UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

CARACTERIZACIÓN GENÉTICA Y MOLECULAR DE MECANISMOS DE REPRESIÓN FLORAL EN ARABIDOPSIS

TESIS DOCTORAL

ANA LÁZARO SOMOZA

MADRID, 2011

UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

CARACTERIZACIÓN GENÉTICA Y MOLECULAR DE MECANISMOS DE REPRESIÓN FLORAL EN ARABIDOPSIS

Memoria presentada por Ana Lázaro Somoza para optar al grado de Doctor por la Facultad de Ciencias de la Universidad Autónoma de Madrid.

Trabajo realizado en el Departamento de Biotecnología del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) bajo la dirección de los Doctores José Antonio Jarillo Quiroga y Manuel Piñeiro Galvín.

VºBº DE LOS DIRECTORES　　VºBº TUTOR　　LA DOCTORANDA

J.A. JARILLO　　M. PIÑEIRO　　RAFAEL RIVILLA　　A. LÁZARO

TESIS DOCTORAL
ANA LÁZARO SOMOZA
MADRID, 2011

AGRADECIMIENTOS

Han pasado ocho años desde que decidí que me iba a dedicar a la investigación y en este tiempo he recibido ayuda y ánimo de un montón de gente. Tengo que dar las gracias a mis directores de Tesis, Jari y Manolo, y a todas las personas que han pasado por el laboratorio durante estos años, Iván, Eugenio, Leti, Laura, Raquel, Angelita, David, Pablo, Silvia, Sandra, Alfonso y Nuria. Una buena parte de esta Tesis se realizó en el Edificio Z del INIA, dónde compartí los primeros años con Bruno, Pepe, Elenita, Ana del Cueto, Mariano, Rafa, Tamara, Iñaki, Carlitos, Fernando Novillo, Gemita, Claudia, Carmen Mansilla, Pablo Lunello, Chuchi, Marga, Adela y Vicky. También tengo mucho que agradecer a Carlos, Conchi, Zamira, Gema, Nacho, Julieta y Rosa, con los que he convivido todo este tiempo, y a Sara, David Carrasco, Kata, Mar y Elena Ramírez, que llegaron más tarde al CBGP. Tampoco quiero olvidarme de otras personas que me han ayudado y enseñado muchas de las técnicas que he usado en estos años, Maremoto, Isra, César Poza, Lars Hennig, Pablo González-Melendi; y de todas las cosas que le he pedido "prestadas" a Julio Salinas.

Gracias a todos por echarme una mano siempre que lo he necesitado, por todo el apoyo en los momentos duros y por aguantarme, que tiene tela.

Y, por supuesto, gracias a David, que es mi refugio, a mi familia y a mi madre en especial, a mis amigos de hace muchos años, María, Lorenzo, Laura Rol, Sara, Gabino, Betty, Raúl, Laura Hillán, y a los amigos que comparto con David estos últimos años. También quiero dar las gracias a la gente que ya no está aunque, de un modo u otro, también han hecho esto posible, mi padre y mis abuelos.

ÍNDICE

Página

SUMMARY 1

ABREVIATURAS Y SIGLAS 2

INTRODUCCIÓN 3

1. La transición floral 5
2. El control genético de la floración en *Arabidopsis thaliana* 6
   2.1. Variación genética natural 6
   2.2. Variación genética inducida 8
      2.2.1. Rutas de inducción de la floración 8
         Ruta del fotoperiodo 9
         Ruta de la vernalización 12
         Ruta dependiente de la temperatura ambiental 14
         Ruta autónoma 14
         Ruta de las giberelinas 15
         Ruta dependiente de la edad 15
      2.2.2. Integradores florales 16
      2.2.3. Represores de la floración 18
3. El control epigenético de la floración en *Arabidopsis thaliana* 19
   3.1. Activación de la expresión de *FLC* 20
   3.2. Represión de la expresión de *FLC* 22
   3.3. Regulación epigenética de otros genes implicados en el control del tiempo de floración 25
4. El papel de la degradación específica de proteínas en la transición floral en *Arabidopsis thaliana* 25

OBJETIVOS 29

RESULTADOS 33

CAPÍTULO 1. 35

EARLY IN SHORT DAYS 1 (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in *Arabidopsis*
(Martin-Trillo y col., 2006).

CAPÍTULO 2. 59
Mutations in the *Arabidopsis SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development (Lazaro y col., 2008).

CAPÍTULO 3. 81
The E3 ubiquitin ligase HOS1 participates in the control of photoperiodic flowering negatively regulating CONSTANS abundance
(Lazaro y col., 2011 en revisión).

DISCUSIÓN 109
1. Papel de la variante histónica H2A.Z en la regulación del tiempo de floración 111
2. Función de HOS1 en la regulación fotoperiódica de la floración 120

CONCLUSIONES 125

BIBLIOGRAFÍA 129

SUMMARY

The coordination of flowering time with seasonal and developmental cues is critical to maximize reproductive success in plants. In this work we have characterised components in two different mechanisms involved in the floral repression in Arabidopsis.

In one hand, we have isolated two early flowering mutations, *esd1/arp6* and *swc6*, affecting putative orthologues of components of the yeast Swr1 chromatin remodelling complex. We found that ESD1/ARP6 and SWC6 are required for maintaining the expression of the *FLC* repressor to levels that inhibit flowering. Genetic and physical interactions between SWC6 and ESD1 have been demonstrated in this study, suggesting that both proteins act in the same complex. Besides, we have established that ESD1/ARP6 and SWC6 are required for both histone H3 acetylation and H3K4 trimethylation on *FLC* chromatin. Altogether, the results obtained suggest that SWC6 and ESD1 are part of an Arabidopsis SWR1 chromatin remodelling complex involved in the regulation of diverse aspects of plant development, including floral repression through the activation of *FLC* and *FLC*-like genes.

On the other hand, we found the *early in short days 6* (*esd6*) mutant in a screening for mutations that accelerate flowering time in Arabidopsis and showed that it was affected in the *HOS1* locus, which encodes a RING finger-containing protein that works as an E3 ubiquitin ligase. The *esd6/hos1* mutation showed a strong requirement of a functional CO protein for its early flowering phenotype under long days. Besides, CO and HOS1 physically interact *in vitro* and *in vivo*, and HOS1 regulates CO abundance, particularly during the daylight period. Accordingly, the *hos1* mutation causes a shift in the typical long day pattern of the *FT* transcript, starting to rise four hours after dawn. In addition, *HOS1* interacts synergistically with *COP1*, another regulator of CO protein stability, in the control of flowering time. Taken together, these results indicate that HOS1 is involved in regulating CO abundance ensuring that CO activation of *FT* occurs only when the light period reaches a certain length and preventing precocious flowering in Arabidopsis.

ABREVIATURAS Y SIGLAS

ADN	Ácido desoxirribonucleico
ARN	Ácido ribonucleico
ARNi	ARN de interferencia
col.	Colaboradores
DC	Día corto
DL	Día largo
GFP	Green fluorescent protein
miR	microRNA
pb	Pares de bases
ZT	Zeitgeber time

INTRODUCCIÓN

1. La transición floral

El desarrollo de las plantas es el resultado de la división de grupos de células pluripotentes denominados meristemos y su posterior diferenciación en los distintos órganos vegetales (Ma, 1998). La porción aérea de la planta se forma a partir del meristemo apical, mientras que el meristemo radicular da lugar a las raíces.

Las plantas son organismos sésiles que tienen la capacidad de percibir multitud de señales ambientales y de adaptar su desarrollo a los cambios que se producen en el medio que las rodea. La transición de la fase de desarrollo vegetativo a la fase reproductiva, lo que se conoce como transición floral, es uno de los procesos más finamente regulado, ya que del momento en que tenga lugar depende el éxito reproductivo de las plantas (Amasino, 2010). La transición floral implica importantes cambios en la identidad del meristemo apical. Durante la fase vegetativa el meristemo da lugar a hojas y meristemos axilares, que a su vez producirán ramas vegetativas. Sin embargo, una vez que se produce la transición floral, comienza la formación de flores a partir de los meristemos reproductivos (Coen y Meyerowitz, 1991).

El tiempo de floración está controlado por multitud de factores, unos endógenos y otros ambientales. Los primeros dependen fundamentalmente del estado de desarrollo de la planta, mientras que los factores ambientales que regulan la floración son el fotoperiodo (relación entre los periodos diarios de luz y de oscuridad), la intensidad y la calidad de la luz que recibe la planta, y la temperatura (Kim y col., 2009; Michaels, 2009; Amasino, 2010; Imaizumi, 2010). En concreto, la aceleración del tiempo de floración que se produce como consecuencia de la exposición de las plantas a periodos prolongados de bajas temperaturas se conoce como vernalización.

Entre las especies sensibles al fotoperiodo se pueden distinguir plantas en las que la floración se induce por exposición a día corto (DC, el periodo de oscuridad es más prolongado que el de luz), como el arroz (*Oryza sativa*), y plantas en las que se induce por condiciones de día largo (DL, el periodo de luz es más prolongado que el de oscuridad), como la avena (*Avena sativa*). En cambio, otras especies vegetales, como el tomate (*Solanum lycopersicum*), son insensibles al fotoperiodo, (Jarillo y col., 2008). De igual modo, hay especies que presentan un requerimiento absoluto de vernalización para florecer, como la remolacha (*Beta vulgaris*), mientras que otras responden a vernalización sin un requerimiento obligado o no responden en absoluto, como diversas especies de cereales (Kim y col., 2009).

2. El control genético de la floración en *Arabidopsis thaliana*

Arabidopsis thaliana presenta una fase de desarrollo vegetativo en roseta caracterizada por la formación reiterada de hojas sin elongación de los entrenudos. La transición entre la fase juvenil y la fase adulta del desarrollo vegetativo determina la adquisición de competencia del meristemo apical para responder al estímulo floral (Poethig, 1990), y se ha asociado con cambios en la morfología foliar y en el patrón de distribución de tricomas en las hojas (Telfer y col., 1997). Cuando se induce la transición floral, se produce la elongación de los entrenudos de las hojas del tallo principal (caulinares) y la formación de una inflorescencia. Los meristemos axilares de las hojas caulinares se desarrollan dando lugar a una inflorescencia lateral o coflorescencia, mientras que los meristemos florales dan lugar a flores.

Arabidopsis es una planta facultativa de DL, es decir, florece de forma más temprana y con menor número de hojas en DL que en DC, y además algunas accesiones son capaces de responder a tratamientos de vernalización (Martínez-Zapater y col., 1994).

El análisis de la variación natural que existe entre las distintas accesiones de Arabidopsis y la caracterización de mutantes afectados en el tiempo de floración que se ha llevado a cabo en los últimos años, ha permitido identificar una serie de genes que participan en la regulación de este proceso.

2.1 Variación genética natural

Arabidopsis presenta una elevada variación genética para el tiempo de floración en poblaciones naturales, probablemente como consecuencia de procesos de adaptación a distintas condiciones ambientales (Koornneef y col., 2004). El análisis genético de la variación existente entre accesiones ha permitido identificar diversos loci que son responsables de esta variación fenotípica para el tiempo de floración.

Se ha descrito que alelos funcionales y dominantes de los loci *FRIGIDA* (*FRI*) y *FLOWERING LOCUS C* (*FLC*) son los responsables del requerimiento de vernalización (Johanson y col., 2000; Shindo y col., 2005; Werner y col., 2005). *FRI* codifica una proteína específica de plantas que es necesaria para retrasar la floración a través de la activación de la expresión de *FLC*, ya que mutaciones de pérdida de función de *FLC* suprimen el efecto de *FRI* sobre el tiempo de floración (Johanson y col., 2000; Michaels y Amasino, 2001). Datos recientes sugieren que FRI activa la transcripción de *FLC* a través de un mecanismo cotranscripcional que implica la interacción de FRI con componentes del complejo de unión al 5´CAP del mensajero de *FLC* (Geraldo y

col., 2009; Crevillen y Dean, 2011). Por su parte, *FLC* codifica un factor de transcripción de la familia MADS que actúa como represor de la floración de forma cuantitativa (Michaels y Amasino, 1999; Sheldon y col., 1999). Los tratamientos de vernalización disminuyen la expresión de *FLC* y hacen a este gen insensible a la activación por *FRI* (He y Amasino, 2005). Una vez que la planta ha sido vernalizada, la represión de *FLC* se mantiene estable durante el resto del ciclo de vida de la planta, y sólo se restablecen niveles elevados de expresión de *FLC* en la siguiente generación (He y Amasino, 2005). En Arabidopsis hay cinco genes parálogos de *FLC*, denominados *MADS AFFECTING FLOWERING 1/FLOWERING LOCUS M* (*MAF1/FLM)*, *MAF2*, *MAF3*, *MAF4* y *MAF5*. Se ha demostrado que al menos dos de ellos, *MAF1/FLM* y *MAF2*, actúan como represores florales (Ratcliffe y col., 2001; Ratcliffe y col., 2003), lo que podría explicar por qué plantas con mutaciones nulas para *FLC* no eliminan totalmente la respuesta a la vernalización. De acuerdo con esta hipótesis, el gen *MAF1/FLM* sufre los mismos cambios epigenéticos que produce la vernalización en la cromatina de *FLC* (Sung y col., 2006a).

Las accesiones de ciclo rápido o de primavera pueden aparecer como consecuencia de la pérdida de una proteína FRI funcional, incapaz de regular positivamente a *FLC*, o de la existencia de un alelo débil o inactivo de *FLC*. Entre las accesiones empleadas en el laboratorio, Columbia (Col) y Landsberg *erecta* (L*er*) poseen una mutación en el locus *FRI* (Grennan, 2006). Además, L*er* presenta un alelo débil de *FLC* debido a la inserción de un trasposón en su primer intrón (Gazzani y col., 2003; Michaels y col., 2003). Por su parte, la accesión C24 también presenta un alelo débil de *FLC* (Grennan, 2006).

El análisis de variantes naturales también ha permitido identificar genes implicados en la regulación del tiempo de floración en respuesta a factores como la temperatura de crecimiento y el fotoperiodo. Entre ellos se encuentran varios loci que codifican para fotorreceptores, como *CHRYPTOCHROME 2*/*EARLY DAYLENGTH INSENSITIVE* (*CRY2*/*EDI*) y *PHYTOCHROME C* (*PHYC*) y *PHYD* (Aukerman y col., 1997; El-Din El-Assal y col., 2001; Balasubramanian y col., 2006b). Cry2 participa en la percepción de la luz azul, que induce la floración, mientras que PhyC y PhyD actúan como receptores de la luz roja, que reprime la transición floral. Además, *MAF1/FLM* codifica un factor de transcripción que actúa modulando la inducción de la floración en respuesta a pequeños aumentos de la temperatura de crecimiento (Balasubramanian y col., 2006a).

2.2 Variación genética inducida

2.2.1 Rutas de inducción de la floración

El análisis genético del tiempo de floración en Arabidopsis se ha basado clásicamente en el estudio de mutantes de floración tardía, que se clasificaron en función de su respuesta al fotoperiodo y a la vernalización. Además, las interacciones genéticas entre los distintos loci permitió postular un modelo genético del control de la floración en el cual una serie de rutas promotoras convergen en la regulación de la expresión de los integradores florales (Moon y col., 2003; Turck y col., 2008). De las seis rutas actualmente aceptadas, tres responden a factores ambientales: la ruta del fotoperiodo, la ruta de la vernalización y la ruta dependiente de la temperatura ambiental, mientras que las otras tres responden a factores endógenos: la ruta dependiente de las giberelinas, la ruta autónoma y la ruta dependiente de la edad (Figura 1) (Fornara y col., 2010). Los integradores florales, que controlan en último término la transición de desarrollo vegetativo a desarrollo reproductivo, son: *FT, TWIN SISTER OF FT* (*TSF*), *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* y *LEAFY* (*LFY*). Estos integradores florales activan la expresión de los genes de identidad de meristemo floral *APETALA 1* (*AP1*), *AP2*, *CAULIFLOWER* (*CAL*), *FRUITFULL (FUL)* y *LFY,* los cuales determinan la identidad del meristemo reproductivo y regulan la expresión de genes de identidad de órgano floral, como son *AP1, AP2, AP3, PISTILLATA (PI), AGAMOUS (AG)* y *SEPALLATA 1-4* (*SEP1-4*) (Abe y col., 2005; Wigge y col., 2005; Jaeger y col., 2006).

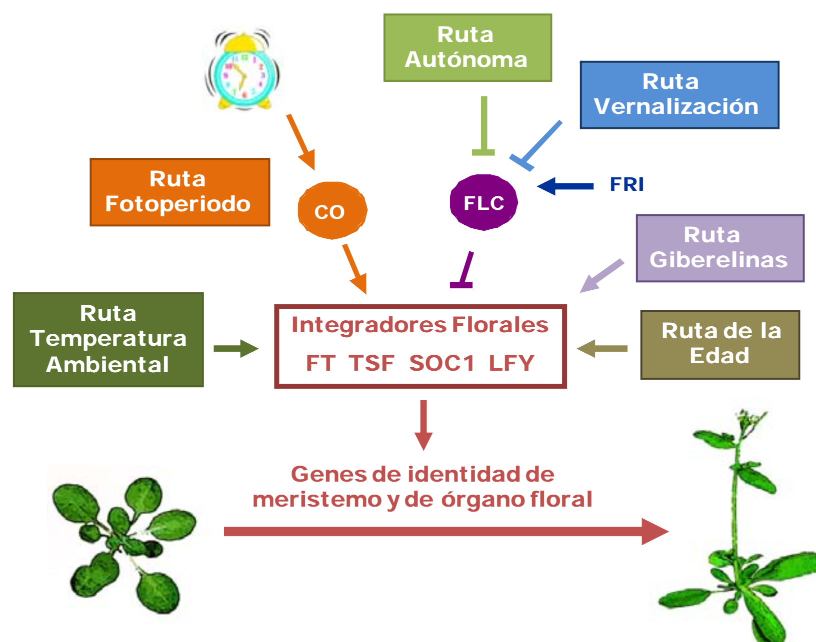


Figura 1. Esquema del control genético de la floración propuesto en Arabidopsis

Ruta del fotoperiodo

Clásicamente, la ruta del fotoperiodo se estableció a partir de la identificación de una serie de mutantes de floración tardía que afectaban a los loci *CONSTANS* (*CO*), *FHA/CRY2*, *GIGANTEA* (*GI*), *FD* y *FT* (Searle y Coupland, 2004). Estos mutantes presentan un retraso en el tiempo de floración en DL aunque en DC florecen al mismo tiempo que plantas de tipo silvestre, además de no mostrar alteraciones en la respuesta a vernalización.

CRY2, como ya hemos descrito, codifica la apoproteína de un fotoreceptor de luz azul (Guo y col., 1998) y *GI* codifica una proteína nuclear que regula, entre otros genes, la expresión de *CO* (Fowler y col., 1999; Rubio y Deng, 2007). Por su parte, *CO* codifica una proteína nuclear con dos dedos de zinc de tipo B-box y un dominio CCT (de CO, CO-LIKE y TOC1) que actúa como un activador floral (Putterill y col., 1995; Robson y col., 2001). *CO* pertenece a una familia génica formada por 17 miembros en Arabidopsis. Se ha descrito que los genes *CO-like 3* (*COL3*) y *COL9* pueden tener un papel como represores florales (Cheng y Wang, 2005; Datta y col., 2006). Por otro lado, *FT* codifica una proteína con homología a inhibidores de Raf quinasas que actúa como un potente inductor floral (Kardailsky y col., 1999; Kobayashi y col., 1999). *FD* codifica un factor de transcripción de tipo b-Zip que se expresa en el meristemo apical (Wigge y col., 2005). Las mutaciones en *FD* suprimen el fenotipo de floración temprana que produce la sobreexpresión del integrador floral *FT,* lo cual sugirió que *FD* participa en la inducción de la floración por debajo de *FT* (Abe y col., 2005; Wigge y col., 2005).

El mecanismo que poseen las plantas para medir la duración del día está basado en un sistema circadiano. Dicho sistema utiliza la información lumínica transmitida por los fotorreceptores para "poner en hora" el mecanismo oscilador central del reloj circadiano (Jarillo y col., 2008). Este mecanismo oscilador consite en una serie de bucles de retroalimentación positiva y negativa entre proteínas que se expresan por la mañana y otras que se expresan al atardecer, y es el encargado de regular el patrón de expresión de multitud de genes con un periodo de oscilación próximo a las 24 horas (de Montaigu y col., 2010; Imaizumi, 2010).

En Arabidopsis, la capacidad para distinguir la longitud de los periodos de luz y oscuridad se basa en la coincidencia de un ritmo interno de la planta, representado por el patrón de expresión de *CO*, con una señal ambiental como es la luz. Los niveles de expresión de *CO* están regulados por el reloj circadiano, de modo que en DL se observan los niveles máximos al amanecer, el atardecer y durante la noche; en

cambio, en DC la expresión de *CO* se limita al periodo de oscuridad (Figura 2) (Suarez-Lopez y col., 2001). Los niveles de la proteína CO no están determinados solamente por el ARN mensajero de *CO,* sino que también están regulados mediante su degradación por el proteosoma (Jang y col., 2008; Liu y col., 2008c). Así, en la oscuridad, la proteína CO no es estable y sólo en DL, cuando la transcripción de *CO* coincide con el periodo de luz, la proteína CO se acumula a niveles capaces de activar la expresión de *FT* (y de *TSF*) (Samach y col., 2000; Suarez-Lopez y col., 2001; Yamaguchi y col., 2005). *FT* es un integrador floral cuyo nivel de expresión es máximo en DL durante la fase de coincidencia de la luz con el pico de expresión de *CO*, condiciones en las que *FT* promueve el inicio de la floración. En cambio, en DC la proteína CO se degrada y el nivel de expresión de *FT* se mantiene bajo, de modo que la floración se retrasa (Figura 2).

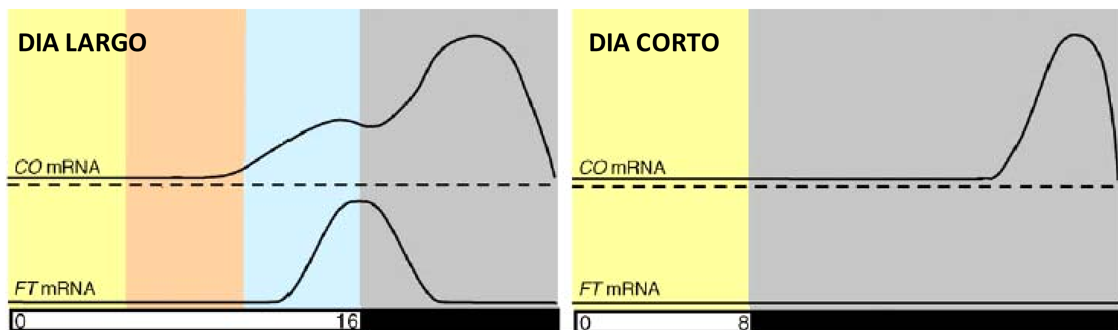


Figura 2. Representación de los niveles de expresión de los mensajeros de *CO* y de *FT* en condiciones de DL y DC en Arabidopsis (adaptada de de Montaigu y col., 2010).

El patrón de expresión de *CO* también está regulado por una serie de proteínas codificadas por genes cuya transcripción, a su vez, está controlada por el reloj circadiano. Varios miembros de la familia de proteínas CYCLING DOF FACTORs (CDFs) reprimen la transcripción de *CO* en la primera parte del día (Imaizumi y col., 2005; Fornara y col., 2009). Hacia el final de la tarde, la expresión de los genes *GI* y *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*) aumenta y se produce la interacción dependiente de luz azul entre ambas proteínas (Sawa y col., 2007). FKF1 contiene un dominio F-box, implicado en degradación de proteínas, y un dominio receptor de luz azul tipo LOV (Demarsy y Fankhauser, 2009). El complejo formado entre FKF1 y GI promueve la degradación de CDF1 y, así, permite eliminar la represión que ejerce esta proteína sobre la expresión de *CO* (Imaizumi y col., 2003; Sawa y col., 2007).

En diversos trabajos se han descrito otros reguladores transcripcionales de *CO* como DAY NEUTRAL FLOWERING (DNF), LONG VEGETATIVE PHASE 1 (LOV1), RED AND FAR-RED INSENSITIVE 2 (RFI2) o SENSITIVE TO FREEZING 6 (SFR6), a los

cuales haremos referencia más adelante (Chen y Ni, 2006b; Yoo y col., 2007; Knight y col., 2008; Morris y col., 2010).

En la regulación post-transcripcional de CO está involucrada la E3 ubiquitina ligasa CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1). Los mutantes *cop1* presentan un fenotipo de floración temprano muy acusado en DC que depende en gran medida de la acumulación de CO (Jang y col., 2008; Liu y col., 2008c). Se ha demostrado que el complejo formado por COP1 y las proteínas SUPRESSOR OF PHYTOCROME A-105 (SPA) degrada a CO en la oscuridad (Laubinger y col., 2006; Jang y col., 2008; Liu y col., 2008c).

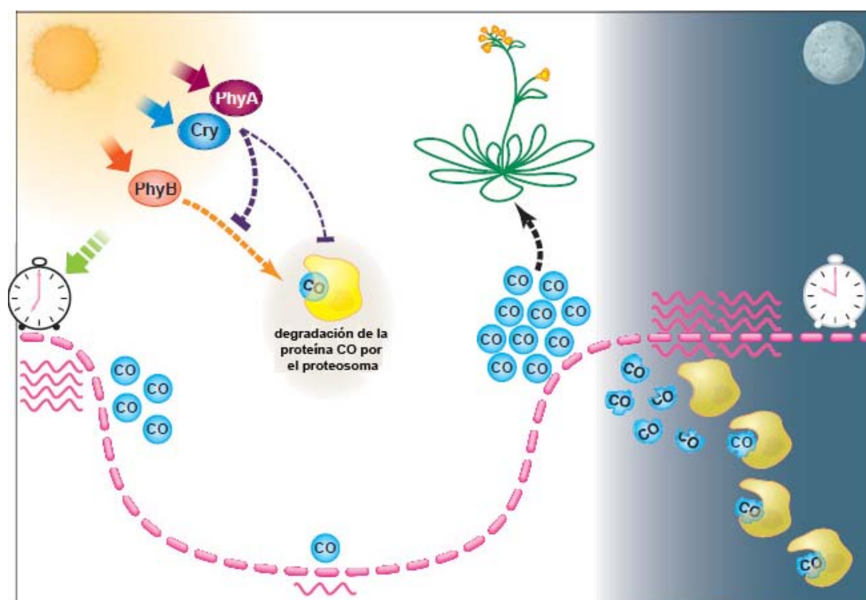


Figura 3. Regulación fotoperiódica del tiempo de floración en Arabidopsis. Los niveles de expresión del mensajero de *CO* en condiciones inductivas de DL están representados por una línea discontínua. La abundancia de la proteína CO está representada por óvalos de color azul. El proteosoma degrada CO durante el periodo inicial de luz del día y en la oscuridad, de modo que la proteína sólo se acumula a niveles capaces de activar la transcripción de *FT* al final del periodo de luz en DL (Klejnot y Lin, 2004).

Distintas calidades de luz tienen un efecto diferente sobre la estabilidad de CO; así, mientras que la luz roja promueve la degradación de CO en un proceso mediado por PhyB, la luz azul y la roja lejana la inhiben a través de Cry2 y PhyA (Figura 3) (Valverde y col., 2004). La luz azul participa en el control fotoperiódico de la transición floral a través de tres mecanismos distintos (Kim y col., 2008). Por un lado, la luz azul promueve la interacción entre FKF1 y GI que es necesaria para la degradación de las proteínas CDF (Sawa y col., 2007). Además, durante el periodo de luz Cry2 impide la proteolisis de CO y GI mediada por COP1 (Liu y col., 2008c; Yu

y col., 2008). Por último, se ha demostrado que Cry2 se une al factor de transcripción CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1 (CIB1) y regula directamente la transcripción de *FT* (Liu y col., 2008b). De forma opuesta al papel de la luz azul, se ha descrito que PhyB participa en la degradación de CO en las primeras horas del día aunque se desconoce el mecanismo molecular que media esta respuesta (Valverde y col., 2004; Jang y col., 2008).

Recientemente, se ha avanzado en el conocimiento del mecanismo de activación de *FT* por parte de CO. Se han identificado varias regiones aguas arriba del gen *FT* que son importantes para la regulación transcripcional de este locus y se ha propuesto que CO contiene dominios tanto de unión a ADN como de activación transcripcional. Estos resultados sugieren que CO se une a un elemento en tándem presente en el promotor de *FT* y que esta unión es suficiente para la activación dependiente de CO (Adrian y col., 2010; Tiwari y col., 2010). Además, también se ha descrito que CO podría actuar como parte de un complejo homólogo al complejo activador de la transcripción Heme Activator Protein (HAP) de levaduras (Ben-Naim y col., 2006; Cai y col., 2007). CO contiene dominios homólogos a HAP2 que sugieren que pueda reemplazar a esta proteína en el complejo HAP y unirse de esta forma al promotor de *FT* en Arabidopsis (Wenkel y col., 2006).

En conclusión, es la interacción entre la luz y el reloj circadiano la que regula la expresión y modula la actividad de CO y, por tanto, la que permite a la planta percibir la duración del día. En fotoperiodos de DL, la expresión de *CO* en el tejido vascular de las hojas induce la expresión del mensajero de *FT* (An y col., 2004). Se ha demostrado que la proteína FT es capaz de moverse desde las hojas hasta el meristemo apical y, por tanto, que actuaría como parte de la señal de larga distancia denominada "florígeno" que inicia el proceso de floración en respuesta a condiciones de DL (Corbesier y col., 2007; Turck y col., 2008; Fornara y col., 2010).

Ruta de la vernalización

La vernalización es el proceso mediante el cual la exposición prolongada a bajas temperaturas hace a las plantas competentes para florecer (Kim y col., 2009). En Arabidopsis esta ruta regula el tiempo de floración a través de la represión de *FLC*. Este mecanismo represor presenta dos características principales: la primera es que tiene carácter cuantitativo, es decir, los niveles de expresión de *FLC* disminuyen de forma gradual a medida que se aumenta el tiempo de exposición a bajas temperaturas (Sheldon y col., 2000). La segunda es que conlleva un cambio

epigenético en el estado de la cromatina de *FLC*, ya que el estado reprimido de *FLC* se mantiene aún cuando la planta es transferida a temperaturas normales de crecimiento (Turck y Coupland, 2011). Se han identificado tres mutantes denominados *vernalization 1* y *2* (*vrn1* y *vrn2)* y *vernalization insensitive 3* (*vin3),* que muestran un fenotipo de floración tardío aún cuando se someten a un tratamiento de vernalización (revisado en Kim y col., 2009).

VRN2 codifica un homólogo de Suppressor of Zeste 12 (Su(z)12), el primer componente identificado del grupo Polycomb (PcG) de *Drosophila* (Gendall y col., 2001). Su(z)12 pertenece al Complejo de Represión Polycomb 2 (PRC2), que está implicado en procesos de represión génica tanto en animales como en plantas (Hennig y Derkacheva, 2009). VRN1, en cambio, presenta dominios implicados en unión a ADN (Levy y col., 2002). Aunque en los mutantes *vrn1* y *vrn2* la exposición a bajas temperaturas reprime transcripcionalmente a *FLC*, este estado no se mantiene cuando la planta deja de ser vernalizada (Sheldon y col., 2006). Por tanto, *VRN1* y *VRN2* no están involucrados en el establecimiento de un estado silenciado de *FLC* en respuesta frío, sino en su mantenimiento cuando la planta se expone a la temperatura normal de crecimiento. Además, se ha demostrado que *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*) o *TERMINAL FLOWER 2* (*TFL2*), homólogo de *HETEROCHROMATIN PROTEIN 1* (*HP1*) de animales y levaduras, también es necesario para mantener el estado reprimido de *FLC* característico de la vernalización (Mylne y col., 2006; Sung y col., 2006b).

El mutante *vin3* no presenta respuesta a la vernalización ni disminución en los niveles de *FLC* tras su exposición al frío (Sung y Amasino, 2004). La expresión de *VIN3* se induce en respuesta a bajas temperaturas, de modo que se ha propuesto que *VIN3* participa en el establecimiento de la represión de *FLC* (Sung y Amasino, 2004). VIN3 posee un dominio tipo Plant Homeo Domain (PHD), característico de componentes de complejos de remodelación de cromatina, y pertenece a una familia de proteínas que tienen la capacidad de dimerizar. Se ha descrito que VIN3-like 1 (VIL1)/VRN5 también participa en la respuesta a vernalización y es capaz de interaccionar con VIN3 (Sung y col., 2006a; Greb y col., 2007).

Por otro lado, durante la exposición a bajas temperaturas se produce un aumento de los niveles de tránscritos no codificantes de *FLC*, denominados *COLD INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COOLAIR*), y *COLD ASSISTED INTRONIC NONCODING RNA* (*COLDAIR*) (Swiezewski y col., 2009; Heo y Sung, 2011). Se ha demostrado recientemente que *COLDAIR* participa en el establecimiento de la

represión epigenética de *FLC* reclutando al complejo PRC2 a la región genómica de este represor floral (Heo y Sung, 2011; Turck y Coupland, 2011).

Ruta dependiente de la temperatura ambiental

Las plantas de Arabidopsis florecen antes cuando se cultivan a una temperatura ambiental de 23°C que cuando se cultivan a 16°C (Blazquez y col., 2003). El gen *SHORT VEGETATIVE PHASE* (*SVP*) juega un papel importante en esta respuesta, ya que los mutantes *svp* son insensibles a estas variaciones en la temperatura de crecimiento (Lee y col., 2007). S*VP* codifica una proteína tipo MADS box que reprime la expresión del integrador floral *FT* y es necesario para retrasar la floración a temperaturas bajas (16°C) (Lee y col., 2007). SVP forma parte de un complejo en el que también se encuentra FLC, y que puede desempeñar un papel central en modular el inicio de la floración en respuesta a la temperatura ambiental (Li y col., 2008).

Ruta autónoma

Los mutantes de floración tardía que definen esta ruta corresponden a los loci *FCA*, *FY*, *FPA*, *LUMINIDEPENDENS* (*LD*), *FLOWERING LOCUS D* (*FLD*), *FVE*, *FLOWERING LOCUS K* (*FLK*) y *RELATIVE OF EARLY FLOWERING 6* (*REF6*). Estos mutantes se caracterizan por presentar un retraso en la floración tanto en DL como en DC, que puede ser revertido cuando se someten a un tratamiento de vernalización (revisado en Amasino, 2010).

