identity genes [97,103]. More recent studies are starting to focus on the role of MADS-box genes also in vegetative development and are uncovering novel functions for these genes [104]. In the following sections, we review the studies that up to our knowledge have uncovered the function of MADS-box genes during *Arabidopsis thaliana* development.

Arabidopsis thaliana as a plant model system

Arabidopsis thaliana (Figure 2) has been the most successful experimental study system among plants because of its relatively short life-cycle (six weeks), its high seed output that enables classical genetic studies, its low incidence of outcrossing under laboratory conditions, its relatively small genome size and the availability of large molecular data sets and resources for molecular genetic studies [105]. In addition, it has a typical sporophyte that shows with the eudicot stereotypical eudicot flower structure and development.

As all higher plants, *Arabidopsis* has alternating haploid (gametophyte) and diploid (sporophyte) life-history stages, with the former depending on the latter. The mature female gametophyte is the embryo sac that is formed before fertilization and is found inside the carpel of the flowering sporophyte (see Chapter by García-Campayo and collaborators in this volume), while the male gametophyte is the germinated pollen grain that is produced within the stamen anther. In this review, we focus mainly on the sporophyte development that starts with embryo development within carpels and seeds (Table 1). But we must mention that a few MADS-box factors have been identified as important regulators of gametophyte development. For example, the *fem111* mutant is affected in central cell development and function. It

corresponds to a T-DNA insertion in *AGAMOUS-LIKE 80* (*AGL80*) [106]. Furthermore, *AGL80* is required for the expression of central cell-expressed genes such as: *DEMETER* and *DD46*, but does not affect *FERTILIZATION-INDEPENDENT SEED2* [106].

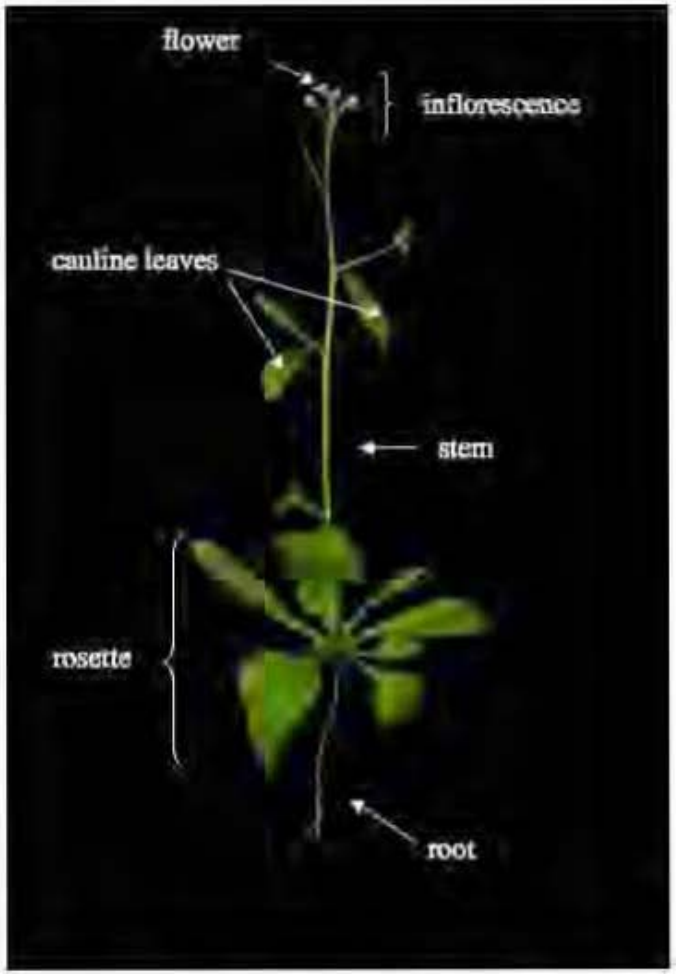

Figure 2. *Arabidopsis thaliana* plant

Table 1. MADS-box genes function in development.

ORGAN/STAGE & Genes	Protein function	Reference
ROOT		
A NO3(-)-INDUCIBLE MADS-BOX GENE (ANR1/AGL44)	Lateral root growth upon nitrogen deficiency.	[107]
XAANTAL1 (XAL1/AGL12)	Pivotal gene for both root and flower development implicated in the photoperiod pathway downstream of CONSTANS (CO) action. Also expressed in embryos.	[104,108]
EMBRYO		
AGAMOUS LIKE 80 (AGL80)	Required for central cell development and function.	[106]
PHERES1 (PHE1/AGL37)	May be involved in pattern formation of the endosperm.	[109]
AGAMOUS LIKE 62 (AGL62)	Regulates cellularization during endosperm development.	[110]
AGAMOUS LIKE 15 (AGL15)	Essential for embryo development. Repressor of *FT* during transition to flowering. Fruit maturation.	[111,112,113]
AGAMOUS LIKE 18 (AGL18)	Essential for embryo development. Repressor of *FT* during transition to flowering. Transcriptional repressor of immature pollen genes downstream of AGL65, AGL66 and AGL104.	[112,113,114]
SEPALLATA1 (SEP1/AGL2)	Expresses during the embryogenesis.	[115]
AGAMOUS LIKE 21 (AGL21)	Expressed during embryogenesis, unknown function.	[108]
TRANSITION TO FLOWERING		
AGAMOUS LIKE 17 (AGL17)	Promoter of flowering and positively regulated by the photoperiod pathway regulator CONSTANS.	[116]
AGAMOUS LIKE 24 (AGL24)	Activator of flowering. Activator of *SOC1* transcription.	[117,118]
FLOWERING LOCUS C (FLC/AGL25)	Repressor of flowering transition. Central role in regulating the response to vernalization. Functional Integrator of flowering transition pathways.	[119,120]

Table 1. Continued.

FLOWERING LOCUS M (FLM)/ MADS AFFECTING FLOWERING 1 (MAF1/ AGL27)	Transition from vegetative to reproductive development. Also expressed in the embryo.	[121]
MADS AFFECTING FLOWERING 2-4 (MAF2/AGL31), (MAF3/AGL70), (MAF4/AGL69)	Floral repressors. May be important for maintenance of a vernalization requirement.	[122,123]
MADS AFFECTING FLOWERING 5 (MAF5/AGL68)	Putative floral promoter.	[123]
SHORT VEGETATIVE PHASE (SVP/AGL22)	Repressor of flowering transition in opposition to *AGL24* and *SOC1*.	[124]
SUPPESSOR OF OVEREXPRESSION OF CONSTANS1(SOC1/AGL20)	Integrator of flowering transition pathways as a positive regulator of flowering. Activator of *AGL24*.	[125,126,127,118]
AGAMOUS LIKE 19 (AGL19)	Expressed after vernalization. Induces flowering transition by up-regulating *LFY*.	[128]
MERISTEM IDENTITY		
CAULIFLOWER (CAL1/AGL10)	Meristem identity.	[129,98]
APETALA1 (AP1/AGL7)	Meristem identity.	[132,97,133]
FLORAL ORGAN INDENTITY		
APETALA 1 (AP1/AGL7)	Class A homeotic gene.	[132,97,133]
APETALA 3 (AP3)	Class B homeotic gene.	[134]
PISTILLATA (PI)	Class B homeotic gene.	[135]
AGAMOUS (AG)	Class C homeotic gene. Important for stamen, carpel, ovule and fruit development, and floral meristem development.	[82,45]
SEPALLATA1/2/3/4 (SEP1/AGL2), (SEP2/AGL4), (SEP3/AGL9,) (SEP4/AGL3),	Regulate activities of B- and C-class homeotic genes.	[76,40,136]
OVULE AND FRUIT		
SEEDSTICK (STK/AGL11) FRUITFUL (FUL/AGL8)	Ovule identity. Fruit development, control of flowering time. Required for normal development of the funiculus.	[137]
SHATTERPROOF (SHP/AGL1), (SHP2/AGL5)	AG-independent carpel development. Ovule, and fruit development dehiscence.	[102,129,137]

Table 1. Continued.

POLLEN		
AGAMOUS LIKE 30 (AGL30)	Apparently, a crucial component during pollen maturation. Pollen tube competitive ability.	[114]
AGAMOUS LIKE 65 (AGL65), *AGAMOUS LIKE 66 (AGL66)*, *AGAMOUS LIKE 104 (AGL104)*	Pollen maturation Pollen tube competitive ability.	[114]
AGAMOUS LIKE 29 (AGL29)	Transcriptional repressor of immature pollen genes downstream of AGL65, AGL66 and AGL104.	[114]

Embryo development

Zygotic embryogenesis in higher plants begins with a double fertilization. The zygote is formed when the egg cell within the embryo sac joins a sperm nuclei, and a second sperm nuclei fuses with the central cell to give rise to the endosperm [138]. The zygote then starts to divide asymmetrically yielding a small apical cell and a large basal cell. The basal region gives rise to the suspensor, which is a structure that supports the embryo, and the apical cell gives rise to the pro-embryo [139]. The pro-embryo goes through diverse cellular stages: globular, heart, torpedo and bent cotyledon or mature stage. During the last stage of embryogenesis, reserves accumulate. Finally, the seed loses water and a quiescent state is established until optimal conditions occur and seeds germinate [140,141].

In animals, embryos at late stages resemble the adult organism, whereas in plants mature embryos prior to germination are very different to adult plants, that grow and complete morphogenesis from the shoot and root meristems. In plant embryos only two different zones can be distinguished: the basal one gives rise to the root and the more apical one to the shoot. Several molecular components of the networks controlling embryo development have been uncovered [142,138,141,139]. Some MADS-box gene factors have been identified as important regulators of this developmental stage (see Table 1).

The MADS-box Type I gene *PHERES1* (*PHE1*), also named *AGL37*, is expressed transiently after fertilization in the embryo and the endosperm. The Polycomb-group (PcG) proteins MEDEA, FERTILIZATION INDEPENDENT ENDOSPERM and FERTILIZATION INDEPENDENT SEED2 regulate seed development in *Arabidopsis* by controlling embryo and endosperm proliferation. These proteins are subunits of a multiprotein PcG complex,

which regulates *PHE1* epigenetically [109] by histone trimethylation on H3K27 residues [143]. It was shown that *medea* mutant plants have a seed-abortion phenotype due to *PHE1* upregulation; *medea* plants that also had a *PHE1* low-expression level rescued the *medea* phenotype. It has been speculated that *PHE1* may be involved in pattern formation of the endosperm [109].

AGL62 encodes another Type I MADS domain protein that has been functionally characterized. It has a strong expression during the syncytial phase of embryo development, in which the endosperm nuclei undergo many rounds of mitosis without cytokinesis, and then is later expressed during the cellularized phase, in which cell walls form around the endosperm nuclei, and finally its expression declines abruptly just before cellularization. Thus, AGL62 is thought to be a component of the mechanisms underlying cellularization during endosperm development [110].

AGL15 is the best characterized MADS-box gene during embryo development. This gene was identified and isolated initially in *Brassica napus* by differential display technique [144]. RNA gel blot analyses and *in situ* hybridization demonstrated that *AGL15* mRNA is accumulated mainly in the developing embryo during all stages. *AGL15* mRNA expression levels were at least 10-fold higher in embryos than in inflorescence apices, young floral buds, young seedlings or vegetative apices [144].

Interestingly, AGL15 protein accumulates in the cell cytoplasm of the egg apparatus and it is translocated into the nuclei during early zygotic embryogenesis [145]. This pattern of accumulation is the same in different types of asexual embryogenesis: apomitic embryogenesis of *Taraxacum officinale* (dandelion), the microspore embryogenesis in *Brassica napus* and the somatic embryogenesis in *Medicago sativa* (alfalfa) [140]. Overexpression of *AGL15* with the constitutive CaMV35S promoter yielded secondary embryonic tissue from cultured zygotic embryos and led to long-term maintenance of the embryonic phase [146]. Also, the longevity of sepals and petals was increased and a delay in the transition to flowering and fruit maturation was observed in these plants [111].

Chromatin immunoprecipitation (ChIP) was used to identify genes that were regulated by AGL15. Those genes were named as *Downstream Targets of AGL15* (*DTA1* and *DTA2*). *DTA1* (*AtGA2ox6*) is a direct downstream target of *AGL15* and encodes a protein with high similarity to gibberellin (GA) 2-oxidases and it was shown to catalyze gibberellins 2β-hydroxylation. Molecular studies showed that the expression of *AtGA2ox6* oxidase is down-regulated in an *agl15* null mutant [147]. On the other hand, *DTA2* encodes a novel protein that is repressed by AGL15 [148].

AGL15 and *AGL18* are sister genes with very similar mRNA spatio-temporal patterns of expression. *AGL18* was detected in the endosperm and embryos by *in situ* hybridization [8] and by RT-PCR and translational fusions with GUS [112]. These assays led Lehti-Shiu et al. (2005) to propose that both genes play an essential role during embryo development. However, no defects in embryo development were observed in single and double mutants of *agl15* and *agl18*, which suggests functional redundancy with other genes [112].