Todos los mutantes de esta ruta presentan niveles altos de expresión del represor floral *FLC* (Michaels y Amasino, 1999; Sheldon y col., 1999; Sheldon y col., 2000; Michaels y Amasino, 2001). La función bioquímica de las proteínas de la ruta autónoma sugiere que puedan participar, bien en mecanismos de unión y procesamiento de ARN, o bien en procesos de remodelación de la cromatina de *FLC* (Amasino y Michaels, 2010). FCA, FPA y FLK contienen dominios de unión a RNA (Macknight y col., 1997; Schomburg y col., 2001; Lim y col., 2004; Manzano y col., 2009) y FY presenta homología con factores de procesamiento de ARNs mensajeros (Simpson y col., 2003). *LD* codifica un factor de transcripción con un dominio homeobox (Lee y col., 1994). Por otro lado, *REF6* codifica una proteína tipo Jumonji y *FLD* una proteína homóloga a la LYSINE-SPECIFIC DEMETHYLASE 1 (LSD1) humana, dos clases diferentes de demetilasas de histonas (He y col., 2003; Noh y col., 2004; Jiang y col., 2007). Por su parte, FVE participa en procesos de deacetilación de histonas sobre la cromatina de *FLC* (Ausin y col., 2004). Recientemente se ha descrito una familia de metiltransferasas de arginina (PRMTs) que se pueden adscribir a esta

ruta y que también participan en la represión de *FLC* (Wang y col., 2007; Niu y col., 2008; Schmitz y col., 2008).

En resumen, la ruta autónoma que controla el tiempo de floración no parece ser una ruta lineal sino que comprende una colección de genes implicados en: (i) la represión de la expresión génica, y (ii) el establecimiento de los niveles basales de expresión de *FLC* (Amasino, 2010).

Ruta de las giberelinas

Las giberelinas son hormonas que promueven la floración en Arabidopsis y que, en DC, resultan imprescindibles para que ocurra la transición de desarrollo vegetativo a reproductivo (Mutasa-Gottgens y Hedden, 2009). Las mutaciones en los loci *GIBBERELLIC ACID 1-5* (*GA1-5)*, que codifican enzimas de la ruta de biosíntesis de giberelinas, retrasan la floración, y en concreto en el caso de *ga1-3*, que carece completamente de giberelinas, se suprime totalmente la floración en DC (Wilson y col., 1992). Además, las mutaciones que provocan una activación constitutiva de la señalización dependiente de giberelinas, como *spindly (spy)*, provocan una aceleración de la floración (Jacobsen y Olszewski, 1993). Las giberelinas regulan la transición floral a través de *SOC1*, aunque se desconoce el mecanismo molecular por el cual estas hormonas activan la expresión de este integrador floral. También se ha descrito que las giberelinas activan la expresión de *LFY*, que presenta elementos de respuesta a giberelinas en su zona promotora (Blazquez y col., 1998; Lee y Lee, 2010).

Ruta dependiente de la edad

En Arabidopsis, se ha descrito que la familia de factores de transcripción *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) regula de forma positiva, tanto la transición de fase juvenil a adulta, como la transición floral. Las proteínas SPL participan en el control del tiempo de floración a través de la regulación de la expresión de los integradores florales *SOC1* y *LFY* (Wang y col., 2009; Yamaguchi y col., 2009; Poethig, 2010).

Recientemente, se ha desvelado el papel que desempeñan algunos microRNAs (miRNAs) en la transición floral. La expresión del miR156 mantiene la fase juvenil y retrasa la floración en Arabidopsis (Wu y Poethig, 2006), mientras que el miR172 muestra un patrón de expresión temporal contrario al del miR156 y aumenta durante la fase adulta del desarrollo. El miR172 promueve la floración a través de un mecanismo de represión post-transcripcional de genes *AP2*-like como *TARGET OF EAT*

1, *2* y *3* (*TOE1*, *2* y *3*), *SCHLAFMÜTZE* (*SMZ*) y *SCHNARCHZAPFEN* (*SNZ*), que actúan como represores de *FT* (Aukerman y Sakai, 2003; Mathieu y col., 2009; Yant y col., 2009). Además, el miRNA172 participa en la regulación fotoperiódica del tiempo de floración mediante un aumento de la expresión de *FT* que es dependiente de *GI* pero independiente de la actividad de CO (Jung y col., 2007; Fornara y Coupland, 2009).

Existe un circuito regulador formado por estos miRNAs y las proteínas SPL. Durante la fase juvenil del desarrollo, las proteínas SPLs están silenciadas por el miR156 (Wu y Poethig, 2006). Las proteínas SPL, a su vez, son reguladores positivos de la expresión del miR172. De este modo, a medida que la planta se desarrolla la expresión del miR156 disminuye y, por tanto, la de las proteinas SPL y la del miR172 aumenta y se promueve la transición floral (Fornara y Coupland, 2009; Wang y col., 2009; Wu y col., 2009a).

2.2.2 Integradores florales

Todas las rutas comentadas con anterioridad convergen en la regulación de la expresión de los integradores florales *FT*, *TSF*, *SOC1* y *LFY*. De los niveles de expresión de estos integradores depende el momento exacto en el que se produce la transición floral (Figura 4) (Lee y Lee, 2010).

Como hemos visto anteriormente, *FT* y *TSF* se expresan en el tejido vascular de las hojas en respuesta a fotoperiodo (Samach y col., 2000; Suarez-Lopez y col., 2001; Yamaguchi y col., 2005). La proteína FT se mueve a través del floema hasta el meristemo apical y, junto con el factor de transcripción FD, activa la expresión de *SOC1* y los genes de identidad de meristemo floral, e induce la transición floral bajo las condiciones ambientales adecuadas (Wigge y col., 2005; Yoo y col., 2005; Jaeger y col., 2006).

Por su parte, *SOC1* es un factor de transcripción tipo MADS box cuya expresión está regulada por todas las rutas inductoras de la floración, bien de forma directa, como es el caso de la ruta dependiente de la edad y de la ruta de las giberelinas, o bien de forma indirecta a través de FT o de FLC (Lee y Lee, 2010). *AGAMOUS-LIKE 24* (*AGL24*) es un factor de transcripción tipo MADS box que actúa como un activador floral similar a *SOC1* (Michaels y col., 2003). El nivel de expresión de *AGL24* depende de las rutas del fotoperiodo, de la vernalización y de la ruta autónoma, lo que sugiere que este gen pudiera actuar como otro integrador floral (Lee y Lee, 2010). AGL24 y SOC1 promueven la floración a través de un bucle de activación transcripcional, ya que cada uno de ellos es capaz de activar la expresión del otro, y ambos activan la del gen *LFY* (Lee y col., 2008; Liu y col., 2008a).

La ruta autónoma y la ruta de la vernalización promueven la floración a través de la represión de *FLC* (Amasino, 2010). FLC se une directamente a los promotores de *SOC1* y *FD*, así como al primer intrón de *FT*, y reprime la expresión de *FT* en la hoja y de *SOC1* y *FD* en el meristemo apical (Searle y col., 2006). Se ha descrito que el complejo formado por *FLC* y *SVP* sería el encargado de reprimir la transcripción de *FT* y *SOC1* (Lee y col., 2007; Li y col., 2008). La expresión de *SVP* está regulada principalmente por las giberelinas, la ruta autónoma y la ruta dependiente de la temperatura ambiental (Li y col., 2008).

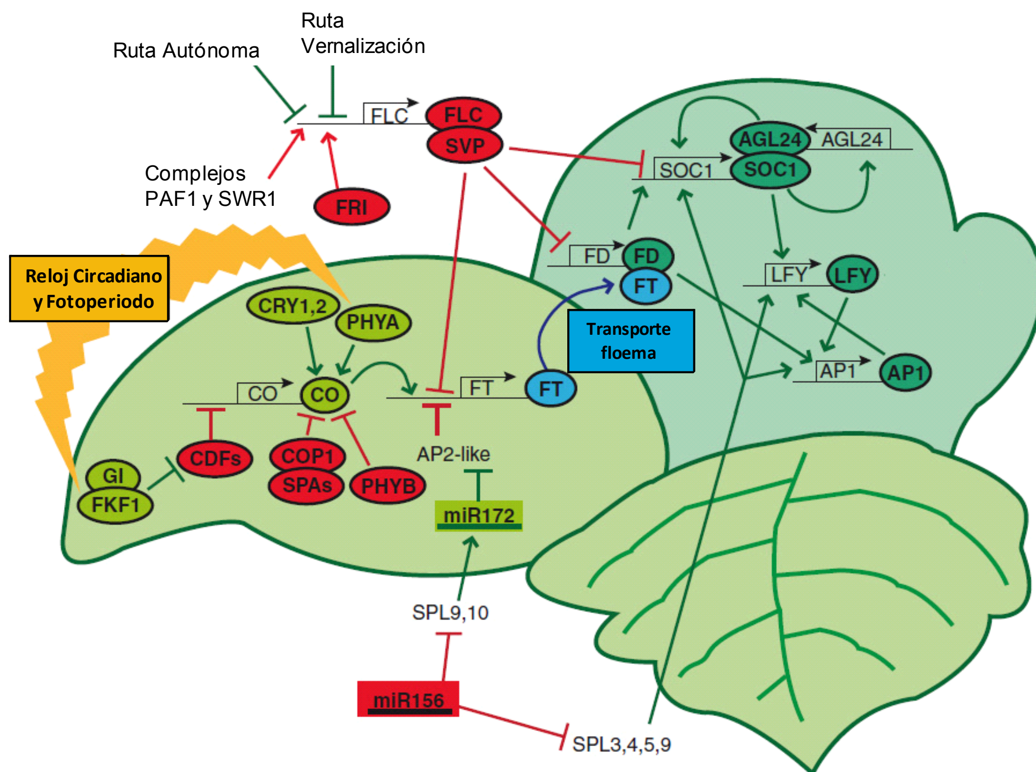


Figura 4. Esquema general de la regulación del tiempo de floración en Arabidopsis. Se representan los genes, proteínas (óvalos) y miRNAs que están implicados en este proceso. Las flechas indican inducción o estabilización, mientras que las líneas cruzadas con una barra perpendicular indican represión o degradación. Los componentes que promueven la floración se representan en color verde y los que la reprimen en color rojo (adaptado de Amasino, 2010).

Por último, aunque el mutante *lfy* se describió por sus defectos en la determinación de la identidad del meristemo floral, se ha demostrado que el locus *LFY* participa en la transición floral (Weigel y Nilsson, 1995; Blazquez y col., 1997) y que podría integrar señales procedentes tanto de la ruta dependiente de la edad como de las giberelinas (Blazquez y col., 1998; Yamaguchi y col., 2009).

Por tanto, el efecto antagonista de *CO* y *FLC* en la regulación de los integradores florales podría proporcionar el mecanismo para coordinar los efectos del fotoperiodo y la temperatura en el control espacio-temporal de la floración. Los mecanismos de activación que existen entre los integradores florales y los genes de identidad de meristemo floral aseguran que, una vez que se ha iniciado la floración, ésta se mantenga aún en ausencia de los estímulos ambientales que la desencadenaron (Kim y col., 2009).

2.2.3. Represores de la floración

El papel que desempeñan los represores florales y cómo estos interaccionan con las rutas de inducción de la floración también contribuye a asegurar que la transición floral tenga lugar en el momento adecuado (Yant y col., 2009). La identificación de mutantes de floración temprana en Arabidopsis ha puesto de manifiesto la variedad de genes y de mecanismos moleculares que participan en la represión de la transición floral (Sung y col., 2003). Muchos de los mutantes tempranos descritos hasta el momento presentan un alto grado de alteraciones pleiotrópicas. Esto puede ser consecuencia de la existencia de distintos procesos generales de regulación génica que convergen sobre dianas clave de la transición floral (Pouteau y col., 2004), o bien revelar la posible existencia de reguladores generales que pueden afectar al control de distintos procesos de desarrollo, además del tiempo de floración (Roux y col., 2006).

A través del análisis de las interacciones genéticas de estos loci con los componentes de las rutas inductoras de la floración, los represores se han ido integrando en el modelo conceptual establecido. Así, se ha descrito una variedad de mutantes tempranos entre los que vamos a citar algunos ejemplos representativos.

Entre los mutantes relacionados con la percepción y transmisión de las señales procedentes de la luz, con el funcionamiento del reloj circadiano o con la ruta del fotoperiodo podemos destacar algunos como *lux arrhytmo* (*lux*) o *early flowering 4* (*elf4*), que afectan a componentes del oscilador central del reloj (Doyle y col., 2002; Hazen y col., 2005) o *cop1* y los mutantes de la familia *SPA*, que afectan a la estabilidad de CO y que discutiremos en detalle más adelante (Laubinger y col., 2006; Jang y col., 2008; Liu y col., 2008c).

También se han descrito mutaciones tempranas que afectan a la expresión de genes responsables de la identidad de meristemo, la identidad de órgano floral, o a la expresión del integrador floral *FT*. Es el caso de los factores de transcripción TEMPRANILLO 1 (TEM1) y TEM2, que reprimen la expresión de *FT* de forma directa

(Castillejo y Pelaz, 2008). Por otro lado, el gen *TERMINAL FLOWER 1 (TFL1*) codifica una proteína similar a los inhibidores de Raf quinasas de animales y presenta un alto grado de similitud con *FT*, por lo que se ha propuesto que ambos genes puedan actuar de forma antagonista en la regulación de las señales de floración por debajo de CO (Kobayashi y col., 1999).

Además, como veremos en el siguiente apartado, se han descrito varias mutaciones tempranas que afectan a la estructura de la cromatina de diversos genes implicados en la regulación del tiempo de floración, como son el represor *FLC* y el integrador floral *FT* (Farrona y col., 2008). Igualmente, hay una serie de mutantes tempranos afectados en reguladores específicos de la expresión de *FLC*, y se ha descrito recientemente que estas proteínas forman un complejo denominado FRI-C (Choi y col., 2011). Al contrario que los mutantes en componentes de complejos de remodelación de la cromatina, que presentan alteraciones pleiotrópicas del fenotipo, los mutantes del FRI-C sólo están afectados en el tiempo de floración (Choi y col., 2011).

Por otro lado, existen mutantes tempranos afectados en otros represores florales tipo MADS box que ya hemos mencionado como *SVP* y los homólogos de *FLC*, *MAF1-MAF5* (Ratcliffe y col., 2001; Ratcliffe y col., 2003; Li y col., 2008). La sobreexpresión de *SVP*, *MAF1*, *MAF2*, *MAF3*, *MAF4* o *MAF5* provoca un retraso en el tiempo de floración, mientras que las mutaciones en *MAF1*, *MAF2* y *SVP* presentan un fenotipo de floración temprana, lo que indica que estos genes actúan como represores florales (Ratcliffe y col., 2001; Ratcliffe y col., 2003; Scortecci y col., 2003; Li y col., 2008).

3. El control epigenético de la floración en *Arabidopsis thaliana*

Los procesos de remodelación de la cromatina desempeñan un papel central en el establecimiento de los patrones de expresión génica que dirigen el desarrollo de las plantas. Además, la organización de la cromatina proporciona un mecanismo que asegura la estabilidad de los patrones de expresión a lo largo de las divisiones mitóticas que tienen lugar en una línea celular (Jarillo y col., 2009). Numerosos trabajos han puesto de manifiesto que la dinámica estructural de la cromatina es esencial en la regulación transcripcional de componentes de las rutas que controlan la transición floral y de los propios integradores florales (Farrona y col., 2008; Crevillen y Dean, 2011; Choi y col., 2011).

La cromatina de las células eucariotas está formada por unas unidades estructurales básicas denominadas nucleosomas. Cada nucleosoma está compuesto por un octámero de histonas (dos dímeros H2A-H2B y un tetrámero H3-H4) y

aproximadamente 140 pb de ADN, que se enrollan alrededor de las histonas (Luger y col., 1997). La cromatina poco condensada o eucromatina contiene la mayor parte de los genes que se expresan activamente puesto que su conformación permite el acceso de la maquinaria de transcripción. Las cadenas de nucleosomas se pueden empaquetar en fibras más compactas, que dan lugar a la heterocromatina, y que generalmente coinciden con zonas transcripcionalmente inactivas. Las proteínas que participan en la remodelación de la estructura de la cromatina pertenecen a tres grandes grupos: (i) complejos remodeladores de la cromatina dependientes de ATP que participan en el desplazamiento de nucleosomas sobre el ADN, por ejemplo los complejos SWI/SNF2, (ii) complejos que intercambian histonas por variantes histónicas y crean regiones genómicas con una estructura y función diferenciada, y (iii) complejos implicados en la modificación post-transcripcional de histonas y ADN que afectan al estado de condensación de la cromatina (Altaf y col., 2009).

Las modificaciones covalentes de las histonas como la acetilación o la metilación conforman el llamado "código de histonas" y suponen un nivel de regulación de la expresión génica adicional al ejercido por los factores de transcripción (He y Amasino, 2005). La acetilación de histonas y la trimetilación de la histona 3 en la lisina 4 y en la 36 (H3K4me3 y H3K36me3) están asociadas a estados transcripcionalmente activos (Carrozza y col., 2003; Rando, 2007; Xu y col., 2008). En cambio, la deacetilación de histonas y la H3K9me3 y H3K27me3 son marcas características de represión de la expresión génica (Carrozza y col., 2003; He y Amasino, 2005; Ringrose y Paro, 2007).

Diversos estudios han desvelado la importancia que tienen los procesos de modificación de la estructura de la cromatina en la activación de la expresión del represor floral *FLC* durante el desarrollo vegetativo y en su posterior silenciamiento previo a la floración. Es por ello que la regulación de este locus es un ejemplo excelente de regulación epigenética de la transcripción en plantas (Deal y Henikoff, 2010; Crevillen y Dean, 2011).

3.1. Activación de la expresión de *FLC*

En las variedades de invierno de Arabidopsis, el nivel de expresión de *FLC* se mantiene elevado en la fase de desarrollo vegetetivo, lo que impide un cambio prematuro a la fase de desarrollo reproductivo (Kim y col., 2009). Niveles altos de expresión del represor *FLC* están asociados a modificaciones activadoras presentes en la cromatina de este gen. Las accesiones que poseen un alelo de *FRI* funcional presentan niveles elevados de trimetilación en H3K4 en la cromatina de *FLC* (He y

Amasino, 2005). Recientemente, se ha descrito la existencia de un complejo en el que participa FRI (FRI-C) que activa la expresión de *FLC* a través del reclutamiento de complejos remodeladores de la cromatina (Choi y col., 2011).

El aislamiento de mutantes de floración temprana en variedades de invierno que presentan una reducción en la expresión de *FLC* ha permitido identificar en Arabidopsis varios de los componentes del complejo PAF1 (RNA Polymerase Associated Factor 1) descrito inicialmente en *Saccharomyces cerevisiae.* En levaduras, el complejo PAF1 se asocia con la ARN polimerasa II y recluta a la metiltransferasa Set 1 del complejo COMPASS (por Complex Proteins Associated with Set 1). Esta metiltransferasa incorpora grupos metilo a la H3K4 y así facilita la transcripción de determinados genes (Ng y col., 2003). Entre los homólogos del complejo PAF1 identificados en Arabidopsis se encuentran VERNALIZATION INDEPENDENCE 4 y 5 (VIP4 y 5*)*, ELF7 y ELF8, y AtCDC73 (Zhang y van Nocker, 2002; Zhang y col., 2003; He y col., 2004; Park y col., 2010b; Yu y Michaels, 2010)*.* De forma análoga a lo que ocurre en levaduras, el complejo PAF1 de Arabidopsis recluta proteínas con actividad metiltransferasa. Dentro de la familia de genes *ARABIDOPSIS TRITHORAX* (*ATX*), se ha demostrado que ATX1 se une directamente a la cromatina de *FLC* y cataliza la metilación de residuos de Lys 4 de la histona H3 (Pien y col., 2008). También se ha identificado en Arabidopsis un homólogo de la metiltransferasa Set 2, que en levaduras trimetila la H3K36, denominada EARLY FLOWERING IN SHORT DAYS/SET DOMAIN GROUP 8 (EFS/SDG8) (Kim y col., 2005). Recientemente se ha demostrado que EFS/SDG8 posee una doble actividad sobre la activación de *FLC*; por un lado recluta un complejo activador que contiene a FRI y, por otro, actúa como un enzima metiltransferasa tanto de H3K4 como de H3K36 en la cromatina de dicho locus (Figura 5) (Ko y col., 2010). Las mutaciones en los componentes del complejo PAF1 son capaces de suprimir el aumento en los niveles de expresión de *FLC* que se producen como consecuencia de la existencia de un locus *FRI* funcional o mutaciones en componentes de la ruta autónoma. Este hecho indica que el complejo PAF1 es necesario para mantener altos los niveles de expresión *FLC* (He y Amasino, 2005).

La monoubiquitinación de la lisina 123 de la histona H2B (H2Bub1) también es una marca asociada a la activación de la expresión génica. En levaduras, un complejo formado por la enzima E2 conjugadora de ubiquitina RAD6 y la E3 ligasa de ubiquitina BRE1 participa en la monoubiquitinación de la histona H2B de genes específicos (Wood y col., 2003). En Arabidopsis existen tres homólogos de RAD6: UBIQUITIN CONJUGATING ENZIME 1, 2 y 3 (AtUBC1, 2 y 3). Se ha descrito que AtUBC1 y 2 participan en la monoubiquitinación de la histona H2B en la cromatina de *FLC* y que

presentan funciones redundantes respecto al control del tiempo de floración puesto que el doble mutante *ubc1 ubc2* florece temprano, mientras que los mutantes simples no están afectados en el tiempo de floración (Cao y col., 2008; Gu y col., 2009; Xu y col., 2009). En cambio, se han identificado dos homólogos de BRE1 en Arabidopsis: HISTONE MONOUBIQUITINATION 1 y 2 (HUB1 y 2) (Cao y col., 2008; Gu y col., 2009; Xu y col., 2009). Se ha demostrado que la H2Bub1 es un importante pre-requisito para el aumento de los niveles de H3K4me3 y para la activación transcripcional de los genes diana en otros organismos (Wood y col., 2003). De acuerdo con estas observaciones, en Arabidopsis los mutantes *hub1* y *hub2* presentan un fenotipo de floración temprana, así como niveles de expresión de *FLC* reducidos y bajos niveles de H3K4me3 en la región promotora de este represor floral (Figura 5) (Cao y col., 2008; Gu y col., 2009).

El intercambio de variantes histónicas en la cromatina de *FLC* también regula los niveles de expresión de este regulador negativo de la floración (Deal y col., 2007). En levaduras, el complejo Swr1 se encarga del reemplazamiento de la histona H2A por la variante histónica H2A.Z (Krogan y col., 2003; Kobor y col., 2004; Mizuguchi y col., 2004). El papel que juega la variante histónica H2A.Z en la regulación de la expresión génica es controvertido, puesto que se encuentra tanto en zonas de heterocromatina como en zonas transcripcionalmente activas en distintos organismos (Draker y Cheung, 2009). Se ha descrito que las zonas ricas en nucleosomas que contienen H2A.Z pueden definir dominios específicos que permitan el acceso de complejos reguladores de la estructura de la cromatina al ADN (Marques y col., 2010). En Arabidopsis se han identificado varias proteínas homólogas a los componentes del complejo Swr1 de levaduras que están implicadas en la activación transcripcional de *FLC*. Entre ellas se encuentra PHOTOPERIOD-INDEPENDENT EARLY FLOWERING (PIE), que es el ortólogo a la subunidad ATPasa del complejo en levaduras (Noh y Amasino, 2003; Deal y col., 2007), y ACTIN RELATED PROTEIN 4 (AtARP4) (Kandasamy y col., 2005). Además, como se expondrá a lo largo de esta Tesis Doctoral, nuestro laboratorio ha contribuido a la identificación de los ortólogos de Arabidopsis de dos nuevas subunidades del complejo SWR1, en concreto las proteínas ESD1/ARP6 y SWC6, confirmando la existencia de este tipo de complejos en plantas (Figura 5) (Choi y col., 2005; Deal y col., 2005; Martin-Trillo y col., 2006; Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008).

3.2. Represión de la expresión de *FLC*

Durante la vernalización, residuos específicos presentes en las colas de las histonas de la cromatina de *FLC* se desacetilan y se produce una disminución en la

metilación de la H3K4 y un aumento en la metilación de la H3K27 y de la H3K9. Todas estas modificaciones de la cromatina participan en el establecimiento del estado reprimido de *FLC* y contribuyen a que esta represión se pueda mantener estable a lo largo de la vida de la planta (Amasino, 2010). Como se comentó anteriormente, *VIN3* se expresa cuando la planta es sometida a bajas temperaturas. En los mutantes *vin3* no se observan ninguna de estas modificaciones sobre la cromatina de *FLC* y, por tanto, se ha propuesto que participa en el establecimiento del estado reprimido de *FLC* (Sung y Amasino, 2004). *VRN2*, *VIN3* y *VIL1/VRN5* forman parte de un PRC2 homólogo al de *Drosophila* (Wood y col., 2006; De Lucia y col., 2008). Tanto en animales como en plantas, los PRC2 están implicados en la trimetilación de la H3K27 (H3K27me3) a través de la función metil transferasa de histonas de la subunidad Enhancer of Zeste [E(Z)]. El PRC2 del que forma parte VRN2 contiene dos homólogos de esta subunidad en Arabidopsis: CLF y SWINGER (SWN) (Wood y col., 2006; De Lucia y col., 2008). Recientemente, se ha demostrado que uno de los ARN no codificantes de *FLC*, *COLDAIR*, interacciona físicamente con componentes del PRC2 y dirige a este complejo a la cromatina de *FLC* (Heo y Sung, 2011). Por otro lado, el dominio PHD que contienen las proteínas de la familia de VIN3, se une a modificaciones histónicas específicas, por lo que es probable que la actividad del complejo PRC2 sobre la cromatina de *FLC* se vea incrementada por la unión de un dímero VIN3-VIL1/VRN5 (De Lucia y col., 2008). Cuando la exposición al frío cesa, la expresión de *VIN3* también lo hace, pero los niveles de H3K27me3 en *FLC* siguen aumentando y la asociación de VIL1/VRN5 a la cromatina de *FLC* se generaliza (Finnegan y Dennis, 2007; De Lucia y col., 2008). Por esta razón, se ha propuesto que VIL1/VRN5 contribuye a mantener el estado reprimido de *FLC* (De Lucia y col., 2008). También la metilación de la H3K9 parece estar involucrada en el mantenimiento de la represión de *FLC* una vez que la planta ha vuelto a la temperatura normal de crecimiento (Bastow y col., 2004; Sung y Amasino, 2004; Sung y col., 2006b; Greb y col., 2007). En los mutantes *vrn1*, la metilación de la H3K27 aumenta durante el tratamiento de frío pero, en cambio, la metilación de la H3K9 no se incrementa y no se mantiene el estado reprimido de *FLC* cuando las plantas se desarrollan a temperatura normal de crecimiento (Levy y col., 2002; Bastow y col., 2004; Sung y Amasino, 2004). De forma análoga, en el mutante *lhp1* tampoco se pueden mantener los niveles de metilación de la H3K9, que sólo aumentan cuando las plantas se someten al frío (Sung y col., 2006b). En animales, el mantenimiento de la represión mediada por PRC2 requiere del Complejo de Represión Polycomb 1 (PRC1), que se une y mantiene la H3K27me3. En plantas no se ha descrito un PRC1 similar al de animales que esté implicado en el control de la

floración, pero es posible que LHP1, e incluso VRN1, realicen funciones similares en Arabidopsis, ya que ambos están implicados en la represión de *FLC* al mantener elevados los niveles de metilación de la H3K9 característicos del estado vernalizado (Figura 5) (Bastow y col., 2004; He y Amasino, 2005; Sung y col., 2006b). Todavía desconocemos la naturaleza molecular de las enzimas que llevan a cabo la metilación de la H3K9 sobre la cromatina de *FLC*, así como el sensor que inicia la respuesta a la vernalización en Arabidopsis.

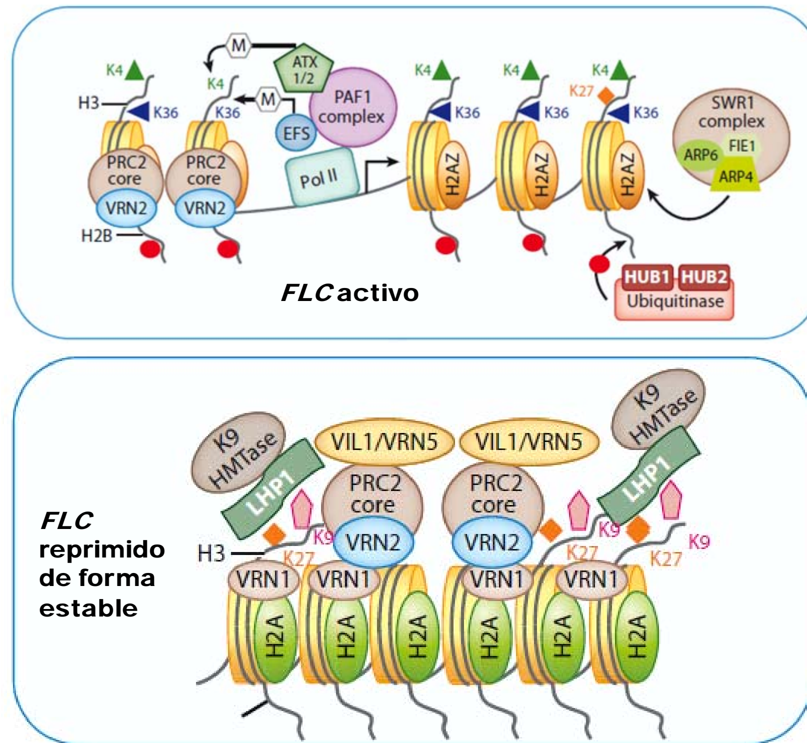


Figura 5. Regulación epigenética del gen *FLC* (adaptado de Kim et al., 2009).

En definitiva, todos estos datos que acabamos de discutir revelan que el nivel de expresión de *FLC* está regulado por un mecanismo conservado en otros eucariotas, que depende del equilibrio entre la actividad represora de las proteínas tipo Polycomb y la actividad activadora de las proteínas Trithorax (Kim y col., 2009).

Algunos de los componentes de la ruta autónoma también regulan a *FLC* a través de cambios en la estructura de su cromatina. El locus *FVE* codifica proteínas similares a las que forman el complejo deacetilasa de histonas (HDAC) en mamíferos y se ha demostrado que los niveles de acetilación en la cromatina de *FLC* están elevados en los mutantes *fve*, *fld* y *ref6* (He y col., 2003; Ausin y col., 2004; Noh y col., 2004). Además, todos los mutantes de la ruta autónoma presentan un aumento en la trimetilación de H3K4 en la cromatina de *FLC*, de modo que los genes de la ruta

autónoma podrían actuar, directa o indirectamente, como represores de este tipo de modificación (He y Amasino, 2005).

3.3 Regulación epigenética de otros genes implicados en el control del tiempo de floración

FLC no es el único gen implicado en la transición floral que está regulado a nivel epigenético (Farrona y col., 2008). Las mutaciones en componentes de los complejos PRC2 como *CLF* y *EMBRYONIC FLOWER 2* (*EMF2*) no sólo afectan a la expresión de *FLC*, sino que también regulan la expresión de *FT* (Jiang y col., 2008). Además, los niveles de expresión de otra proteína tipo MADS box, AGL19, que actúa como un inductor floral, también están regulados por un mecanismo dependiente del PRC2 (Schonrock y col., 2006).

Otros genes que regulan la estructura de la cromatina de *FT* incluyen a *LHP1*/*TFL2* y *EARLY BOLTING IN SHORT DAYS* (*EBS*). Las mutaciones en ambos loci presentan un fenotipo de floración temprana y altos niveles de expresión del integrador floral *FT* (Kotake y col., 2003; Pineiro y col., 2003; Takada y Goto, 2003). Por otro lado, AtBRAHMA (AtBRM), una proteína de la familia de complejos remodeladores de cromatina dependientes de ATP SWI/SNF2, está implicada en la represión de la transición floral mediante la inhibición de la activación de *CO* y *FT* (Farrona y col., 2004).

4. El papel de la degradación específica de proteínas en la transición floral en *Arabidopsis thaliana*

En los últimos años se ha realizado un gran avance en el estudio de la implicación que tiene la ruta de degradación de proteínas dependiente de ubiquitina/proteosoma 26S en la regulación de distintos procesos en organismos eucariotas. En las plantas, más del 50% de las proteínas totales están sometidas a un recambio semanal. Entre los procesos regulados por esta ruta de degradación de proteínas en plantas se incluyen, entre otros, embriogénesis, fotomorfogénesis, floración, señalización hormonal, resistencia a enfermedades o senescencia (Smalle y Vierstra, 2004).

La función general de esta ruta es conjugar polímeros de ubiquitina a las proteínas diana en un residuo de Lys y, de este modo, marcarlas para su posterior degradación por el proteosoma. La ubiquitina es un polipéptido de 76 aminoácidos que se une covalentemente a las proteínas que va a degradar mediante la acción de tres enzimas: la activadora de ubiquitina (E1), la conjugadora de ubiquitina (E2) y la ubiquitina ligasa (E3) (Moon et al., 2004). La enzima E1 forma un enlace tioéster con el extremo carboxilo terminal de la ubiquitina en una reacción dependiente de ATP y

transfiere la ubiquitina así activada a la enzima E2. A su vez, la enzima E2 puede transferir directamente la ubiquitina a la E3 en el caso del tipo HECT (Homologous with E6-associated protein C-Terminus), o unirse a la E3 y entonces transferir la ubiquitina a la proteína diana. En ambos casos la enzima E3 es la que confiere especificidad por el sustrato. Generalmente este proceso se repite sucesivas veces y permite la unión de múltiples moléculas de ubiquitina al sustrato (Figura 6). Se ha demostrado que la poliubiquitinación de las proteínas es necesaria para su degradación por el proteosoma (Moon y col., 2004).

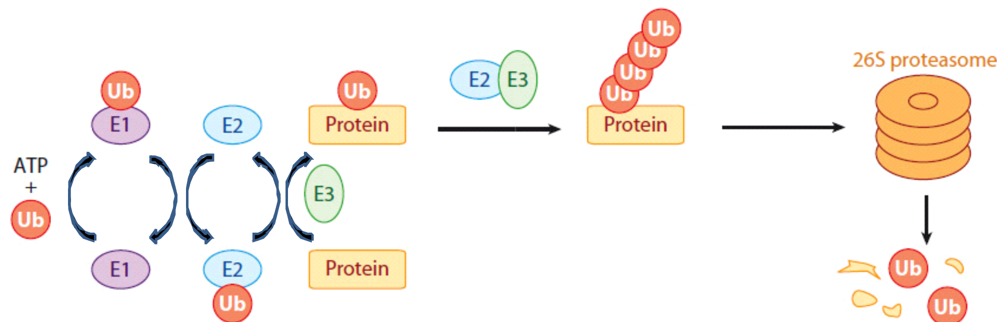


Figura 6. Ruta de ubiquitinación y degradación de proteínas por el proteosoma (adaptada de Deshaies y Joazeiro, 2009).

El proteosoma 26S es un complejo proteico formado por un núcleo cilíndrico 20S con actividad proteasa, flanqueado en cada extremo por una partícula reguladora 19S. La partícula 19S es la encargada de reconocer los sustratos ubiquitinados y de eliminar la cadena de ubiquitina de la proteína que se va a degradar (Moon y col., 2004).

En Arabidopsis aproximadamente el 5% del proteoma codifica componentes relacionados con la ruta ubiquitina/proteosoma 26S. De ellos, unos 1200 genes podrían codificar posibles E3 ligasas (Santner y Estelle, 2010). Las E3 ubiquitina ligasas engloban una amplia y diversa familia de proteínas que contienen, o bien un dominio HECT, o un dominio RING/U-box. Las E3 tipo RING (por Really Interesting New Gene) se pueden subdividir a su vez en aquellas compuestas por una sola subunidad RING/U-box, y en las E3 tipo RING formadas por varias subunidades, que incluyen las de tipo SCF (por SKP1, Cullin y F-box) o los complejos APC (Anaphase Promoting Complex) (Moon y col., 2004; Stone y Callis, 2007). En las RING E3 que actúan individualmente la especificidad de sustrato reside en la propia proteína RING. En cambio, en los complejos E3 la subunidad encargada del reconocimiento del sustrato es otra, y la proteína con el dominio RING participa en la interacción con la enzima E2 (Deshaies y Joazeiro, 2009).

Dentro de los mecanismos que regulan la transición floral en Arabidopsis, la degradación específica de proteínas a través de la ruta ubiquitina/26S proteosoma ha cobrado mayor relevancia en los últimos años. En concreto, se han descrito varias mutaciones en componentes de E3 ligasas que afectan a la regulación fotoperiódica del tiempo de floración. Como ya hemos discutido anteriormente, los complejos COP1-SPA participan en la degradación del promotor floral CO durante el periodo de oscuridad (Laubinger y col., 2006; Jang y col., 2008; Liu y col., 2008c). COP1 es una proteína que contiene un dominio RING, un dominio coiled-coil y un dominio WD40, y que está conservada en plantas superiores y vertebrados. En plantas, COP1 actúa como una E3 ligasa que degrada tanto fotoreceptores como factores de transcripción implicados en la transducción de las señales lumínicas (Yi y Deng, 2005). COP1 interacciona con las proteínas SPA1-4 para formar complejos E3 ligasa funcionales que reprimen la fotomorfogénesis en plántulas cultivadas en oscuridad, así como la transición floral (Laubinger y col., 2006). Recientemente, se ha demostrado que las proteínas CULLIN 4 (CUL4) y DAMAGED DNA BINDING PROTEIN 1 (DDB1), que forman el esqueleto de una variedad de complejos SCF, se unen a los complejos COP1-SPA y participan en la regulación de la fotomorfogénesis y del tiempo de floración (Chen y col., 2010). De forma análoga, el mutante temprano *red and far red insensitive 2* (*rfi2*) también fue descrito por estar afectado en las respuestas de fotomorfogénesis (Chen y Ni, 2006a). *RFI2* codifica una proteína nuclear con un dominio RING que, al contrario que COP1, afecta a los niveles de expresión de *CO* (Chen y Ni, 2006b). Más recientemente, se ha descrito otro represor de CO que actúa en DC, DAY NEUTRAL FLOWERING (DNF). DNF es una proteína unida a membrana que también presenta un dominio RING (Morris y col., 2010). Las dianas de RFI y DNF que participan en el control de la transición floral no se han identificado hasta el momento. Por otro lado, los receptores de luz azul ZEITLUPE (ZTL), LOV KELCH PROTEIN 2 (LKP2) y FKF1 son proteínas tipo F box que pertenecen a complejos SCF y que también están involucradas en el control del tiempo de floración (Nelson y col., 2000; Somers y col., 2000; Jarillo y col., 2001; Schultz y col., 2001). Como hemos descrito anteriormente, estas proteínas F box participan en la degradación de CDF1 y CDF2, que son represores transcripcionales de CO (Imaizumi y col., 2003; Sawa y col., 2007; Fornara y col., 2009).

También se ha descrito que el represor floral FLC está regulado por la ruta ubiquitina/26S proteosoma. La proteína SINAT5 es una E3 tipo RING que interacciona con FLC y participa en su degradación en ensayos de ubiquitinación in vitro (Park y col., 2007). De forma adicional, se ha descrito que SINAT5 también participa en la

regulación del tiempo de floración a través de la degradación de uno de los componentes del reloj circadiano (Park y col., 2010a).

La relación entre los procesos de degradación específica de proteínas y las rutas de señalización hormonal en plantas se ha caracterizado más en detalle. Como ya hemos discutido, en Arabidopsis las giberelinas controlan el tiempo de floración, así como otros procesos del desarrollo (Mutasa-Gottgens y Hedden, 2009). Las proteínas DELLA, que regulan negativamente las respuestas a giberelinas, son degradadas por un complejo SCF específico en respuesta a la presencia de esta hormona (Santner y Estelle, 2010).

Por otra parte, el genoma de Arabidopsis contiene una familia de 27 enzimas con actividad proteasa de ubiquitina (UBP) que están involucradas en la deubiquitinación de proteínas (Liu y col., 2008d). Se ha descrito que las mutaciones en *UBP15* y *UBP26* presentan un fenotipo de floración temprano entre otros defectos del desarrollo (Liu y col., 2008d; Schmitz y col., 2009). Además, datos recientes confirman que la enzima UBP26 participa en la deubiquitinación de la histona H2B sobre la cromatina de *FLC* (Schmitz y col., 2009).

De forma análoga a la ubiquitinación, hay otros procesos de señalización de proteínas mediante la adición de una pequeña molécula entre los que se encuentra la sumoilación (Wilkinson y Henley, 2010). El mecanismo de conjugación y deconjugación de una molécula de SUMO (Small Ubiquitin-related Modifier) a una proteína es similar al que se produce en el caso de la ubiquitina. Al contrario que la poliubiquitinación, la sumoilación no señaliza a las proteínas para su degradación, sino que puede afectar a su estabilidad, actividad, localización subcelular, etc. (Miura y col., 2007a; Miura y Hasegawa, 2010). En Arabidopsis, se ha demostrado que la enzima E3 ligasa de SUMO SIZ1 está implicada en el control del tiempo de floración y en la aclimatación de las plantas al frío, entre otros procesos (Miura y col., 2007b; Jin y Hasegawa, 2008). Por un lado, SIZ1 participa en la regulación de los niveles de *FLC* a través de la represión de la actividad del componente de la ruta autónoma FLD (Jin y Hasegawa, 2008). Por otro, SIZ1 participa en la sumoilación y estabilización de ICE1, que es un regulador positivo de la respuesta de aclimatación a bajas temperaturas (Miura y col., 2007b). En cambio, la E3 ligasa de ubiquitina HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) reprime la respuesta de las plantas al frío mediante la degradación de ICE1 (Dong y col., 2006). Curiosamente, la sumoilación mediada a través de SIZ1 reduce la poliubiquitinación que lleva a cabo HOS1 sobre ICE1 (Miura y col., 2007b).

OBJETIVOS

Además de las rutas genéticas promotoras de la floración, otro aspecto central de la regulación de la transición floral es el papel funcional que desempeñan los represores florales y cómo estos interaccionan con las rutas de inducción de la floración para asegurar que este cambio del desarrollo tenga lugar en el momento más apropiado. En este trabajo, nuestro interés principal ha sido profundizar en el conocimiento de los mecanismos moleculares que regulan el tiempo de floración y, en particular, la caracterización de algunos factores que inhiben el inicio de la floración hasta que la planta se encuentra en las condiciones medioambientales óptimas, o alcanza el nivel de desarrollo adecuado para florecer. Por ello, en la presente Tesis se planteó como objetivo general la caracterización genética y molecular de varios represores del tiempo de floración en Arabidopsis mediante el aislamiento inicial de mutantes de floración temprana. Dicha caracterización ha conducido a la identificación de proteínas implicadas en dos procesos clave en el control de la transición floral: los procesos de remodelación de la estructura de la cromatina y los mecanismos de degradación específica de proteínas. Como se expone a lo largo de este trabajo, hemos identificado dos ortólogos en Arabidopsis de los componentes del complejo de remodelación de cromatina Swr1 de levaduras: las proteínas ESD1/ARP6 y SWC6, que participan en la regulación de la expresión del represor *FLC*. Por otro lado, también hemos abordado la caracterización del papel que juega la E3 ligasa de ubiquitina HOS1 en el control fotoperódico del tiempo de floración en Arabidopsis a través de la regulación de la estabilidad de la proteína CO.

Para ello, hemos desarrollado los siguientes objetivos concretos:

1. Caracterización genética y molecular del gen *ESD1/ARP6* como represor del tiempo de floración en Arabidopsis.

2. Análisis funcional del papel de ESD1/ARP6 en los procesos de remodelación de la estructura de la cromatina de *FLC*.

3. Caracterización genética del locus *SWC6* y análisis de la relación funcional de SWC6 con ESD1/ARP6.

4. Caracterización genética y molecular del locus *ESD6/HOS1* como represor del tiempo de floración en Arabidopsis.

5. Análisis funcional de ESD6/HOS1 en relación con su papel en la degradación específica del promotor floral CO.

RESULTADOS

CAPÍTULO 1: *EARLY IN SHORT DAYS 1* (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in *Arabidopsis*.

Development 133, 1241-1252 (2006)

EARLY IN SHORT DAYS 1 (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in *Arabidopsis*

Mar Martin-Trillo[1,*], Ana Lázaro[2,*], R. Scott Poethig[3], Concepción Gómez-Mena[1,†], Manuel A. Piñeiro[2], Jose M. Martinez-Zapater[1] and Jose A. Jarillo[2,‡]

[1]Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, C/ Darwin 3, Madrid 28049, Spain.
[2]Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Ctra. de A Coruña, km 7, Madrid 28040, Spain.
[3]Plant Science Institute, Department of Biology, University of Pennsylvania, PA 19104, USA.

*These authors contributed equally to this work
†Present address: Department of Cell and Developmental Biology, John Innes Centre, Norwich NR4 7UH, UK
‡Author for correspondence (e-mail: jarillo@inia.es)

We have characterized *Arabidopsis esd1* mutations, which cause early flowering independently of photoperiod, moderate increase of hypocotyl length, shortened inflorescence internodes, and altered leaf and flower development. Phenotypic analyses of double mutants with mutations at different loci of the flowering inductive pathways suggest that *esd1* abolishes the *FLC*-mediated late flowering phenotype of plants carrying active alleles of *FRI* and of mutants of the autonomous pathway. We found that *ESD1* is required for the expression of the *FLC* repressor to levels that inhibit flowering. However, the effect of *esd1* in a *flc-3* null genetic background and the downregulation of other members of the *FLC*-like/*MAF* gene family in *esd1* mutants suggest that flowering inhibition mediated by *ESD1* occurs through both *FLC*- and *FLC*-like gene-dependent pathways. The *ESD1* locus was identified through a map-based cloning approach. *ESD1* encodes ARP6, a homolog of the actin-related protein family that shares moderate sequence homology with conventional actins. Using chromatin immunoprecipitation (ChIP) experiments, we have determined that ARP6 is required for both histone acetylation and methylation of the *FLC* chromatin in *Arabidopsis*.

KEY WORDS: Flowering time, Floral repression, Chromatin remodelling, *Arabidopsis*

INTRODUCTION

The floral transition is highly regulated in many plant species to modulate flowering time in response to environmental and endogenous factors, and to ensure reproductive success. *Arabidopsis thaliana* is a facultative long-day (LD) species in which winter and summer annual accessions can be distinguished. In winter annual accessions, flowering time is regulated by the vernalization, photoperiod and gibberellin (GA) pathways (Boss et al., 2004; Komeda, 2004; Puterill et al., 2004; Amasino, 2005). Winter annuals require exposure to an extended period of cold (vernalization) to become flowering competent, thus preventing premature flowering in the fall (Michaels and Amasino, 2000; Henderson and Dean, 2004). This requirement is mainly conferred by dominant alleles at the *FRIGIDA* (*FRI*) (Johanson et al., 2000) and *FLOWERING LOCUS C* (*FLC*) loci (Michaels and Amasino, 1999; Sheldon et al., 1999), as well as by other *FLC*-related genes within the *MAF* clade (Scortecci et al., 2001; Ratcliffe et al., 2003; Werner et al., 2005). Active alleles of *FRI* increase *FLC* expression to levels that delay flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). FLC is a MADS box transcription factor that acts to delay flowering, in part by suppressing the expression of the floral promoters *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), which function as integrators of flowering signals (Kobayashi et al., 1999; Samach et al., 2000). Vernalization promotes flowering by overcoming the

effect of *FRI* and repressing *FLC* expression; this repression is stably maintained after plants are returned to warm growth conditions, allowing plants to flower (Michaels and Amasino, 1999; Sheldon et al., 1999). The photoperiod pathway promotes flowering in response to LD through the activation of the floral integrators *FT* and *SOC1*. Mutations in photoperiod-pathway genes [e.g. *constans* (*co*), *fd*, *fe*, *fha*/*cryptochrome2* (*cry2*), *ft*, *fwa* and *gigantea* (*gi*)] delay flowering in LD but have little effect on flowering time under short days (SD) (Searle and Coupland, 2004). The GA pathway is required for flowering in non-inductive photoperiods, and mutants with reduced GA levels are extremely delayed in flowering time under SD (Wilson et al., 1992).

Many summer annual accessions of *Arabidopsis* lack an active *FRI* allele (Johanson et al., 2000; Gazani et al., 2003; Shindo et al., 2005). Under these circumstances, *FLC* expression is low and flowering occurs rapidly without vernalization. In these accessions, the reduction of *FLC* expression depends on the function of the autonomous pathway (Michaels and Amasino, 2001). In fact, mutations in autonomous pathway genes [*fca*, *flowering locus d* (*fld*), *fpa*, *fve*, *fy*, *flowering locus k* (*flk*) and *luminidependens* (*ld*)] cause a flowering delay under any photoperiod (Boss et al., 2004) that is associated with higher *FLC* expression, and can be rescued by vernalization (Michaels and Amasino, 1999; Sheldon et al., 1999; Michaels and Amasino, 2001).

Thus, transcriptional regulation of the *FLC* repressor is a central checkpoint in both winter and summer annual accessions of *Arabidopsis*. Recently, the involvement of chromatin modification in *FLC* regulation has been described (for a review, see He and Amasino, 2005). In non-vernalized winter annual plants, *FLC* chromatin is in an active conformation and is enriched in modifications, such as the acetylation of histones 3 (H3) and 4 (H4), and the trimethylation of lysine 4 of H3 (H3-K4), which are hallmarks of active genes (He et al., 2003; Ausin et al., 2004; He et al., 2004). Late-flowering autonomous pathway mutants also have increased levels of H3-K4 trimethylation and histone acetylation compared with the rapid-flowering parental line (He et al., 2003; Ausin et al., 2004; He et al., 2004; Kim et al., 2005). Many early flowering mutations suppressing the late flowering phenotype of *FRI*-containing lines have identified components that are required to maintain high levels of *FLC* expression. This is the case of mutants such as *early flowering in short days* (*efs*), *photoperiod independent early flowering 1* (*pie1*), *early flowering 5* (*elf5*), *vernalization independence3* (*vip3*) and *frigida-like1* (*frl-1*), and mutants in genes encoding components of the PAF1 complex (*ELF7*, *VIP4*, *VIP5* and *VIP6*/*ELF8*) (Zhang and Van Nocker, 2002; Noh and Amasino, 2003; Zhang et al., 2003; Noh et al., 2004; He et al., 2004; Michaels et al., 2004; Oh et al., 2004; Kim et al., 2005). Most of these mutations also appear to affect flowering in an *FLC*-independent manner.

After exposure to an extended winter and the completion of vernalization, the level of modifications associated with 'active' chromatin is reduced, and the histone tails of *FLC* chromatin are deacetylated and become enriched in methylation of lysine 9 (K9) and 27 (K27) of H3 (Bastow et al., 2004; Sung and Amasino, 2004), which are hallmarks of repressed genes (Orlando, 2003). Mutants that are unable to reduce *FLC* transcript levels by vernalization or to maintain the vernalised state have permitted the identification of some of the proteins participating in this process, such as the chromatin remodelling factors VERNALIZATION INSENSITIVE 3 (VIN3) and VERNALIZATION 2 (VRN2) (Gendall et al., 2001; Sung and Amasino, 2004), and a plant-specific DNA-binding protein, VRN1 (Levy et al., 2002).

In summer annual accessions, reduced expression of *FLC* depends on the autonomous pathway, and is associated with lower histone acetylation of *FLC* chromatin as a result of *FVE* and *FLD* function (He et al., 2003; Ausin et al., 2004). Mutations in both genes cause *FLC* chromatin to

become more acetylated at H3 and H4 concomitantly with an increase in *FLC* expression (He et al., 2003; Ausin et al., 2004).

Here, we report the identification of *EARLY IN SHORT DAYS1* (*ESD1*), a gene that is required for the maintenance of *FLC* expression. The *esd1* mutation causes early flowering through the reduction of *FLC* expression, although the mutation also appears to affect flowering through other *FLC*-like repressors. Using a map-based approach, we have determined that *ESD1* encodes ARP6, a member of the actin-related protein family that share moderate sequence homology and basal structure with conventional actins. Recently, ARPs and actins have been discovered in the nucleus as integral components of several chromatin remodelling and histone acetyltransferase (HAT) complexes (Schafer and Schroer, 1999; Galarneau et al., 2000; Rando et al., 2000; Shen et al., 2000; Olave et al., 2002; Blessing et al., 2004). We present evidence that *ESD1* is needed to achieve the levels of both H3 acetylation and H3-K4 methylation required for high *FLC* expression.

MATERIALS AND METHODS

Genetic stocks and growth conditions

Mutant seed stocks used were in the Landsberg *erecta* (L*er*) and Columbia (Col) genetic backgrounds, and were obtained from the Arabidopsis Biological Resource Centre (ABRC) of Ohio State University (Columbus, USA), the Nottingham Arabidopsis Centre (NASC) in UK and personal donations. The monogenic *fve-1*, *fca-1*, *co-2* and *gi-3* mutants were described by Koornneef et al. (Koornneef et al., 1991); *flc-3* was described by Michaels and Amasino (Michaels and Amasino, 2001) and the Col *FRISf2* lines were described by Lee et al. (Lee et al., 1995). GA-deficient *ga1-3* and *ga2-1* mutants were described by Koornneef and van der Veen (Koornneef and van der Veen, 1980) and *spy-5* by Jacobsen and Olszewski (Jacobsen and Olszewski, 1993). The origin of the *esd1-1* to *esd1-9* alleles is summarized in Table 1. The *esd1-10* allele in Col corresponds to the T-DNA line Wisc Ds-Lox 289_29L8, and was kindly provided by the ABRC. We confirmed that all *esd1* mutations were allelic by their failure to complement the early flowering phenotype in F1 plants derived from crosses between them. Plants were grown in plastic pots containing a mixture of substrate and vermiculite (3:1). Controlled environmental conditions were provided in growth chambers at 21°C and 80% relative humidity. Plants were illuminated with cool-white fluorescent lights (approximately 120 $\mu E\ m^{-2}\ second^{-1}$). LD conditions consisted of 16 hours of light followed by 8 hours of darkness; SD conditions consisted of 8 hours of light followed by 16 hours of darkness.

Phenotypic analyses

Total leaf number was scored as the number of main leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time of opening of the first flower (Koornneef et al., 1991). Cauline, adult and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered as juvenile leaves (Telfer et al., 1997). Floral organs were analyzed by scanning electron microscopy as described (Ruiz-Garcia et al., 1997).

Genetic analysis

Double mutants were constructed by crossing the monogenic *esd1-2* mutant with lines carrying the mutations *co-2*, *gi-3*, *fve-1*, *fca-1*, *ga1-3*, *ga2-1* or *spy-5*. *esd1-3* was crossed with a line carrying the *flc-3* mutation in Col and with Col *FRI Sf-2* (Lee and Amasino, 1995). Double mutants were isolated from selfed F2 progeny that showed the *esd1* phenotype and that segregated for the second mutation.

Molecular characterization of the *esd1* alleles and map-based cloning

The *esd1-2* mutation was initially mapped to chromosome 3 between markers GAPab and nga6, using the cleaved-amplified polymorphic sequence (CAPS) and the simple sequence length polymorphism (SSLP) molecular markers indicated in Table S1 in the supplementary material. Additional analysis of 925 *esd1*-like

F2 plants allowed us to locate *ESD1* to a pericentromeric region of 1.4 cM, between the T8N9 and ATA1 markers (see Table S2 in the supplementary material). To fine map the *esd1* mutation within the interval deleted in the *esd1-1* and *esd1-6* mutant plants, which is located between the 5F21A14 and 1T14A11 markers, we designed specific PCR molecular markers (see Table S3 in the supplementary material) that were used to amplify the genomic DNA of each *esd1* mutant allele, in order to score the presence or absence of the amplified product. Southern blot hybridizations with genomic DNA were performed to confirm the PCR results (data not shown).

Plant transformation

Four overlapping binary TAC clones (JAtY74I04, JAtY64M05, JAtY54G02, JAtY49O18) spanning the minimum deleted region in the *esd1* alleles were obtained from the Genomic Arabidopsis Resource Network (GARNET) and introduced into the *esd1-3* allele by *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent, 1998). The *Agrobacterium* strain used was C58C1. Transformant plants were selected on soil by spraying seedlings with BASTA.

Only *esd1*-3 mutant plants transformed with the JAtY T74I04 TAC clone that spans from position 28823 bp of T4P3 BAC clone to 78776 bp of T14A11 BAC clone, showed complementation of the early flowering phenotype. To check whether the integration of the TAC clone was complete in transformed *esd1-3* plants, we used a set of specific molecular markers (see Table S3 in the supplementary material) contained in the deleted region. We chose markers that amplify PCR products over genomic DNA extracted from wild-type plants, but not from *esd1-3* mutant plants. In this way, we demonstrated that the genomic region of the JAtYT74I04 TAC clone integrated in the complementing transgenic plants contained only two ORFs predicted to encode proteins, *At3g33520* and *At3g33530*. The rest of the ORFs present in this region correspond to pseudogenes and retrotransposon elements.

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen-Gibco), electrophoresed and transferred onto Hybond N+ membranes (Amersham), following described protocols. For the *FLC* probe, we used a 700 bp *Eco*RI/*Sph*I fragment from pFLC lacking the MADS-box domain (Michaels and Amasino, 1999). As loading controls, we used a 305-bp *Eco*RI fragment of the cauliflower 18S ribosomal DNA gene. *ARP6* transcript levels were assayed by RT-PCR. cDNA was prepared by reverse transcription of total RNA from *Arabidopsis* roots, stems, cauline leaves, floral buds and flowers, according to described procedures (Piñeiro et al., 2003). *ARP6* gene-specific primers, 5´-GAGCTTCGACCACTTGTCCCAGAT-3´ and 5´-GCATTACAATAT ACGACAAATAATGTG-3´, were designed to amplify the C-terminal end of the coding region, including the last intron and a portion of the 3´ untranslated region. For low abundance mRNAs, such as the *MAF*, *FT* and *SOC1* genes, we also performed reverse transcriptase-mediated PCR, according to described procedures (Scortecci et al., 2001; Piñeiro et al., 2003; Ratcliffe et al., 2003). *UBIQUITIN 10* (*UBQ10*) was used as control in these experiments.

Histochemical β-glucuronidase assays

esd1-2 fca-1 FLC:GUS plants were obtained by crossing *esd1-2* with *fca-1* plants carrying a 6 kb *FLC:GUS* translational fusion construct (Sheldon et al., 2002). GUS activity in *fca-1* and *esd1-2 fca-1 FLC*:*GUS* plants was revealed by incubation in 100 mM $NaPO_4$ (pH 7.2), 2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.25% Triton X-100. Plant tissue was incubated at 37°C for 20 hours. After staining, chlorophyll was cleared from the samples by dehydration through an ethanol series.

ChIP assays and PCR

ChIP assays were carried out as described (Ausin et al., 2004). Chromatin proteins and DNA were cross linked in 10-day-old Col, *esd1*-3, *FRI*, *esd1*-3*FRI*, Ler, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1* and *esd1-2 fve-1* seedlings by formaldehyde fixation. After chromatin isolation, the H3 acetylated and methylated fractions were immunoprecipitated using specific antibodies to acetylated K9 and K14, and trimethylated K4, residues

(06-599 and 07-473 from Upstate Biotechnology, respectively). Cross-links were reversed by incubations at 65°C for 2 hours, and DNA was purified with QIAquick spin columns (QIAGEN) and eluted in 40 μl of TE (pH 8.0). Semiquantitative PCR was used to amplify six different fragments of the *FLC* gene (Michaels and Amasino, 1999) (details and primer sequences are available on request). All PCR reactions and quantification of the amplified DNA were done as described previously (Ausin et al., 2004). We carried out three independent experiments and data provided in Fig. 7 are from one representative. *UBQ10* served as an internal control for the ChIP analysis. To calculate the fold enrichment in H3 acetylation or methylation, *FLC* was first normalized to *UBQ10* in each sample, and, subsequently, these values were normalized against their respective wild-type controls.

RESULTS

esd1 mutants are early flowering and display a pleiotropic vegetative and reproductive phenotype

Mutants at the *ESD1* locus were independently identified in screens for L*er* mutations conferring early flowering under SD (*esd1-1* and *esd1-2*) or for Col mutations that accelerate developmental phase transitions (*esd1-3* to *esd1-9*) (Table 1). All of the selected alleles produced a similar array of phenotypes, independently of their genetic background. Plants homozygous for *esd1* mutations were early flowering under both LD and SD photoperiods (Table 1), showing a more extreme phenotype under SD (Table 1, Fig. 1A,B). The fact that *esd1* mutants flower earlier under inductive photoperiods indicates that the mutations do not abolish the photoperiod responses. Earliness was associated with a reduction in the length of all developmental phases of the plant (Fig. 1C), based on leaf shape and leaf trichome distribution (Telfer et al., 1997). This reduction was more dramatic in the case of adult rosette leaves, which were almost absent from *esd1* mutants grown under LD and highly reduced in *esd1* mutant plants under SD (Fig. 1C). This behaviour is similar to that exhibited by other early flowering mutants such as *esd4* and *ebs*, which also show a major reduction of the adult vegetative phase (Gomez-Mena et al., 2001; Reeves et al., 2002).

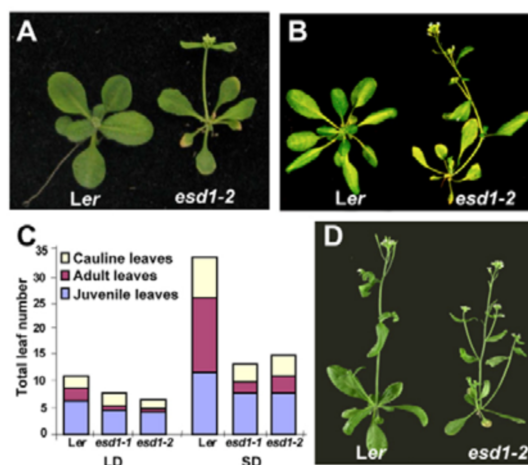


Fig. 1. The flowering phenotype of *esd1* mutants. (**A**) Wild-type L*er* and *esd1-2* 2-week-old plants grown under LD. (**B**) L*er* and *esd1-2* 4-week-old plants grown under SD. (**C**) Histogram comparing the number of juvenile, adult and cauline leaves in L*er* and *esd1* mutants. Plants were grown under both LD and SD. (**D**) L*er* and *esd1-2* 3.5-week-old plants grown under LD.

Table 1. Flowering time of wild-type and *esd1* mutant plants, indicating the nature of each allele

Genotype	Long days	Short days	Mutagen	Origin
L*er*	9.87±0.81	32.91±1.57		
esd1-1	6.81±0.48	14.10±1.40	Gamma rays	Coupland (1995)
esd1-2	7.03±0.57	15.10±1.84	Fast neutron	This work
Col	14.13±0.96	66.00±2.12		
esd1-3	9.47±0.64	28.09±1.95	Fast neutron	This work
esd1-4	9.13±0.62	29.10±1.79	Fast neutron	This work
esd1-5	9.73±0.53	31.20±1.97	Ionizing carbon	This work
esd1-6	9.71±0.64	26.81±1.75	Fast neutron	This work
esd1-7	9.67±0.61	29.96±1.94	Fast neutron	This work
esd1-8	9.48±0.51	30.83±1.90	Fast neutron	This work
esd1-9	9.47±0.51	31.05±1.81	Fast neutron	This work

Flowering times are shown as mean leaf number±s.d. of the mean.

Apart from their flowering time phenotype, *esd1* mutants also displayed a complex pleiotropic vegetative and reproductive phenotype. *esd1* mutants show a moderate increase in hypocotyl length but have shortened inflorescence internodes. Furthermore, *esd1* leaves are smaller and more curled than wild-type leaves, and usually have serrated margins (Fig. 1D). *esd1* flowers are smaller than wild-type ones (Fig. 2C) and frequently bear extra perianth organs. This phenotype was more extreme under SD, where *esd1* flowers contained an average of two extra sepals and two extra petals per flower (Fig. 2B,E,G). Mutant flowers showed a reduced fertility that was associated with a reduction in the amount of pollen and approximately a 50% reduction in seed set when compared with wild-type plants. Mutant carpels were much smaller and the filaments of the stamens of mutant flowers were shorter than those of wild-type plants (Fig. 2E). Concomitantly, siliques were approximately 50% shorter in *esd1* mutants than in wild-type plants (Fig. 2F). Thus, mutations at the *ESD1* locus directly or indirectly alter multiple aspects of plant development.

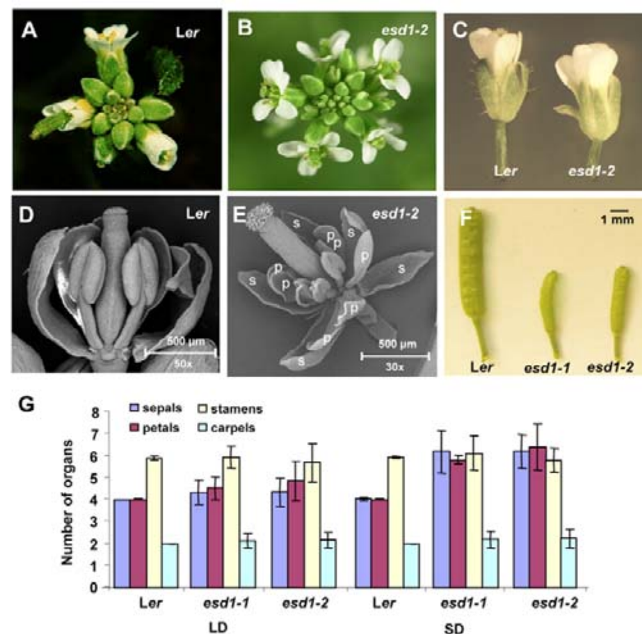


Fig. 2. The inflorescence phenotype of *esd1* mutants.

(**A**) Apex of 3-week-old L*er* plants. (**B**) Apex of 2.5-week-old *esd1* mutant plants, showing open flowers with extra sepals and petals. (**C**) Detached flowers showing the increased number of sepals and petals in *esd1* mutant lowers. (**D**,**E**) Scanning electron micrographs of L*er* (D) and *esd1* (E) flowers of plants grown under SD. s, sepal; p, petal. (**F**) A comparison of silique shape and length in L*er*, *esd1-1* and *esd1-2* plants. (**G**) Number of sepals, petals, stamens and carpels in L*er* and *esd1* mutants. Plants were grown under both LD and SD. Bars represent the standard error.

Genetic interactions between *esd1* and mutations affecting flowering time regulatory pathways

The early flowering phenotype of *esd1* mutants suggested that *ESD1* could negatively interact with a flowering promoting pathway in *Arabidopsis*. To test this hypothesis, we analyzed the phenotype of double mutants carrying *esd1* and mutations causing a delay in flowering time. We chose representative mutations for each of the photoperiod, GA and autonomous pathways. Within the photoperiod pathway, mutations at the *CO* and *GI* loci delay flowering mainly under LD (Koornneef et al., 1998) (Table 2). *esd1-2 co-2* and *esd1-2 gi-3* double mutants flowered later than *esd1-2* mutants, and earlier than *co-2* and *gi-3* plants, and thus displayed an additive phenotype (Table 2). Similar to *co-2* and *gi-3* single mutants, *esd1-2 co-2* and *esd1-2 gi-3* double mutants lack the capacity to respond to inductive photoperiods, and flowered with a similar number of leaves under both LD and SD photoperiods (Table 2).

Mutations affecting GA synthesis, such as *ga1-3* (Sun and Kamiya, 1994) and *ga2-1* (Yamaguchi et al., 1998), delay flowering in both LD and SD (Wilson et al., 1992). By contrast, mutations in *SPINDLY* (*SPY*) cause constitutive GA signalling and accelerated flowering time (Jacobsen and Olszewski, 1993). To determine whether the GA synthesis and response pathways are required for the early flowering phenotype of *esd1*, we analyzed the phenotype of *esd1-2 ga1-3*, *esd1-2 ga2-1* and *esd1-2 spy-5* double mutants. Under LD, the *esd1-2 ga1-3* and *esd1-2 ga2-1* double mutants showed an additive flowering time phenotype, in that they flowered earlier than their late parent and later than their early parent (Table 2). Under SD conditions, *esd1-2 ga2-1* also showed an intermediate flowering time phenotype; however, the *esd1-2 ga1-3* double mutant was unable to flower under SD. This is similar to the phenotype of the *ga1*-3 mutant, and indicates that the early flowering of *esd1* mutants requires GA biosynthesis under SD. In agreement with these results, *esd1-2 spy-5* double mutants also display an additive early flowering phenotype that is more readily observed under SD (Table 2).

Table 2. Flowering time of *esd1* double mutants

Genotype	Long days	Short days
Ler	8.18±0.72	32.91±1.57
esd1-2	6.11±0.42	15.10±1.84
gi-3	24.10±1.97	39.36±2.16
esd1-2 gi-3	14.52±0.79	15.68±1.49
co-2	23.15±1.28	35.63±1.75
esd1-2 co-2	16.17±0.87	16.44±1.53
ga1-3	17.8±2.2	>65
esd1-2 ga1-3	11.00±1.10	>65
ga2-1	22.01±2.81	56.31±2.83
esd1-2 ga2-1	12.81±1.56	39.30±2.31
spy-5	6.41±0.50	14.60±1.55
esd1-2 spy-5	5.20±0.51	6.60±0.90
fve-1	20.23±0.81	61.93±1.87
esd1-2 fve-1	7.32±0.61	15.96±2.18
fca-1	27.80±1.07	61.83±2.32
esd1-2 fca-1	8.84±0.80	20.56±1.73

To test the interaction between *ESD1* and autonomous pathway genes, we analyzed the flowering phenotype of *esd1-2 fve-1* and *esd1-2 fca-1* double mutants (Table 2; Fig. 3A). Under LD, some of the *esd1-2 fve-1* and *esd1-2 fca-1* double mutants were indistinguishable from *esd1*, although, on average, *esd1-2 fve-1* and *esd1-2 fca-1* produced one and two leaves more than *esd1*, respectively (Table 2; Fig. 3A). Under SD, *esd1-2 fve-1* and *esd1-2 fca-1* mutants were also very similar to *esd1-2* single mutants (Table 2), producing a few more leaves (16 and 21 leaves, respectively) than the early flowering parental plants (15 leaves); this result indicates that the late

flowering phenotype of autonomous pathway mutations requires *ESD1*. In summary, these results suggest that *ESD1* does not interact with the photoperiod and GA floral induction pathways, but shows an almost epistatic interaction with genes in the autonomous pathway.