SEP1 (*AGL2*, [76]) mRNA is accumulated in embryos after fertilization but it is also expressed at similar or higher levels in other tissues and stages of development, as ovules [115,76]. There are other members of this family, *FLM* and *MAF1* [122], as well as *XAL1* (*AGL12*) and *AGL21* [108] that have been detected in embryos, but their functions are unknown.

After germination, all aerial *Arabidopsis* structures form from the shoot apical meristem (SAM), while the adult root apical meristem (RAM) develops from the meristem at the tip of this structure after seed germination. In the following sections we focus on the role of MADS-box genes during sporophyte development after germination. The role of these genes during seedling and vegetative development is largely unexplored. We therefore focus on the transition to flowering and flower morphogenesis.

MADS-box genes are key components of flowering transition networks

In wild and cultivated annual plant species, flowering time is an important life-history trait that coordinates life cycle with environmental conditions [149]. Plants initially undergo a period of vegetative development, characterized mainly by the production of rossette leaves from the shoot meristem. Later in development, the meristem undergoes a change in fate and enters a reproductive stage producing flowers and differentiating the germ line. Plant species exhibit variability in flowering time, and the timing of this floral switch is controlled by multiple environmental and endogenous cues [150]. Four different flowering control pathways have been described in *Arabidopsis thaliana* based on genetic data, however it is important to note that molecular data is clearly showing that these pathways crosstalk and are integrated by a complex module of feedback interactions [151] (see Figure 3).

The photoperiod pathway perceives light and responds to it [154,155]. Mutant plants in this pathway are late flowering under long day conditions [156]. The vernalization pathway comprehends genes involved in the response to long periods of cold exposure which accelerates flowering transition [119,157,158]. The gibberellin (GA) pathway, promotes flowering

by the induction and action of this plant hormone [159]. Finally, the autonomous pathway responds to developmental signals independently of the external signals and the GA action, and mutant plants in this pathway are late flowering under long- and short-day photoperiods, but can be rescued by vernalization [160,119,161].

Details on the molecular components of all these pathways have been extensively reviewed (see for example: [162,163,164,127,165]; among others). In this Chapter we will focus on the role of MADS-box genes in such pathways and integrating module. There are two key MADS-box functional integrators of these pathways that have been most thoroughly characterized: *FLOWERING LOCUS C (FLC / AGL25)*, which acts as a repressor of flowering [119,120], and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1 / AGL20)*, that promotes this developmental process [125,126,166].

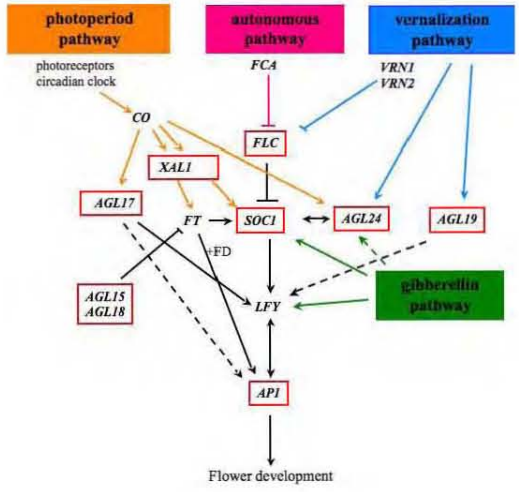

Figure 3. Simplified scheme of the four different flowering control pathways described for *Arabidopsis thaliana* and the integrator module starting to be characterized and in which MADS-box genes are key components. MADS-box genes are framed in red. Dotted lines represent hypothetical interactions. Genetic tests still have to be done to demonstrate XAL1's precise position in the network (Modified from [152] and [153]; for specific protein interactions see also references from text).

FLC is a central repressor of flowering

Plants have different ways to repress flowering until the appropriate seasonal and developmental cues overcome this repression. A central player in this process is *FLC*, which blocks flowering by inhibiting genes such as *FLOWERING LOCUS T* (*FT*) and *SOC1* that are required to induce genes which turn the vegetative meristem into a reproductive one [119,167]. Regulation of the expression of *FLC* has become a key system in plant molecular genetics to uncover transcriptional and epigenetic regulatory mechanisms.

Natural winter-annual *Arabidopsis* accessions need to pass through a long period of cold temperature or vernalization in order to be able to flower during the following spring–summer season. Genetic studies have demonstrated that a single dominant gene *FRIGIDA* (*FRI*) is able to increase the levels of *FLC* expression in these accessions, and only vernalization overcomes this effect by epigenetic repression of *FLC* [168]. Under vernalization, *FLC* expression is progressively reduced by the action of VERNALIZATION INSENSITIVE 3 (VIN3), VERNALIZATION1 and 2 (VRN1/2) and the *Polycomb* Repressive Complex 2 (VRN-PRC2) [169,54]. After this, it remains stably low during subsequent growth in warm conditions by the action of a heterochromatin protein1-like (LHP1) enabling the plant to flower [119,120,165]. It is vital that this memory is lost in the next generation so that the vernalization requirement is re-established [168]. On the other hand, summer-annual *Arabidopsis* accessions have a recessive *fri* allele often produced by a loss-of-function mutation in this gene and these plants do not need vernalization to flower [170].

In the absence of *FRI*, the autonomous pathway negatively regulates *FLC* expression. However, autonomous late-flowering mutants are overcome by vernalization, meaning that both pathways function in parallel [171]. Seven genes have been implicated in the autonomous pathway. Interestingly, three of them (*FCA*, *FPA* and *FLOWERING LATE KH MOTIF* or *FLK*) codify for RNA-binding proteins, and a fourth one, *FY*, codifies for a polyadenylation factor which collaborates with FCA in RNA processing [172]. Two other members identified in this pathway have been implicated in histone deacetylation complexes, FVE and FLOWERING LOCUS D (FLD) [173,174]. Finally, *LUMINIDEPENDENS* (*LD*) encodes a homeodomain protein with an unknown function [175]. It seems plausible to think that the autonomous pathway is conformed by functional redundant genes all targeting *FLC* [165,171].

In order to maintain flowering repression under the vegetative state, several activators of *FLC*, other than FRI, are also part of chromatin-remodeling complexes. For example, *EARLY FLOWERING IN SHORT DAYS* (*EFS*) and *ARABIDOPSIS TRITHORAX1* (*ATX1*) are necessary for the H3K4 trimethylation at *FLC* chromatin domains, which activates this gene's expression. As expected, mutations in *EFS* or *ATX1* produce early-flowering plants due to low transcriptional levels of *FLC* [176,165,177].

SOC1 / AGL20 an integrator and activator of flowering transition

The *soc1* mutant was independently isolated after a screening for suppressors of the early phenotype induced by *CONSTANS* (*CO*) overexpression [178,166], and from an activation screening in the *FRI/FLC* background [126], and using reverse genetics [125]. *soc1* mutants flower late in both long and short day conditions [125,126,127].

It has been demonstrated that FLC binds to the CArG-box in the promoter region of *SOC1* inhibiting its expression, and therefore repressing flowering [32,179]. Vernalization and the autonomous pathways allow the up-regulation of *SOC1*, after lowering the levels of *FLC* [126,180]. However, null mutations in *FLC* are not sufficient to induce high transcript levels and other signals are required to up-regulate *SOC1* during vegetative growth, particularly in the shoot apical meristem [125,126,166,18]. These signals come from the photoperiod and/or the gibberellin pathways [125,181]. In the first one, CO induces *SOC1* expression, partially through FT [166,18,182] as the input pathway. No GA regulatory elements have been found in the *SOC1* promoter [18] even though GA has been demonstrated to be crucial to promote flowering by regulating *SOC1* and *LEAFY* (*LFY*), particularly in short day conditions [183,151,181]. SOC1 induces the expression of *LFY* [184], another flowering integrator that is also a key flower meristem identity gene that activates flower development and the key floral organ identity MADS-box ABC genes that are reviewed below.

AGL24 and *SVP* participate as activator and repressors of flowering, respectively

AGL24 shares many characteristics with SOC1 as an important protein that promotes flowering [117,185]. Like *soc1*, *agl24* mutant is late flowering in both long and short day conditions. This gene is also regulated by GA, and the double mutant *soc1-2 agl24-1* is capable of flowering only after the addition of this hormone [118]. Its overexpression leads to early flowering,

indicating that *AGL24* is another important flowering regulator. Contrary to *SOC1*, *AGL24* is induced by vernalization and by the autonomous pathways, in a *FLC* independent way [117]. During the photoperiod pathway, CO also induces *AGL24* expression but not through FT, whereas *SOC1* is mainly regulated by the FT protein, as mentioned before [185].

It was recently established that *AGL24* and *SOC1* directly regulate each other at the transcriptional level by binding each other's promoters (but not their own), thus establishing a positive-feedback regulation that probably forms part of a larger and more complex module which integrates all the signals known to promote flowering transition [118]. It is also important to note, that Liu and colleagues (2008) demonstrated that SOC1, but not AGL24, binds directly to the *LFY* promoter [118].

SHORT VEGETATIVE PHASE (*SVP* / *AGL22*) is the sister MADS-box gene and closest homolog of *AGL24*, however it acts as a repressor of flowering transition in opposition to *AGL24* and *SOC1* [124]. Concordantly, the *svp* loss-of-function mutant is early flowering, and *SVP* overexpression causes a late flowering phenotype. Interestingly, both mutants are insensitive to cold acclimation, a phenomenon different from vernalization in which plants become tolerant to freezing temperatures by being previously exposed to short periods of cold (16 °C). *SVP* mediates the temperature-dependent functions of *FCA* and *FVE* within the "thermo-sensory pathway" and negatively regulates *FT* by directly binding this gene's promoter at its CArG motifs [186]. Interestingly, the flowering time regulators *AGL24*, *SVP* and *SOC1* have shown to be down-regulated and kept away from the floral meristems by the floral identity genes *LFY* and *API* that are in turn key flower meristem identity genes [187,188].

Other MADS-box genes implicated in flowering transition

Additional MADS-box genes have been recently implicated in the flowering transition regulatory network. Among the positive flowering regulators, *XAANTAL1* (*XAL1* / *AGL12*) [104] and *AGL17* [116] have been implicated in the photoperiod pathway downstream of CO action. Also, *AGL19* is normally repressed by the polycomb complex, but after vernalization its repression marks are reduced allowing *AGL19* to be highly expressed, which in turn induces flowering transition by up-regulating *LFY* [128]. These three genes are also strongly expressed in the roots. On the other hand, FLOWERING LOCUS M (FLM) / MADS AFFECTING FLOWERING (MAF1) is thought to act as a co-regulator with SVP inhibiting flowering transition [189]. Interestingly, temperature might

suppress the repressive effect of *FLM* on flowering, or temperature might act downstream of FLM to bypass its repressive effect. This has been described in a new pathway that involves the thermal induction of flowering when plants are shifted from 23 to 27°C under short day conditions [190].

Other flowering repressors are AGL15 and AGL18 that act redundantly to down regulate *FT* expression [113]. It is very likely that the number and complexity of MADS-box interactions involved in the regulation of flowering transition is still much larger and rapid progress in understanding their specific functions is expected in the coming years in this exciting and important field for plant development.

The MADS story of flower development

Arabidopsis thaliana is a self-fertilizing plant that has a simple flower structure typical of the Brassicaceae and with a basic floral plan shared by highest eudicots. The flower has two external sterile organs (whorls 1 and 2) surrounding the reproductive ones (whorls 3 and 4): a calyx of four sepals (whorl 1) and a corolla of four petals (whorl 2) whose positions are alternate and interior to those of the sepals. The androecium (whorl 3) consists of four medial, long stamens, and two lateral short stamens with a superior sessile gynoecium (whorl 4) in the center of the flower that consists of two fused carpels [105]; Figure 4.

Based on morphological evidence, flower development can be divided into several stages. Flower meristems arise from the flanks of the inflorescence meristem in a phyllotactic spiral (stage 1), and soon become isolated from the inflorescence meristem by tissue that will later become the flower pedicel (stage 2). Then, the sepals begin to arise from the outermost cells of the flower meristem (stage 3) and elongate (stage 4). The next stage is characterized by sepal growth and emergence of primordia of petals and stamens (stage 5). Subsequently, sepals cover the flower bud (stage 6) and at this stage stamens and carpels become clearly differentiated (stage 7). Stamen primordia elongate (stage 8) and carpels differentiate (stage 9) [105]; for a review see [194]).

Studies at the molecular level suggest that there is an overall conservation among key regulators of floral organ identity and arrangement [195,196,18]. The flowering genes reviewed in the previous section induce the meristem identity genes and these, in turn, regulate the floral organ specification genes among which, MADS-box genes are also key players. Upon induction to flowering, the inflorescence meristem identity genes (such as *TFL1*), that specify the inflorescence shoot as indeterminate and non-floral [197,198] are repressed, while the floral meristem identity genes (*API*,

Figure 4. *Arabidopsis thaliana*'s flower structure is determined by the combinatorial action of different MADS-box proteins. The "quartet model" [191] proposes that the transcription factors complex binds DNA at the promoter regions of their target genes. According to the model, two dimers of each tetramer recognize two conserved DNA sites termed CArG-boxes on the same strand of DNA (with specific separation between them), which are brought into close proximity by DNA bending. Binding of the first dimer is thought to facilitate the binding of the second one. Proteins in the tetramer interact through their different motifs: The MADS-domain binds to the DNA, the I and K domains are involved in dimer formation. The C-domains are supposed to be the transactivation sites, but some of the MADS proteins seem to lack these activity [18]). The exact structures of the MADS-box protein tetramers that control the identity of flower organs are still hypothetical, though several studies on MADS-box protein interactions have been done (e.g., [192,50,37]). CArG boxes represented by blue boxes in bent DNA (blue line), MADS-domain proteins are represented as circles. A-function protein: AP1, APETALA1; B-function proteins: AP3, APETALA3; PI, PISTILLATA; C-function protein: AG, AGAMOUS, E-function proteins: SEP, SEPALLATA. (Figure adapted from [193]; Arabidopsis picture by ER Alvarez-Buylla).

AP2, *CAL*, *LFY*), [129,199,18] are turned on [198]. Mutations in the floral meristem identity genes cause primordia that would develop into flowers to acquire inflorescence meristem identity thus becoming indeterminate. Indeed, *TFL1* is a repressor of the expression of at least two of the floral meristem identity genes, *LFY* and *AP1*. The flower meristem identity genes activate downstream floral homeotic ABC genes (*AP1*, *AP2*, *AP3*, *PI* and *AG*), which are transcription factors necessary for floral organ identity [46,200,77,201]. All of these, except *AP2*, are Type II MADS-box genes.

In the classical ABC model, three different types of homeotic genes of overlapping activities have been proposed to control the development of wild type flowers as follow: sepal (A), petal (A+B), stamen (B+C) and carpel identity (C) [46]. The A and C function genes negatively regulate each other and the B function is restricted to the second and third whorls independently of A and C functions [82,202]. Originally, the function of these genes was inferred by the characterization of their homeotic mutants, which have altered floral organ positions: A class mutants have flowers consisting of carpel-stamen-stamen-carpel; B class mutant flowers bear sepals-sepals-carpel-carpel and the C class mutant flowers have sepals-petals-petals-sepals [46]. Finally, mutations in all three functions lead to the transformation of all floral organs into leaf-like organs, suggesting that flowers are transformed leaves (reviewed in [203]).

Arabidopsis A function genes are: *AP1* and *APETALA2* (*AP2*). *AP1* is a MADS-box gene expressed in the two outer whorls of the floral meristem [97]. Strong *ap1* alleles (*ap1-1*) often lack the second whorl while weaker alleles of this gene do not have a full homeotic conversion of floral organs [132]. This gene is transcriptionally regulated by the B class genes *AP3/PI* [33] and by LFY [201]. On the other hand, *AP2* encodes a putative transcription factor that is a member of a plant specific gene family of genes (AP2/EREBP family) with diverse functions [204,205]. Mutants in the *AP2* gene rarely develop petals and, additionally, their sepals are transformed into carpelloid structures due to ectopic *AG* expression which is negatively regulated by AP2 itself [202].

The B class genes are also MADS-box (*AP3* and *PI*). These two genes are expressed in the second and third whorls and mutant flowers for any of these two genes are identical lacking petals and stamens as predicted by the ABC model [18]. It has also been shown that the proteins encoded by these two genes form heterodimers to exert their function and both are required to activate each other and perform the B function during petal and stamen determination [72,135,206]. Furthermore, these proteins move to the nucleus to function as transcriptional regulators only after they form a heterodimer [42].

Another MADS-box gene is the only C-type gene discovered up to now: *AG*. It has been shown that there was an ancestral *AG*-like MADS-box gene that duplicated before the angiosperm radiation, producing two paralogous lineages [134]: C and D. Even though these two functions are not mutually exclusive, the D class function is primarily involved in ovule identity [207]. Mutant *ag* flowers lack stamens and carpels, and also bear indeterminate

flowers with reiterating sepals and petals, suggesting that *AG* is important for floral meristem determinancy, besides stamen and carpel identity [208].

Interestingly, all MADS-box genes, have expression patterns that correlate with the site where these are necessary. In contrast, *AP2* mRNA is expressed in all four whorls throughout flower development but mutations in *AP2* only affect identity of whorls 1 and 2. Recent data has shown that AP2 is repressed at the translational level by microRNA, which is active only in whorls 3 and 4 [199], thus explaining that its role is delimited to the two first flower organs.

MADS-box genes are also crucial during ovule development. In *Arabidopsis*, ovules develop inside two fused carpels and the MADS-box genes *AG*, *SHATTERPROOF1/2* (*SHP1* and *SHP2*), and *SEEDSTICK* (*STK*) promote the identity of this organ [137,209]. It has been shown that the double mutant *shp1* and *shp2* does not affect ovule development but, as the name of the genes suggest, affect the dehiscence zone inhibiting the carpels shattering [102]. However, the triple mutant *shp1 shp2 stk* shows clear alterations in ovule development with these converted into carpel-like structures [137].

Stamen development is also under the control of the overlapping activities of B and C MADS-box genes. Little is known, however, about additional molecular components that participate in this developmental process. Recent transcriptomic analyses are starting to uncover additional components of this developmental process [210,211,114]. Verelst and collaborators (2007) compared the pollen grain transcriptome of an *agl65/agl66/agl104* triple mutant (which is altered in pollen tube competitiveness, but shows normal pollen grain morphology) against the transcriptome of wild type plants, and found that these MADS-box genes are important regulators of pollen maturation. They also compared their results against those reported by Honys and Twell (2003) on different stages of wild type pollen development and found that the absence of these MADS-box proteins and the complexes they usually form, alters gene expression during several stages of pollen maturation. Verelst and collaborators (2007) also analyzed the role of some double mutants in pollen transcription profiles, and inferred some interactions of complex regulatory network controlling pollen development. Importantly, they found that AGL65/66, AGL65/104, AGL30/66, AGL94/66 and AGL30/104 protein complexes repress immature pollen-specific transcription factor genes and activate mature pollen-specific transcription factors such as the MADS-box genes, *AGL18* and *AGL29*. The latter MADS box genes, in turn, are transcriptional repressors that are highly expressed during immature tricellular pollen grain stages. In addition, these

complexes also repress *AGL30* and *AGL65* in a negative feedback loop, whereas AGL18 acts to fine tune the expression level of *AGL29*. This study suggest that different combinations of MADS proteins have distinct roles during pollen grain development, that seems to be a nice system to uncover the complexity of MADS-protein complexes and their role during cell differentiation processes. Indeed, it has been postulated that MADS proteins exert their regulatory function as multimeric complexes.

Higher-order MADS-domain protein complexes

Using *Antirrhinum majus*, in which the ABC model was also discovered [46], a ternary complex between A and B function proteins that binds DNA more efficiently in comparison to single proteins, was described. A complex of SQUAMOSA (SQUA, the AP1 ortholog) and DEFICIENS/GLOBOSA (DEF/GLO; the AP3/PI orthologs) bound DNA more efficiently compared with DEF/GLO or SQUA alone [50]. Thus suggesting that transcriptional complexes that combine A and B function proteins are more stable than those formed with any of these functions alone.

Using a yeast three-hybrid experiment, it was shown that *SEP3* (another MADS-box gene) and *AP1* are able to interact with the heterodimer *AP3/PI* but not with *AP3* or *PI* alone [37]. Moreover, they described that this interaction was essential for the function of the MADS proteins because the heterodimer *AP3/PI* lacks the activation domain necessary for a transcription factor to function and that both *SEP3* and *AP1*, possess it [37]. These findings suggest that the inclusion of *SEP3* or *AP1* together with *AP3/PI* could result in an active tetrameric transcriptional complex. Concomitantly, our laboratory in collaboration with M. Yanofsky [40] demonstrated that the ABC proteins on their own or combined (A, AB, BC or C) were not sufficient to determine floral organs when expressed in leaves under the action of the 35S constitutive promoter. However, floral organs could indeed be recovered combining ABC and *SEP* genes were expressed in leaves [40,37].

Another example in which the SEP proteins are necessary for the formation of a ternary (or quaternary) complex is during ovule development. AG, SHP1, SHP2 and STK form ternary complexes among them only when SEP proteins are present [209]. Interestingly, the *SEP* genes, *SEP1*, *SEP2* and *SEP3*, received their names because the floral organs that develop in any of the four whorls in triple *sep* mutant plants resemble sepals, and the flowers become indeterminate [76]. This *sep1 sep2 sep3* triple mutant phenotype is markedly similar to that of double mutants that lack both B and C class

activity, such as *pi ag* and *ap3 ag* [212,76]. Single or double mutants for these genes yielded flowers undistinguishable from wild type, thus suggesting that the three *SEP* genes are functionally redundant and are important for the determination of three of the four floral organs: petals, stamens and carpels [37,40,203]. Since the triple *sep1 sep2 sep3* mutant does not show alterations in sepal identity, an additional MADS-box gene could be also involved during specification and development of these floral organs. Indeed, another *SEP*-like MADS-box gene (*SEP4* previously *AGL3*) was characterized [136]. The quadruple *sep1 sep2 sep3 sep4* mutant plants produce flowers that have leaf-like organs in all whorls, thus validating the contributions of *SEP* genes to flower organ identity in all floral organs. Coincidently, *SEP* genes are expressed in the whole floral meristem during flower development, they regulate B and C genes at the transcriptional level and encode proteins that interact with all the ABC proteins [213].

Based on the *SEP* results, it was proposed that MADS proteins form tetrameric complexes during floral organ determination ([18,191,193,195]; Figure 4). The model suggests that within each transcriptional complex, there would be two MADS dimers, each one of them would bind a single CArG binding site causing the promoter DNA region to bend and enable the MADS dimers to act cooperatively. For example, binding of one dimer of the tetramer to DNA could increase affinity for local binding of the second dimer in the tetramer. Besides, one of the dimers could function as an activation domain of the tetramer allowing an efficient transcriptional activation [37]. In a recent study of the complete MADS-domain protein family provided two-yeast hybrid data to document the complete MADS protein-protein interactome for *Arabidopsis* [192]. Several dimers and potential tetramers can be formed from this database and it will be interesting to test which of them are functional and what is their role during *Arabidopsis* development.

There are few examples of MADS proteins that interact or form complexes with members outside this family. To our knowledge only four examples of complexes that involve MADS-box proteins and unrelated polypeptides have been reported in the literature. Our laboratory was the first group to report non-MADS proteins as interactors of a MADS-domain protein (AG): a phosphatase (VSP1) and a Leucine rich protein called FLOR1 [214]; see also [215]. A second report documented an interaction of histone fold protein NF-YB with OsMADS18 from rice [216]. A more recent report showed that AP1 and SEP3 could form a complex with the transcription co-repressors LEUNIG (LUG) and SEUSS (SEU) [217]. Finally, AGL15 was shown to interact with a protein that forms part of the

SWI-independent 3-histone deacetylase (SIN3/HDAC19) complex (SAP18) and with HDAC19 itself [218].

An integrative model of the gene regulatory network underlying floral organ determination

Analytical molecular approaches have been, and will continue to be, successful in producing a wealth of data on specific genes, their most immediate interactors and some cell functions. However, understanding the concerted action of many interacting molecular components, the resulting behavior of complex and integrated biological systems, as well as the consequences of intervening in them, presents serious challenges to contemporary biologists. We are meeting these challenges by combining experimental molecular approaches with dynamic mathematical/computational models [219,58,59,60,220,61].

We have put forward a dynamic gene regulatory network model which steady states or attractors correspond to the multi-gene expression configurations characteristic of each of the four types of primordial cells during early flower development, those of: sepals, petals, stamens and carpel primordia [58]. Interestingly, simulations of loss of function mutations of the nodes corresponding to the ABC genes recovered observed results. For example, when the activation state of *AP1* is set to "0" at all interactions, in order to simulate a homeotic mutant, the steady states configuration that corresponds to the combinations of gene activation typical of primordial sepal and petal cells is not recovered any more. Instead, all the initial states of the network that used to lead to that steady-state configuration now go to the configuration characteristic of stamen and carpel primordial cells. Thus, the model recovers the profile characteristic of the observed homeotic flower lacking sepals and petals. The same was true for all mutations that have been characterized experimentally in *Arabidopsis*, thus verifying the proposed model [58].

Finally, such type of dynamic computational models are also useful to evaluate how robust are the gene activation combinations that characterize each studied primordial cell-type. Indeed, the basic floral plan consisting of whorled sepals, petals, stamens and carpels, which sequentially appear from the outermost to the inner of the flower during development, is quite conserved among angiosperms (specially among higher eudicot species). This pattern suggests that the mechanisms underlying the determination of such primordial cell types should be robust. Concordantly, our simulations of the proposed model confirmed that the network's steady states are robust to

initial states (all of the more than 130,000 initial conditions converge to the gene expression configurations observed in primordial cells during early flower development), but also to small alterations in the rules of interaction among genes that could correspond to genetic alterations [58,59,60].