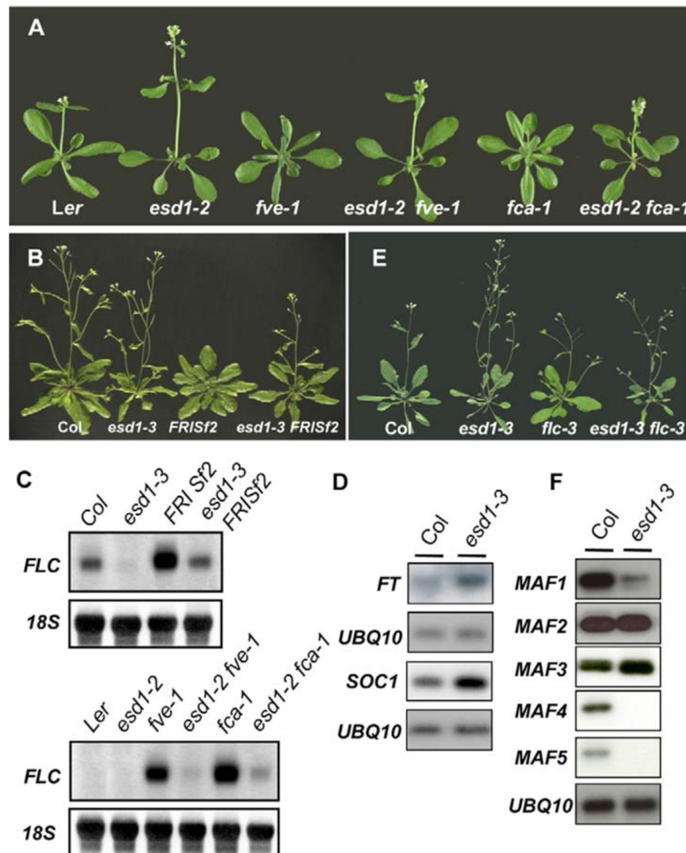


Fig. 3. Suppression of *FLC*-dependent late flowering by *esd1* mutations. (**A**) Photograph illustrating the flowering phenotype of double mutant *esd1 fve* and *esd1 fca* plants grown under LD. (**B**) Flowering phenotype of lines where an active allele of *FRI* is combined with *esd1* grown under LD. (**C**) Analysis of the expression of *FLC* in the late-flowering genotypes *FRI*, *fve* and *fca* combined with *esd1.* RNA blot hybridizations were performed using total mRNA from 9-day-old Col, *esd1-3*, *FRI*, *esd1-3 FRI*, L*er*, *esd1-2*, *fve-1*, *esd1-2 fve-1*, *fca-1* and *esd1-2 fca-1* plants grown under LD. (**D**) Analysis of the expression of *FT* and *SOC1* genes in *esd1* mutants. RT-PCR assays comparing *FT* and *SOC1* expression in 9 day-old Col and *esd1*-3 plants. The samples were taken at the time of the day with the maximum expression; for *FT* expression analysis, before dusk, and for *SOC1* analysis, 8 hours after dawn. (**E**) Flowering phenotype of *esd1 flc* double mutant plants grown under LD. (**F**) Analysis of the expression of *MAF* genes in *esd1* mutant plants. Total RNA was extracted from pools of 50 9-day-old seedlings grown under LD conditions. Expression was monitored by RT-PCR over 32 cycles for *MAF1*, 28 cycles for *MAF2*, and 35 cycles for *MAF3*, *MAF4* and *MAF5*. For the *UBQ10* control, we amplified during 22 cycles. RT- PCR products were blotted and hybridized with specific probes for each *MAF* gene.

The epistatic interaction with mutations in the autonomous pathway suggest that *esd1* might cause early flowering either by increasing the activity of the autonomous pathway downstream of *FCA* and *FVE*, or by bypassing the requirement for the autonomous pathway by reducing *FLC* expression. Because other flowering pathways, besides the autonomous pathway, converge on the regulation of *FLC* expression, it was of interest to evaluate the interaction of *esd1* with *FRI*, a dominant-positive regulator of *FLC* (Lee and Amasino, 1995; Michaels and Amasino, 1999; Johanson et al., 2000), and to study *FLC* expression in the different mutant backgrounds. When

the *FRI* allele introgressed from the San Feliu-2 ecotype (*FRI*-Col) (Lee and Amasino, 1995) was combined with the *esd1-3* mutation in a Col genetic background, plants showed an additive phenotype in which the *FRI* late-flowering phenotype was only partially suppressed by *esd1-3* (Table 3, Fig. 3B).

Table 3. Flowering time of *esd1 flc* double mutant and *FRI* combined with *esd1*

Genotype	Long days	Short days
Col	14.13±0.96	66.00±2.12
esd1-3	9.47±0.64	28.09±1.95
FRI	61.70±9.60	83.57±5.22
esd1-3 FRI	13.50±1.10	62.30±2.30
flc-3	11.70±0.91	55.20±1.75
esd1-3 flc-3	8.54±0.64	32.93±2.15

In order to check if *esd1* suppresses the effect of the autonomous pathway mutations and *FRI* by reducing *FLC* mRNA levels, we compared the abundance of the *FLC* mRNA in wild-type, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1*, *esd1-2 fve-1*, *FRI* and *esd1-3 FRI* seedlings (Fig. 3C). *FLC* mRNA was present at higher levels in both *fca-1* and *fve-1* mutants and in *FRI*-containing lines than in wildtype plants, as has been previously shown (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* transcript levels in *FRI* and in *fca-1* and *fve-1* mutants were decreased by the *esd1* lesion (Fig. 3C). *FLC* mRNA levels were also compared between wild-type plants and *esd1* mutants. Because *FLC* is expressed at a low level in L*er* wild-type plants, we could not observe a clear reduction in its expression in the *esd1* alleles isolated in L*er* background. However, we were able to detect a reduction in the *FLC* expression in the *esd1* alleles isolated in Col background, which bears an *FLC* allele expressed at higher levels (Fig. 3C). In summary, *ESD1* is required to maintain high *FLC* expression levels, either as promoted by *FRI* or by mutations that impair the autonomous pathway, and, consistent with the genetic analysis, *esd1* mutations suppress the increase in *FLC* expression caused by autonomous pathway mutations more effectively than that caused by active *FRI* alleles. In agreement with this scenario, the expression of the floral integrator genes *FT* and *SOC1*, normally repressed by *FLC* (Moon et al., 2003), was upregulated in the *esd1* mutants (Fig. 3D).

Although the effects of *esd1* mutations on flowering time are more readily observed in the late-flowering *FRI* and autonomous pathway mutant backgrounds, as discussed above, the fact that *esd1* mutants also flower earlier than the rapid-flowering wild-type strains L*er* and Col (Fig. 1 and Table 1) suggests that, in addition to regulating *FLC* expression, *ESD1* plays other roles in the control of flowering time. To determine the fraction of the *esd1* early-flowering phenotype that is independent of the effect of the *esd1* mutation on *FLC* expression, we analyzed the phenotypic effect of the *esd1-3* mutation in an *flc* null (*flc-3)* genetic background (Michaels and Amasino, 1999) under both LD and SD. When combined with *flc-3*, the *esd1* mutation significantly reduces the number of leaves produced by *flc-3* under both photoperiods (Fig. 3E and Table 3), confirming that *esd1* mutations have an *FLC*-independent effect on flowering time. Indeed, loss of function of *ESD1* also resulted in downregulation of some other members of the *FLC*/*MAF* gene family, particularly *MAF1*, *MAF4* and *MAF5* (Fig. 3F). RT-PCR analysis indicated a modest but reproducible decrease in *MAF1* gene expression and a marked silencing of the *MAF4* and *MAF5* genes, suggesting that these *MAF* gene family members represent additional regulatory targets of *ESD1*.

ESD1 encodes ACTIN-RELATED PROTEIN 6 (ARP6)

To understand the molecular function of *ESD1*, we decided to identify the gene responsible for the observed phenotypes in the mutant by a map-based cloning approach. For this, 925 *esd1* F2 plants derived from the cross between *esd1-2* and Col were screened with the markers shown in Table S1 in the supplementary material. This allowed us to locate *ESD1* south of the T8N9 marker and north of the ATA1 marker, close to the pericentromeric region of chromosome 3 (see markers used in Table S2 in the supplementary material). Owing to the severe suppression of recombination in the vicinity of the centromere and because this chromosomal region is almost completely sequenced, we designed an alternative strategy to complete the identification of the *ESD1* gene, based on the observation that all of the isolated alleles harbour a deletion in the pericentromeric region of chromosome 3. We identified the shorter overlapping genomic region that was deleted in all of the *esd1* alleles by using PCR molecular markers to amplify specific genomic DNA fragments from all of the *esd1* alleles, and looked for the presence or absence of an amplified product (see Table S3 in the supplementary material). In this way, we delimited the *ESD1* locus to a deleted genomic region between the 5F21A14 and 1T14A11 markers. This region spans three overlapping BAC clones, F21A14, T4P3 and T14A11 (Fig. 4A), and is enriched in retrotransposon and transposase elements, pseudogenes and highly repeated sequences.

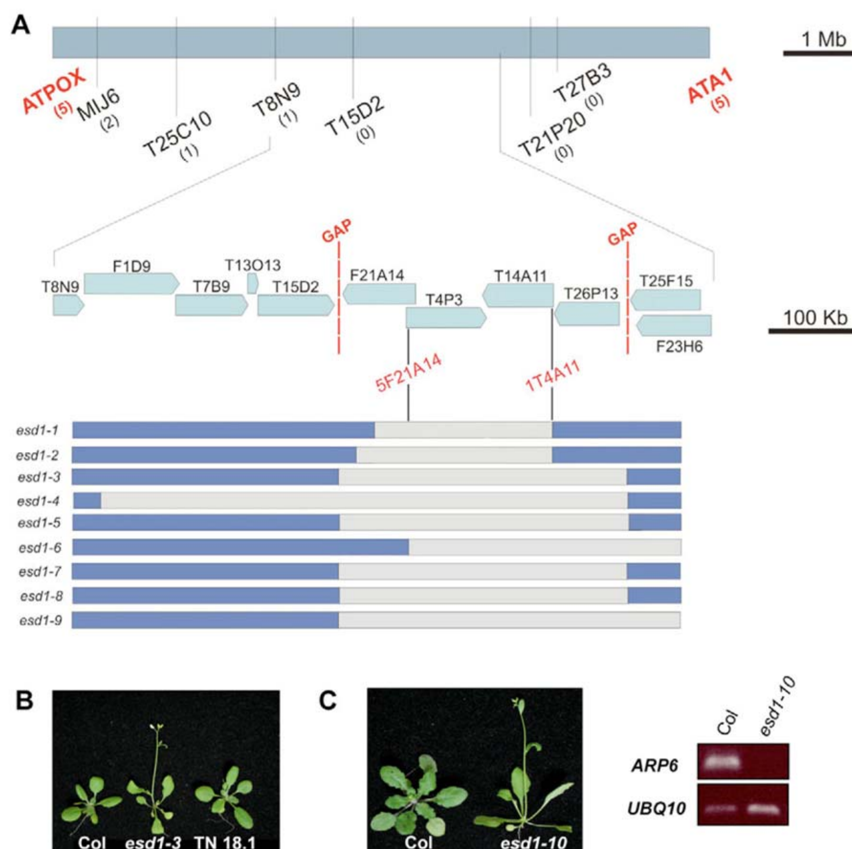


Fig. 4. Identification of *ESD1*. (**A**) Map-based cloning of *ESD1*. The genetic interval, molecular markers and BAC clones in the *ESD1* region are shown. The number of recombinant events between molecular markers is given in parentheses. The centromere is located between the T15D2 and T25F15 markers (http://www.arabidopsis.org/info/agicomplete.jsp). GAP indicates the existence of genomic regions of unknown size, where it was not possible to get overlapping BAC clones. Gray bars correspond to the deleted region in each *esd1* allele. The *ESD1* locus was delimited to a deleted overlapping genomic region between the 5F21A14 and 1T14A11 molecular markers. Mb, megabases. (**B**) Complementation of the *esd1* mutant.

Col, *esd1-3* and TN 18.1, a transgenic *esd1-3* plant containing the genomic region harbouring open reading frames *At3g33520* y *At3g33530*, shown at the time of bolting initiation. (**C**) Flowering phenotype of *esd1-10*, a T-DNA insertion allele. Left, Col plant; right, a homozygous plant for the T-DNA insertion within the *At3g33520* gene (Wisc Ds-Lox 289 line), showing an early flowering phenotype. RT-PCR analyses of the expression of *At3g33520 in esd1-10* show no expression of this gene in the T-DNA mutant, indicating that it is a loss-of-function allele.

Subsequently, different overlapping binary TAC clones spanning the deleted region were identified and introduced into the *esd1-3* allele by *Agrobacterium*-mediated transformation, in order to identify those that complemented the mutant phenotype. Only certain transgenic *esd1-3* mutant plants transformed with JAtYT74I04 TAC flowered at a similar time as wild-type plants, and had lost the pleiotropic phenotype of *esd1* mutant plants (Fig. 4B). The analysis of these transgenic plants showed that TAC clone integration had been incomplete (see Materials and methods), and that only in those lines that contained the genomic region harbouring open reading frames *At3g33520* y *At3g33530* had the mutant phenotype been complemented, suggesting that one of those ORFs represent *ESD1*. To determine which one of them corresponded to *ESD1*, we searched for T-DNA insertions within the *At3g33520* and *At3g33530* ORFs and identified the Wisc Ds-Lox 289_29L8 line for *At3g33520* and the SALK_003098 line for *At3g33530* (Alonso et al., 2003). We obtained seeds of these lines and identified plants homozygous for the T-DNA insertions. Only the plants that harbour a T-DNA insertion in *At3g33520* flowered early under both LD and SD (producing around nine and 29 leaves, respectively; wild-type plants produce 14 leaves in LD and 66 in SD), and showed a pleiotropic phenotype similar to that of *esd1* mutants regarding leaf shape, extra perianth organs and small siliques (Fig. 4C). Reverse-transcription (RT-PCR) analyses showed no expression of the *At3g33520* mRNA in these insertional mutant plants (Fig. 4C), indicating that the T-DNA insertion causes a loss-of-function allele. Complementation tests confirmed that this T-DNA mutation was allelic to *esd1*. Thus, we refer to the Wisc Ds-Lox 289_29L8 line as the *esd1-10* allele, and conclude that the *ESD1* locus corresponds to the *At3g33520* gene.

To determine the genomic structure of *ESD1*, a cDNA was identified and sequenced. The *ESD1* gene possesses six exons and five introns, and encodes a protein of 421 amino acids (Fig. 5A). This protein corresponds to ARP6, a member of the actin-related protein family that shares moderate sequence homology and basal structure with conventional actins, but it has two peptide insertions that seemingly provide divergent surface features from actins (Fig. 5B). ARPs are normally grouped into several classes or subfamilies that are highly conserved in a wide range of eukaryotes, from yeast to plants and humans (Goodson and Hawse, 2002). Database searches with the AtARP6 protein sequence identified eight potential ARP proteins in *Arabidopsis* (ARP2-ARP9) (McKinney et al., 2002). In particular, AtARP6 is a likely ortholog of a group of less-characterized ARPs, including ARP6s from yeast, *C. elegans*, fruit fly and humans (Fig. 5B). RT-PCR analysis revealed that *ARP6* mRNA is detected in most plant organs, with the highest levels found in roots and floral buds (Fig. 5C). Lower levels were detected in cauline leaves, stems and flowers. These results indicate that ARP6 is expressed ubiquitously.

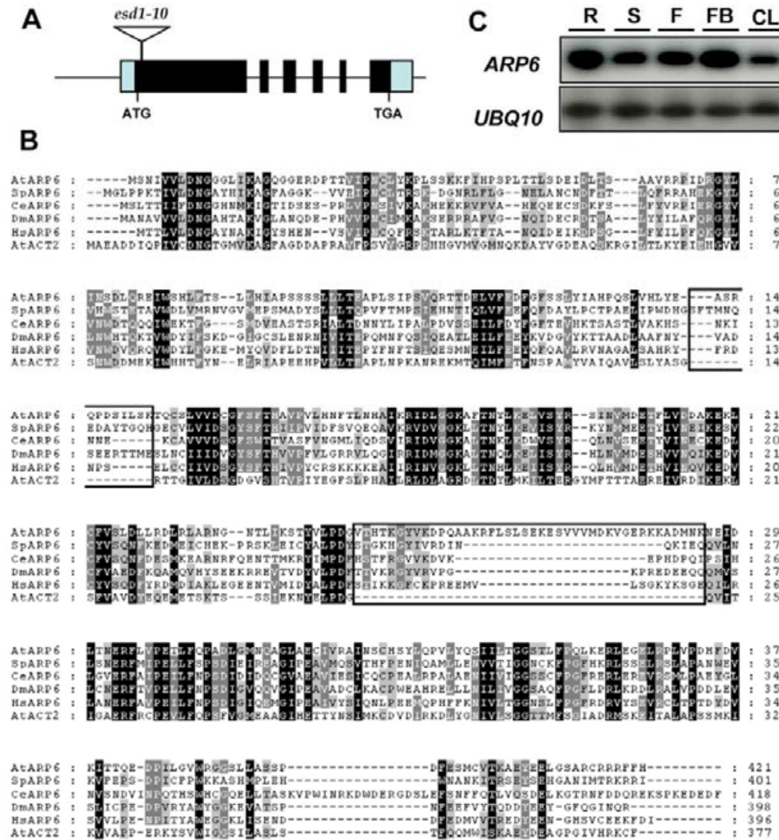


Fig. 5. *ESD1* encodes ARP6. (**A**) Scheme of the *ARP6* gene showing the position of the T-DNA insertion in the *esd1-10* mutant. Exons are shown as black boxes. The position of the start and stop codon are indicated. (**B**) Sequence comparison of AtARP6 with yeast (Sp), *C. elegans* (Ce), *Drosophila* (Dm) and human (Hs) ARP6s, and *Arabidopsis* Actin2. Amino acid residues in black are functionally similar in all sequences and those in gray are similar in at least four of them. Boxed regions indicate the two peptide insertions in ARP6s, which do not disrupt the conserved actin fold structure. GenBank Accession numbers are NP_566861 for AtARP6, AAF4849 for Dm ARP6, AAK14934 for Hs ARP6, CAA19116 for Sp ARP6, NP_495681 for Ce ARP6, and BAB01806 for AtACT2. (**C**) *APR6* expression in different organs of Col plants. RT-PCR assays were performed with RNA prepared from different tissues. R, roots; S, main stems; F, flowers; FB, flower buds; CL, cauline leaves. RT-PCR products were blotted and hybridized with a specific probe for *ARP6*. *UBQ10* was used as a loading control.

ESD1 is required to activate *FLC* transcription through both histone acetylation and methylation mechanisms

FLC gene expression integrates signals coming from different pathways involved in the regulation of the floral transition (Michaels and Amasino, 1999; Sheldon et al., 2000; Amasino, 2005). Recent work has demonstrated the role of histone modification in the regulation of *FLC* expression through *FRI*, the autonomous and the vernalization pathways (He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004; Kim et al., 2005). These results have also identified the first intron of *FLC* as a relevant region for histone modification (He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004) and transcriptional regulation (Gendall et al., 2001; Sheldon et al., 2002).

We first analyzed the effect of *esd1* mutations on a *FLC:GUS* translational fusion containing all the *FLC* promoter and intron elements required for proper regulation (Sheldon et al., 2002). For

this purpose, we introduced the *FLC:GUS* construct into the *fca-1 esd1-2* background and analyzed five independent lines for *GUS* expression. In contrast to the pattern of *GUS* expression in the *fca-1* background, all of the *fca-1 esd1-2 FLC:GUS* lines we examined showed undetectable *FLC:GUS* expression in the shoot apical meristem (SAM) and in the root apical meristem (RAM) (Fig. 6). These results indicate that *ARP6* is required for the high level of *FLC* expression in the SAM and the RAM.

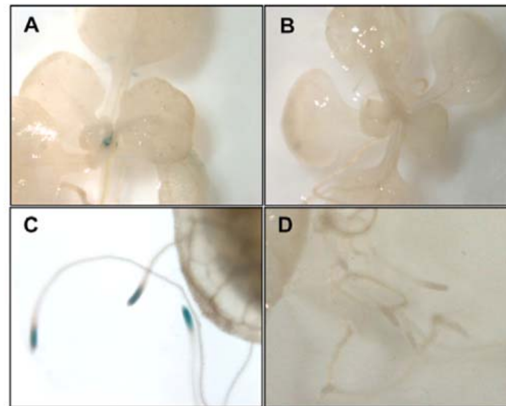


Fig. 6. Histochemical β-glucuronidase assays in *fca-1 FLC:GUS* and *esd1-2 fca-1 FLC:GUS* plants. (**A-D**) Gus staining is shown in the shoot apical meristem region (A,B) and the root tip (C,D) of representative *fca-1 FLC:GUS* (A,C) and *esd1-2 fca-1 FLC:GUS* (B,D) seedlings grown for 10 days under LD conditions.

Because *esd1* suppresses the late-flowering phenotype of autonomous pathway mutants such as *fve* and *fca*, and *FVE* represses *FLC* transcription through a histone deacetylation mechanism, we initially speculated that *ARP6* could be required for the acetylation of histones necessary to activate *FLC* expression. In fact, as mentioned above, there is considerable evidence implicating nuclear ARPs in chromatin remodelling complexes (Cairns et al., 1998; Boyer and Peterson, 2000; Olave et al., 2002).

To determine whether ARP6 promotes histone acetylation of the *FLC* chromatin, we used chromatin immunoprecipitation (ChIP) assays (Fig. 7B). High levels of expression of *FLC* in the *fve* mutant were correlated with H3 and H4 hyperacetylation at the *FLC* locus (He et al., 2003; Ausin et al., 2004). Chromatin of *esd1-2*, *fve-1* and *fve-1 esd1-2* plants was immunoprecipitated by using antibodies against acetylated H3, and PCR was used to amplify six DNA fragments spanning the promoter, the first exon and the first intron of *FLC* from the precipitated chromatin. For five out of the six probes assayed, *FLC* amplified sequences were consistently less abundant in DNA from precipitated chromatin of *fve-1esd1-2* double mutants than from chromatin of the *fve-1* mutant plants (Fig. 7B). Thus, in *fve-1 esd1-2* plants, *FLC* chromatin shows a reduction in acetylated H3 in comparison to the *fve-1* mutant, indicating that ARP6 affects the levels of H3 acetylation of *FLC*. We concluded that ARP6 is required to activate *FLC* expression through a mechanism involving the histone acetylation of *FLC* chromatin. We extended this assay to other genetic backgrounds with high levels of *FLC* expression, such as *fca-1* and *FRI*. For this, we focused our analysis on the *FLC V* and *FLC IX* probes, because they were among those that consistently showed the biggest effect of *esd1* on the histone acetylation of *FLC* chromatin in a *fve* background. In agreement with previous data, we only detected very small changes in acetylated H3 in *fca-1* and *FRI* backgrounds, when compared with those observed in the *fve-1* mutant (He et al., 2003; Ausin et al., 2004). These differences were suppressed to a certain degree when *fca-1* or *FRI* was combined with *esd1* (Fig. 7C).

Because *esd1* mutations reduced *FLC* expression in the *fca* and *FRI* background as shown, we hypothesized that ARP6 might be required for other chromatin modifications, in addition to histone acetylation, that are involved in the regulation of *FLC* expression. To further explore this hypothesis, we examined if ARP6 has an effect on histone methylation at the *FLC* locus. It has been shown recently that H3-K4 hypertrimethylation is associated with actively transcribed *FLC* chromatin (He et al., 2004), being elevated in *FRI* containing winter annuals and autonomous pathway mutants. Given the fact that *esd1* mutations reduce *FLC* expression in these backgrounds, we wondered whether *ESD1* was required for the elevated trimethylation of H3-K4 in *FLC* chromatin. Compared with wild-type plants, the trimethylated H3-K4 levels were elevated in a *FRI*-containing line and in autonomous pathway mutants, as reported previously (He et al., 2004; Kim et al., 2005) (Fig. 7D). Introduction of *esd1* into *FRI*, *fca* and *fve* consistently eliminated the H3-K4 trimethylation increase in *FLC* chromatin associated with *FRI* and the autonomous pathway mutations (Fig. 7D). These data indicate that ESD1 is also required for the hypertrimethylation of H3-K4 in *FLC* chromatin.

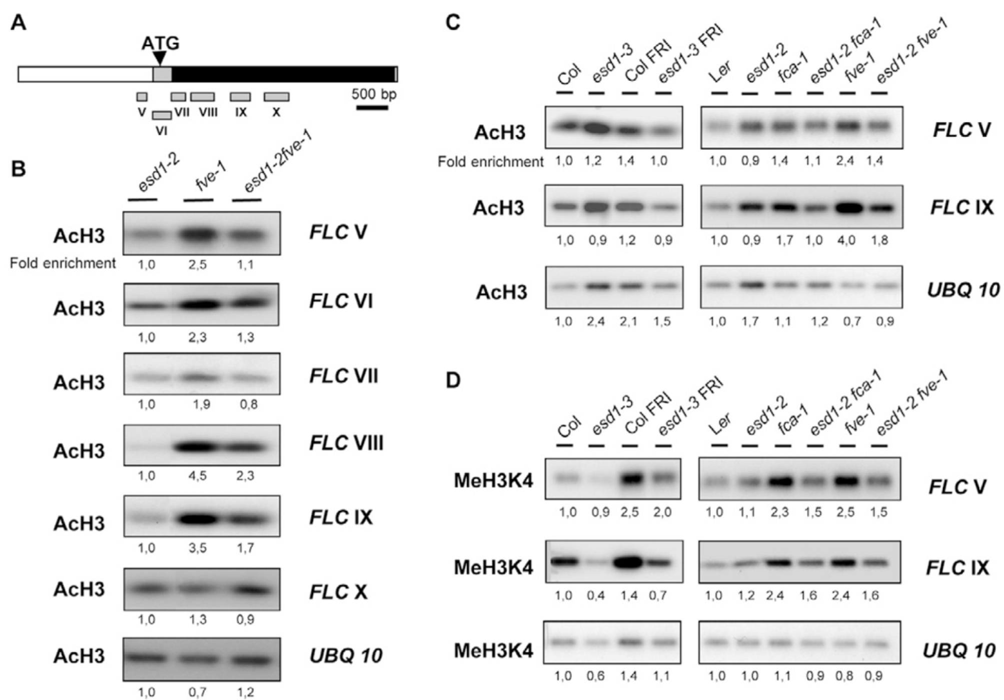


Fig. 7. Effect of *esd1* mutation on histone H3 acetylation and methylation in the *FLC* genomic region by ChIP analysis. (**A**) *FLC* genomic region analyzed by ChIP. The white box corresponds to the promoter *FLC* region, gray boxes to exons and the black box to the first intron. The six *FLC* fragments analyzed by semi-quantitative PCR are depicted and numbered. (**B**) PCR products after 25 cycles of *esd1-2*, *fve-1* and *esd1-2 fve-1* mutants, using as a template DNA purified from chromatin inmunoprecipitated with antibodies against acetylated H3 (AcH3). *UBQ10* was amplified during 22 cycles and used as control for DNA quantification. Fold enrichment in H3 acetylation of *fve-1* over *esd1-2* and *esd1-2 fve-1* double mutant is shown. (**C**) PCR products after 25 cycles of Col, *esd1-3*, *FRI*, *esd1-3FRI*, L*er*, *esd1-2*, *fca-1*, *esd1-2 fca-1*, *fve-1*, and *esd1-2 fve-1* plants, using as a template DNA purified from chromatin inmunoprecipitated with antibodies against acetylated H3 (AcH3). *UBQ10* was amplified during 22 cycles and used as control for DNA quantification. Fold enrichment in H3 acetylation of mutants over wild-type ecotypes is shown. (**D**) PCR products as in C, but using a as template DNA purified from chromatin inmunoprecipitated with antibodies against trimethylated H3-K4 (MeH3-K4). Fold enrichment in H3-K4 methylation of mutants over wild-type ecotypes is shown.

DISCUSSION

esd1 mutants were selected from multiple screens for early flowering and accelerated phase change plants in *Arabidopsis*. Phenotypical analyses of these mutants revealed a complex pleiotropic phenotype affecting vegetative and reproductive development, together with a reduction in flowering time and phase length. The results of our genetic analyses revealed that the early flowering phenotype of *esd1* mutants is almost completely epistatic over the flowering time delay caused by mutations in the autonomous pathway, and that *esd1* partially suppresses the late flowering phenotype conferred by active *FRI* alleles (Fig. 3, Tables 2, 3). These epistatic effects correlate at the molecular level with a decrease in the steady state levels of *FLC* mRNA in lines carrying *esd1* mutant alleles. Together, these results indicate that *ESD1* is required for the expression of *FLC.* Thus, mutations of *ESD1* behave like mutations at the *EFS*, *ESD4*, *PIE1*, *ELF5*, *VIP3*, *ELF7*, *VIP4*, *VIP5* and *VIP6*/*ELF8* loci (Soppe et al., 1999; Reeves et al., 2002; Zhang and Van Nocker, 2002; Noh and Amasino, 2003; Zhang et al., 2003; Noh et al., 2004; He et al., 2004; Oh et al., 2004; Kim et al., 2005), all of which are also required for high *FLC* expression and flowering repression.

Additionally, the residual early flowering phenotype observed in *esd1-3 flc-3* double mutants, especially under SD, indicates an additional role of *ESD1* in the repression of flowering time that is independent of *FLC*. The most conservative hypothesis is that *ESD1* is also required for the expression of *FLC*-related repressors, such as some of the *MAF* genes, which is consistent with our results showing a decreased expression of *MAF1*, *MAF4* and *MAF5*, previously shown to play a role in flowering repression in *Arabidopsis* under certain environmental conditions (Scortecci et al., 2001; Ratclife et al., 2003). Finally, until triple and quadruple mutants carrying lesions at *FLC*, *ESD1* and these *MAF* genes are analyzed, we cannot discard possible additional effects of *ESD1* on flowering time through additional genes. The pleiotropic phenotype of *esd1* mutants together with the broad expression pattern detected for this gene suggest that its function could be required in other developmental processes apart from flowering time.

Positional identification of the genomic region deleted in *esd1* alleles and the complementation of the *esd1* phenotype by a genomic clone containing both *At3g33520* and *At3g33530* ORFs, together with the lack of genetic complementation between a T-DNA insertion line in *At3g33520* and *esd1-3*, identified *ESD1* as encoding ARP6. Recently, two publications have also described the characterization of early flowering mutants affected in the *ARP6* gene, proposing its role in the maintenance of *FLC* expression and repression of flowering in *Arabidopsis* (Choi et al., 2005; Deal et al., 2005). ARP6 belongs to the actin-related protein family that shares moderate sequence homology and basal structure with actins. In *Arabidopsis* and rice, four divergent ARP classes (ARP4, ARP5, ARP6 and ARP9) are sequence homologs of ARPs, which are nuclear located in animals and fungi (McKinney et al., 2002; Kandasamy et al., 2004). Most of the nuclear ARPs are essential components of large multiprotein chromatin-modifying complexes (Blessing et al., 2004). The fruit fly ARP6-related protein ARP13E is associated with heterochromatin and may also play a role in chromatin structure (Frankel and Mooseker, 1996; Kato et al., 2001). ARP13E colocalizes with heterochromatin protein 1 (HP1) (Frankel et al., 1997), which is also linked to heterochromatin-mediated gene silencing and chromatin structure (Eissenberg and Elgin, 2000). Moreover, in nuclei expressing mutant forms of HP1, the localization patterns of HP1 and dARP6 are altered in a parallel fashion (Frankel et al., 1997), implying that dARP6 interacts with HP1 directly or indirectly, and that they play a role in the organization of heterochromatin together. Mutants with a defect in an *Arabidopsis HP1* ortholog, *LIKE-HETEROCHROMATIN PROTEIN 1* (*LHP1*), also show an early flowering phenotype (Gaudin et al., 2001), raising the possibility that both proteins might be involved in the same chromatin-remodelling complexes in *Arabidopsis*.

Covalent modification of chromatin histones constitutes a code for maintaining states of gene activation and repression, and is a major component in the transcriptional regulation of *FLC* (Gendall et al., 2001; He et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung et al., 2004). High levels of expression of *FLC* in autonomous pathway mutants are correlated with H3 and H4 hyperacetylation and trimethylation of H3-K4 at the *FLC* locus (He et al., 2003; Ausin et al., 2004; He et al., 2004; Kim et al., 2005). Furthermore, ARP6-like proteins have been found in other organisms as part of large protein complexes involved in chromatin remodelling (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). Because *esd1* mutations suppress the late-flowering phenotype of *fve* mutants, and *FVE* represses *FLC* transcription through a histone deacetylation mechanism, we initially hypothesized that *ESD1* could be required to activate *FLC* expression to levels that inhibit flowering, participating in chromatin remodelling complexes involved in histone acetylation of *FLC* chromatin. The lack of expression of *GUS* in *esd1 fca* plants expressing the *FLC:GUS* translational fusion, already suggested that if *ESD1* was required for active expression of *FLC*, this had to take place at the *FLC* sequences present in the construct used (promoter, first exon and first intron) (Sheldon et al., 2002). In fact, the results of ChIP experiments directed to that chromosomal region of *FLC* demonstrated that it is hypoacetylated in the *esd1 fve* mutant compared with the *fve* mutant (Fig. 7B). Thus, we conclude that ESD1 is required for histone acetylation at *FLC*, probably through its participation in HAT complexes. However, *esd1* mutations also reduce both the late-flowering phenotype and *FLC* expression in *FRI*-containing lines and *fca* mutants, despite the fact that in these backgrounds the levels of acetylated H3 of *FLC* chromatin did not show significant changes in comparison to *fve* (Fig. 7C). This raised the possibility that ARP6 would participate in other mechanisms besides histone acetylation; our results indicate that the hypermethylation of H3-K4 in *FLC* chromatin is one of these mechanisms (Fig. 7D). It remains to be determined whether the effect of *esd1* on the expression of other *MAF* genes takes place through similar mechanisms.