Perspectives on the role of MADS-box genes in plant development

As reviewed here, plant MIKC genes have been mostly characterized as regulators of the transition to flowering [99] and flower, fruit or seed development [82,130,129,221,137]. They are fairly specific meristem- [97,103], cell- [102] or organ-identity [20,76] genes. The first studies suggested that the function of these genes was specific to certain cell types, tissues or stages of development at which these genes were expressed at the transcriptional level.

However, genome-wide studies are suggesting that most MADS-box genes are expressed at several stages of the plant's life cycle and in a variety of organs, tissues and cell types ([14]; for a review see [93]), suggesting that these genes may have developmental roles that affect multiple stages of development and plant organs. Such recent studies are challenging previous phylogenetic analyses, that had suggested that the genes clustered within each clade shared structure, expression pattern and gene function (*e.g.*, [11]). For example, three groups of genes belonging to the clades of *AG, SOC1* and *ANR1* seemed to be specific to roots [8,108], but we now know that they are expressed in several other organs and may have diverse developmental roles or the same function at different tissues and developmental stages [104]. Interestingly, a transcriptional regulation map for *Arabidopsis* development has revealed that at least one of the ABC MADS-box genes (*PI*), that was supposed to be specific to flowers, could be expressed in the roots as well [192,222].

Indeed, in *Arabidopsis, XAANTAL1* (*XAL1; AGL12*), the sister gene of the group where the first cloned plant MADS-box gene was found (*AG*), is a pivotal gene for both root and flower development [104]. This was unexpected because all the genes in this clade are reported to be specifically expressed and functional only in reproductive tissues [137].

Given the high sequence conservation of MADS domains among plant and animal proteins within each lineage (I and II), we have hypothesized that some of their functions exerted in various plant organs and at different life-stages, may have been also conserved. Such conserved functions may be, for example, related to the animal MEF-related MADS proteins roles, which

have been implicated in the regulation of cellular homeostasis and linked to cell-cycle control [223]. Indeed, *XAL1* seems to be an important modulator of cell proliferation versus differentiation decisions.

The analyses of MADS-box gene function in the root, that is a transparent organ with a relatively simple cellular structure, may enable quantitative analyses of cell dynamics of mutants of these genes [224,225]. Indeed, the root has become a very useful system for unraveling general features of multicellular developmental mechanisms [226,227,228], and specifically for understanding the links between cellular dynamics and cell type specification during normal morphogenesis of a complex organ *in vivo* [229,228,230]. Some components of the molecular mechanisms involved in stem-cell niche patterning and behavior [231,232], as well as in the patterns of cell proliferation along morphogenetic gradients, that in the root are importantly determined by auxins, have been characterized as well [233,234,235]. It will be very interesting to unravel the role of other MADS-box genes in such networks.

Our data strongly suggest that *XAL1* is an important regulator of cell proliferation in the root. *XAL1* mutant alleles have short roots with an altered cell production rate, meristem size and cell-cycle duration. Thus *XAL1* is the first MADS-box gene that is shown to be involved in cell-cycle regulation [104]. Auxins have been implicated in cell-cycle regulation [236,237] and our data interestingly also show that *XAL1* is induced by auxins. On the other hand, as it was reviewed above, *xal1* alleles are also late flowering and our data suggest that *XAL1* could be an important promoter of the flowering transition through up-regulation of *SOC, FT* and *LFY* [104].

Finally, several studies indicate that MADS-box genes are able to integrate environmental and internal signals and, consequently, are very likely important components of the mechanisms underlying the plastic developmental responses of plants to environmental conditions [119,181,162,187]. For example, recent results appear to indicate that MADS-box genes are the targets of both GA signaling [187] and biosynthesis [148]. It remains to be determined whether the regulation of hormone homeostasis is also one of the many MADS-box gene functions. If such were the case, at least some aspects of the MADS-box phenotypes would be mediated by hormone activity and thus mimic phenotypes of lines with altered hormone activities. Additional recent data has demonstrated that *AG, PI, AP3* and *AGL15* [148,187] are direct targets of GA signaling (*AG, PI* and *AP3*) and biosynthesis (*ALG15*). Our recent studies suggest that *XAL1* may be an important component of the regulatory networks that respond to light in the flowering transition control and is a pivotal element of the developmental

pathways that underlie root development, where it seems to mediate hormone activities as well [104].

Another root MADS-box gene, *ANR1*, has been identified as a gene implicated in nitrate signaling pathway and to be responsible for lateral root growth upon nitrogen deficiency [107]. Indeed, nutritional deficiency also triggers flowering [238], and might be sensed at the root [239]. Several important regulators of the transition to flowering are MADS-box genes [127]. Interestingly most of them are expressed in roots but their role in this tissue and how this relates to their role in root development and the flowering pathways is unknown. Therefore, one of the most fascinating challenges in MADS studies will be to continue elucidating their complex integrative roles during coordinated plastic developmental responses occurring at distant parts of the plant.

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Flower Development

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

1. INTRODUCTION: WHEN DID THE FLOWER EVOLVE?

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

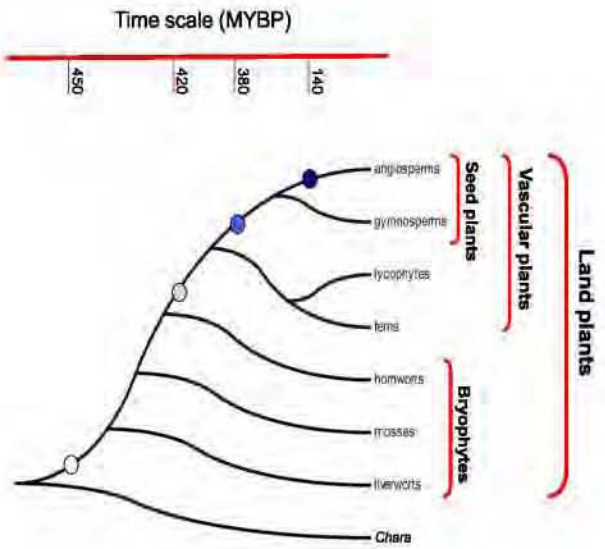


Figure 1. Phylogenetic context of *Arabidopsis thaliana*: Evolutionary history of land plants.

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

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

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

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

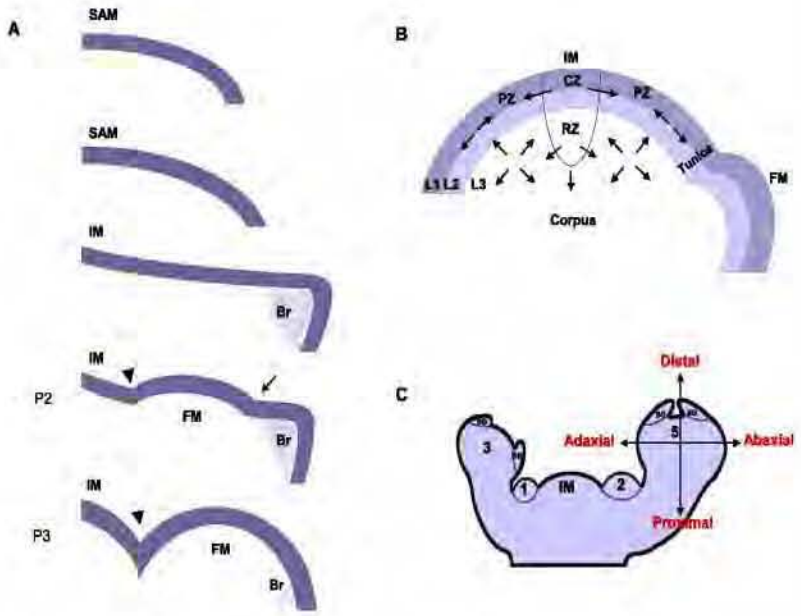


Figure 2. Schematic representation of the shoot apical meristem (SAM): the inflorescence shoot apical meristem and floral meristem.

(A) Diagram outlining the geometry of the inflorescence shoot apical meristem (IM) and flower meristem (FM) during the first stages of development of the latter. On the flank of the IM a first bulge that corresponds to the rudimentary bract (Br) appears. In its axil, a second bulge forms and this continues to grow engulfing the first one and forming the FM proper. Theses stages of FM development correspond to P2 and P3 according to Reddy et al. (2004). The arrow and arrowhead indicate the first and second visible grooves respectively (see section 2.3 for further detail).

(B) Three distinctive zones make up the IM: the central zone (CZ) which contains the stem cells; the peripheral zone (PZ) on the flanks of the CZ that gives rise to the bract and floral primordia; and the rib zone (RZ) underneath the CZ that yields stem tissue. Three cell layers are distinguished: L1 and L2 layers constitute the tunica and include portions of both the CZ and the PZ. The rest of the cells form the L3 layer or corpus. In L1 and L2, cell divisions are anticlinal, while in L3 they occur in all directions (arrows, direction of cell division). The structure is maintained in the FM.

(C) Schematic representation of the boundary zones (blue lines) and axes of polarity during floral development with the differentiation of sepals (se) from the floral primordium illustrated.

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

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

2. STRUCTURAL ASPECTS OF ARABIDOPSIS FLOWER DEVELOPMENT

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

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

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

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

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

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

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

2.2 Floral Organ Primordia

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

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

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

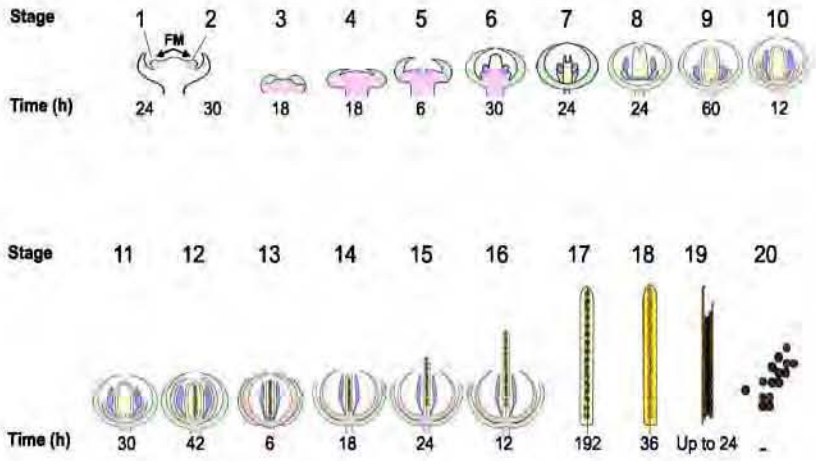


Figure 3. Summary of the 20 stages of flower development.

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

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

2.3 Stages of Flower Development

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

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

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

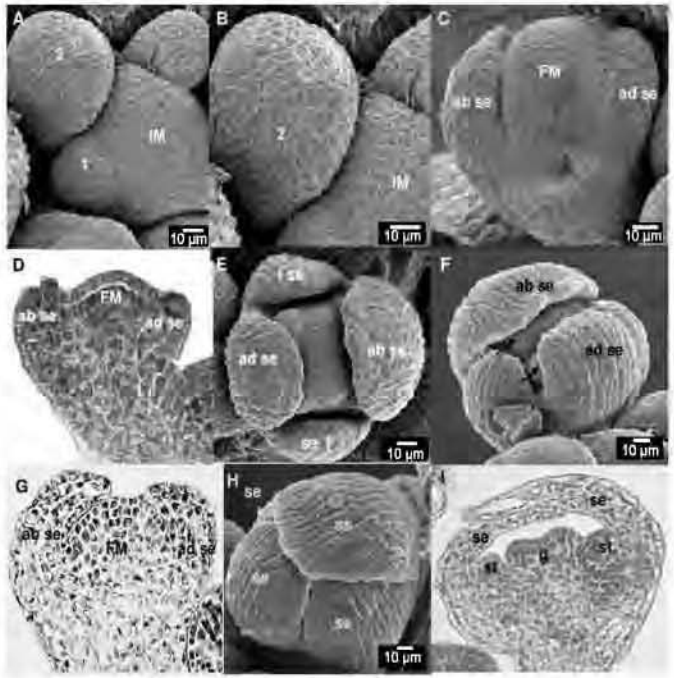


Figure 4. Stages 1 to 6 of Arabidopsis flower development.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.4 Morphology, Histology and Development of Floral Organs

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

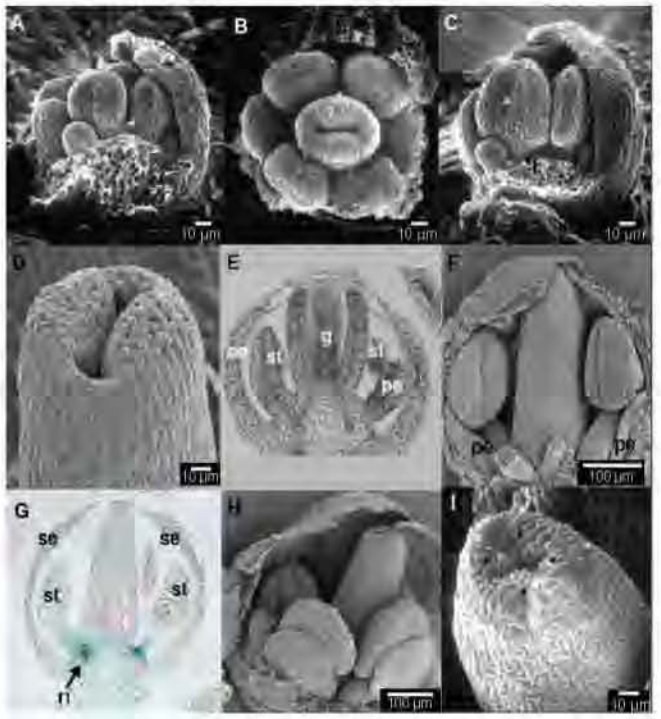


Figure 5. Stages 7 to 10 of Arabidopsis flower development.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. MOLECULAR GENETICS OF ARABIDOPSIS FLOWER DEVELOPMENT