Our observation that ARP6 regulates the activation of *FLC* expression by promoting both histone acetylation and methylation is consistent with a role for plant ARPs in chromatin-mediated transcriptional regulation. ARP4 is also likely to be involved in transcriptional regulation via chromatin remodelling, as it is a component of the human SWI/SNF and yeast INO80 complexes that are involved in chromatin remodelling, transcriptional regulation and DNA damage repair (Zhao et al., 1998; Shen et al., 2003). Other ARP4-containing complexes, such as yeast NuA4 and human TIP60, are suggested to have roles in chromatin-mediated epigenetic control of transcription through modifications of core histones (Galarneau et al., 2000; Ikura et al., 2000). Yeast Arp4 interacts with all four core histones (Harata et al., 1999), and recent findings have shown that Arp4 and Arp6 are also part of the Swr1 chromatin-remodelling complex, which catalyzes the exchange of conventional histone H2A for the histone H2A.Z variant in nucleosome arrays (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2004). These histone variants are involved in the regulation of gene expression and the establishment of a buffer to the spread of silent heterochromatin (Meneghini et al., 2003). Indeed, a human H2A.Z complex, equivalent to the yeast Swr1 complex has histone acetyl transferase activity, which might help to understand the role of *ESD1* in histone acetylation (Owen-Hughes and Bruno, 2004). In the same way, the fact that components of the Swr1 complex were found to interact genetically with the PAF1 complex might explain the role of *ESD1* in the trimethylation of H3-K4 in *FLC* chromatin (Krogan et al., 2002; Mueller and Jaehning, 2002; Squazzo et al., 2002; Krogan et al., 2003; Krogan et al., 2004). Like the yeast PAF1 complex, the PAF1-like complex in *Arabidopsis* may also recruit an H3-K4 methyl transferase to *FLC* to regulate its expression (Kim et al., 2005). Indeed, mutations in *Arabidopsis* homologs of the components of the PAF1 complex cause a decrease in the trimethylation of H3-K4 in *FLC* chromatin, and provoke early flowering and small leaves, similar to

the *esd1* mutation (He et al., 2004), raising the possibility that all of these genes are in the same pathway and regulate similar targets.

In agreement with the pleiotropic phenotype of *esd1* mutants, the general pattern of expression of *ESD1/ARP6* suggests that this gene is required in additional vegetative and reproductive developmental processes in which protein complexes harbouring ARP6 might play a relevant regulatory role. Given the molecular identity of *ESD1*, it seems reasonable to propose that loss-of-function alleles will cause a great effect on transcription, interfering with the expression of genes controlling various developmental pathways and thereby provoking changes in the morphology of different organs throughout the development of *Arabidopsis*. Among them, organ number in the perianth, which increases in *esd1* mutants, is affected in a similar way in *pie1* mutants. *PIE1* encodes a protein similar to the ATPdependent, chromatin remodelling proteins of the ISWI and SWI/SNF2 family, and it is a close homolog to the Swr1 ATPase, the core subunit of the yeast Swr1 complex that harbours Arp6 (Mizuguchi et al., 2004). Loss of function of the *PIE1* gene causes strikingly similar phenotypes to those of the *esd1* mutant (Noh and Amasino, 2003), apart from the development of extra petals. In addition, *pie1* mutations also cause early flowering and suppress *FLC*-mediated delay of flowering as a result of the presence of *FRI* or of mutations in autonomous pathway genes, suggesting that PIE1 and ARP6 may act in the same genetic pathways and might be part of the same protein complexes. However, in contrast to *esd1* mutations, which suppress *FLC* expression in both SAM and RAM (Fig. 6), the effect of *pie1* lesions is restricted to the shoot apex (Noh and Amasino, 2003), suggesting that the root tip expression of *FLC* requires *ARP6* and probably other root-expressed relatives of *PIE1*, and that the level of *FLC* expression in the shoot apex, but not in the root apex, influences flowering behaviour.

Recent analyses of knockdown *AtARP4* expression in *Arabidopsis* have also revealed dramatic pleiotropic phenotypes, both similar to and entirely different from those of *esd1/arp6* (Kandasamy et al., 2005a). For example, silencing of the expression of *ARP4* or loss of function of *ARP6* caused early flowering; however, silencing of the expression of *ARP4* but not *ARP6* induced specific phenotypes, such as the altered organization of plant organs, delayed flower senescence and high levels of sterility (Kandasamy et al., 2005a), suggesting that both of these proteins may also be involved in the same and in different chromatin modifying complexes in *Arabidopsis*. Another ARP member, AtARP7 is required for normal embryogenesis, plant architecture, root growth and floral organ abscission (Kandasamy et al., 2005b), and may be also involved in chromatin-remodelling complexes.

In summary, our results demonstrate that *ESD1*/*ARP6* is required for both *FLC* and *FLC*-like gene expression in the shoot and the root apex, and for the activity of a floral repressor pathway. The role of *ESD1* in *FLC* regulation is to ensure competence for a high level of expression of this gene. We propose that ARP6 is required to activate *FLC* transcription through mechanisms involving both histone acetylation and methylation. We have determined that *FLC*, and maybe the *FLC* paralogs *MAF1*, *MAF4* and *MAF5* are targets of ARP6-containing chromatin-remodelling complexes, and that some components of the autonomous pathway might affect the activity of such complexes. Moreover, the pleiotropic phenotype observed for *esd1* mutants suggests a crucial role for the *Arabidopsis* ARP6 protein in the regulation of several leaf and flower development stages, probably through chromatin-mediated regulation of gene expression. Further functional studies, such as the identification of the proteins within ARP6-containing complexes, as well as the identification of additional genes regulated by these complexes, will help us to understand the crucial role of ARP6 in *Arabidopsis* development.

We are grateful to Dr George Coupland for providing us with *esd1-1* seeds and Dr Jean Finnegan for *fca-1* seeds harbouring the *FLC: GUS* construct. We would like to thank to Dr Richard Amasino for kindly providing *flc*-3 and *FRI*Col lines, to the ABRC and NASC for supplying the T-DNAs lines used in this study, to the GARNET for providing us with the binary TAC clones and to GENOSCOPE for the *ARP6* cDNA clone. This work was supported by grants BIO2001-00391 to J.M.M.-Z. and BIO2002-00753 to J.A.J. from the Spanish Ministerio de Ciencia y Tecnología. A.L. is funded by a predoctoral fellowship from Direccion General de Investigación, Secretaria General de Política Científica y Tecnológica (Spain). We thank to all members of Jarillo's lab for helpful discussions.

References

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301**, 653-657.

Amasino, R. M. (2005). Vernalization and flowering time. *Curr. Opin. Biotechnol.* **16**,154-158.

Ausin, I., Alonso-Blanco, C., Jarillo, J. A., Ruiz-Garcia, L. and Martinez-Zapater, J. M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nat. Genet.* **36**,162-166.

Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A. and Dean, C. (2004). Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* **427**, 164-167.

Blessing, C. A., Ugrinova, G. T. and Goodson, H. V. (2004). Actin and ARPs: action in the nucleus. *Trends Cell Biol.* **14**, 435-442.

Boss, P. K., Bastow, R. M., Mylne, J. S. and Dean, C. (2004). Multiple pathways in the decision to flower: enabling, promoting, and resetting. *Plant Cell Suppl.* **16**, S18-S31.

Boyer, L. A. and Peterson, C. L. (2000). Actin-related proteins (ARPs): conformational switches for chromatin-remodeling machines? *BioEssays* **22**, 666-672.

Cairns, B. R., Erdjument-Bromage, H., Tempst, P., Winston, F. and Kornberg, R. D. (1998). Two actin-related proteins are shared functional components of the chromatin-remodeling complexes RSC and SWI/SNF. *Mol. Cell* **2**, 639-651.

Choi, K., Kim, S., Kim, S. Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.-G., Michaels, S. and Lee, I. (2005). *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in *Arabidopsis*. *Plant Cell* **17**, 2647-2660.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

Coupland, G. (1995). Genetic and environmental control of flowering time in Arabidopsis. *Trends Genet.* **11**, 393-397.

Deal, R. B., Kandasamy, M., McKinney, E. C. and Meagher, R. B. (2005). The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of *FLOWERING LOCUS C* expression and repression of flowering in Arabidopsis. *Plant Cell* **17**, 2633-2646.

Eissenberg, J. C. and Elgin, S. C. (2000). The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* **10**, 204-210.

Frankel, S. and Mooseker, M. S. (1996). The actin-related proteins. *Curr. Opin. Cell Biol.* **8**, 30-37.

Frankel, S., Siegel, E. A., Craig, C., Elgin, S. C., Mooseker, M. S. and Artavanis-Tsakonas, S. (1997). An actin-related protein in Drosophila colocalizes with heterochromatin protein 1 in pericentric heterochromatin. *J. Cell Sci.* **110**, 1999-2012.

Galarneau, L., Nourani, A., Boudreault, A. A., Zhang, Y., Heliot, L., Allard, S., Savard, J., Lane, W. S., Stillman, D. J. and Cote, J. (2000). Multiple links between the NuA4 histone acetyltransferase complex and epigenetic control of transcription. *Mol. Cell* **5**, 927-937.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, D. and Grandjean, O. (2001). Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affect flowering time and plant architecture in Arabidopsis. *Development* **128**, 4847-4858.

Gazzani, S., Gendall, A. R., Lister, C. and Dean, C. (2003). Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. *Plant Physiol.* **132**, 1107-1114.

Gendall, A. R., Levy, Y. Y., Wilson, A. and Dean, C. (2001). The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. *Cell* **107**, 525-535.

Gomez-Mena, C., Piñeiro, M., Franco-Zorrilla, J. M., Salinas, J., Coupland, G. and Martinez-Zapater, J. M. (2001). *Early bolting in short days*: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of *leafy*. *Plant Cell* **13**, 1011-1024.

Goodson, H. V. and Hawse, W. F. (2002). Molecular evolution of the actin family. *J. Cell Sci.* **115**, 2619-2622.

Harata, M., Oma, Y., Mizuno, S., Jiang, Y. W., Stillman, D. J. and Wintersberger, U. (1999). The nuclear actin-related protein of *Saccharomyces cerevisiae*, Act3p/Arp4, interacts with core histones. *Mol. Biol. Cell* **10**, 2595-2605.

He, Y. and Amasino, R. M. (2005). Role of chromatin modification in floweringtime control. *Trends Plant Sci.* **10**, 30-35.

He, Y., Michaels, S. D. and Amasino, R. M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. *Science* **302**, 1751-1754.

He, Y., Doyle, M. D. and Amasino, R. M. (2004). PAF1 complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter annual habit in Arabidopsis. *Genes Dev.* **18**, 2774-2784.

Henderson, I. R. and Dean, C. (2004). Control of Arabidopsis flowering: the chill before the bloom. *Development* **131**, 3829-3838.

Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J. and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**, 463-473.

Jacobsen, S. E. and Olszewski, N. E. (1993). Mutations at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. *Plant Cell* **5**, 887-896.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R. and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. *Science* **290**, 344-347.

Kandasamy, M. K., Deal, R. B., McKinney, E. C. and Meagher, R. B. (2004). Plant actin-related proteins. *Trends Plant Sci* **9**, 196-202.

Kandasamy, M. K., Deal, R. B., McKinney, E. C. and Meagher, R. B. (2005a). Silencing the nuclear actin-related protein AtARP4 in Arabidopsis has multiple effects on plant development, including early flowering and delayed floral senescence. *Plant J.* **41**, 845-858.

Kandasamy, M. K., McKinney, E. C., Deal, R. B. and Meagher, R. B. (2005b). Arabidopsis ARP7 is an essential actin-related protein required for normal embryogenesis, plant architecture, and floral organ asbscission. *Plant Physiol.* **138**, 2019-2032.

Kato, M., Sasaki, M., Mizuno, S. and Harata, M. (2001). Novel actin-related proteins in vertebrates: similarities of structure and expression pattern to Arp6 localized on Drosophila heterochromatin. *Gene* **268**, 133-140.

Kim, S.-Y., He, Y., Jacob, Y., Noh, Y.-S., Michaels, S. and Amasino, R. (2005). Establishment of the vernalization-responsive winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. *Plant Cell* **17**, 3301-3310.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**, 1960-1962.

Kobor, M. S., Venkatasubrahmanyam, S., Meneghini, M. D., Gin, J. W., Jennings, J. L., Link, A. J., Madhani, H. D. and Rine, J. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS Biol.* **2**, 587-599.

Komeda, Y. (2004). Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annu. Rev. Plant Biol.* **55**, 521-535.

Koornneef, M. and van der Veen, J. H. (1980). Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) *Heynh. Theor. Appl. Genet.* **58**, 257-263.

Koornneef, M., Hanhart, C. J. and van der Veen, J. H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **229**, 57-66.

Koornneef, M., Alonso-Blanco, C., Peeters, A. J. and Soppe, W. (1998). Genetic control of flowering time in Arabidopsis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 345-370.

Krogan, N. J., Kim, M., Ahn, S. H., Zhong, G., Kobor, M. S., Cagney, G., Emili, A., Shilatifard, A., Buratowsky, S. and Grennblatt, J. F. (2002). RNA pol II elongation factors of *Sacharomyces cereviseae*: a targeted proteomics approach. *Mol. Cell. Biol.* **22**, 6979-6992.

Krogan, N. J., Keogh, M.-C., Datta, N., Sawa, C., Ryan, O. W., Ding, H., Haw, R. A., Pootoolal, J., Tong, A., Canadien, V. et al. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Mol. Cell* **12**, 1565-1576.

Krogan, N. J., Baetz, K., Keogh, M.-C., Datta, N., Sawa, C., Kwok, T. C. Y., Thompson, N. J., Davey, M. G., Pootoolal, J., Hughes, T. R. et al. (2004). Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4. *Proc. Natl. Acad. Sci. USA* **101**, 13513-13518.

Lee, I. and Amasino, R. M. (1995). Effect of vernalization, photoperiod, and light quality on the flowering phenotype of Arabidopsis plants containing the *FRIGIDA* gene. *Plant Physiol.* **108**, 157-162.

Levy, Y. Y., Mesnage, S., Mylne, J. S., Gendall, A. R. and Dean, C. (2002). Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. *Science* **297**, 243-246.

McKinney, E. C., Kandasamy, M. K. and Meagher, R. B. (2002). Arabidopsis contains ancient classes of differentially expressed actin-related protein genes. *Plant Physiol.* **128**, 997-1007.

Meneghini, M. D., Wu, M. and Madhani, H. D. (2003). Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. *Cell* **112**, 725-736.

Michaels, S. D. and Amasino, R. M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.

Michaels, S. D. and Amasino, R. M. (2000). Memories of winter: vernalization and the competence to flower. *Plant Cell Environ.* **23**, 1145-1154.

Michaels, S. D. and Amasino, R. M. (2001). Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-941.

Michaels, S. D., Bezerra, I. C. and Amasino, R. M. (2004). *FRIGIDA*-related genes are required for the winter-annual habit in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **101**, 3281-3285.

Mizuguchi, G., Shen, X., Landry, J., Wu, W. H., Sen, S. and Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* **303**, 343-348.

Moon, J., Suh, S. S., Lee, H., Choi, K. R., Hong, C. B., Paek, N. C., Kim, S. G. and Lee, I. (2003). The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. *Plant J.* **35**, 613-623.

Mueller, C. L. and Jaehning, J. A. (2002). Ctr9, Rtf1 and Leo1 are components of the Paf1/RNA polymerase II complex. *Mol. Cell. Biol.* **22**, 1971-1980.

Mullins, R. D. and Pollard, T. D. (1999). Structure and function of the Arp2/3 complex. *Curr. Opin. Struct. Biol.* **9**, 244-249.

Neely, K. E. and Workman, J. L. (2002). The complexity of chromatin remodelling and its links to cancer. *Biochim. Biophys. Acta* **1603**, 19-29.

Noh, Y. S. and Amasino, R. M. (2003). PIE1, an *ISWI* family gene, is required for *FLC* activation and floral repression in Arabidopsis. *Plant Cell* **15**,1671-1682.

Noh, Y. S., Bizzell, C. M., Noh, B., Schomburg, F. M. and Amasino, R. M. (2004). *EARLY FLOWERING 5* acts as a floral repressor in Arabidopsis. *Plant J.* **38**, 664-672.

Oh, S., Zhang, H., Ludwig, P. and van Nocker, S. (2004). A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis *FLC/MAF* MADS box gene family. *Plant Cell* **16**, 2940-2953.

Olave, I. A., Reck-Peterson, S. L. and Crabtree, G. R. (2002). Nuclear actin and actin-related proteins in chromatin remodeling. *Annu. Rev. Biochem.* **71**, 755-781.

Orlando, V. (2003). Polycomb, epigenomes, and control of cell identity. *Cell* **112**, 599-606.

Owen-Hughes, T. and Bruno, M. (2004). Breaking the silence. *Nature* **303**, 324-325.

Piñeiro, M., Gómez-Mena, C., Schaffer, R., Martínez-Zapater, J. M. and Coupland, G. (2003). *EARLY BOLTING IN SHORT DAYS* is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. *Plant Cell* **15**, 1552-1562.

Putterill, J., Laurie, R. and Macknight, R. (2004). It's time to flower: the genetic control of flowering time. *BioEssays* **26**, 363-373.

Rando, O. J., Zhao, K. and Crabtree, G. R. (2000). Searching for a function for nuclear actin. *Trends Cell Biol.* **10**, 92-97.

Ratcliffe, O. J., Kumimoto, R. W., Wong, B. J. and Riechmann, J. L. (2003). Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. *Plant Cell* **15**, 1159-1169.

Reeves, P. H., Murtas, G., Dash, S. and Coupland, G. (2002). *Early in short days 4*, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. *Development* **129**, 5349-5361.

Ruiz-García, L., Madueño, F., Wilkinson, M., Haughn, G., Salinas, J. and Martínez-Zapater, J. M. (1997). Different roles of flowering time genes in the activation of floral initiation genes in Arabidopsis. *Plant Cell* **9**, 1921-1934.

Samach, A., Onouchi, H., Gold, S. E., Ditta, G. S., Schwarz-Sommer, Z., Yanofsky, M. F. and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. *Science* **288**, 1613-1616.

Schafer, D. A. and Schroer, T. A. (1999). Actin-related proteins. *Annu. Rev. Cell Dev. Biol.* **15**, 341-363.

Scortecci, K. C., Michaels, S. D. and Amasino, R. M. (2001). Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. *Plant J.* **26**, 229-236.

Searle, I. and Coupland, G. (2004). Induction of flowering by seasonal changes in photoperiod. *EMBO J.* **23**, 1217-1222.

Sheldon, C. C., Burn, J. E., Perez, P. P., Metzger, J., Edwards, J. A., Peacock, W. J. and Dennis, E. S. (1999). The *FLF* MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *Plant Cell* **11**, 445-458.

Sheldon, C. C., Rouse, D. T., Finnegan, E. J., Peacock, W. J. and Dennis, E. S. (2000). The molecular basis of vernalization: the central role of *FLOWERING LOCUS C* (*FLC*). *Proc. Natl. Acad. Sci. USA* **97**, 3753-3758.

Sheldon, C. C., Conn, A. B., Dennis, E. S. and Peacock, W. J. (2002). Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *Plant Cell* **14**, 2527-2537.

Shen, X., Mizuguchi, G., Hamiche, A. and Wu, C. (2000). A chromatin remodelling complex involved in transcription and DNA processing. *Nature* **406**, 541-544.

Shen, X., Ranallo, R., Choi, E. and Wu, C. (2003). Involvement of actin-related proteins in ATP-dependent chromatin remodeling. *Mol. Cell* **12**, 147-155.

Shindo, C., Aranzana, M. J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M. and Dean, C. (2005). Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of Arabidopsis. *Plant Physiol.* **138**, 1163-1173.

Soppe, W. J., Bentsink, L. and Koornneef, M. (1999). The early-flowering mutant *efs* is involved in the autonomous promotion pathway of *Arabidopsis thaliana*. *Development* **126**, 4763-4770.

Squazzo, S. L., Costa, P. J., Lindstrom, D. L., Kumer, K. E., Simic, R., Jeennings, J. L., Link, A. J., Arndt, K. M. and Hartzog, G. A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. *EMBO J.* **21**, 1764-1774.

Sun, T. P. and Kamiya, Y. (1994). The Arabidopsis *GA1* locus encodes the cyclise ent-kaurene synthetase A of gibberellin biosynthesis. *Plant Cell* **6**, 1509-1518.

Sung, S. and Amasino, R. M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **427**, 159-164.

Telfer, A., Bollman, K. M. and Poethig, R. S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana. Development* **124**, 645-654.

Werner, J. D., Borevitz, J. O., Warthmann, N., Trainer, G. T., Ecker, J. R., Chory, J. and Weigel, D. (2005). Quantitative trait locus mapping and DNA array hybridization identify an FLM deletion as a cause for natural flowering-time variation. *Proc. Natl. Acad. Sci. USA* **102**, 2460-2465.

Wilson, R. N., Heckman, J. W. and Somerville, C. R. (1992). Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiol.* **100**, 403-408.

Yamaguchi, S., Sun, T., Kawaide, H. and Kamiya, Y. (1998). The *GA2* locus of *Arabidopsis thaliana* encodes ent-kaurene synthase of gibberellin biosynthesis. *Plant Physiol.* **116**, 1271-1278.

Zhang, H. and van Nocker, S. (2002). The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of *FLOWERING LOCUS C*. *Plant J.* **31**, 663-673.

Zhang, H., Ransom, C., Ludwig, P. and van Nocker, S. (2003). Genetic analysis of early flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for expression of the flowering-time switch *FLOWERING LOCUS C*. *Genetics* **164**, 347-358.

Zhao, K., Wang, W., Rando, O. J., Xue, Y., Swiderek, K., Kuo, A. and Crabtree, G. R. (1998). Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell* **95**, 625-636.

Supplementary material

Table S1. Molecular markers of chromosome 3 used in the chromosomal location of the *esd1* mutation

Marker	Type	Location
GAPab	SSLP	43.77 cM
GL1	CAPS	48.45 cM
ATPOX	CAPS	52.40 cM
Centromere		
ATA1	CAPS	53.87 cM
NIT1.2	SSLP	55.12 cM
TOPP5	CAPS	59.20 cM
ASN1	CAPS	61.42 cM
nga6	SSLP	86.41 cM

Table S2. Markers used to fine map the *esd1* mutation

Marker	Type	Restriction enzyme	Forward	Reverse
ATPOX	CAPS	*Msp*I	TCTGCTGCGGTGGGAATACAAAAG	GCCATCATTCCCCGGTTCTCATAG
MIJ6*	CAPS	*Dde*I	CATTCCGCATTATTATCTACCC	CAAGTGACAATACAGCGGAAC
T25C10	SSLP	–	TAGGGGACATATCAAACCAAC	GTCTAAAACCATCTTCACCATAAT
T8N9*	SSLP	–	TGCTCGTCATCTCTTGTGTGTG	CTTCTCATTCGTGTAACCGCA
T15D2*	CAPS	*Xba*I	TGAGGTAGTGGAGGAGAAAACG	TTGATGTATGGGGGATTGATTT
T21P20	CAPS	*Mse*I	CAAGCTTCATGGGGACTAGCTAG	TAATACGGGACAATCTACAACAC
T27B3*	CAPS	*Taq*I	AGAATCAATGCCTATCAAGACAC	CAACTAAGAGACTGATCATAAATAA
ATA1	CAPS	*Nla*III	ATGATCAAAGGGGGACGAGG	AAGGAAACACCACCAAACGAAAAC

*Molecular markers developed during this work.

Table S3. Molecular markers used for the analysis of the deleted regions in the *esd1* alleles

BAC	Marker	Forward	Reverse	Deleted in allele
F1D9	F1D9	CACTCTTGCTGTTGTTTTTCTTA	GAAAGTTGAGATGACGTATTG	*4*
T7B9	1.T7B9	TACTCTAACCCATCCTCAGCAAC	TTCCACCATCATCCACCACTC	*4*
	2.T7B9	CTTTGGTTGCGAGAGACAGG	GTTGTTCATCCTGTTATTGTTCTG	*4*
	3.T7B9	TTGGCTTGGATGAGTTCGG	ATCCGTCTTACCACATCCACTC	*4*
	4.T7B9	TCCTCCTCGTCGTCGTGGTC	ACTCCACGGGCATTTCGGT	*4*
T13O13	1.T13O13	TCTCAAATGGCTCTACTCGTGC	ACGGTTTCCTTCGGCTTTC	*4*
	5.T13O13	GCAACTGCTACAGCCCCG	CGAATCTGTGGCTGGCCACGCGG	*4*
	2.T13O13	ATGACTTCTACTTCCGCTGC	GCTATGGTCAGTTGTCCGTT	*4*
	3.T13O13	ATGGAGAGACAGAAAAAGAAGTTG	TTTCAGCCGCATTTGTATTC	*4*
	4.T13O13	ATGGTTACCAGATTTAAGTGTTCT	TCTGGATCATCTTCGTCTTCC	*4*
T15D2	1.T15D2	AAGCTTGACAAGCTAGTAAACCTA	AAGTCTATTCAGAAATTGACTAAGTG	*4*
	2.T15D2	CCACAACAACGGAGGTATCG	GCTGTGGGTCATACTCCTTTTT	*4*
	3.T15D2	AACCTTGTCTTTTTTTGTGATTAG	TACCAACCATCATCCTCTTCTC	*4*
	4.T15D2	ATTTGGTGTATGTTCTTTCGGTCC	TAGATAGTGAACGCAACAAATAGA	*4*
	5.T15D2	TTTTCACGCCTCCCTCAAGC	AACTCTCCCGTCTTCTTGTCTACC	*4*
	6.T15D2	TATTCTGGGCTTGAGGGTAGTA	CACAACTACTCTCTCCCATCCTCA	*4*
F21A14	3.F21A14	ATTCCTCCATCTTTCCTTCCATCA	CGAGCACTTGTTCTTGGACTTCAT	*1-5, 7-9*
	4.F21A14	TGGTCCGCACAACATCTTAT	TGCCGAACTCCATCTCCACATC	*1-5, 7-9*
	5.F21A14	AATCCTCACCTTTCCCTTGCCA	TCTTCTTCTCTTCCTTGTGCTTGA	*1-5, 7-9*
	2.F21A14	TCCTCCGATTCCTTGCCGA	ACTTGGGTCGGATTCAGGGC	*1-9*
	1.F21A14	ATTGTTTTGGGGGATGGGC	CGAGTGTCTGTTGAGTGCGGAA	*1-9*
T4P3	4.T4P3	ATGATGATGCCCTCGTAGTTA	ACATACACGCCACTTGCCATTT	*1-9*
	1.T4P3	TAAATGTGAGCAATCCCTGAG	ACTTGCCTGCTCCTTTCTCTGTCG	*1-9*
	2.T4P3	CGGTCTCTTCGCCTTTGCCTCT	GCTGCCTCTCTTTCCAACCTCGG	*1-9*
	3.T4P3	TTTTGGGTCGGTTTTTGAATCCTA	TAGGATTTTTCTGCTTCATACTTA	*1-9*
T14A11	4.T14A11	TCAGCATCTTCTTGTCGAGGA	ATCCCGACTTCTCATTCACCTC	*1-9*
	3.T14A11	ATCGTGTCTTTCCAGGTTTTGA	TAACCTCCAACCTTCTCCAATCTA	*1-9*
	2.T14A11	TTCTTCCCCTCTTCTCTTCCTTG	GGCGTAGTCCTTGTCCATTGTTCA	*1-9*
	1.T14A11	TCCCAACCTTTATCTCATTACTGT	CATTCTTTAGTGTCTGATTTCGC	*3-9*
T26P13	4.T26P13	AAGATTGTAGGGAGATTGTGATAA	GCGTTTGGTAGTGATGCTCCGATT	*3-9*
	3.T26P13	ACTGATGTTGAGGCGGGAAGGTTT	GCTTTGCGGTAATGGTCGTTCTTC	*3-9*
	2.T26P13	AGAAGGGTTGAAGCGGGTGTA	GCTACAGGTTTTGGTGGGAAGAA	*3-9*
	1.T26P13	ACCACTGCTGTCATTGCTCGGGAA	CGCCTCTCTTTGTCTTCCTTCTTC	*3-9*
T25F15	1.T25F15	ATATTTTTTCAGTCAACAGAGTG	CGAGTGGGTATGCTTCAGGAGAT	*6, 9*

CAPÍTULO 2: Mutations in the *Arabidopsis SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development.

Journal of Experimental Botany, Vol. 59, No. 3, pp. 653-666, 2008

Mutations in the *Arabidopsis SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development

Ana Lázaro*, Ángeles Gómez-Zambrano*, Leticia López-González, Manuel Piñeiro and Jose A. Jarillo†

Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria-Centro de Biotecnologia y Genómica de Plantas (INIA-UAU), Ctra. de A Coruña, Km 7,5, Madrid 28040, Spain

* These authors contributed equally to this work.
† To whom correspondence should be addressed. E-mail: jarillo@inia.es

Received 31 October 2007; Revised 24 November 2007; Accepted 28 November 2007

Abstract

Mutations affecting the *Arabidopsis SWC6* gene encoding a putative orthologue of a component of the SWR1 chromatin remodelling complex in plants have been characterized. *swc6* mutations cause early flowering, shortened inflorescence internodes, and altered leaf and flower development. These phenotypic defects resemble those of the *photoperiod independent early flowering 1* (*pie1*) and *early in short days 1* (*esd1*) mutants, also affected in homologues of the SWR1 complex subunits. SWC6 is a ubiquitously expressed nuclear HIT-Zn finger-containing protein, with the highest levels found in pollen. Double mutant analyses suggest that *swc6* abolishes the *FLC*-mediated late-flowering phenotype of plants carrying active alleles of *FRI* and of mutants of the autonomous pathway. It was found that SWC6 is required for the expression of the *FLC* repressor to levels that inhibit flowering. However, the effect of *swc6* in an *flc* null background and the down-regulation of other *FLC*-like/*MAF* genes in *swc6* mutants suggest that flowering inhibition mediated by SWC6 occurs through both *FLC*- and *FLC*-like gene-dependent pathways. Both genetic and physical interactions between SWC6 and ESD1 have been demonstrated, suggesting that both proteins act in the same complex. Using chromatin immunoprecipitation, it has been determined that SWC6, as previously shown for ESD1, is required for both histone H3 acetylation and H3K4 trimethylation of the *FLC* chromatin. Altogether, these results suggest that SWC6 and ESD1 are part of an *Arabidopsis* SWR1 chromatin remodelling complex involved in the regulation of diverse aspects of plant development, including floral repression through the activation of *FLC* and *FLC*-like genes.

Key words: *Arabidopsis*, chromatin remodelling, floral repression, HIT-Zn finger, phase transition, SWR1 complex.

Introduction

To ensure that flowering occurs in optimal conditions, plants integrate both environmental and endogenous signals before switching to reproductive development. To select the right season for flowering, plants rely fundamentally on environmental factors such as light and temperature that suffer predictable changes through the year. *Arabidopsis thaliana* is a facultative long-day (LD) species in which winter and summer annual accessions can be distinguished. In winter annual accessions, flowering time is regulated by the vernalization, photoperiod, and gibberellin (GA) pathways (Baurle and Dean, 2006; Imaizumi and Kay, 2006; Schmitz and Amasino, 2007). The photoperiod pathway promotes flowering in response to LD through the activation of the floral integrators *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*). Indeed, FT protein has been recently proposed as an essential component of the systemic signal that mediates photoperiodic induction of flowering (Corbesier et al., 2007). The GA pathway is required for

flowering in non-inductive photoperiods, and mutants with reduced GA levels are extremely delayed in flowering time under short days (SD) (Wilson et al., 1992). In addition, winter annuals require exposure to an extended period of cold (vernalization) to become flowering competent, thus preventing premature flowering in the autumn (Michaels and Amasino, 2000; Sung and Amasino, 2006). This requirement is mainly conferred by dominant alleles at the *FRIGIDA* (*FRI*) (Johanson et al., 2000) and *FLOWERING LOCUS C* (*FLC*) loci (Michaels and Amasino, 1999; Sheldon et al., 1999), as well as by other *FLC*-related genes within the *MAF* clade (Scortecci et al., 2001; Ratcliffe et al., 2003; Werner et al., 2005). Active alleles of *FRI* increase *FLC* expression to levels that delay flowering (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC* suppresses the expression of *FT* and *SOC1* genes, which function as integrators of flowering signals; therefore, *FLC* confers a flowering response to vernalization in *Arabidopsis* by repressing both the generation of flowering-inductive systemic signals and the meristem competence to respond to such signals (Searle et al., 2006). Vernalization promotes flowering by overcoming the effect of *FRI* and repressing *FLC* expression; this repression is stably maintained after plants are returned to warm growth conditions, allowing them to flower (Michaels and Amasino, 1999; Sheldon et al., 1999).

Many summer annual accessions of *Arabidopsis* lack an active *FRI* allele (Johanson et al., 2000; Gazzani et al., 2003; Shindo et al., 2005). Under these circumstances, *FLC* expression is low and flowering occurs rapidly without vernalization. In these accessions, the reduction of *FLC* expression depends on the function of the autonomous pathway (Michaels and Amasino, 2001). In fact, mutations in autonomous pathway genes cause a flowering delay under any photoperiod (Boss et al., 2004) that is associated with higher *FLC* expression, and can be rescued by vernalization (Michaels and Amasino, 1999, 2001; Sheldon et al., 1999). Several genes have been classically ascribed to the autonomous pathway, including either factors involved in the binding and processing of mRNAs or proteins associated with chromatin remodelling processes (for reviews see Baurle and Dean, 2006; Schmitz and Amasino, 2007). *FVE* and *FLOWERING LOCUS D* (*FLD*) belong to the last group. The homologue of *FVE* in animals is found in nucleosome Remodelling Factor (NuRF) and histone deacetylase (HDAC) complexes and is likely to act as a histone chaperone (Ausin et al., 2004). FLD and related proteins, such as *swp1*, are highly homologous to human KIAA0601/lysine demethylase 1 (LSD1) (He et al., 2003; Krichevsky et al., 2007), also present in HDAC complexes (Lee et al., 2006). Consistent with the nature of these proteins, the increased expression of *FLC* in *fve*, *fld,* and *swp1* mutants is correlated with hyperacetylation of histones H3 and H4 (He et al., 2003; Ausin et al., 2004; Krichevsky et al., 2007), a modification associated with transcriptionally active chromatin conformations.

During vernalization, histone modifications associated with active genes such as acetylation of histone H3 and H4 and methylation at H3K4 decrease in *FLC* chromatin but the level of repressive markers such as H3K9 and H3K27 trimethylation increase (Sung and Amasino, 2004; Sung et al., 2006). This vernalization-dependent repressed state of *FLC* is mitotically stable; upon passing to the next generation, *FLC* expression is reset to the active state, suggesting the involvement of a mechanism conferring cellular memory for remembering winter. VERNALIZATION INSENTIVE 3 (VIN3) appears to be required for histone deacetylation in the *FLC* region following vernalization (Sung and Amasino, 2004) and none of the repressive markers associated with vernalization is present in *vin3* mutants. A polycomb group (PcG) complex containing VERNALIZATION 2 (VRN2) and VIN3 may bring histone deacetylase and histone methyltransferase activities together at *FLC* chromatin, providing a coordinated mechanism for the epigenetic modifications associated with the vernalization-mediated repression of the *FLC* gene (Wood et al., 2006).