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

3.1 Shoot Apical Meristem Proliferation and Maintenance

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

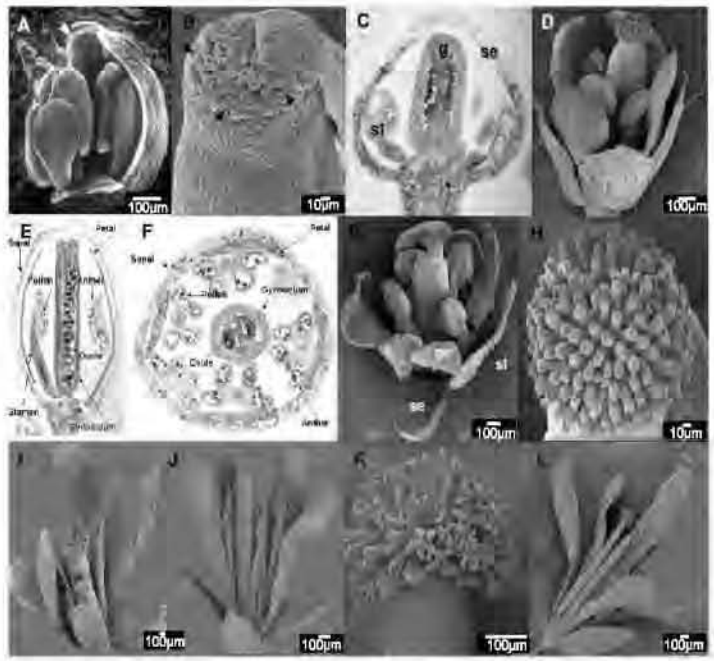


Figure 6. Stages 11 to 16 of Arabidopsis flower development.

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

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

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

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

3.2 Floral Meristem Specification and Determination

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

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

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

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

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

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

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

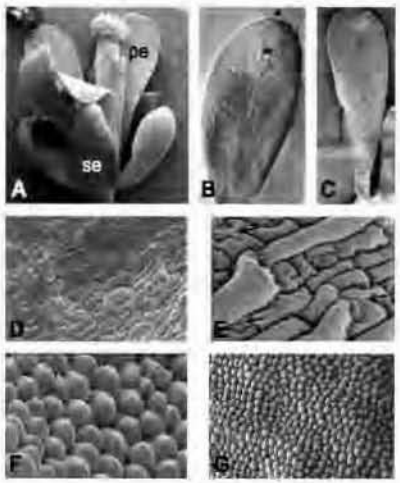


Figure 8. Sepal and petal cell types.

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

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

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

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

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

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

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

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

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

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

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

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

3.3 Specification of Floral Organs: The ABC Genes

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

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

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

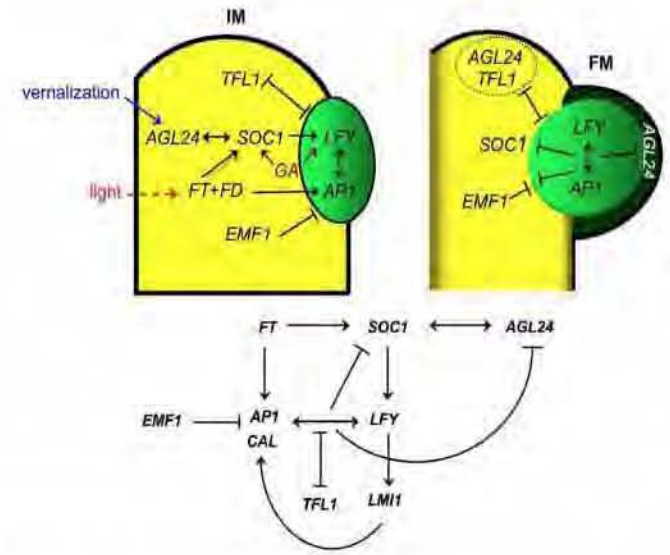


Figure 9. Inflorescence shoot apical meristem (IM) versus flower meristem (FM).

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

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

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

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

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

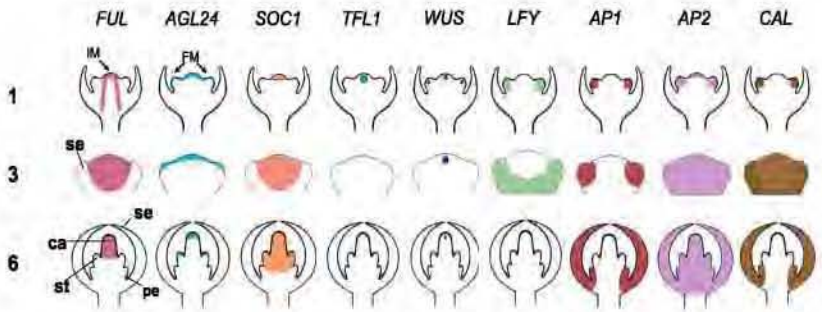


Figure 10. Schematic representation of some inflorescence shoot apical (IM) and flower (FM) meristem gene expression patterns at stages 1, 3 and 6.

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

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

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

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

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

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

AG is repressed by a transcriptional co-repressor complex formed by *LEUNIG* (*LUG*) and *SEUSS* (*SEU*) (Figure 15; Franks et al., 2002). *LUG* encodes a transcription protein similar to *TUP1* from yeast and interacts with *SEU*, which encodes a plant specific protein (see Table S1; Conner and Liu, 2000; Franks et al.,

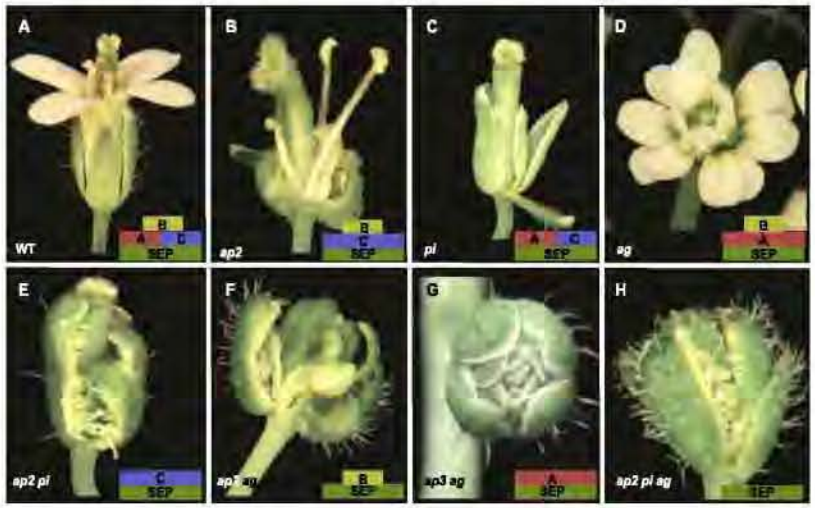


Figure 11. Arabidopsis ABC homeotic floral mutants.

Photos of single, double and triple ABC gene mutant flowers. Each photo is accompanied by a small diagram where rectangles represent the A (*AP1* and *AP2*), B (*AP3* and *PI*), and C (*AG*) combinatorial transcriptional regulatory functions and the *SEP* (*1, 2, 3, 4*) genes active in these mutants. Organs are listed below from the outer to the inner whorl unless stated otherwise.

(A) Wild-type (WT) flower.

(B) Single *ap2* mutant flower composed of carpelloid sepals, stamens, stamens and carpels.

(C) The *pi* mutant has flowers composed of sepals, sepals, carpels and carpels.

(D) The *ag* flower has the stamens transformed into petals and the carpels are replaced by another flower repeating the same pattern.

(E) The *ap2 pi* double mutant displays flowers composed only of sepalloid carpels.

(F) The *ap2 ag* flowers have leaf-like organs in the first and fourth whorls and mosaic petal/stamen organs in the second and third whorls.

(G) *ap3 ag* double mutants produce flowers composed of repeated whorls of sepals.

(H) The *ap2 pi ag* mutant has leaf-like organs with some residual carpel properties. (Photographs provided by Dr. J. Bowman).

2002; Sridhar et al., 2004). Neither of these proteins are able to bind DNA sequences and AP1 and SEP3 recruit SEU/LUG to the second intron of *AG* to perform their inhibitory function and prevent the ectopic expression of *AG* (Sridhar et al., 2006). Recently, another transcriptional repressor of *AG* was identified, *LEUNIG_HOMOLOG* (*LUH*). This gene is the closest homolog of *LUG* and its inhibitory function on *AG* is completely dependent on SEU (Sitaraman et al., 2008).

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

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

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

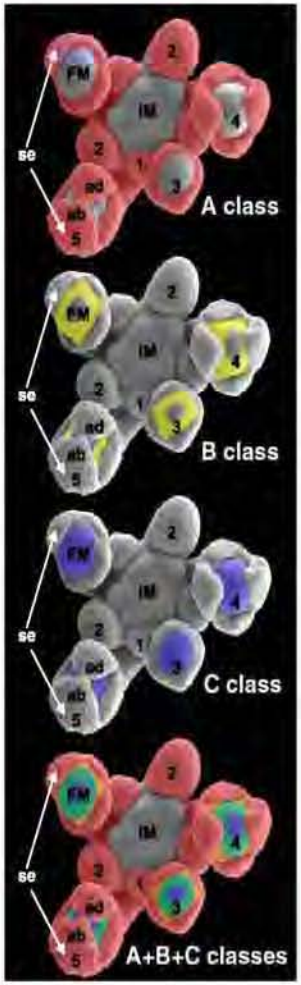


Figure 12. Expression patterns of the ABC genes during early stages of *Arabidopsis* flower development.

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

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

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

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

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

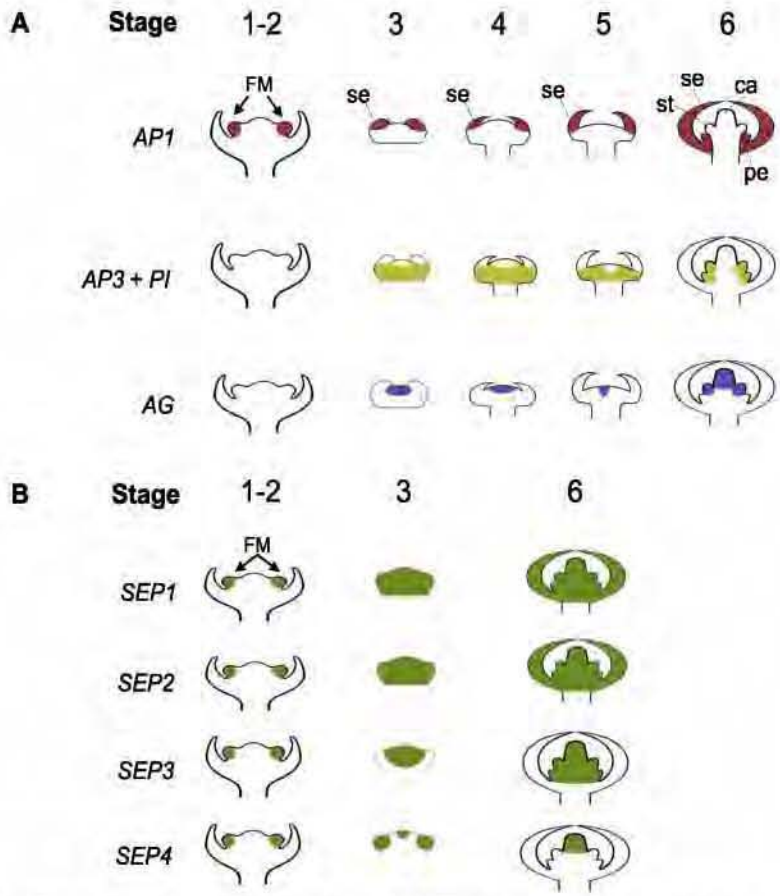


Figure 13. Diagram illustrating mRNA expression patterns of Arabidopsis ABC and SEP genes during different stages of flower development.

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

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

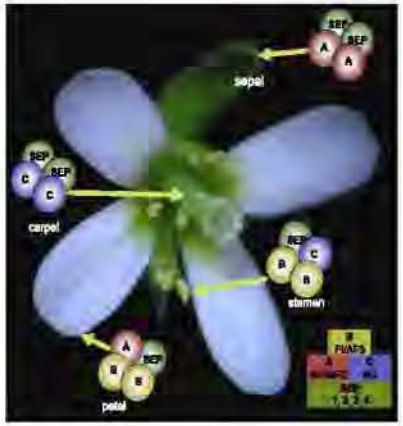


Figure 14. Schematic representation of the interaction of ABC and SEP proteins in the quartet model for *Arabidopsis* floral organ specification.

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

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

3.3.1 *Target genes of the ABCs*

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

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

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

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

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

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

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

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

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

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

3.4 Floral Organogenesis

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

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

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

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

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

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

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

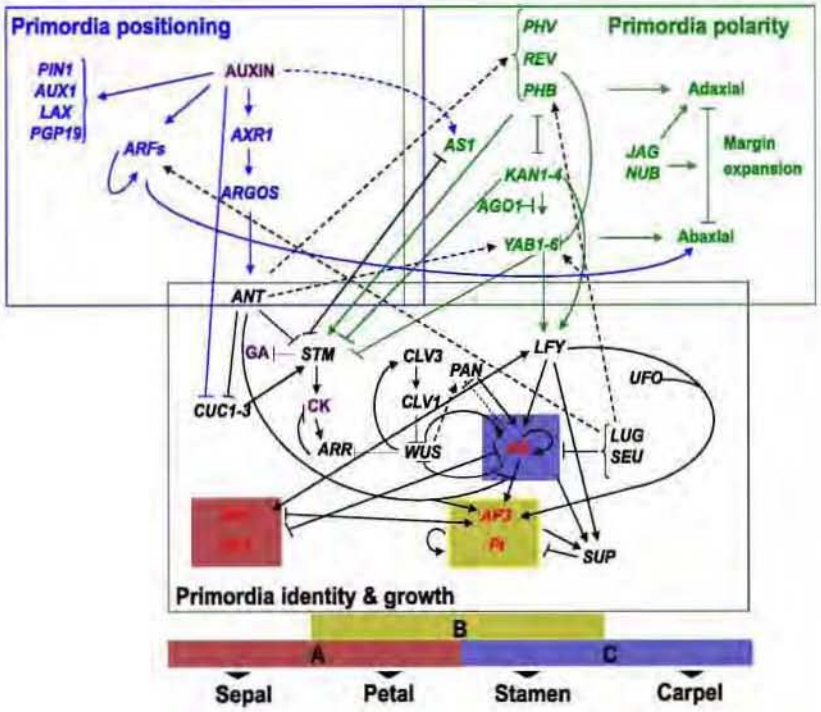


Figure 15. Functional gene regulatory modules during early flower development.

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

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

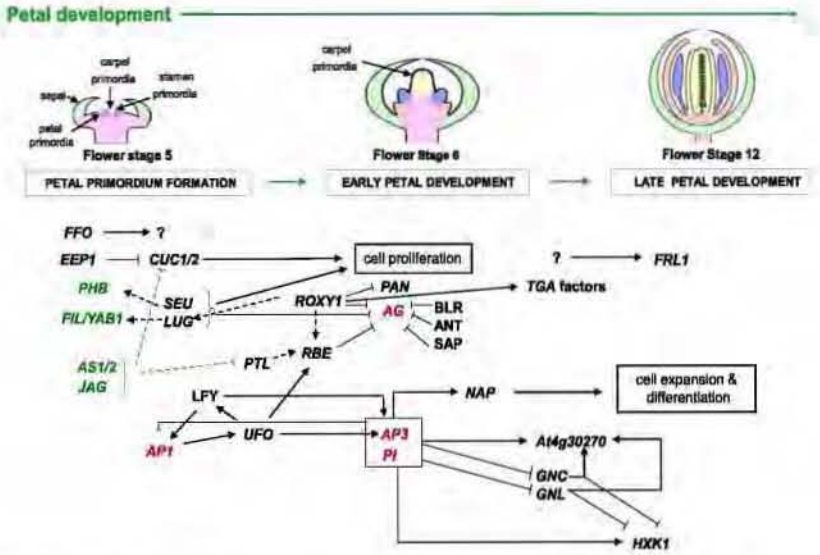


Figure 16. Main stages of petal development and some genes involved.

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

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

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

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

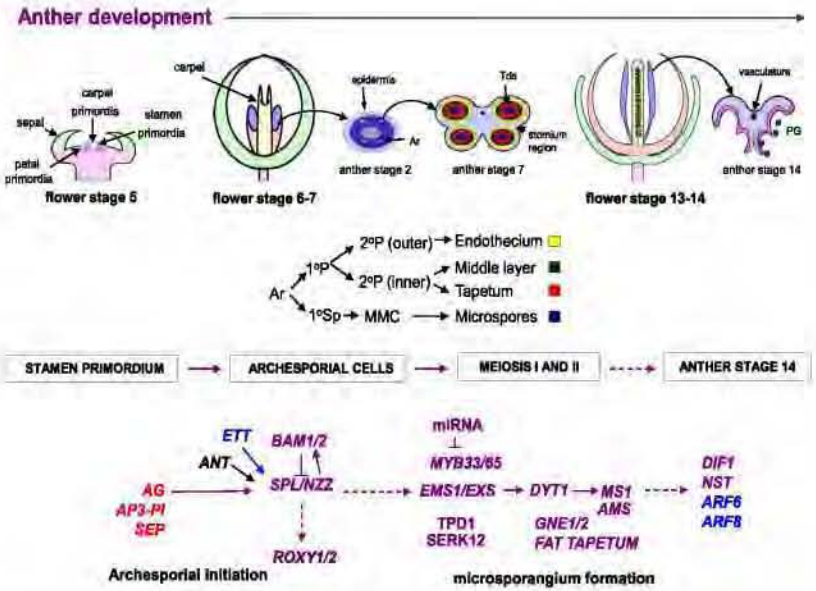


Figure 17. Stages of stamen development with emphasis on the genes implicated in anther formation.

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

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

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

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

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

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

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

3.4.2. Floral organ primordia number, size, and boundaries

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

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

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

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

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

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

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

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

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

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

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

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

3.4.3. *Floral organ polarity*

Establishing organ polarity is an important aspect of morphogenesis and it is sometimes clearly associated with specific functions of plant organs. Both, adaxial-abaxial and proximal-distal polarities are regulated by genetic circuits that are similar for all lateral organs (Figure 2C; Fang and Dickinson, 2007), although each organ type has distinct cell types and morphogenesis in the abaxial versus adaxial surfaces, and in the proximal versus distal regions (Figures 2C and 6). Organ polarity is also linked to the establishment of hormone gradients.

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

YABBY proteins (YAB) are transcription factors with a Zn-finger and a helix-loop-helix (YABBY) domain that are promoters of abaxial cell fate in all lateral organs, among other functions (Bowman 1999; Sawa et al., 1999; Siegfried et al., 1999). During flower development they participate in establishing the primordium domain and meristem patterns, and later in maintaining abaxial polarity (Siegfried et al., 1999; Goldshmidt et al., 2008). *FIL/YAB1*, *YAB2*, and *YAB3* are expressed in a polar manner in all lateral organs of the flower meristem, while *CRABS CLAW* (*CRC/YAB5*) is only expressed in carpels and nectaries, and *INNER NO OUTER* (*INO/YAB4*) is restricted to outer integuments (see section 3.4.6 and 3.4.7; Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Villanueva et al., 1999).

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

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

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

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

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

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

3.4.4 *Sepals and petals*

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

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

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

As it was said before sepal and petal boundary and organ number establishment are controlled by the *CUC* and *FFO2* genes (see Figure 18 and section 3.4.2; Aida et al., 1997; Levin et al., 1998). *CUC* gene expression is regulated by the miR164c (encoded by *EEP1*) in an organ specific manner (Laufs et al., 2004; Baker et al., 2005).

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

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

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

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

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

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

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

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

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

Final sepal and/or petal morphology is also determined by *FRL1* (Hase et al., 2000; Hase et al., 2005), *TSO1* (Hauser et al., 1998), the AP3/PI regulated genes *GNC*, *GNL*, *AtMg30270*, *HXK1* (Mara and Irish, 2008), and *NAP* (Sablowski and Meyerowitz, 1998). Except for FRL1, which is involved in endoreduplication control, and TSO1, which is likely involved in chromatin remodeling, the position of these genes in the petal GRN has already been established (see Figure 16).

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

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

3.4.5 Stamens

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

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

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

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

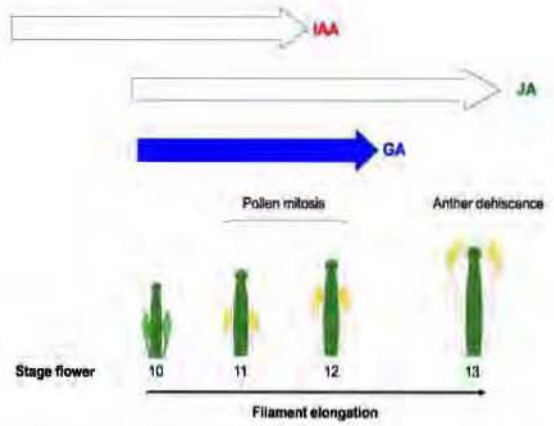


Figure 16. Hormones in late stages of stamen development.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

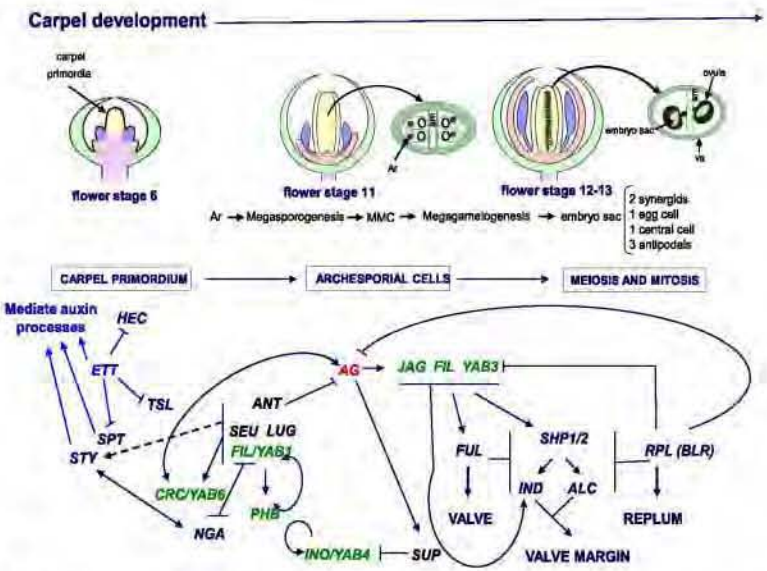


Figure 19. Main stages of carpel development and some genes involved.

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

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

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

3.4.6 *Carpels and ovules*

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

3.4.7 Nectaries

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

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

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

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

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

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

4.1 Gene Regulatory Network Models

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

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

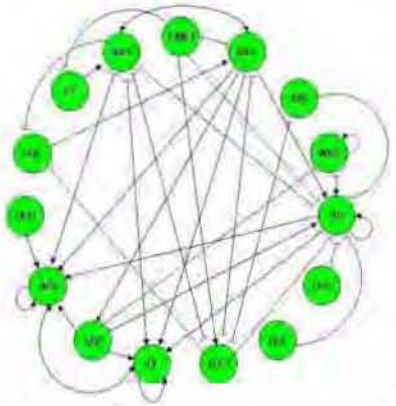


Figure 20. Floral organ specification gene regulatory network (FOS-GRN) model.

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

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

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

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

4.1.2 Functional Modules in Flower Development

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

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

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

4.2 Arabidopsis Flower Organ Specification GRN (FOS-GRN)

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

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

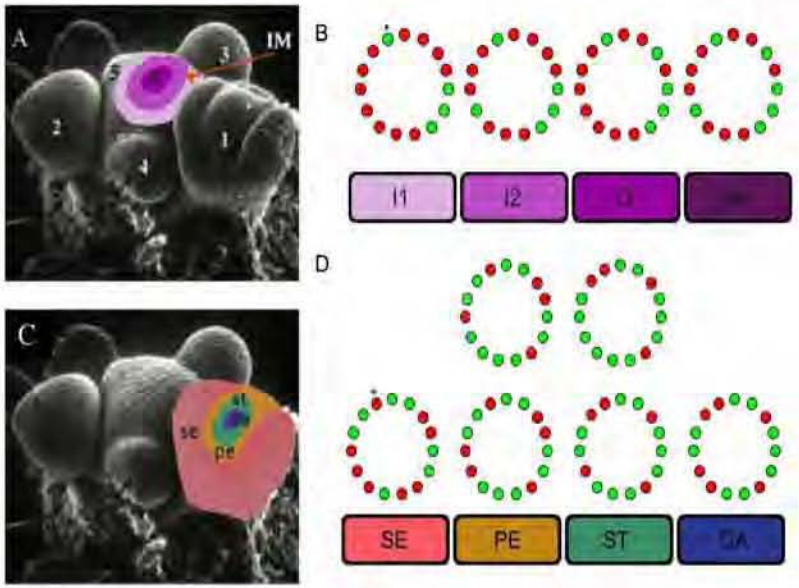


Figure 21. *Arabidopsis* inflorescence and flower development and FOS-GRN.