The establishment of the winter-annual habit of *Arabidopsis* requires that *FLC* is expressed at high levels in the first growing season to block flowering before winter. High levels of acetylation of histone H3 and H4 and H3K4 methylation contribute to an active chromatin conformation at the *FLC* locus during initial stages of development (He et al., 2004; Sung and Amasino, 2004; Kim et al., 2005; Martin-Trillo et al., 2006; Sung et al., 2006). The isolation of mutants capable of flowering early in winter-annual backgrounds has led to the identification of genes required to activate *FLC* at the beginning of the life cycle and that encode components of putative chromatin remodelling complexes. Most of these mutants can be classified into two different groups, affecting putative orthologues of either the SWR1 or the PAF1 complexes. Mutations in genes encoding proteins related to components of the yeast transcriptional-activating PAF1 complex [*early flowering 7* (*elf7*), *elf8*, and *vernalization independent 4* (*vip4*)] (He et al., 2004; Oh et al., 2004) cause an acceleration of flowering. In yeasts, this complex interacts with SET1 and SET2 histone methyltransferases involved in methylation of H3K4 and H3K36, respectively (Krogan et al., 2003). Mutants in the *Arabidopsis* histone methyltransferase *EARLY FLOWERING IN SHORT DAYS* (*EFS*/*SDG8*) also flower early and display reduced levels of *FLC* expression, like PAF1 complex mutants (Kim et al., 2005; Zhao et al., 2005), suggesting that the PAF1 complex and EFS may act directly on *FLC* to maintain high levels of expression. Consistent with this, two different studies have provided evidence that this protein is required for high levels of either H3K4me3 or H3K36me2 in the region of *FLC* (Kim et al., 2005; Zhao et al., 2005).

In the same way, mutations in putative orthologues of the yeast SWR1 complex, including EARLY IN SHORT DAYS 1/SUPPRESSOR OF FRIGIDA 3/ACTIN RELATED PROTEIN 6 (ESD1/SUF3/ARP6) (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006), the SWI/SNF ATPase PHOTOPERIOD INDEPENDENCE1 (PIE1) (Noh et al., 2003), and SEF (SERRATED AND EARLY FLOWERING)/AtSWC6 (Choi et al., 2007; March-Diaz et al., 2007) have been described recently. The SWR1 complex in yeast catalyses the replacement of nucleosomal H2A with the H2A.Z variant, ensuring full activation of underlying genes. Recently H2A.Z was identified within *FLC* and *FLC*-like chromatin (Deal et al., 2007). Loss of H2A.Z from *FLC* chromatin in *esd1*/*suf3*/*arp6* and *pie1* mutants results in reduced *FLC* expression and premature flowering, indicating that this histone variant is required for a high level of expression of *FLC* (Deal et al., 2007). In addition, H2A.Z interacts with both PIE1 and AtSWC2, and knockdown of the H2A.Z genes by RNA interference or artificial microRNA caused a phenotype similar to that of *esd1*/*suf3*/*arp6* (Choi et al., 2007). These observations support the existence of a SWR1-like complex in plants that is targeted to different loci including *FLC*, and show that H2A.Z can enhance transcriptional activation in plants. The fact that H2A.Z remains associated with chromatin throughout mitosis suggests that it may serve as an epigenetic memory function by marking active genes and poising silenced genes for reactivation (Deal et al., 2007).

This work reports the characterization of *SWC6*, a putative component of the SWR1 complex of *Arabidopsis*, required for the maintenance of *FLC* expression. *swc6*, like mutations in other putative components of this complex, causes early flowering mainly through the reduction of *FLC* expression, although it also appears to affect flowering through other *FLC*-like repressors. It is demonstrated here that SWC6 interacts both genetically and physically with another crucial subunit of the complex such as ESD1/SUF3/ARP6 and that both proteins are needed to achieve the levels of both H3 acetylation and H3K4me3 required for high *FLC* expression. Taken together, the data indicate that SWC6 and ESD1/SUF3/ARP6 might form a molecular complex in *Arabidopsis* related to the SWR1/SRCAP complex identified in other eukaryotes, which regulates diverse aspects of plant development, including floral repression.

Materials and methods

Genetic stocks and growth conditions

Mutant seed stocks used were in the Columbia (Col) genetic background, and were obtained from the Arabidopsis Biological Resource Center (ABRC) of Ohio State University (Columbus, USA) and personal donations. The *fve-3* mutant was described by Ausin et al. (2004); *flc-3* was described by Michaels and Amasino (2001); *esd1-10* was described by Martin-Trillo et al. (2006); the Col *FRI-Sf2* lines were described by Lee and Amasino (1995). The origin of the *swc6-1* and *swc6-2* alleles is described in the text. The same alleles were identified previously and denoted as *sef-2* and *sef-1*, respectively (March-Diaz et al., 2007). *swc6-1* and *swc6-2* mutations were confirmed to be allelic by their failure to complement the early flowering phenotype in F1 plants derived from crosses between them. Plants were grown in plastic pots containing a mixture of substrate and vermiculite (3:1). Controlled environmental conditions were provided in growth chambers at 21 ºC and 80% relative humidity. Plants were illuminated with cool-white fluorescent lights (~120 $\mu E\ m^{-2}\ s^{-1}$). LD conditions consisted of 16 h of light followed by 8 h of darkness; SD conditions consisted of 8 h of light followed by 16 h of darkness.

Phenotypic analyses

Total leaf number was scored as the number of main leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time of opening of the first flower; for each experiment the average flowering time of at least 20 plants ± SE is error is given (Martin-Trillo et al., 2006). Cauline, adult, and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered as juvenile leaves (Telfer et al., 1997).

Genetic analysis

Double mutants were generated by crossing the monogenic *swc6-1* mutant with lines carrying the *fve-3* (Ausin et al., 2004), *flc-3* (Michaels and Amasino, 2001), and *esd1-10* (Martin-Trillo et al., 2006) mutations and with Col *FRI Sf-2* (Lee and Amasino, 1995). Double mutants were isolated from selfed F2 progeny using molecular markers associated with each mutation.

Molecular characterization of the swc6 *alleles*

The T-DNA insertion *swc6-1* (SAIL_1142_C03) and *swc6-2* (SAIL_536_A05) mutant lines were obtained from NASC. Two specific primers or one specific primer and a T-DNA left border (LBA SAIL) primer were used for amplification of wild-type or T-DNA insertion alleles, respectively (LBA SAIL, 5’-TTCATAACCAATCTCGATACAC-3’). T-DNA borders were determined by sequencing PCR products obtained with T-DNA border primers and gene-specific primers.

Generation of transgenic plants

Transgenic plants expressing *SWC6* and *ESD1*/*SUF3*/*ARP6* fulllength cDNAs under the control of the *35S* cauliflower mosaic virus promoter or expressing a promoter fragment of the *SWC6* gene fused to the *GUS* gene (353 bp upstream of the ATG) were generated following *Agrobacterium tumefaciens*-mediated transformation using the floral-dip method (Clough and Bent, 1998). The *Agrobacterium* strain used was C58C1. Transformant plants were selected on GM medium containing appropriate antibiotics. Levels of overexpressed genes were tested by northern blots using *SWC6*- and *ESD1*/*SUF3*/*ARP6*-specific probes. As loading controls, a 305 bp *Eco*RI fragment of the cauliflower 18S rDNA gene was used.

Histochemical β-glucuronidase assays

GUS activity in *pSWC6:GUS* plants was revealed by incubation in 100 mM $NaPO_4$ (pH 7.2), 2.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.25% Triton X-100. Plant tissue was incubated at 37 ºC overnight. After staining, chlorophyll was cleared from the samples by dehydration through ethanol.

Whole-mount anther preparation for microscopy

Anthers were collected and incubated overnight at 4 ºC in coloration buffer, containing equal volumes of extraction buffer (0.1% Nonidet P40, 10% dimethyl sulphoxide, 5 mM EGTA, pH 7.5, 50 mM PIPES, pH 6.9) and DAPI solution (1 mg DAPI ml^{-1} dimethyl sulphoxide).

Yeast two-hybrid analysis

Yeast two-hybrid interaction analyses were conducted in the Y190 strain with the MatchMaker two-hybrid system (Clontech). pGBT-8 or pGAD plasmids were used for GBD or GAD fusion constructs, respectively. cDNAs for SWC6 and ESD1/SUF3/ARP6 were obtained by standard PCR techniques and cloned into the abovementioned vectors using Gateway recombinant technologies (Clontech). Selection was performed on synthetic complete (SC) minimal medium without His, Leu, and Trp, supplemented with 5–25 mM 3-amino-1,2,4-triazole (3-AT).

Protein expression, purification, and pull-down assays

The *SWC6* expression construct was prepared in the pGEX-6P-3 vector (Amersham Biosciences) and expressed in *Escherichia coli BL21* Rosetta. Standard PCR techniques were used for GST tagging of SWC6. Proteins were purified on gluthatione 4B Sepharose beads (GE Amersham) and kept on beads as GST–SWC6 or GST alone. *In vitro* transcription/translation ESD1/SUF3/ARP6 reactions were performed with the TNT Quick Coupled Transcription/Translation system (Promega) in the presence of [^{35}S]methionine (Amersham Biosciences). For pull-down assays, 500 ng of GST or GST–SWC6 bound to beads were incubated in 200 μl of binding buffer (20 mM TRIS-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40) with 15 μl of the TNT reaction and rinsed with binding buffer supplemented with 500 mM NaCl. Samples were boiled with Laemmli buffer and analysed by SDS–PAGE.

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen-Gibco). cDNA was prepared by reverse transcription of total RNA from *Arabidopsis* roots, stems, rosette and cauline leaves, floral buds, and flowers, according to described procedures (Martin-Trillo et al., 2006). *SWC6* transcript levels were assayed by reverse transcription (RT)-PCR, with specific primers, 5'-ATGGAGGAAGAGATGTCGAACC-3' and 5'-CGAGATCATCATCTTCATCAAGAG-3', designed to amplify the N-terminal end of the coding region. For the rest of genes analysed, such as *FLC*, the *MAF* family, *FT* RT–PCR and *SOC1* genes, was performed, according to described procedures (Scortecci et al., 2001; Pin˜eiro et al., 2003; Ratcliffe et al., 2003; Martin-Trillo et al., 2006). *UBIQUITIN 10* (*UBQ10*) was used as the control in these experiments.

Chromatin immunoprecipitation (ChIP) assays and PCR

ChIP assays were carried out as described (Ausin et al., 2004). Chromatin proteins and DNA were cross-linked in 10-d-old Col, *esd1-10*, and *swc6-1* seedlings by formaldehyde fixation. After chromatin isolation, the H3 acetylated and methylated fractions were immunoprecipitated using specific antibodies; one of them recognizes both acetylated K9 and K14 residues, and the second one recognizes K4me3 residues (06-599 and 07-473 from Upstate Biotechnology, respectively). Cross-links were reversed by incubations at 65 ºC for 2 h, and DNA was purified with QIAquick spin columns (QIAGEN) and eluted in 40 μl of TE (pH 8.0). Semiquantitative PCR was used to amplify two different fragments of the *FLC* gene as described previously (Martin-Trillo et al., 2006; details and primer sequences are available on request). All PCR and quantification of the amplified DNA were done as described previously (Martin-Trillo et al., 2006). Three independent experiments were carried out. *UBQ10* served as an internal control for the ChIP analysis. To calculate the fold decrease in H3 acetylation or methylation, *FLC* was first normalized to *UBQ10* in each sample, and, subsequently, these values were normalized against their respective wild-type controls.

Gene sequences described in this article can be found in GenBank under accession numbers NM_123064 (SWC6) and NM_114070 (ESD1/SUF3/ARP6).

Results

swc6 mutants are early flowering and display pleiotropic defects in both vegetative and reproductive development

Previously, *esd1*, an *Arabidopsis* early flowering mutant affected in an orthologue of ACTIN-RELATED PROTEIN 6 (ARP6), had been identified (Martin-Trillo et al., 2006). The yeast ARP6 protein is a component of the SWR1 complex, which consists of 13 subunits including the ATPase component SWR1 and SWC6/VSP71 (Kobor et al., 2004; Mizuguchi et al., 2004). A physical interaction between ARP6 and SWC6 has been proposed in yeast (Wu et al., 2005). A search for an *Arabidopsis* protein homologue of the yeast SWC6/VSP71 subunit led to the identification of a related protein, encoded by the At5g37055 gene. To investigate the role of *Arabidopsis* SWC6 in plant development, T-DNA insertion lines were searched in different collections, and two different lines were identified (Fig. 1A) and designated as *swc6-1* and *swc6-2*. Line SAIL_1142_C03 (*swc6-1*) bore an insertion in exon 2 of the At5g37055 locus, in a position corresponding to nucleotide 146 of the coding region. RT-PCR analysis, using primers forward (F) and reverse (R), upstream of the T-DNA insertion, demonstrated no expression of *SWC6* mRNA in homozygous *swc6-1* plants (Fig. 1B). In the same way, line SAIL_536_A05 (*swc6-2*) contained a T-DNA inserted in the promoter region, upstream of the 5' UTR of *SWC6* mRNA. RT-PCR analysis was unable to detect *SWC6* mRNA in *swc6-2* plants (data not shown).

Fig. 1. Isolation of *SWC6* loss-of-function mutations. (A) Genomic structure of the *SWC6* gene and locations of T-DNA insertions. Primers F and R used for RT-PCR experiments are indicated. (B) *SWC6* expression in the *swc6-1* mutant. RT-PCR analysis showing the level of expression of *SWC6* mRNA in Columbia wild-type and *swc6-1* plants grown under LD photoperiods. *UBQ10* was used as an internal control.

Because both alleles produced a similar array of phenotypes, *swc6-1* was chosen to carry out all the genetic and phenotypic analyses. Heterozygous plants displayed a wild-type phenotype, indicating that both *swc6-1* and *-2* were recessive. Plants homozygous for *swc6-1* mutations were early flowering mainly under non-inductive SD photoperiods (Fig. 2A, B, Table 1). The fact that *swc6-1* mutants flower earlier under inductive photoperiods indicates that this mutation does not abolish the flowering photoperiodic response. Earliness was associated with a reduction in the length of all developmental phases of the plant (Fig. 2C), based on leaf shape and leaf trichome distribution (Telfer et al., 1997). This reduction was more dramatic for adult rosette leaves, which were highly reduced in *swc6* mutant plants under both LD and SD (Fig. 2C). This behaviour is similar to that exhibited by other early flowering mutants such as *esd1*, *esd4*, and *ebs*, which also show a major reduction in the adult vegetative phase (Gomez-Mena et al., 2001; Reeves et al., 2002; Martin-Trillo et al., 2006).

Apart from their flowering-time phenotype, *swc6* mutants also displayed complex pleiotropic alterations of both vegetative and reproductive development. Mutant plants produced more coflorescence shoots than Col. This was accompanied by a shortening of inflorescence internodes, resulting in a reduction in inflorescence length and apical dominance (Fig. 2D). Furthermore, *swc6* leaves are smaller and more curled than wild-type leaves, and frequently have serrated margins (Fig. 2E, F). As shown in Fig. 2G–J, *swc6* flowers displayed several developmental abnormalities, including a reduction in size as compared with wild-type flowers.

Petals of mutant plants were smaller than wild-type petals and slightly wrinkled (Fig. 2H); mutant anthers were also smaller than those of the wild type and often presented a heart shape characteristic of immature anthers (Fig. 2I); indeed the *swc6-1* mutant showed a reduced fertility associated with a reduction in the amount of pollen. In the same way, mutant carpels (Fig. 2J) and siliques (Fig. 2K) were approximately half the length in *swc6* mutants of those in wild type plants. In addition, *swc6* flowers frequently bear extra perianth organs. This phenotype was more extreme under SD, where *swc6* flowers contained 5.8±0.8 sepals and 5.7±0.9 petals per flower (Fig. 2G). Similar phenotypes have been described in *pie1* and *esd1*/*suf3*/*arp6* mutant plants, suggesting that these proteins may form part of a complex that regulates multiple aspects of *Arabidopsis* development.

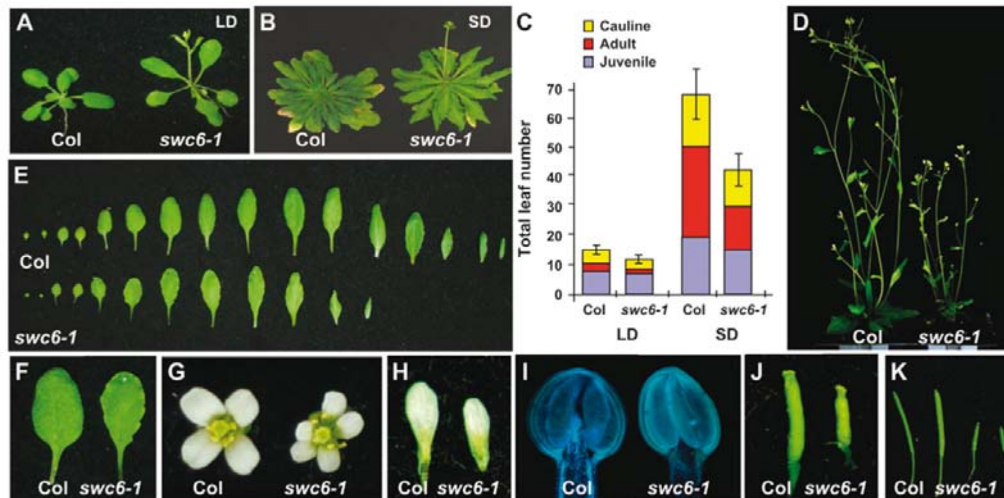


Fig. 2. *swc6* mutants are early flowering and display pleiotropic alterations in vegetative and reproductive development. (A) Flowering-time phenotype of *swc6* mutants under LD. Wild-type Col and *swc6-1* 2-week-old plants are shown. (B) Flowering phenotype of *swc6* mutants under non-inductive photoperiods. Col and *swc6-1* 4-week-old plants grown under SD. (C) Histogram comparing the number of juvenile, adult, and cauline leaves in Col and *swc6-1* plants grown under both LD and SD photoperiods. (D) Col and *swc6* 4-week-old plants grown under LD, showing a reduction in inflorescence length and weak apical dominance in the mutant. (E) Rosette and cauline leaves of wild-type and *swc6-1* plants grown under LD conditions. All leaves, including cotyledons, are shown in order of production from the first true leaf. (F) Rosette leaves of Col and *swc6-1* plants grown under SD conditions. (G) Detached flowers showing the increased number of petals in *swc6* mutant flowers. (H) Detached petals showing the size reduction and wrinkled appearance of *swc6* mutant petals. (I) DAPI staining of wild-type (left) and *swc6-1* mutant (right) anthers. (J) Wild-type (left) and *swc6-1* (right) flower gynoecium. (K) Wild-type (left) and *swc6-1* (right) siliques.

To complement the mutant *swc6* phenotype, an At5g37055 cDNA driven by the *35S* promoter was introduced into *swc6-1* mutants. Several transgenic lines of *35S:SWC6* were generated (Fig. 3A). Northern blot analysis showed high accumulation of *SWC6* mRNA in the transgenic lines that complemented all the developmental defects observed in the mutant plants (Fig. 3A, Table 1). It is significant that overexpression of *SWC6* in *swc6* or overexpression of *ARP6* in *esd1* (Fig. 3B) did not cause an additional delay in flowering time. Consistently, the *SWC6* or the *ARP6* overexpression lines in the Col genetic background did not show any additional flowering phenotype.

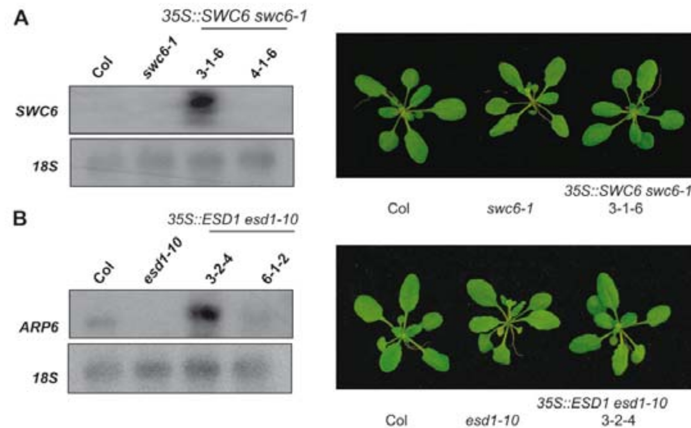


Fig. 3. Phenotype of *SWC6* and *ESD1*/*SUF3*/*ARP6* overexpression lines. (A) Col, *swc6-1*, and *35S::SWC6 swc6-1* transgenic *Arabidopsis* (line 3-1-6) plants grown for 2 weeks under LD conditions. In the left panel a northern blot hybridization shows the level of expression of *SWC6* in these plants and in the *35S::SWC6 swc6-1* 4-1-6 line, a representative that did not show complementation of the *swc6* mutant phenotype. (B) Col, *esd1-10*, and *35S::ESD1 esd1-10* transgenic *Arabidopsis* (line 3-2-4) plants grown for 2 weeks under LD conditions. In the left panel a northern blot hybridization shows the level of expression of *ESD1* in these plants together with the *35S::ESD1 esd1-10* 6-1-2 line, which did not show complementation of the *esd1* mutant phenotype. *18S* risobomal probe was used as a control of loading and integrity of RNAs in the northern blots.

Genotype	Long days	Short days
Col	13.3±1.5	69.5±4.5
swc6-1	9.5±0.6	38.5±4.6
esd1-10	8.9±0.7	31.8±8.3
swc6-1 esd1-10	7.9±0.3	31.3±6.3
fve-3	34.3±3.9	>110
swc6-1 fve-3	13.7±1.6	62.9±5.3
FRI	34.7±7.1	>110
swc6-1 FRI	21.2±3.7	79.3±9.1
flc-3	12.1±1.4	62.7±4.1
swc6-1 flc-3	8.7±0.7	40.5±3.3
35S::SWC6 swc6-1(3-1-6)	14.6±1.2	ND
35S::SWC6 swc6-1(4-1-6)	8.9±0.5	ND
35S::ESD1 esd1-10(3-2-4)	12.7±0.8	ND
35S::ESD1 esd1-10(6-1-2)	8.7±0.9	ND

Table 1. Flowering time of *swc6*, double mutants with *swc6*, and *35S::SWC6* transgenic plants

SWC6 *encodes a HIT-type zinc-finger protein*

To confirm the genomic structure of *SWC6*, a cDNA of 516 bp was identified and sequenced. *SWC6* is a single gene in *Arabidopsis*; it possesses four exons and encodes a nuclear HIT-type zinc-finger protein of 171 amino acids (Choi et al., 2007), whose homologues, SWC6 and ZNHIT1, are subunits of the yeast SWR1 and mammalian SRCAP (SWI2/SNF2-related CBP activator protein) complexes, respectively (Mizuguchi et al., 2004; Cai et al., 2005). AtSWC6 is also closely related to *Nicotiana benthamiana* CIBP1, identified as a Plum pox virus cylindrical inclusioninteracting protein, and to a SWC6 rice protein (OsSWC6). All these proteins have seven cysteines and one histidine highly conserved in a C-terminal region, which are part of a HIT-type zinc finger domain (Fig. 4A).

Semi-quantitative RT-PCR experiments demonstrated that *SWC6* transcript was present at variable levels in all the tissues tested (Fig. 4B). *AtSWC6* expression was more strongly detected

in roots, flowers, and flower buds (Fig. 4D). Similar expression profiles of *SWC6* are obtained from Genevestigator (http://www.genevestigator.ethz.ch; Zimmermann et al., 2004). In the transgenic plants expressing a 353 bp transcriptional fusion of the *AtSWC6* promoter region with *GUS* (*AtSWC6p:GUS*), GUS expression was detected in actively dividing cells such as root and shoot apices, lateral root primordia, trichomes, inflorescences, flowers, etc. (Fig. 4D). Interestingly, GUS expression was particularly high in anthers (Fig. 4D). By contrast, GUS expression was rarely detected in stems, leaves, seeds, and siliques (data not shown). Analysis of the *SWC6* promoter region fused to *GUS* shows the presence of various cis-acting elements, including sequences known to confer anther/pollen-specific gene expression (Fig. 4C).

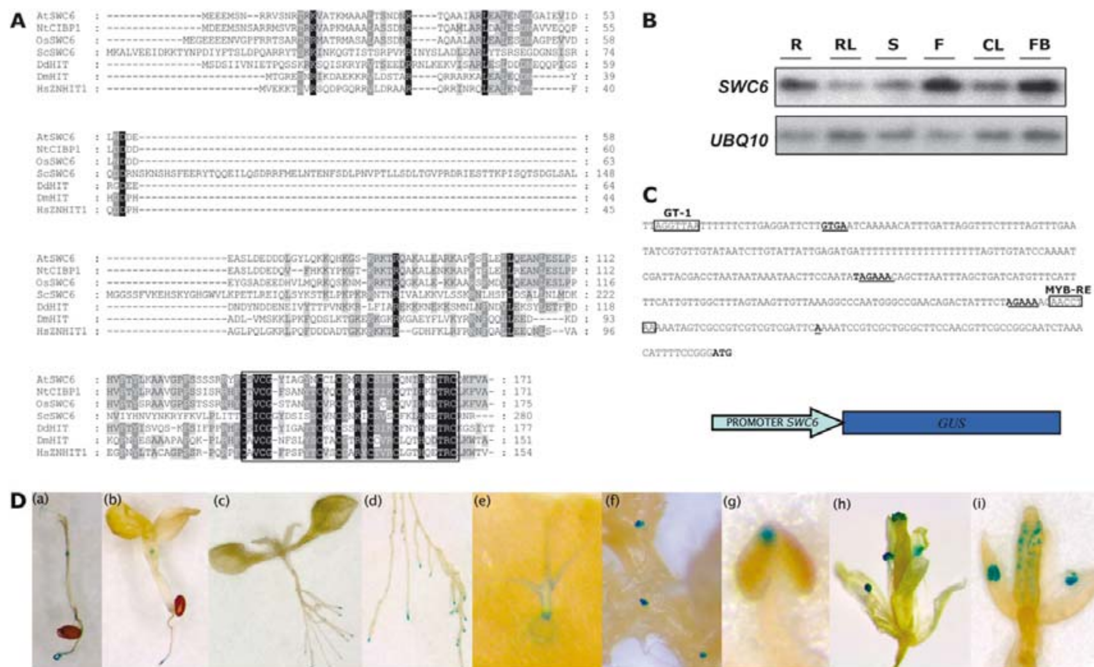


Fig. 4. *SWC6* encodes a nuclear HIT-type zinc-finger protein. Sequence comparisons of AtSWC6 with tobacco (NtCIBP1), rice (Os), yeast (Sc), *Dyctiostelium* (Dd), *Drosophila* (Dm), and human (HsZNHIT1) SWC6 homologues. Amino acid residues in black are identical and those in grey are functionally similar in all sequences. Boxed regions indicate the HIT-type zinc-finger domain, with seven cysteines and one histidine highly conserved. (B) *SWC6* expression in different organs of 25-d-old Col plants. RT-PCR assays were performed with RNA prepared from different tissues. R, Roots; RL, rosette leaves; S, main stem; F, flowers; CL cauline leaves; FB, flower buds. RT-PCR products were blotted and hybridized with a specific probe for the *SWC6* gene. *UBQ10* was used as a loading control. (C) Nucleotide sequence of promoter region and 5' UTR of the *SWC6* gene. ATG at the end of the sequence represents the initiation codon. The first base of the transcript (+1) is bold and underlined. The GTGA and AGAAA sequences in the regulatory regions, which are similar to the conserved motifs in the promoter regions of some other anther/pollen-specific genes are indicated in bold and underlined. A GT-1 motif and a MYB-response element are boxed. (D) Tissue expression pattern of *SWC6*. Spatial expression patterns of *SWC6* were examined by GUS staining in the *SWC6p:GUS* transgenic plants. Histochemical GUS staining was performed in whole (a) 2-, (b) 4-, and (c) 8-d-old seedlings, in (d) lateral root, (e) trichomes, (f) apical meristem region, (g) anthers, and (h) and(i) flowers.

The* swc6-1 *mutation suppresses the late flowering of* FRI *and autonomous pathway mutants

The early-flowering phenotype of *swc6* mutants suggested that *SWC6* could negatively interact with a flowering promoting pathway or alternatively interact positively with a flowering-repressor pathway in *Arabidopsis*. Mutations affecting *ESD1*, another member of the SWR1 complex, suppress the late flowering of *FRI* and autonomous pathway mutants (Martin-Trillo et al., 2006) and, for that reason, the genetic analysis was focused initially in combinations between *swc6* and these genotypes. To test the possible interaction between *SWC6* and autonomous pathway genes, the flowering phenotype of the *swc6*
fve-3 double mutant was analysed (Fig. 5A). Under LD, some of the *swc6 fve-3* double mutants were indistinguishable from *swc6*, although, on average, *swc6 fve-3* produced a few more leaves than *swc6* (Table 1); this result indicates that the late-flowering phenotype of *fve* mutations requires *SWC6*.

The quasi-epistatic interaction of *swc6* with *fve* mutations suggests that *swc6* might cause early flowering, either by increasing the activity of the autonomous pathway downstream of *FVE* or by bypassing the requirement for the autonomous pathway causing a reduction of *FLC* expression. When the *swc6* mutation was introduced into *FRI*-containing Col (Col:*FRI SF2*, referred to as *FRI* below) (Michaels and Amasino, 1999), which displays a very late-flowering phenotype, *swc6* partially suppressed *FRI*-mediated late flowering (Fig. 5B, Table 1). The plants harbouring the *swc6 FRI* combination showed much earlier flowering than *FRI* (Table 1).

In order to test if *swc6* suppresses the effect of the autonomous pathway mutations and *FRI* by reducing *FLC* mRNA levels, the abundance of the *FLC* mRNA in wildtype and *swc6* seedlings was compared. *FLC* transcript levels were reduced by the *swc6* lesion (Fig. 5D), suggesting that SWC6 is required to maintain high *FLC* expression levels, either as promoted by *FRI* or by mutations that impair the autonomous pathway. In agreement with this scenario, the expression of the floral integrator genes *FT* and *SOC1*, normally repressed by *FLC* (Moon et al., 2003), was up-regulated in the *swc6* mutants under SD conditions where the early-flowering phenotype of the mutant is more conspicuous (Fig. 5D).

Although the effects of *swc6* mutations on flowering time are more readily observed in the late-flowering *FRI* and *fve* mutant backgrounds, as discussed above, the fact that *swc6* mutants also flower earlier than the rapid-flowering wild-type strain Col (Fig. 2A, Table 1) suggests that, in addition to regulating *FLC* expression, SWC6 plays other roles in the control of flowering time. To determine the fraction of the *swc6* early-flowering phenotype that is independent of *FLC*, the phenotypic effect of the *swc6-1* mutation in an *flc* null (*flc-3*) background (Michaels and Amasino, 1999) was examined. When combined with *flc-3*, the *swc6* mutation reduces the number of leaves produced by *flc-3* (Fig. 5C, Table 1). In addition, as for *ESD1*, loss of function of *SWC6* also resulted in down-regulation of some other members of the *FLC*/*MAF* gene family, particularly *MAF4* and *MAF5* (Fig. 5D), suggesting that these *MAF* genes represent additional regulatory targets of *SWC6* and confirming that *swc6* mutations have an *FLC*-independent effect on flowering time.

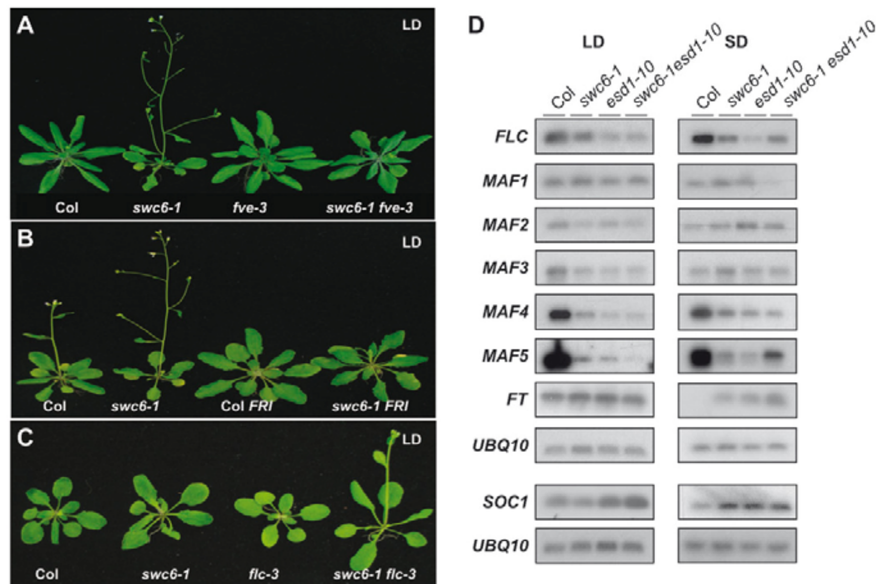


Fig. 5. Suppression of *FLC*-dependent late flowering by *swc6* mutations. (A) Flowering phenotype of double mutant *swc6 fve* plants grown under LD. (B) Flowering phenotype of lines where an active allele of *FRI* is combined with *swc6* grown under LD. (C) Flowering phenotype of *swc6 flc* double mutant plants grown under LD conditions. (D) Analysis of the expression of *FLC*, *FT*, *SOC1*, and the *MAF* genes in Col, *swc6*, *esd1*, and *swc6 esd1* double mutant plants. Total RNA was extracted from pools of fifty 8-d-old (LD) and 23-d-old (SD) seedlings collected 8 h and 16 h after dawn under SD and LD, respectively. For *SOC1* expression, samples were taken 4 h and 8 h after dawn for SD and LD, respectively. Expression was monitored by RT-PCR over 25 cycles for *FLC*, 32 cycles for *FT*, 25 cycles for *SOC1*, 30 cycles for *MAF1*, 28 cycles for *MAF2*, and 35 cycles for *MAF3*, *MAF4*, and *MAF5* genes. The *UBQ10* control was amplified during 20 cycles. RT-PCR products were blotted and hybridized with specific probes for each gene.

Genetic and physical interaction between SWC6 and ESD1/SUF3/ARP6

Previous observations indicate that the *swc6* mutant displays a number of phenotypic characteristics similar to those of *esd1* plants (Fig. 1) (Martin-Trillo et al., 2006). One possibility is that SWC6 and ESD1/SUF3/ARP6 may act in the same pathway or alternatively participate in different parallel pathways controlling similar processes. To investigate this aspect further, *swc6 esd1* double mutants were generated. As shown in Fig. 6A, *swc6 esd1* plants were indistinguishable from *esd1* plants. The flowering time of *swc6 esd1* double mutants was identical to that of *esd1* plants (Table 1), and the expression of floral integrator genes in *swc6 esd1* plants was in general similar to that observed for each parental mutant (Fig. 5D). In addition, vegetative and reproductive phenotypes of the *swc6 esd1* double mutant were quite similar to those observed in *esd1* mutants (Fig. 6B). Taken together, the above results are consistent with *SWC6* and *ESD1* acting in the same genetic pathway.

Since yeast homologues of these proteins are part of the SWR1 complex and several lines of evidence have suggested the existence of this complex in plants, a possible physical interaction between these *Arabidopsis* proteins was analysed by yeast two-hybrid assays. To do this, full-length SWC6 protein was expressed as bait, fused to the GAL4 DNA binding domain (GBD), and full-length ESD1/SUF3/ARP6 protein as the prey, fused to the GAL4 activation domain (GAD). As shown in Fig. 6C, yeast co-expressing the GAD–ESD1 and GBD–SWC6 fusion proteins were able to grow in selective medium without His plus 3-AT, due to the activation of the *GAL1::HIS3* reporter gene. To confirm this interaction further, *in vitro* pull-down experiments using glutathione

S-transferase (GST)–SWC6 and *in vitro*-translated ESD1 protein were performed. As shown in Fig. 6D, GST–SWC6 was able to interact with ESD1, but not GST alone. Together, the results show interaction among SWC6 and ESD1, providing further evidence for the existence of a SWR1 complex in *Arabidopsis*.

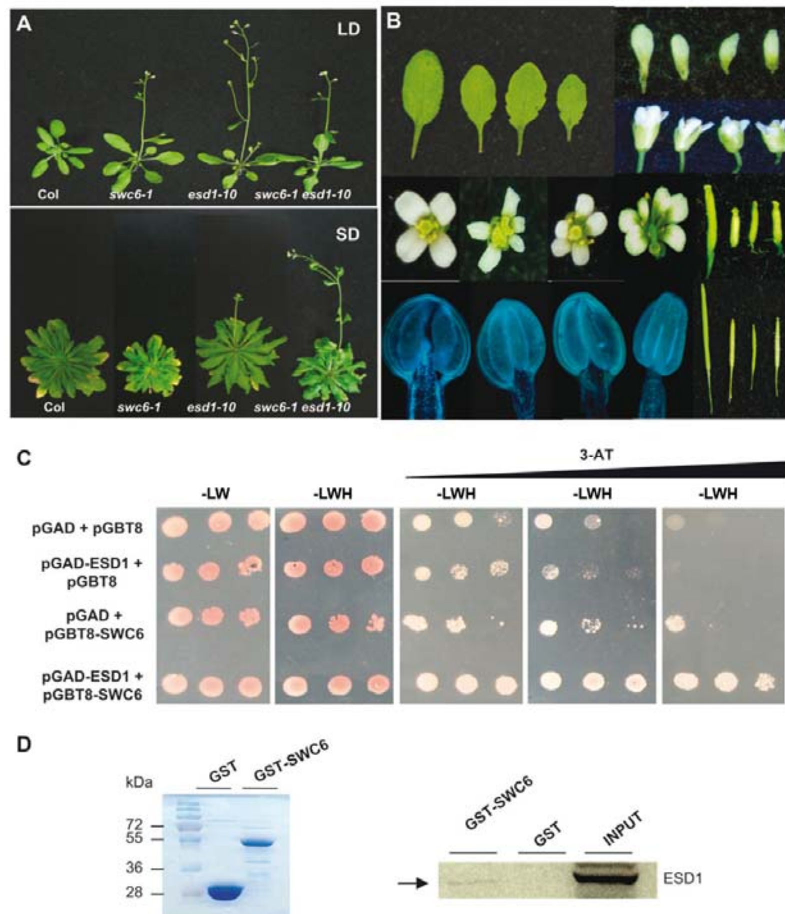


Fig. 6. Genetic and physical interaction between SWC6 and ESD1/SUF3/ARP6. (A) Flowering phenotype of Col, *swc6*, *esd1*, and *swc6 esd1* double-mutant plants under both LD and SD photoperiods. (B) Pleiotropic defects in vegetative and reproductive organs displayed by *swc6 esd1* mutant plants. Photographs illustrating leaves, petals, flowers, carpels, DAPI-stained anthers, and siliques of wild type (left), *esd1* (middle left), *swc6* (middle right), and *swc6 esd1* mutants (right). (C) SWC6 and ESD1 interact in yeast. Full-length SWC6 and ESD1 proteins were fused to the GAL4 DNA binding and activation domain, respectively. Yeast transformed with these constructs or empty vectors (pGBT8 and pGAD) were grown in nonselective (SC-L,-W) or selective media (SC-L,-W,-H) with increasing concentrations of 3-AT (5, 10, and 25 mM). Five-fold yeast dilutions were plated left to right in each panel. (D) Pull-down assay with SWC6 and ESD1 proteins. Bead-bound GST or GST–SWC6 fusion proteins were incubated with [^{35}S]Met-labelled ESD1 protein. Retained ESD1 protein was visualized after exposure and autoradiography of the dried gel.

SWC6 is required to activate FLC *transcription through both histone acetylation and methylation mechanisms*

FLC gene expression integrates signals coming from different pathways involved in the regulation of the floral transition (Schmitz and Amasino, 2007). Recent work has demonstrated the role of histone modification in the regulation of *FLC* expression through *FRI*, the autonomous, the vernalization, and the *PAF1* pathways (He et al., 2003, 2004; Ausin et al., 2004; Bastow et al.,

2004; Sung and Amasino, 2004; He and Amasino, 2005; Kim et al., 2005). These results have also identified the first intron of *FLC* as a relevant region for histone modification (He et al., 2003, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004) and transcriptional regulation (Gendall et al., 2001; Sheldon et al., 2002). Because *swc6* suppresses the late-flowering phenotype of *fve* autonomous pathway mutants and FVE represses *FLC* transcription through a histone deacetylation mechanism, it was speculated that SWC6 could be required for the acetylation of histones necessary to activate *FLC* expression. In fact, another putative component of the SWR1 complex, ESD1/SUF3/ARP6, has been previously reported to be required for setting this epigenetic marker in *FLC* chromatin (Martin-Trillo et al., 2006).

To determine whether *SWC6* promotes histone acetylation of the *FLC* chromatin, ChIP assays were performed (Fig. 7). Chromatin of Col, *esd1-10*, and *swc6-1* plants was immunoprecipitated by using antibodies against acetylated H3, and PCR was used to amplify two DNA fragments spanning regions of the promoter and the first intron of *FLC*, respectively, from the precipitated chromatin (Fig. 7A). These probes were among those that consistently showed the biggest differences in previous experiments involving the *esd1* mutant (Martin-Trillo et al., 2006). For the probes assayed, *FLC*-amplified sequences were consistently more abundant in DNA from precipitated chromatin of Col than from chromatin of the *swc6* and *esd1* mutant plants (Fig. 7B), indicating that both SWC6 and ESD1/SUF3/ARP6 affect the levels of H3 acetylation of *FLC*. Therefore, both proteins are required to activate *FLC* expression through a mechanism involving the histone acetylation of *FLC* chromatin.

This assay was extended to explore further if *SWC6* also has an effect on histone methylation at the *FLC* locus, as does *ESD1*. It has been shown recently that H3K4 hypertrimethylation is associated with actively transcribed *FLC* chromatin (He et al., 2004), and we wondered whether SWC6 was required for the setting of this epigenetic marker on *FLC* chromatin. Compared with wild-type plants, the trimethylated H3K4 levels in the *FLC* probes assayed were lower in *swc6* and *esd1* mutant plants than in Col (Fig. 7C), indicating that SWC6 is also required for the hypertrimethylation of H3K4 in *FLC* chromatin. Although in *swc6* background a decrease in both H3 acetylation and H3K4 trimethylation was consistently observed with the probes assayed, this effect was always less pronounced than that observed in *esd1* mutants, suggesting a stronger involvement of ESD1 in these modifications as compared with SWC6.

Discussion

In *Arabidopsis*, flowering time is regulated by a complex genetic network where the floral repressor *FLC* has a pivotal role integrating the autonomous and vernalization pathways and down-regulating the expression of *FT* and *SOC1* floral integrators (Searle et al., 2006). The expression level of these integrators is mainly responsible for the correct flowering time (Baurle and Dean, 2006). Transcriptional regulation of *FLC* is a central checkpoint in both winter and summer annual accessions of *Arabidopsis*. Recently, the regulation of *FLC* through chromatin modifications has been intensively demonstrated (reviewed by Reyes, 2006; Sung and Amasino, 2006).

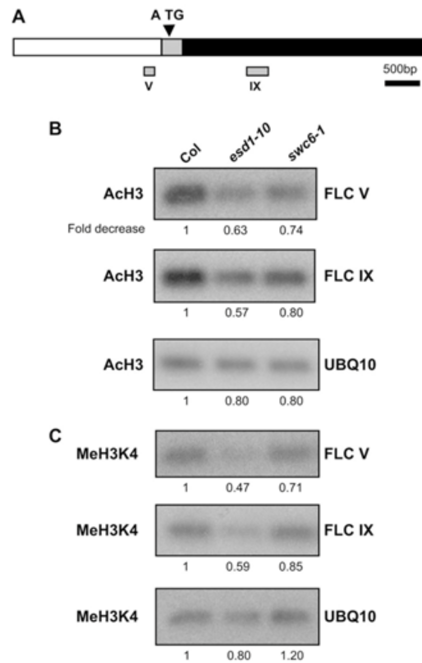


Fig. 7. Effect of *swc6* mutation on histone H3 acetylation and methylation in the *FLC* genomic region by ChIP analysis. (A) *FLC* genomic region analysed by ChIP. The white box corresponds to the promoter *FLC* region, grey boxes to exons, and the black box to the first intron. The two *FLC* fragments analysed by semiquantitative PCR are depicted and numbered. (B) PCR products after 25 cycles of Col, *esd1-10*, and *swc6-1* mutant plants using as template DNA purified from chromatin inmunoprecipitated with antibodies against acetylated H3 (AcH3). *UBQ10* was amplified during 22 cycles and used as control for DNA quantification. Fold decrease in H3 acetylation of *swc6* and *esd1* mutants over Col is shown. (C) PCR products after 25 cycles of Col, *esd1-10*, and *swc6-1* mutant plant PCR products as in (B) but using as template DNA purified from chromatin inmunoprecipitated with antibodies against trimethylated H3K4 (MeH3K4). Fold decrease in H3K4 methylation of mutants over the wild-type ecotype is shown. The data provided are representative and are from one of three independent experiments.

In this work, *Arabidopsis swc6* mutants that are affected in a putative orthologue of the SWR1 chromatin remodelling complex have been characterized. *SWC6* is the only *Arabidopsis* gene homologue of yeast *SWC6*/*VPS73* (Krogan et al., 2003; Kobor et al., 2004; Wu et al., 2005). Recently, the function of the ATP-dependent chromatin remodelling complex SWR1 has been intensively studied in yeast (Wu et al., 2005). The subunits of SWR1 and of the mammalian homologue SRCAP complexes have been biochemically identified and analysed (Kobor et al., 2004; Mizuguchi et al., 2004; Cai et al., 2005; Wu et al., 2005), and the evidence for the presence of a homologous complex in plants has been provided (Choi et al., 2007; Deal et al., 2007). In addition, how SWR1 homologues affect development in higher eukaryotes remains largely unknown. Homologues of most SWR1 components are present in *Arabidopsis*, and thus the function of this complex is being genetically dissected.

Phenotypical analyses of *swc6* mutants revealed a complex array of pleiotropic defects affecting vegetative and reproductive development, including a reduction in flowering time and phase length (Fig. 2). *swc6* causes early flowering mainly through the reduction in *FLC* expression (Fig. 5), suggesting a role for the SWR1 complex in the regulation of flowering time. Genetic analyses have revealed that the early flowering phenotype of *swc6* mutants is almost completely epistatic over the flowering time delay caused by *fve* mutation in the autonomous pathway, and that *swc6* partially suppresses the late flowering phenotype conferred by active *FRI* alleles (Fig. 5). These

genetic interactions correlate at the molecular level with a decrease in the steady-state levels of *FLC* mRNA in *swc6* mutant alleles (Fig. 5D). Together, these results indicate that SWC6 is required for the proper expression of *FLC*. The phenotypic analysis of *swc6* mutants indicates that this protein also controls other developmental processes such as leaf and flower morphology (Fig. 2), suggesting that SWC6 regulates other genes involved in plant development. For instance, *swc6* plants show smaller leaves with serrated margins, which might reflect defects in cell proliferation along the margins of leaf primordia. In addition, *swc6* mutant flowers display extra floral perianth organs, suggesting a role for the SWR1 complex in the control of floral development.

Mutations in different *Arabidopsis* homologues of SWR1 components such as *PIE1*, *ESD1*/*SUF3*/*ARP6*, and *SWC6*/*SEF* cause similar developmental defects (Noh and Amasino, 2003; Choi et al., 2005, 2007; Martin-Trillo et al., 2006; March-Diaz et al., 2007; this study), and these genes display similar expression patterns, although *SWC6* is more highly expressed in anthers (Fig. 4D). This may be explained by the presence of various *cis*-acting elements (AGAAA and GTGA) in the *SWC6* promoter region, known to confer anther/pollen-specific gene expression (Gupta et al., 2007), and is consistent with a role for SWC6 in anther and/or pollen development (March-Diaz et al., 2007; this study) (Fig. 4C). All three mutants cause suppression of late flowering in autonomous pathway mutants as well as in *FRI*-containing lines and other developmental defects including leaf serration, weak apical dominance (bushy aspect), flowers with extra petals, and short siliques. They also show earlier flowering than an *flc*-null mutant, suggesting that flowering inhibition mediated by these proteins occurs through both *FLC*- and *FLC*-like gene-dependent pathways (Noh and Amasino, 2003; Choi et al., 2005, 2007; Martin-Trillo et al., 2006). However, some phenotypes are more dramatic in *pie1* plants than in *swc6*/*sef* and *esd1*/*suf3*/*arp6* plants (Noh and Amasino, 2003). *pie1* displays a stronger reduction in fertility, a very notable reduction in primary inflorescence elongation, and smaller and deformed leaves (Noh and Amasino, 2003; March-Diaz et al., 2007), phenotypes that were not obvious in *esd1*/*suf3*/*arp6* or in *swc6*/*sef* (Noh and Amasino, 2003; Choi et al., 2005, 2007; Deal et al., 2005; Martin-Trillo et al., 2006; March-Diaz et al., 2007). Moreover, *pie1* plants show a stronger down-regulation of *FLC* and *MAF4* transcript levels than the *esd1* and *swc6* plants (Deal et al., 2007; March-Diaz et al., 2007). Furthermore, the *MAF5* gene was deregulated in the *pie1*, *esd1*/*suf3*/*arp6*, and *swc6*/*sef* mutant (Fig. 5D; Martin-Trillo et al., 2006; March-Diaz et al., 2007). Altogether, these observations suggest that PIE1 might fulfil functions that are at least partially independent from those of SWC6 and ESD1; a tempting possibility is that PIE1 may participate in other chromatin remodelling complexes besides SWR1.

SWC6 and ARP6 yeast homologues are tightly associated in SWR1C, being necessary for the interaction with the SWC2 subunit and for nucleosome binding (Wu et al., 2005). According to this, *Arabidopsis SWC6* and *ESD1*/*SUF3*/*ARP6* have a similar developmental function. The phenotypes of *swc6* and *esd1* mutants are quite comparable, and the *esd1 swc6* double mutant has the same phenotype and causes similar alterations in gene expression as any single mutant (Figs 5D, 6B), indicating that both genes act in the same genetic pathway. Together with the absence of any obvious developmental phenotype in plants overexpressing either *ESD1* or *SWC6*, the results described in this study strongly suggest that ESD1 and SWC6 act together as a protein complex. The protein interaction analyses confirmed that both proteins can physically interact (Fig. 6C, D). Biochemical characterization of the yeast SWR1 complex indicates that removal of either arp6 or swc6 results in the reciprocal loss of the other subunit from the complex and also in the loss of two other proteins, Swc2 and Swc3, suggesting that Arp6, Swc6, Swc2, and Swc3 form a subcomplex associated with Swr1 (Wu et al., 2005). Similarly, *Arabidopsis* ARP6 and SWC6, together with SWC2 and other unidentified factors, may form a subcomplex that associates with PIE1 (Choi et

al., 2007; March-Diaz et al., 2007). Again, this is consistent with a very similar phenotype of the *swc6* and *esd1*/*suf3*/*arp6* mutants but a slightly different phenotype of the *pie1* mutant.

The SWR1 complex in yeast catalyses the replacement of nucleosomal H2A with the H2A.Z variant, ensuring full activation of underlying genes (Guillemette et al., 2005; Li et al., 2005; Raisner et al., 2005; Zhang et al., 2005). Recent studies have shown that two H2A.Z nucleosomes flank a nucleosome-free region containing the transcription initiation site in promoters of both active and inactive genes in yeast and that H2A.Z-bearing nucleosomes facilitate transcription activation through their susceptibility to loss, thereby helping to expose promoter DNA (reviewed in Raisner and Madhani, 2006). In *Arabidopsis*, the histone variant H2A.Z has been identified within *FLC*, *MAF4*, and *MAF5* chromatin, occupying regions near both the transcription start and termination sites on the three genes examined (Deal et al., 2007). In addition, H2A.Z interacts with both PIE1 and AtSWC2, and knockdown of H2A.Z caused a phenotype similar to that of *pie1*, *esd1*/*suf3*/*arp6*, and *swc6* (Choi et al., 2007). Loss of H2A.Z from *FLC* chromatin in *esd1*/*suf3*/*arp6* and *pie1* mutants results in reduced *FLC* expression and premature flowering, indicating that this histone variant is required for a high level of expression of *FLC* (Deal et al., 2007). These observations support the existence of an SWR1-like complex in plants that is targeted to different loci including *FLC*, and show that H2A.Z can poise transcriptional activation in plants. Interestingly, the spatial distribution and the overall levels of H2A.Z on *FLC* was the same in samples that had a 10-fold higher level of *FLC* expression (Deal et al., 2007), suggesting that H2AZ by itself does not activate *FLC* gene expression and that the replacement of nucleosomal H2A with H2A.Z may form a variant nucleosome with unique tails that might bind specific regulatory proteins to help promote *FLC* gene activation.

High levels of *FLC* expression are correlated with H3 and H4 hyperacetylation and trimethylation of H3K4 and H3K36 at the *FLC* locus (He et al., 2003, 2004; Ausin et al., 2004; Bastow et al., 2004; Sung and Amasino, 2004; Zhao et al., 2005). Martin-Trillo et al. (2006) have recently reported that *esd1*/*suf3*/*arp6* mutants present low levels of histone H3 acetylation and H3K4 methylation in the *FLC* locus; this work demonstrates a comparable behaviour in the *swc6* mutant, although the effect was consistently more conspicuous for *esd1* (Fig. 7). Whether ARP6 and SWC6, and, consequently, the SWR1 complex are directly involved in setting these epigenetic markers or whether these alterations are secondary consequences is still unclear. Moreover, it remains to be determined whether the effect of *esd1*/*suf3*/*arp6* and *swc6* on the expression of other *MAF* genes takes place through similar mechanisms.

A human H2A.Z complex equivalent to the yeast SWR1 complex has histone acetyltransferase activity (Owen-Hughes and Bruno, 2004), and the Swr1 complex shares several subunits with the NuA4 histone acetyltransferase. Furthermore, mutants of these two complexes share several phenotypes, suggesting that they may work together, which might help to understand the role of ESD1/SUF3/ARP6 and SWC6 in histone acetylation. In the same way, the fact that components of the Swr1 complex were found to interact genetically with the PAF1 complex in yeast might explain the role of ESD1 and SWC6 in the trimethylation of H3K4 in *FLC* chromatin (Mueller and Jaehning, 2002; Squazzo et al., 2002; Krogan et al., 2003, 2004). Like the yeast PAF1 complex, the PAF1-like complex in *Arabidopsis* may also recruit an H3K4 methyl transferase to *FLC* to regulate its expression (Kim et al., 2005). Indeed, mutations in *Arabidopsis* homologues of the components of the PAF1 complex cause a decrease in the trimethylation of H3K4 in *FLC* chromatin, and provoke early flowering and small leaves, similar to the *esd1* and *swc6* mutations (He et al., 2004), raising the possibility that all of these genes are in the same pathway and regulate similar targets.

We propose that the H2A.Z variant may serve to poise the *FLC* gene, and maybe other related genes, in a state competent for activation by other factors, rather than activating transcription directly (Deal et al., 2007). This may reflect the ability of H2A.Z to facilitate nucleosome remodelling (Santisteban et al., 2000) and/or to recruit the transcription machinery (Adam et al., 2001) or other chromatin remodelling complexes to allow high-level transcription under certain conditions. Thus, in the absence of H2A.Z in *swc6*, *esd1*/*suf3*/*arp6*, and *pie1* mutants, *FLC* levels remain low even in the presence of strong activators such as *FRI* (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005, 2007; Martin-Trillo et al., 2006; this study), resulting in early flowering.

Biochemical characterization of the SWR1C homologue and functional studies using transcriptomic analyses and ChIP-to-chip hybridization will help to identify additional genes regulated by this complex and to understand the crucial role of the SWR1 complex plant homologue in chromatin remodelling processes related to leaf and flower development and to the control of flowering time.

Acknowledgements

We would like to thank Dr Richard Amasino for kindly providing *flc-3* and *FRI-Col* lines, the ABRC and NASC for supplying the T-DNAs lines used in this study, and GENOSCOPE for the ESD1/SUF3/ARP6 and SWC6 cDNA clones. GST–SWC6 was a gift of Dr Jose Carlos Reyes. This work was supported by grants BIO2005-00251 to JAJ and BIO2004-00494 to MP from the Spanish Ministerio de Ciencia y Tecnología. AL and AG-Z were funded by a predoctoral fellowship from Direccion General de Investigacioón, Secretaria General de Política Científica y Tecnológica (Spain), and LL-G by an INIA predoctoral fellowship. We thank to all members of Jarillo's and Piñeiro's laboratories for helpful discussions, and D Saez for his skilful technical assistance.

References

Adam M, Robert F, Larochelle M, Gaudreau L. 2001. H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions. *Molecular and Cellular Biology* **21**, 6270–6279.

Ausin I, Alonso-Blanco C, Jarillo JA, Ruiz-Garcia L, Martinez-Zapater JM. 2004. Regulation of flowering time by FVE, aretinoblastoma-associated protein. *Nature Genetics* **36**, 162–166.

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C. 2004. Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* **427**, 164–167.

Baurle I, Dean C. 2006. The timing of developmental transitions in plants. *Cell* **125**, 655–664.

Boss PK, Bastow RM, Mylne JS, Dean C. 2004. Multiple pathways in the decision to flower: enabling, promoting, and resetting. *The Plant Cell* **16** (Suppl.), S18–S31.

Cai Y, Jin J, Florens L, Swanson SK, Kusch T, Li B, Workman JL, Washburn MP, Conaway RC, Conaway JW. 2005. The mammalian YL1 protein is a shared subunit of the TRRAP/TIP60 histone acetyltransferase and SRCAP complexes. *Journal of Biological Chemistry* **280**, 13665–13670.

Choi K, Kim S, Kim SY, Kim M, Hyun Y, Lee H, Choe S, Kim SG, Michaels S, Lee I. 2005. *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in Arabidopsis. *The Plant Cell* **17**, 2647–2660.

Choi K, Park C, Lee J, Oh M, Noh B, Lee I. 2007. Arabidopsis homologs of components of the SWR1 complex regulate flowering and plant development. *Development* **134**, 1931–1941.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.

Corbesier L, Vincent C, Jang S, et al. 2007. FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science* **316**, 1030–1033.

Deal RB, Kandasamy MK, McKinney EC, Meagher RB. 2005. The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of *FLOWERING LOCUS C* expression and repression of flowering in Arabidopsis. *The Plant Cell* **17**, 2633–2646.

Deal RB, Topp CN, McKinney EC, Meagher RB. 2007. Repression of flowering in Arabidopsis requires activation of *FLOWERING LOCUS C* expression by the histone variant H2A.Z. *The Plant Cell* **19**, 74–83.

Gazzani S, Gendall AR, Lister C, Dean C. 2003. Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. *Plant Physiology* **132**, 1107–1114.

Gendall AR, Levy YY, Wilson A, Dean C. 2001. The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. *Cell* **107**, 525–35.

Gomez-Mena C, Pineiro M, Franco-Zorrilla JM, Salinas J, Coupland G, Martinez-Zapater JM. 2001. *early bolting in short days*: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. *The Plant Cell* **13**, 1011–1024.

Guillemette B, Bataille AR, Gevry N, Adam M, Blanchette M, Robert F, Gaudreau L. 2005. Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. *PLoS Biology* **3**, E384.

Gupta V, Khurana R, Tyagi AK. 2007. Promoters of two antherspecific genes confer organ-specific gene expression in a stagespecific manner in transgenic systems. *Plant Cell Reports* **26**, 1919–1931.

He Y, Amasino RM. 2005. Role of chromatin modification in flowering-time control. *Trends in Plant Science* **10**, 30–35.

He Y, Doyle MR, Amasino RM. 2004. PAF1-complex-mediated histone methylation of FLOWERING LOCUS C chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. *Genes and Development* **18**, 2774–2784.

He Y, Michaels SD, Amasino RM. 2003. Regulation of flowering time by histone acetylation in Arabidopsis. *Science* **302**, 1751–1754.

Imaizumi T, Kay SA. 2006. Photoperiodic control of flowering: not only by coincidence. *Trends in Plant Science* **11**, 550–558.

Johanson U, West J, Lister C, Michaels S, Amasino R, Dean C. 2000. Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. *Science* **290**, 344–347.

Kim SY, He Y, Jacob Y, Noh YS, Michaels S, Amasino R. 2005. Establishment of the vernalization-responsive, winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. *The Plant Cell* **17**, 3301–3310.

Kobor MS, Venkatasubrahmanyam S, Meneghini MD, Gin JW, Jennings JL, Link AJ, Madhani HD, Rine J. 2004. A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. *PLoS Biology* **2**, 587–599.

Krichevsky A, Gutgarts H, Kozlovsky SV, Tzfira T, Sutton A, Sternglanz R, Mandel G, Citovsky V. 2007. C2H2 zinc finger-SET histone methyltransferase is a plant-specific chromatin modifier. *Developmental Biology* **303**, 259–269.

Krogan NJ, Baetz K, Keogh MC, et al. 2004. Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4. *Proceedings of the National Academy of Sciences, USA* **101**, 13513–13518.

Krogan NJ, Keogh MC, Datta N, et al. 2003. A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. *Molecular Cell* **12**, 1565–1576.

Lee I, Amasino RM. 1995. Effect of vernalization, photoperiod, and light quality on the flowering phenotype of Arabidopsis plants containing the *FRIGIDA* gene. *Plant Physiology* **108**, 157–162.

Lee MG, Wynder C, Bochar DA, Hakimi MA, Cooch N, Shiekhattar R. 2006. Functional interplay between histone demethylase and deacetylase enzymes. *Molecular and Cellular Biology* **26**, 6395–6402.

Li B, Pattenden SG, Lee D, Gutierrez J, Chen J, Seidel C, Gerton J, Workman JL. 2005. Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. *Proceedings of the National Academy of Sciences, USA* **102**, 18385–18390.

March-Diaz R, Garcia-Dominguez M, Florencio FJ, Reyes JC. 2007. SEF, a new protein required for flowering repression in Arabidopsis, interacts with PIE1 and ARP6. *Plant Physiology* **143**, 893–901.

Martin-Trillo M, Lazaro A, Poethig RS, Gomez-Mena C, Pineiro MA, Martinez-Zapater JM, Jarillo JA. 2006. *EARLY IN SHORT DAYS 1* (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in Arabidopsis. *Development* **133**, 1241–1252.

Michaels SD, Amasino RM. 1999. *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *The Plant Cell* **11**, 949–956.

Michaels SD, Amasino RM. 2000. Memories of winter: vernalization and the competence to flower. *Plant, Cell and Environment* **23**, 1145–1154.

Michaels SD, Amasino RM. 2001. Loss of FLOWERING LOCUS C activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *The Plant Cell* **13**, 935–941.

Mizuguchi G, Shen X, Landry J, Wu WH, Sen S, Wu C. 2004. ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. *Science* **303**, 343–348.

Moon J, Suh SS, Lee H, Choi KR, Hong CB, Paek NC, Kim SG, Lee I. 2003. The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. *The Plant Journal* **35**, 613–623.

Mueller CL, Jaehning JA. 2002. Ctr9, Rtf1 and Leo1 are components of the Paf1/RNA polymerase II complex. *Molecular and Cellular Biology* **22**, 1971–1980.

Noh YS, Amasino RM. 2003. *PIE1*, an *ISWI* family gene, is required for *FLC* activation and floral repression in Arabidopsis. *The Plant Cell* **15**, 1671–1682.

Oh S, Zhang H, Ludwig P, van Nocker S. 2004. A mechanism related to the yeast transcriptional regulator Paf1c is required for expression of the Arabidopsis *FLC/MAF* MADS box gene family. *The Plant Cellular* **16**, 2940–2953.

Owen-Hughes T, Bruno M. 2004. Breaking the silence. *Nature* **303**, 324–325.
Piñeiro M, Gomez-Mena C, Schaffer R, Martinez-Zapater JM, Coupland G. 2003. *EARLY BOLTING IN SHORT DAYS* is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. *The Plant Cell* **15**, 1552–1562.
Raisner RM, Hartley PD, Meneghini MD, Bao MZ, Liu CL, Schreiber SL, Rando OJ Madhani HD. 2005. Histone variant H2A.Z marks the 5´ ends of both active and inactive genes in euchromatin. *Cell* **123**, 233–248.
Raisner RM, Madhani HD. 2006. Patterning chromatin: form and function for H2A.Z variant nucleosomes. *Current Opinion in Genetics and Development* **16**, 119–124.
Ratcliffe OJ, Kumimoto RW, Wong BJ, Riechmann JL. 2003. Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. *The Plant Cell* **15**, 1159–1169.
Reeves PH, Murtas G, Dash S, Coupland G. 2002. *Early in short days 4*, a mutation in Arabidopsis that causes early flowering and reduces the mRNA abundance of the floral repressor *FLC*. *Development* **129**, 5349–5361.
Reyes JC. 2006. Chromatin modifiers that control plant development. *Current Opinion in Plant Biology* **9**, 21–27.
Santisteban MS, Kalashnikova T, Smith MM. 2000. Histone H2A.Z regulates transcription and is partially redundant with nucleosome remodeling complexes. *Cell* **103**, 411–422.
Schmitz RJ, Amasino RM. 2007. Vernalization: a model for investigating epigenetics and eukaryotic gene regulation in plants. *Biochimica et Biophysica Acta* **1769**, 269–275.
Scortecci KC, Michaels SD, Amasino RM. 2001. Identification of a MADS-box gene, *FLOWERING LOCUS M*, that represses flowering. *The Plant Journal* **26**, 229–236.
Searle I, He Y, Turck F, Vincent C, Fornara F, Krober S, Amasino RA, Coupland G. 2006. The transcription factor *FLC* confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes and Development* **20**, 898–912.
Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES. 1999. The *FLF MADS* box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. *The Plant Cell* **11**, 445–458.
Sheldon CC, Conn AB, Dennis ES, Peacock WJ. 2002. Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. *The Plant Cell* **14**, 2527–2537.
Shindo C, Aranzana MJ, Lister C, Baxter C, Nicholls C, Nordborg M, Dean C. 2005. Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of Arabidopsis. *Plant Physiology* **138**, 1163–1173.
Squazzo SL, Costa PJ, Lindstrom DL, Kumer KE, Simic R, Jeennings JL, Link AJ, Arndt KM, Hartzog GA. 2002. The Paf1 complex physically and functionally associates with transcription elongation factors *in vivo*. *EMBO Journal* **21**, 1764–1774.
Sung S, Amasino RM. 2004. Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **427**, 159–164.
Sung S, Amasino RM. 2006. Molecular genetic studies of the memory of winter. *Journal of Experimental Botany* **57**, 3369–3377.
Sung S, Schmitz RJ, Amasino RM. 2006. A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. *Genes and Development* **20**, 3244–3248.
Telfer A, Bollman KM, Poethig RS. 1997. Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* **124**, 645–654.
Werner JD, Borevitz JO, Warthmann N, Trainer GT, Ecker JR, Chory J, Weigel D. 2005. Quantitative trait locus mapping and DNA array hybridization identify an *FLM* deletion as a cause for natural flowering-time variation. *Proceedings of the National Academy of Sciences, USA* **102**, 2460–2465.
Wilson RN, Heckman JW, Somerville CR. 1992. Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiology* **100**, 403–408.
Wood CC, Robertson M, Tanner G, Peacock WJ, Dennis ES, Helliwell CA. 2006. The *Arabidopsis thaliana* vernalization response requires a Polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. *Proceedings of the National Academy of Sciences, USA* **103**, 14631–14636.
Wu WH, Alami S, Luk E, Wu CH, Sen S, Mizuguchi G, Wei D, Wu C. 2005. Swc2 is a widely conserved H2AZ-binding module essential for ATP-dependent histone exchange. *Nature Structural and Molecular Biology* **12**, 1064–1071.
Zhang H, Roberts DN, Cairns BR. 2005. Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. *Cell* **123**, 219–231.
Zhao Z, Yu Y, Meyer D, Wu C, Shen WH. 2005. Prevention of early flowering by expression of *FLOWERING LOCUS C* requires methylation of histone H3 K36. *Nature Cell Biology* **7**, 1256–1260.
Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W. 2004. GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiology* **136**, 2621–2632.

CAPÍTULO 3: The E3 ubiquitin ligase HOS1 participates in the control of photoperiodic flowering negatively regulating CONSTANS abundance.

Under revision

The E3 ubiquitin ligase HOS1 participates in the control of photoperiodic flowering in Arabidopsis negatively regulating CONSTANS abundance

Ana Lazaro[1], Federico Valverde[2], Manuel Piñeiro[1], Jose A. Jarillo[1,3]

[1] Centro de Biotecnología y Genómica de Plantas (CBGP), INIA-UPM. Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Campus de Montegancedo, 28223 Madrid, Spain.

[2] Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas y Universidad de Sevilla. Américo Vespucio 49, 41092 Sevilla, Spain.

[3] To whom correspondence should be addressed. e-mail: jarillo@inia.es

Telephone: 34-91-3364576; Fax 34-91-7157721

Key words: HOS1, E3 ubiquitin ligase, CONSTANS, flowering time, floral repression, Arabidopsis

We have isolated the *early in short days 6* (*esd6*) mutant in a screening for mutations that accelerate flowering time in Arabidopsis. *esd6* displays early flowering in both long and short day conditions among other developmental alterations. Fine mapping of the mutation showed that *esd6* was affected in the *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1* (*HOS1*) locus, which encodes a RING finger-containing protein that works as an E3 ubiquitin ligase. *esd6*/*hos1* mutation causes decreased expression of the *FLC* gene and shows a strong requirement of a functional CO protein for its early flowering phenotype under long days. Besides, CO and HOS1 physically interact in vitro and in vivo, and HOS1 is regulating CO abundance, particularly during the daylight period. Accordingly, the *hos1* mutation causes a shift in the typical long day pattern of *FT* transcript, starting to rise four hours after dawn. In addition, HOS1 interacts synergistically with COP1, another regulator of CO protein stability, in the control of flowering time. Taken together, these results indicate that HOS1 is involved in regulating CO abundance, ensuring that CO activation of *FT* occurs only when the light period reaches a certain length and avoiding precocious flowering in Arabidopsis.