(A) SEM colored where four regions I1, I2, I3 and I4 are distinguished within the IM. FMs are also seen arising from the flanks of the IM,1 the oldest and 5 the youngest.

(B) I1, I2, I3 and I4 regions of the IM correspond to four of the FOS-GRN attractors. Expressed genes for each attractor are represented as green circles, while non-expressed genes correspond to red circles (nodes are in the same relative position as in Figure 20. * marks the position of the *EMF1* node for further reference). Note that this model recovers the respective regions in the IM with both *WUS* and *UFO*, with either one of these two genes, or with neither expressed.

(C) SEM colored to distinguish four types of primordial cells in young flower meristems. Each will eventually develop into the different flower organs, from the flower periphery to the center, sepals (se), petals (pe), stamens (st) and carpels (ca).

(D) The six attractors of the FOS-GRN model match gene expression profiles characteristic of sepal, petal (p1 and p2), stamen (st1 and st2) and carpel primordial cells. The gene activation profiles of the attractors are congruent with the combinatorial activities of A, B, and C genes described in the ABC model of floral organ determination (adapted from Alvarez-Buylla et al., 2008).

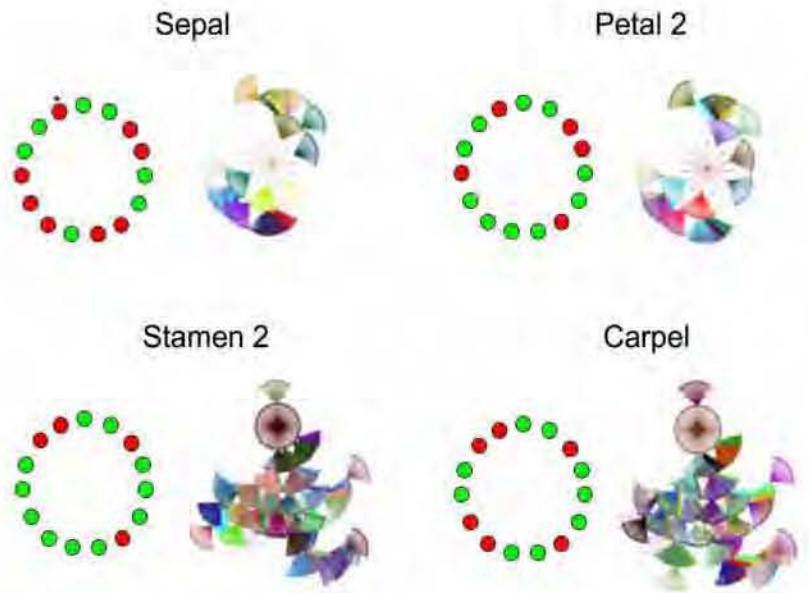


Figure 22. Basins of attraction for the four flower organ FOS-GRN attractors.

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

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

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

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

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

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

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

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

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

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

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

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

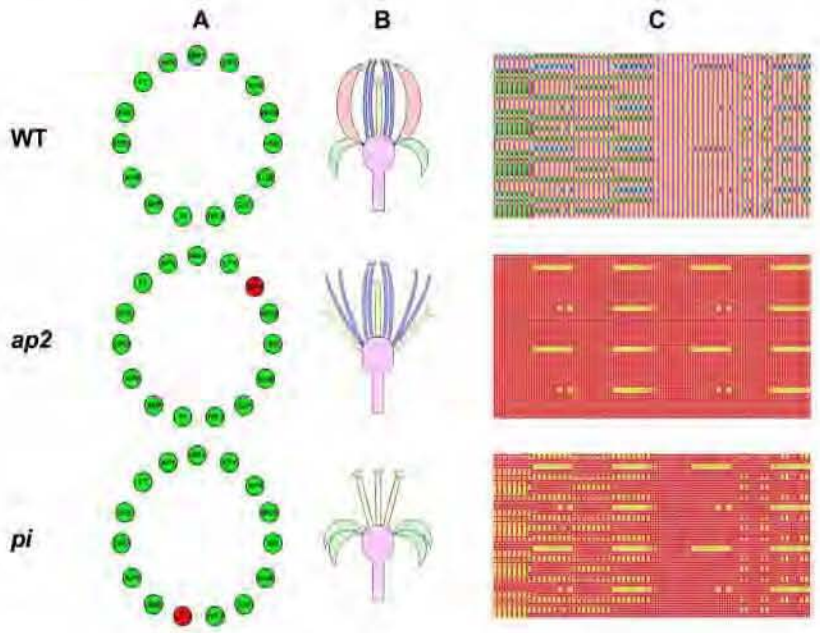


Figure 23. Simulation results for wild type (WT) and two mutants.

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

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

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

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

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

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

(Continued)

Table 1. (continued)

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
LFY+*UFO* (AT1G30950) → *AP3*	Both the amount and the domain of *AP3* expression are reduced in *lfy-6* mutants. *ufo-2* plants have less AP3 protein and less AP3 mRNA. Both *LFY* and *UFO* have to be overexpressed to induce ectopic expression of *AP3*. EMSA show that LFY binds directly to sequences in the *AP3* promoter. ChIP shows that UFO associates with the *AP3* promoter. This association was abolished when ChIP was performed using extracts from *lfy-6* plants harboring the *35S:UFO-Myc* transgene.	Weigel and Meyerowitz, 1993; Meyerowitz, 1995; Parcy et al., 1998; Lamb et al., 2002; Levin and Chae, 2006.
SEP (AT5G15800, AT5G02310, AT1G24260, AT2G03710) → *AP3*	In *AP3:GUS 35S:PI 35S:AP3 35S:AP1* mutants, AP3-GUS is expressed throughout the plant supporting the idea that full activation of the B-function genes requires tetramer formation to include SEP. The ectopic expression of *SEP3* resulted in the induction of ectopic *AP3* expression. Stronger *35S:SEP3* lines are also capable of activating *AP3:GUS* ectopically	Honma and Goto, 2001; Castillejo et al., 2005.
LFY ⊣ *EMF1* (AT5G11530)	Ectopic *LFY* expression in *emf-1* mutants increases the severity of the *emf* phenotype.	Chen et al., 1999.
EMF1 ⊣ *FT*	*FT RNA* levels are higher in the *emf1-1* mutant and are detected earlier than in the wild type.	Moon et al., 2003.
AP1 ⊣ *FUL* (AT5G60910)	*FRUITFULL* is ectopically expressed in *ap1* mutants.	Mandel and Yanofsky, 1995b; Fernández et al., 2000a.
TFL1 ⊣ *FUL*	TFL1 has been postulated to be an inhibitor but it also is possible that other factors have this posttranscriptional inhibitory role. This interaction is necessary as when the negative posttranscriptional regulation of FUL by TFL1 is not considered, the nonfloral gene steady states disappear. No experimental evidence.	Espinosa-Soto et al., 2004.
AP1 → *LFY*	In *ap1* and *ap1 cal* double mutants, *LFY* expression is reduced. Additionally, *LFY* is activated earlier in *35S:AP1* plants than in the wild type.	Bowman et al., 1993; Kempin et al., 1995; Weigel and Nilsson, 1995; Pinheiro and Coupland, 1998; Liljegren et al., 1999.
EMF1 ⊣ *LFY*	Double mutants of the weak *emf1-1* allele and *lfy-1* bear *lfy*-like flowers suggesting that, for this trait, *lfy* is epistatic. These genes have antagonistic activities.	Yang et al., 1995.
FUL → *LFY*	Even though *LFY* expression is similar in wild type and *LFY:GUS ful-2* plants, there is less expression in *ful ap1 cal* triple mutants than in *ap1 cal* double mutants, suggesting that the role of *FUL* in *LFY* upregulation is only important when *AP1* is inactive.	Fernández et al., 2000a.
TFL1 ⊣ *LFY*	In *tfl1* mutant plants *LFY* is ectopically expressed in the shoot apex.	Weigel et al., 1992; Ratcliffe et al., 1999.
LFY→ *PI* (AT5G20240)	Amount and domain of *PI* expression are reduced in *lfy-6* mutants. There is no *GUS* expression in early *lfy PI:GUS* flowers.	Weigel and Meyerowitz, 1993; Honma and Goto, 2000.
PI → *PI*	AP3 and PI co-immunoprecipitate. *AP3* and *PI* mRNA levels are not maintained in *ap3-3 pi-1* double mutants. In *AP3:GUS 35S:PI 35S:AP3 35S:AP1* mutants, *AP3:GUS* is expressed throughout the plant supporting the idea that full activation of the B-function genes requires PI	Jack et al., 1992; Goto and Meyerowitz, 1994; Honma and Goto, 2001.
LFY → *SEP*	Microarray experiments show that the group of *LFY* dependent genes includes the homeotic collectors *SEP1-3*.	Schmid et al., 2003.
AP1 ⊣ *TFL1*	In *35S:AP1*, *TFL1* expression is greatly diminished. *TFL1* is ectopically expressed in *ap1 cal* double mutants.	Liljegren et al., 1999.
AP2 ⊣ *TFL1*	The *tfl1-1* mutation partially suppresses the *ap2-1 ap1-1* inflorescence phenotype.	Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993.
EMF1 → *TFL1*	In *emf1-2 tfl1* double mutants, the *emf1-2* mutation is epistatic with respect to flower initiation. These genes do not have antagonistic activities. This suggests that EMF1 upregulates *TFL1*.	Chen et al., 1997.
LFY – *TFL1*	The *35S:LFY* plants resemble the *tfl1* mutant and have no *TFL1* expression. *LFY* can inhibit *TFL1* at the transcriptional level. *TFL1* is also ectopically expressed in *lfy* mutants.	Weigel and Nilsson, 1995; Liljegren et al., 1999; Ratcliffe et al., 1999.
AG ⊣ *WUS*	There is strong *WUS* expression in the center of *ag* floral meristem.	Lenhard et al., 2001; Lohmann et al., 2001.

Table 1. (continued)

INTERACTIONS	EXPERIMENTAL EVIDENCE	REFERENCE
SEP –| *WUS*	*SEP* activity is required for *WUS* downregulation by *AG* because *sep1 sep2 sep3* triple mutant plants bear indeterminate flowers.	Pelaz et al., 2000.
WUS → *WUS*	No experimental evidence. Assumption of model.	Espinosa-Soto et al., 2004; Chickarmane et al., 2006
UPDATES (Chaos et al., 2006 and this chapter)		
EMF1 –| *AG*	In ChIP experiments, *EMF1* is associated with sites in the promoter and second intron of *AG*. *EMF1* interferes with transcription by RNA polymerase II and T7 RNA polymerase *in vitro*.	Calonje et al., 2008.
AP3 –| *AP1*	*AP1* transcript levels are significantly higher in *ap3-3* mutant plants than in both *WT* and *35S:AP3*.	Sundström et al., 2006.
PI –| *AP1*	ChIP shows that PI binds to target sequences in the *AP1* promoter	Sundström et al., 2006
MIR172 (AT2G28056, AT3G04275, AT3G11435) + *HEN1* (AT4G20910) –| *AP2*	Elevated *miR172* accumulation results in floral organ identity defects similar to those in loss-of-function *ap2* mutants. On the other hand, the *miR172* abundance depends on the activity of DICER-like protein HUA ENHANCER 1 (HEN1), which is expressed through the plant. This observation suggests that a cofactor expressed in the inner floral whorls is required to give specificity to the HEN1-dependent repression of *AP2*. The need for *AG* inactivity for *AP2* function is added to the *AP2* logical rules	Chan et al., 2002; Park et al., 2002; Chen et al., 2004; Zhao et al., 2007.
LFY → *SEP1-3*	Microarray experiments show that the group of *LFY* dependent genes includes the homeotic cofactors *SEP1-3*.	Schmid et al., 2003.
INTERACTIONS NOT INCLUDED IN THE MODEL		
AGL24 (AT4G24540) + *SVP* (AT2G22540) –| *AG*	In the *ag24 svp* double mutant, *AG* mRNAs are detected in the inflorescence and floral meristems as early as stage 1, indicative of early *AG* expression. In later stages, *AG* is still expressed in all floral organs. Probably, this interaction is part of a different GRN that occurs before the cell fate determination	Gregis et al., 2006.
BLR (AT5G02030) –| *AG*	*AG* is expressed ectopically in *blr* mutants. *BLR* directly binds to *AG cis* elements (identified by EMSA). This interaction is probably important in organogenesis.	Bao et al., 2004.
RBE (AT5G03070) –| *AG*	In *rbe* mutants, there is ectopic expression of *AG* in second-whorl cells. This interaction may be important in organogenesis.	Krizek et al., 2006.
SEU (AT1G43850) –| *AG*	The direct *in vivo* association of SEUSS (SEU) with the *AG cis*-regulatory element was shown by ChIP. SEU interacts with LUG in a repressor complex to regulate *AG*, and LUG is already considered in the GRN model.	Sridhar et al., 2006.
AGL24+*SVP* –| *AP3*	An *in situ* analysis shows that in the *ag24 svp* double mutant, *AP3* is expressed in all parts of the floral meristem and late in all floral organs. Probably, this interaction is part of a different GRN occurring before the cell fate determination.	Gregis et al., 2006.
LFY → *CAL* (AT1G28310)	Using posttranslational activation of LFY-GR, it is demonstrated that CAL is a direct LFY target. *cis*-regulatory elements in the putative CAL promoter are bound by LFY. AP1 forms heterodimers with CAL, and AP1 is already included.	William et al., 2004.
AP3 –| *FUL*	The domain of *FUL* expression is expanded to the third whorl in stage-3 *ap3* mutants, but no direct interaction is detected by ChIP analysis.	Mandel and Yanofsky, 1995b; Sundström et al., 2006.
FT –| *FUL*	*FUL* is expressed at higher levels in 35S:FT-VP16. It is not considered because this interaction could be mediated by TFL1 and LFY.	Teper-Bamnolker and Samach, 2005.
PNY (AT5G02260) → *LFY* *PNF* (AT2G27990) → *LFY*	The transcripts of *LFY* are substantially reduced in shoot apices of *pny pnf* double mutants after floral induction. *pny pnf* double mutants do not produce flowers but, *35S:LFY pny pnf* plants do produce flowers. This interaction is part of a different GRN.	Ansar et al., 2006.
AP2 → *PI*	*In situ* hybridization shows there is less *PI* RNA occupying a smaller area in *ap2-2* flowers than in wild type. Probably an indirect effect.	Zhao et al., 2007.
AG — *SEP3*	ChIP shows that AG interacts *in vivo* with predicted regulatory sequences of *SEP3*. Insufficient experimental data.	Gómez-Mena et al., 2005.
FT → *SEP3*	Overexpression of *FT* causes ectopic expression of *SEP3* in leaves. No further experimental evidence.	Teper-Bamnolker and Samach, 2005.

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

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

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

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

5. CONCLUSIONS AND PERSPECTIVES

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

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

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

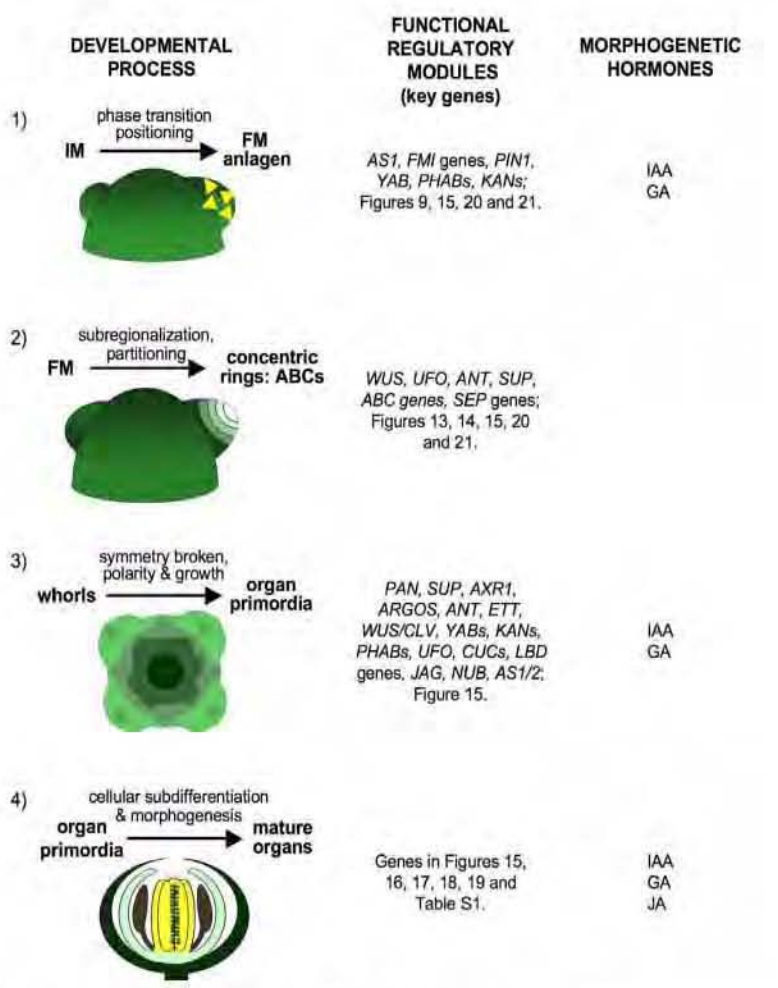
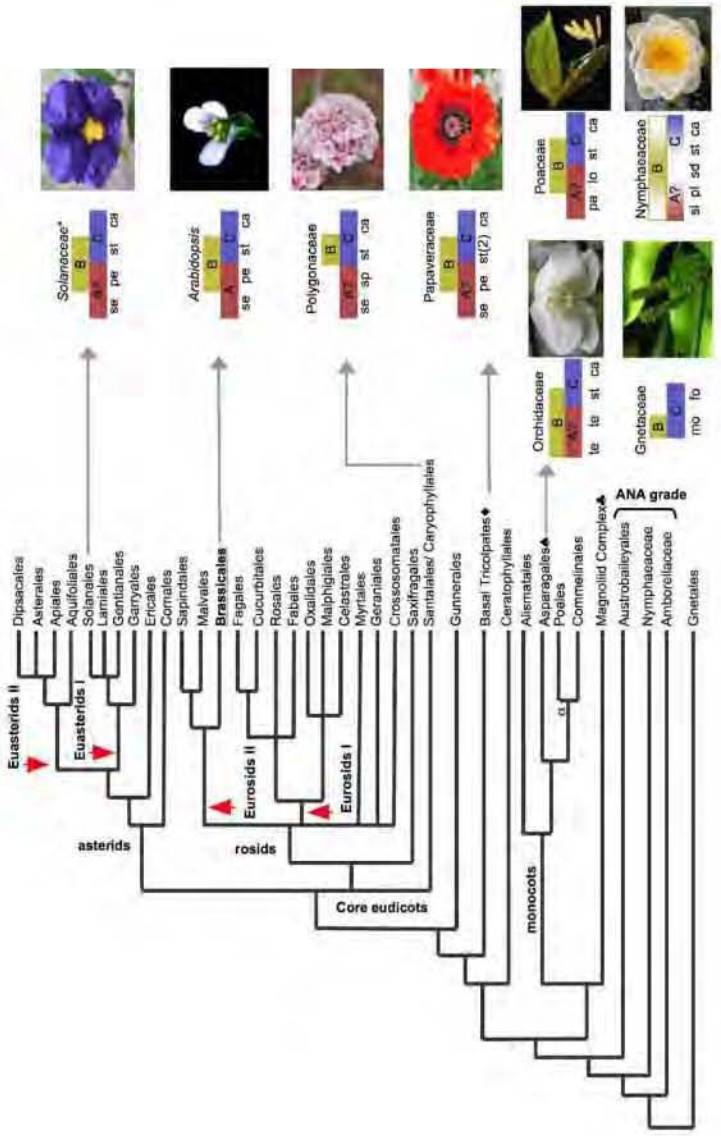


Figure 24. The main regulatory gene modules and hormone signaling pathways during flower developmental processes.

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



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

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

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

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

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

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ANEXO C: Artículos de divulgación realizados durante el doctorado y en los que participé como co-autor (sólo portadas).

Apéndice III

Alvarez-Buylla, E.R., Anaya, AL., Barradas, V., Benítez, M., Cruz-Ortega, R., Gamboa, A., Garay, A., García Ponce de León, B., Mendoza ; A., Campo, J., **Pérez, R.**, Orozco, A., Sánchez-Jiménez, MP. y Solis, E. **(2011)** De los genes al cambio climático: nuevos paradigmas y retos de la ecología funcional. Revista Ciencias 103:54-64.

Apéndice IV

Garay-Arroyo, García-Ponce B., **Pérez-Ruiz, R.,** Piñeyro-Nelson, A y Sánchez MP. **(2011)**. La genética de la flor y la sexualidad de las plantas. Oikos 4:14-21.

De los genes al cambio climático

Es evidente el deterioro ambiental que se percibe a escala local y a corto plazo, así como el que se vislumbra por efecto del cambio climático global. Ambos constituyen retos fundamentales para las ciencias ecológicas y la supervivencia de la vida sobre la Tierra. Para hacer frente a estos retos se requiere nuevos paradigmas en biología, tales como la ecología y evolución del desarrollo, el estudio de los sistemas complejos y las ciencias de la sustentabilidad. Estos tres ejes de estudio de los sistemas ecológicos son sustento conceptual y motor de las investigaciones que se realizan en nuestro departamento.

E. Álvarez-B., A. L. Anaya, V. Barradas, M. Benítez, J. Campo, R. Cruz, A. Gamboa,

Los sistemas complejos

Gracias al avance en el estudio de los sistemas biológicos ha quedado demostrado que el mundo vivo no se puede entender con modelos simples, relaciones de causalidad que relacionan una a una las variaciones en el comportamiento de los múltiples componentes de cualquier sistema vivo y de manera proporcional o lineal, en función del ambiente y sus perturbaciones. Esto es válido para todos los niveles de organización de la vida: desde los genes, las redes reguladoras o metabólicas y el funcionamiento y la diferenciación de las células, hasta los fenómenos ecosistémicos y globales –por ejemplo, aquellos que rigen la relación de los vegetales con los cambios atmosféricos, uno de los temas importantes de nuestras investigaciones.

Históricamente, la ciencia se ha dedicado principalmente a descomponer los sistemas en sus partes más pequeñas y a estudiar su comportamiento de manera aislada o, cuando mucho, en interacciones pareadas. A este enfoque se le conoce como "reduccionismo" y es útil, pero no suficiente para entender el comportamiento de los complejos sistemas biológicos.

La ciencia ahora se aboca también al estudio de las interacciones de muchos o todos los componentes de un sistema y de su comportamiento colectivo. Esto ha permitido estudiar cómo se autoorganizan los elementos que interactúan en un sistema y llegan a dar lugar a ciertos patrones y regularidades emergentes, una característica de los sistemas complejos. Por ejemplo, el desarrollo de los organismos es regular, pero no es simple, y sus patrones emergen como resultado de la acción concertada y compleja de muchos componentes genéticos, epigenéticos (cambios heredables aunque no codificados en el ADN), celulares, etcétera.

Desde la perspectiva de los sistemas complejos se puede analizar procesos micro, meso y macroscópicos. Por ejemplo, es posible elaborar algoritmos que ayuden a entender el efecto de los genes en rasgos visibles (en el fenotipo), y así poder entender el desarrollo, la ecología y la evolución.

Ecofisiología y evolución del desarrollo

El desarrollo es el proceso mediante el cual una célula fecundada o un cúmulo de células indiferenciadas (como las meristemáticas en las plantas) se diferencian y arreglan espacio-temporalmente en las estructuras tridimensiona-

A. Garay, B. García, A. Mendoza, R. Pérez, A. Orozco, M. Sánchez, M. Sánchez y E. Solís

La genética de la flor y la sexualidad de las plantas

Adriana Garay-Arroyo, Berenice García-Ponce, Rigoberto V. Pérez-Ruiz, Alma Piñeyro-Nelson y María De La Paz Sánchez

La sexualidad y la evolución de la mayor parte de las plantas que nos rodean y alimentan (las angiospermas) gira alrededor de sus flores; sin embargo, aún quedan muchas interrogantes sobre las bases genéticas que favorecen que las flores sean al mismo tiempo tan conservadas en su estructura básica y tan variables en sus detalles. Nuestra curiosidad por entender a las flores no es nada nuevo; desde tiempos remotos el ser humano se ha preguntado sobre su origen, su modo de desarrollo y las fuerzas naturales que dieron paso a la gran diversidad vegetal existente. Al inicio, el interés del humano se concentraba en conocer las plantas en función de su valor de uso, pero luego le fueron surgiendo preguntas en torno a su desarrollo y relaciones de parentesco. Dichas preguntas datan desde la época de los griegos y diferentes culturas fueron creando sus propios sistemas de organización y descripción de la flora con la que convivían. Este fenómeno se exacerbó en Europa desde el Renacimiento, cuando se comenzó a tener registro de la flora y fauna exótica procedente de otros continentes. Sin embargo, la flor como estructura no adquirió la importancia botánica que tiene ahora sino hasta que Carl von Linné estableció, en 1753, un sistema de clasificación taxonómica (*Species Plantarum*) con base fundamentalmente en la estructura de la flor. A partir de ese momento, el entender cómo se generaba esta estructura y sus variantes en diferentes linajes de angiospermas ocupó el tiempo y mente de los morfólogos de los siguientes dos siglos, entre ellos, de Johann Wolfgang von Goethe.

Foto: Alma Piñeyro Nelson

De todas las plantas, sólo las espermatofitas, que agrupan a las gimnospermas y a las angiospermas, tienen estructuras reproductivas con semillas en sentido estricto y de éstas, sólo las angiospermas tienen flores. Las flores tienen un conjunto de características que las hacen únicas, como son: 1) la presencia de un carpelo, conocido también como gineceo o pistilo, que constituye el órgano femenino de las flores y dentro del cual se desarrollan primero los óvulos y después de la fertilización, los embriones (Fig. 1). Este carpelo tiene como característica fundamental el estar conformado por dos integumentos que protegen a los óvulos. Otras caracterís-