INTRODUCTION

The integration of complex signals from environmental and endogenous cues is necessary to enable plants to time the floral transition at the most advantageous moment (Michaels, 2009; de Montaigu et al., 2010; Imaizumi, 2010). Plants growing at northern latitudes adapt their developmental program to the varying daylengths and temperatures that occur along the year (Jackson, 2009). Arabidopsis is a facultative long-day (LD) plant in which flowering time is controlled by a network of six major pathways: information about daylength, low winter temperatures and growth temperature are mediated by the photoperiod, the vernalization and the ambient temperature pathway, respectively. In contrast, the aging, the autonomous and the gibberellin pathways act more independently of ambient conditions (Fornara et al., 2010). A potent repressor of flowering, *FLOWERING LOCUS C* (*FLC*), integrates signals coming from both the vernalization and the autonomous pathway (Amasino, 2010). Eventually, the whole network converges in the regulation of the floral integrators: *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Fornara et al., 2010).

The photoperiod pathway comprises several genes, including *GIGANTEA* (*GI*), *CONSTANS (CO)*, and *FT* (Kobayashi and Weigel, 2007; Turck et al., 2008). Mutations in any of these genes cause a delay in flowering mainly under LDs, whereas their overexpression accelerates flowering independently of daylength (Turck et al., 2008). CO is a B-box-type protein that acts in the vascular tissue of the leaves to activate *FT* and *TSF* transcription (An et al., 2004; Jackson, 2009; Tiwari et al., 2010). CO may induce *FT* expression by forming a DNA binding complex with NUCLEAR FACTOR Y (NF-Y)/HEME ACTIVATOR PROTEIN (HAP) proteins (Wenkel et al., 2006; Kumimoto et al., 2010) and by binding the *FT* promoter directly at CO-responsive elements (Tiwari et al., 2010). FT protein, and possibly TSF, are part of the florigen that moves to the shoot apical meristem to induce flowering in response to LDs (Corbesier et al., 2007; Jaeger and Wigge, 2007; Jang et al., 2009).

Plants have developed a sophisticated molecular mechanism to measure daylength based on the coincidence of an internal rhythm, set by the circadian clock, with an external cue, such as light. The ability to distinguish LDs from short days (SDs) is largely the result of the complex regulation of *CO*, both at the transcriptional and post-translational level. Under LDs, *CO* mRNA shows two peaks of expression, the first following the expression of *GI* at the end of a LD, when plants are still exposed to light; and the second during the night. Under SDs, only the night peak of *CO* expression takes place (Suarez-Lopez et al., 2001). The precise timing of *CO* also requires the degradation of a family of repressors, the cycling DOF transcription factors (CDFs), by the F-Box protein FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) in conjunction with GI (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009).

The increased expression of *CO* in the light under LDs but not SDs is crucial for the promotion of flowering, because exposure to light is required for stabilization of CO protein (Valverde et al., 2004; Jang et al., 2008). The high *CO* transcript levels detected during the dark phase of both LD and SDs do not correlate with CO protein accumulation because the RING finger protein CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) promotes CO degradation in the dark (Jang et al., 2008; Liu et al., 2008b). Mutations in *COP1*, a component of an ubiquitin ligase complex, cause extreme early flowering under SDs. This early flowering phenotype is largely dependent on CO activity and correlates with an increase in *FT* transcription in *cop1* mutant (Jang et al., 2008; Liu et al., 2008b). COP1 and CO interact both *in vivo* and *in vitro*, and it has been proposed that COP1 contributes to daylength perception by reducing the abundance of the CO protein during the night (Jang et al., 2008; Liu et al., 2008b; Chen et al., 2010). However, in the morning CO degradation occurs independently of COP1 (Jang et al., 2008). Therefore, it has been suggested that an unidentified E3 ubiquitin ligase must collaborate in CO degradation during the early part of the day to ensure that CO induction of *FT* only takes place in LDs (Jang et al., 2008).

In addition to the duration of the daily light/dark periods, plants also perceive light quality. Blue and far-red light promote flowering, while red light (RL) delays it (Valverde et al., 2004). Far-red light can increase CO protein levels independently of transcription (Kim et al., 2008). Blue light mediates photoperiodic control of the floral initiation at least by three different mechanisms: first, it promotes the interaction of FKF1 and GI necessary for the CDFs degradation (Sawa et al., 2007); second, the blue light receptor Cryptochrome 2 (Cry2) prevents GI and CO proteolysis by COP1 (Liu et al., 2008b; Yu et al., 2008); and third, Cry2 modulates *FT* transcription directly (Liu et al., 2008a). On the other hand, the red light photoreceptor Phytochrome B (PhyB) has been implicated in the degradation of CO during the first part of the day (Valverde et al., 2004; Jang et al., 2008).

Screenings devoted to the isolation of early flowering mutants have revealed the existence of genes that repress the floral transition (Pouteau et al., 2004). Floral repressors are essential to

safeguard against premature flowering, and knowledge of how these repressors interact with the floral promotion pathways is just emerging (Pouteau et al., 2004; Roux et al., 2006). Here we demonstrate that the *esd6* early flowering mutant is affected in the *HOS1* gene. *HOS1* encodes a protein with E3 ubiquitin ligase activity, previously described as a negative regulator of cold acclimation responses (Lee et al., 2001; Dong et al., 2006a). The early flowering phenotype of *hos1* is completely suppressed by mutations in *CO* gene in Landsberg *erecta* (L*er*) background and notably delayed by *co* mutations in Columbia (Col) background. In addition, we show that HOS1 physically interacts with CO and regulates CO protein abundance during the daylight period, indicating the participation of another RING finger-containing protein, besides COP1, in the photoperiodic control of flowering time in Arabidopsis. Thus, we propose that HOS1 is required to modulate precisely the timing of CO accumulation, and that this regulation is essential to maintain *FT* levels low during the first part of the day and, subsequently, a correct photoperiodic response in Arabidopsis.

METHODS

Genetic stocks and growth conditions

Arabidopsis thaliana (L.) mutant seed stocks used were in L*er*, Col and C24 genetic backgrounds, and were obtained from the Arabidopsis Biological Resource Centre (ABRC) of Ohio State University (Columbus, USA), the Nottingham Arabidopsis Centre (NASC) in the UK, and personal donations. C24 accession and mutant *hos1-1* seeds were kindly donated by Dr. J.K Zhu (Lee et al., 2001). The monogenic mutants used in this work were described previously: *fca-1*, *ft-1*, *co-2* and *gi-3* (Koornneef, 1991); *fve-3* (Ausin et al., 2004); *flc-3* (Michaels and Amasino, 1999); *phyB-1* (Reed et al., 1993); *fha-1* (Guo et al., 1998); *vrn1-2 fca-1* (Levy et al., 2002); *vin3-4 FRI* Sf-2 (Sung and Amasino, 2004); *fld-1* (He et al., 2003); *siz1-2* (Miura et al., 2005); *fkf1-1* (Nelson et al., 2000); *cop1-4* (Deng et al., 1991); *co-10* (Laubinger et al., 2006); *soc1-1* (Samach et al., 2000); and the Col *FRI* Sf-2 line was described by Lee and Amasino (Lee and Amasino, 1995).

Plants were grown in plastic pots containing a mixture of substrate and vermiculite (3:1) or in MS (Murashige and Skoog) medium supplemented with 1% (w/v) sucrose and 0,8% (w/v) agar for *in vitro* culture. Controlled environmental conditions were provided in growth chambers at 22°C and 70% relative humidity. Plants were illuminated with cool-white fluorescent lights (approximately 120 μmol m^{-2} s^{-1}). LD conditions consisted of 16 h of light followed by 8 h of darkness; SD conditions consisted of 8 h of light followed by 16 h of darkness.

Phenotypic analysis

Total leaf number was scored as the number of leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time of opening of the first flower (Koornneef, 1991). Cauline, adult and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered as juvenile leaves (Telfer et al., 1997). Data are shown as mean ± standard deviation.

Root length was measured at different developmental stages in seedlings grown in MS medium supplemented with 1% (w/v) sucrose and 1% (w/v) plant agar in Petri dishes placed vertically.

Total chlorophyll content (Ct) was calculated as described by Moran (Moran, 1982).

Map-based cloning of *esd6* mutation and molecular characterization of the *hos1* alleles

A mapping population was generated from the crossing of the *esd6* mutant, in L*er* background, and a Col wild-type plant. The analysis of 550 early flowering plants with several polymorphic molecular markers (Supplemental Table 1) located the *esd6* mutation to the upper arm of chromosome 2, between markers C005 and T5I7. Mutations *hos1-1*, in C24 background, *hos1-2*, in L*er* background, and *hos1-4*, in Col background, generate premature stop codons in the seventh, fifth and first exon of the *HOS1* locus respectively. The T-DNA insertion mutant *hos1-3*, isolated in Col background, was obtained from NASC (SALK_069312).

Genetic analysis

Double mutants were constructed by crossing the monogenic *hos1* mutants with lines carrying the mutations *flc-3, fca-1, fve-3, fld-1, siz1-2, fha-1*, *gi-3*, *co-2*, *co-10*, *cop1-4*, *fkf1-1*, *ft-1*, *soc1-1, vrn1-2* or *vin3-4*. Double mutants were isolated from selfed F2 progeny using molecular markers. A dCAP marker was designed for the *hos1-2* mutation (PCR amplification using 5´-TTTTTACATGGCCGGTTCAGATC-3´ and 5´-GCAATGTAATGTGAAACTAGGCGA-3´ primers followed by *Bgl*II digestion). For the *hos1-3* mutation we used 5´-GGTTTCTGGACCGCATATTTC-3´, 5´- GGCTTCTGACCAGAGAGTGTT-3´ and the SALK LB1 primer. *hos1-3* was also crossed with lines carrying the *FRI* Sf-2 allele (Lee and Amasino, 1995) and the *35S*::*CO* transgene (Simon et al., 1996).

Expression analysis

Total RNA was isolated using TRIzol (Invitrogen-Gibco) and reverse transcriptase-mediated PCR was performed according to described procedures (Martin-Trillo et al., 2006). For semiquantitative RT-PCR analysis the *HOS1* specific primers, 5´-TTGTCCTCTATTTGCGTTTGT-3´ and 5´-TCAAATTGGGGAAGAAGTTATG-3´, were designed to amplify the N-terminal part of the *HOS1* coding region. The *FLC*, *CO*, *FT* and *SOC1* probes used were described elsewhere (Pineiro et al., 2003; Lazaro et al., 2008). *UBIQUITIN 10* (*UBQ10*) was used as a loading control in these experiments. Quantitative real-time PCR (Q-PCR) analyses were performed using FastStart Universal SYBR Green Master (Roche) and protocols and primers already described for analyzing the expression of *CO*, *FT*, *SOC1* and *β-ACTIN* (*ACT*) genes (Chiang et al., 2009; Morris et al., 2010).

In vitro pull-down assays

The pMAL and the pMAL-HOS1 constructs were expressed in *Escherichia coli BL21* Rosetta strain and the proteins, Maltose Binding Protein (MBP) or MBP-HOS1, were purified on amylose resin (New England Biolabs). *In vitro* transcription/translation *CO* reactions were performed with the TNT Quick Coupled Transcription/Translation System (Promega) in the presence of ^{35}S-methionine (Amersham Biosciences). For pull-down assays, 1 mg of MBP or MBP-HOS1 bound to beads was incubated with 15 µl of the TNT reaction in 200 µl of binding buffer containing 50mM Hepes (pH 7,4), 1mM EDTA, 150mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Tween-20, and 0.5mM DTT (Dong et al., 2006a). The mixture was incubated at room temperature for 1h and then washed five times with washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.2% (v/v) Nonidet P-40). Samples were boiled in the presence of Laemmli buffer, analyzed by SDS-PAGE and autoradiography.

Bimolecular fluorescence complementation (BiFC) studies

HOS1 and CO complete ORFs were cloned in pYFPN43 and pYFPC43 vectors to produce amino-HOS1 fused to the N terminal part of the Yellow Fluorescent Protein (YFNHOS1), and carboxy-CO fused to the C terminal part of the YFP (COYFC). These constructs were introduced into *Agrobacterium tumefaciens* strain C58C1. 5-week old *Nicotiana benthamiana* plants were leaf-inoculated with COYFC and YFNHOS1, the negative control pairs (COYFC and YFN alone, and YFC alone and YFNHOS1) or the positive control (amino and carboxy parts of AKINβ and AKIN10 Sucrose non fermenting (Snf1)-related kinases (SnRK) (Ferrando et al., 2001), following protocols previously described (Voinnet et al., 2003). Fluorescent interactions were visualized under a Leica TCS SP2 confocal microscopy set at 550 nm. Images were analysed employing Leica LCSLite software.

Nuclear protein extraction and immunological experiments

Nuclei were isolated from frozen Arabidopsis seedlings grown in MS plates for two weeks. Plants were grinded with mortar and pestle in the presence of liquid nitrogen and 30 ml of nuclei isolation buffer containing 50 mM MES-KOH pH 8.0, 1 mM EDTA pH 8.0, 30% (v/v) glycerol, 5% (w/v) sucrose, 50 mM KCl, 10 mM MgCl2, 10 mM PMSF, 0,1% (v/v) Triton-100 and plant protease inhibitor cocktail (SIGMA). The slurry was filtered through 100 µm mesh and centrifuged sequentially at 6.000 rpm for 20 min; 5.000 rpm for 10 min and 4.000 rpm for 10 min in a Beckman Avanti J-26 XP centrifuge at 4°C employing JA-25.50 rotor, being the

supernatant discarded in each step and the pellets resuspended in the same nuclei isolation buffer. The final pellet was resuspended in 1,5 ml of the same buffer omitting the detergent and centrifuged at 2.000 g in a microfuge at 4ºC. The nuclei pellet was disrupted in the presence of 6M guanidine chlorhydrate with circular stirring at 4°C, sonicated in a Brandson sonifier set at 10 W force level and centrifuged at 20.000 g 10 min in a microfuge at 4°C. The supernatant was precipitated with 90% (v/v) ethanol, recentrifuged at the same speed for 10 min and washed 3 times in 90% (v/v) ethanol. The final pellet was dried and resuspended in Laemmli loading buffer and loaded into 4-12% (w/v) acrylamide gels. Immunoblots were performed as described before using CO antibodies (Valverde et al., 2004), and anti-H3 antibodies (AbCAM) as loading controls. Immuno-chemiluminescence signals were visualized and quantified using a ChemiDoc system (Bio-Rad).

Luciferase activity assays

A *35S*::*CO-LUC* construct was transformed in Col plants and homozygous lines were established. Several independent transgenic plants exhibiting early flowering phenotype were selected and one representative line was crossed with *hos1-3* plants. For non-invasive *in vivo* luciferase (LUC) imaging, 10 day-old Col and *hos1-3* seedlings harbouring the *35S::CO-LUC* construct were grown in MS plates and sprayed with 100 μM luciferin (Biotium) 3 h after dawn. The imaging system consisted of a PHOTON COUNTING I-CCD VIDEO CAMERA C2400-32 (Hammamatsu Photonics) mounted in a dark chamber. Image acquisition and processing were performed with the WASABI software provided by the camera manufacturer.

Quantification of luciferase activity was assayed on seedlings grown in the same conditions described above with a MicroBeta TriLux Luminometer (PerkinElmer). Seedlings were grinded in liquid nitrogen and resuspended in Steadylite Plus Reagent (PerkinElmer). The luciferase activity was measured as a mean of three independent experiments and expressed as luciferase counts per second (LCPS) in serial dilutions of fresh tissue in Steadylite Plus Reagent (mg/ml).

Subcellular localization of HOS1.

hos1-3 mutant plants were transformed with a *35S::HOS1-GFP* construct and the selected transgenic plants were grown in MS medium supplemented with 1% (w/v) sucrose and 1% (w/v) plant agar in Petri dishes placed vertically. 10 day-old transgenic plants grown under continuous light or dark conditions were analyzed by confocal microscopy (Zeiss LSM 710). DAPI staining of the nuclei was done at a final concentration of 10 μg/ml with 0,1% Tween-20.

RESULTS

esd6 mutant is early flowering and displays pleiotropic defects in both vegetative and reproductive development

A recessive mutation that accelerated flowering time, named *early in short days 6* (*esd6*), was identified in a screening of a L*er* mutagenized population. Plants homozygous for *esd6* were selected as early flowering under LD conditions, although the *esd6* mutation also accelerated flowering under non inductive SD photoperiods (Figure 1A and B and Table 1). Earliness of *esd6* was mainly associated to the production of fewer leaves during the adult vegetative phase (Figure 1D) based on leaf trichome distribution (Telfer et al., 1997).

Besides their flowering phenotype, *esd6* mutant plants also displayed complex pleiotropic alterations of both vegetative and reproductive development. Mutant plants were smaller than wild type (Figure 1A and B) and showed a reduced leaf size compared to L*er* (Figure 1E). Moreover, *esd6* primary root was shorter and produced less secondary roots than the wild type (Figure 1G and Supplemental Figure 1). In contrast, the stem length was not noticeably affected by the *esd6*

mutation (Figure 1C). *esd6* flowers also displayed some developmental abnormalities including a reduced size in comparison to wild-type flowers (Figure 1F and Supplemental Figure 1). Besides, siliques were approximately 30% shorter in *esd6* mutant than in L*er* (Figure 1F and Supplemental Figure 1).

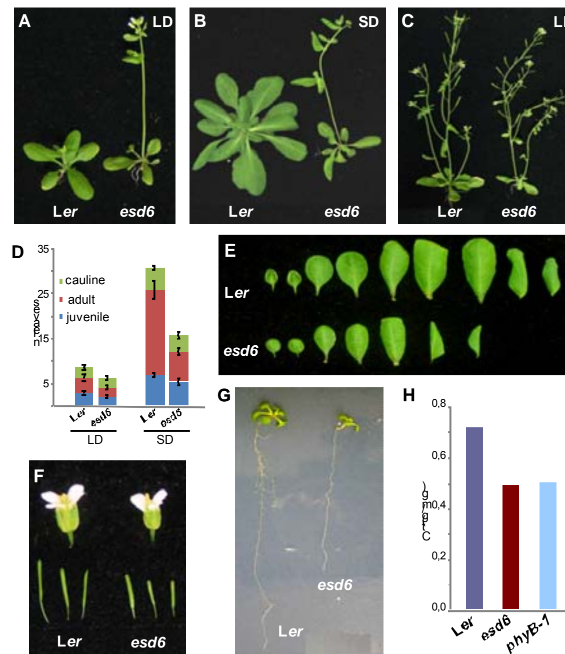


Figure 1. Phenotypic characterization of the *esd6* mutant.
(A,B) Flowering time phenotype of L*er* and *esd6* plants grown in LD conditions for 23 days (A) or in SD conditions for 54 days (B). (C) Phenotype of L*er* and *esd6* plants grown in LD conditions for 35 days (D) Histograms comparing the number of juvenile, adult and cauline leaves in L*er* and *esd6* plants grown under both LD and SD photoperiods. (E) Rosette and cauline leaves of Ler and *esd6* plants grown in LDs. (F) Detached L*er* and *esd6* flowers and siliques from plants grown under LD conditions. (G) Root elongation in 11 day-old L*er* and *esd6* seedlings. (H) Total chlorophyll content (Ct) in L*er* and *esd6* and *phyB-1* mutant seedlings.

Because *esd6* mutant looked paler than L*er* (Figure 1H), we decided to measure the total chlorophyll content (Ct) present in both genotypes. We included *phyB-1* as a control in this experiment, since *phyB* mutants display a reduced chlorophyll accumulation (Reed et al., 1993). As expected, both *phyB-1* and *esd6* mutant showed less Ct than L*er* (Figure 1H), indicating an additional role of *ESD6* gene in the control of chlorophyll biosynthesis.

The *ESD6* gene encodes HOS1, an E3 ubiquitin ligase

esd6 was identified in a L*er* transposon-mutagenized population generated from the genetic cross between two transgenic L*er* plants, one containing the *Ds (Dissociation)* element, and the other the transposase gene, capable of mobilizing the *Ds* element. The selection of *esd6* mutant was carried out in the F2 population where plants with different phenotypes were observed due to the mobilization of the transposon. We first noticed that the *esd6* early flowering phenotype did not cosegregate with the selection resistance gene. For this reason, we considered that the mutation was originated due to a second mobilization event of the *Ds* element that left a fingerprint in the genome. Consequently, to understand the molecular function of *ESD6,* we carried out a map-based cloning approach. *ESD6* was initially located in the upper arm of chromosome 2 and further

linkage analyses allowed us to define a candidate region between C005 and T5I7 molecular markers, which encompassed eight open reading frames (ORFs) (Figure 2A). Among these loci, *HOS1* (*At2g39810*) had already been described as a negative regulator of cold acclimation responses also affecting flowering time (Lee et al., 2001). The sequencing of this transcription unit in *esd6* revealed a single nucleotide deletion in the position 2212 (fifth exon) which generated a premature stop codon (Figure 2B). To confirm that *esd6* was indeed affecting the same locus as the *hos1-1* mutation, we performed an allelism test. The F1 plants derived from the cross between *hos1-1*, in C24 background, and *esd6* mutant resulted to be early flowering, indicating that both mutations were allelic (Table 1). *esd6* mutant was referred to hereafter as *hos1-2*. In addition, we searched for T-DNA insertional alleles within the *HOS1* locus and identified the line SALK_069312, which carried an insertion in the fifth exon of *HOS1* gene (Figure 2B). This T-DNA mutant allele was named *hos1-3* and, an additional allele, *hos1-4* was obtained later on in our laboratory during the screening of an EMS-mutagenized population of Col plants. *hos1-4* mutation created a single nucleotide deletion in the position 88 of the *HOS1* genomic annotation, which generated a premature stop codon in the first exon of the gene (Figure 2B). All *hos1* alleles analysed display an early flowering phenotype both in LD and SD photoperiods, but the fact that they flower earlier under inductive photoperiods indicates that the mutation does not completely abolish the plant photoperiod response (Figure 2C and Table 1).

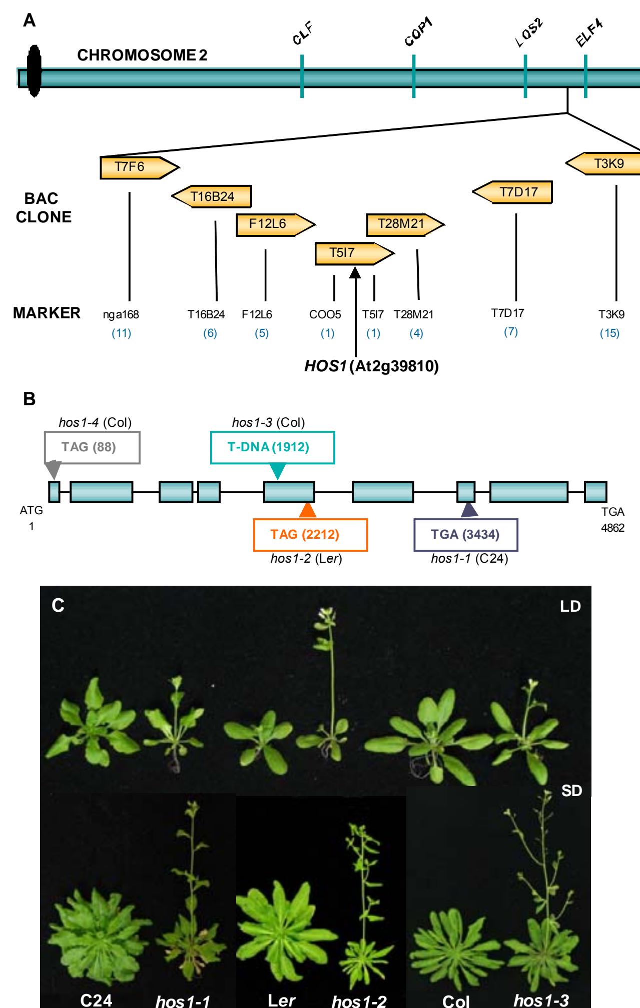


Figure 2. Identification of *ESD6*.

(A) Map-based cloning of *ESD6*. The genetic interval and the bacterial artificial chromosome (BAC) clones in the genomic region surrounding *ESD6* are shown. The number of recombinant events between molecular

markers is given in parentheses. The position of *HOS1* ORF in T5I7 BAC clone is indicated. (B) Scheme of the *ESD6/HOS1* gene structure showing the polymorphisms associated to the different *hos1* mutant alleles isolated. Exons are represented by squared boxes, while introns are drawn by a line. (C) Pictures illustrating the flowering time of *hos1* mutants and their respective wild-type genotypes in LD and SD conditions. Plants were grown for 23 days under LD conditions (upper panel). SD pictures (lower panel) were taken after 64 days for C24 and *hos1-1*, 58 days for L*er* and *hos1-2* and 60 days for Col and *hos1-3*.

The *AtHOS1* gene is around 5,5 Kb long, bears 9 exons and encodes a protein of 915 amino acids that contains a non-canonical RING finger domain in the N-terminal region and a putative nuclear localization signal in the C-terminal part (Figure 2B and Supplemental Figure 2). At*HOS1* is a unique gene in Arabidopsis and putative orthologues have only been found in plants. The cysteine residues present in the RING finger domain are totally conserved between all AtHOS1 orthologues (Supplemental Figure 2). RING finger domains are found in proteins with E3 ubiquitin ligase activity that participate in the ubiquitin/26S proteasome pathway (Deshaies and Joazeiro, 2009). Previously, it has been described that AtHOS1 can function as an E3 ubiquitin ligase in ubiquitination assays (Dong et al., 2006a).

hos1 mutations affect *FLC* expression and have an *FLC*-independent effect in the control of flowering time

The early flowering phenotype of *hos1* mutants suggested that HOS1 could be a negative regulator of the floral transition in Arabidopsis. To test this hypothesis, we analyzed the phenotype of double mutants carrying *hos1* and different mutations affecting flowering time. It had been previously described that *FLC* expression levels were reduced in the *hos1-1* mutant compared to C24 accession (Lee et al., 2001). In order to check if the *hos1* early flowering phenotype was fully dependent on *FLC*, we decided to analyse the effect of *hos1-3* mutation in an *flc* null genetic background (*flc-3)* (Michaels and Amasino, 1999). Both *hos1-3* and *hos1-3 flc-3* double mutant plants flowered with the same number of leaves, irrespectively of photoperiodic conditions, although the *hos1-3 flc-3* plants bolted consistently earlier than *hos1-3* under LD (Figure 3A and Table 1). This result may indicate that there is no additional effect of *flc* null mutation on the acceleration of flowering time caused by *hos1*. Besides, both *hos1-3* and *hos1-3 flc-3* plants flowered clearly earlier than *flc-3* plants under both LD and SD conditions (Figure 3A and Table 1), indicating that the effect of the *hos1* mutation on flowering time could not be exclusively dependent on *FLC* activity, and that there is an *FLC*-independent effect responsible for the early flowering phenotype of *hos1*. To find out whether *FLC* expression was altered in the *hos1* mutant alleles isolated in different backgrounds, semiquantitative RT-PCR analyses were performed in *hos1-1*, *hos1-2*, and *hos1-3* mutants and the corresponding wild-type genotypes. Consistently with previous results (Lee et al., 2001), in all *hos1* mutants assayed, *FLC* transcript was clearly down-regulated (Figure 3B), and therefore we cannot rule out that this change in *FLC* expression has an effect on the early flowering time of the *hos1* alleles.

Dominant alleles of the *FRIGIDA (FRI)* gene confer a vernalization requirement that delays flowering through the up-regulation of *FLC* (Johanson et al., 2000). In order to find out the genetic relationship between *HOS1* and *FRI*, the mutant *hos1-3* was crossed with a Col plant bearing an active *FRI* allele introgressed from the San Feliu-2 (Sf-2) accession (Lee and Amasino, 1995). Under LD conditions, the *hos1-3 FRI* Sf-2 line showed an additive phenotype, the *FRI* late-flowering phenotype being only partially suppressed by *hos1-3* (Figure 3D upper panel and Table 1). This suggests that *HOS1* and *FRI* do not regulate *FLC* expression through the same pathway in LDs. In contrast, the *hos1-3 FRI* Sf-2 plant flowered with approximately the same number of

leaves as the Col *FRI* Sf-2 plants under SD conditions, abolishing the effect of the *hos1* mutation (Figure 3D lower panel and Table 1).

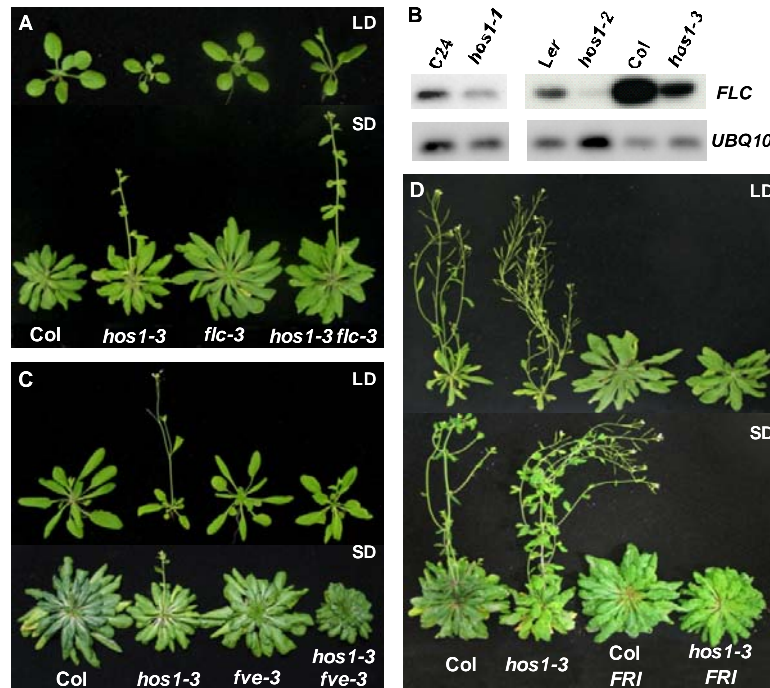


Figure 3. *hos1* mutations downregulate *FLC* expression and have an *FLC*-independent effect in the control of flowering time.

(A) Flowering time phenotype of *hos1-3 flc-3* double mutant in LD (upper panel) and SD (lower panel) conditions. (B) Analysis of the expression of *FLC* in 14 day-old *hos1* mutant seedlings and their corresponding wild-type genotypes. *FLC* expression was monitored by semiquantitative RT-PCR analysis over 22 cycles for C24 and *hos1-1* and over 28 cycles for L*er*, *hos1-2*, Col and *hos1-3*. For the *UBQ10* control 22 cycles were used. (C) Flowering time phenotype of *hos1-3 fve-3* double mutant plants grown in LD (upper panel) and SD (lower panel) conditions. (D) Flowering time phenotype of *hos1-3* plants bearing an active allele of *FRI* in LDs (upper panel) and SDs (lower panel).

Because *HOS1* locus is involved in cold signal transduction (Lee et al., 2001) and vernalization regulates *FLC* expression (Amasino, 2010), we hypothesized that *HOS1* could be controlling *FLC t*ranscript levels through the vernalization pathway. To analyze this, we generated combinations between *hos1* and two other mutants impaired in the vernalization response such as *vernalization 1* (*vrn1)* and *vernalization-insensitive 3 (vin3)* (Levy et al., 2002; Sung and Amasino, 2004), both in late flowering backgrounds that allowed us to observe the acceleration of flowering due to the vernalization treatment. The *hos1* mutation did not impair the acceleration of flowering caused by vernalization when combined with the late flowering *fca-1* or *FRI* Sf-2 plants (Supplemental Table 2). Besides, we found no difference in flowering time for the *hos1-2 vrn1-2 fca-1* triple mutant grown after either 1 or 4 weeks of vernalization treatment (Supplemental Table 2). The same result was observed for *hos1-3 vin3-4* carrying an active *FRI* allele, as both 1 and 4 week-vernalized plants flowered with approximately the same number of leaves (Supplemental Table 2). Thus, we concluded that *HOS1* does not regulate *FLC* expression through the vernalization pathway.

Considering that the autonomous pathway also converges on the regulation of *FLC* expression, we analyzed the flowering phenotype of double mutants combining *hos1* and

mutations in representative autonomous pathway genes, in particular the *hos1-3 fve-3*, *hos1-2 fca-1* and *hos1-3 fld-1* double mutants. Under LD, these double mutant plants showed an additive phenotype because the late-flowering phenotype of autonomous pathway mutants was only partially suppressed by *hos1* (Figure 3C upper panel and Table 1). In contrast, under SDs, flowering time of these double mutants was very similar to the one displayed by the autonomous pathway mutants, as they produced only a few leaves less than *fve-3*, *fca-1* and *fld-1* respectively (Figure 3C lower panel and Table 1).

Altogether, these results suggest that the *hos1* mutation cannot accelerate flowering in SD when combined with genetic backgrounds that have very high *FLC* expression levels, such as mutations of the autonomous pathway or active alleles of *FRI*. In contrast, under LD the repressive effect of *HOS1* on flowering time may be mediated by additional pathways that remain inactive in SD.

Table 1. Flowering time of *hos1* double mutants

	LD	SD
C24	25,6 ± 3,7	56,3 ± 7,2
hos1-1 (C24)	11,9 ± 2,2	38,7 ± 7,0
L*er*	8,4 ± 0,9	24,1 ± 3,3
hos1-2 (L*er*)	6,7 ± 0,9	17,6 ± 1,9
Col	13 ± 1,1	71,8 ± 6.5
hos1-3 (Col)	7,2 ± 0,5	32,5 ± 6
hos1-1 x hos1-2 (F1)	8,1 ± 1	
flc-3 (Col)	9,1 ± 0,6	61,9 ± 12,7
hos1-3 flc-3	6,8 ± 0,4	33,7 ± 6,7
Col FRI Sf-2	61,7 ± 9,6	121,3 ± 21,6
hos1-3 FRI Sf-2	40,9 ± 6,8	129,9 ± 16,4
fca-1 (L*er*)	33,3 ± 4,5	84,6 ± 11,2
hos1-2 fca-1	15,4 ± 1,8	79,9 ± 10,8
fve-3 (Col)	42,8 ± 6,1	108,4 ± 11,8
hos1-3 fve-3	13,6 ± 1,1	94,6 ± 9,2
fld-1 (Col)	36,6 ± 6	117,2 ± 8,5
hos1-3 fld-1	16,8 ± 3,3	110,4 ± 4,9
siz1-2 (Col)	10,2 ± 1,1	16 ± 2,3
hos1-3 siz1-2	7,4 ± 0,6	13,7 ± 3,1
fha-1 (L*er*)	12,5 ± 0,8	
hos1-2 fha-1	8,7 ± 0,8	
gi-3 (L*er*)	25 ± 2	
hos1-2 gi-3	18,6 ± 1,2	
co-2 (L*er*)	21,8 ± 5,9	23,4 ± 2,7
hos1-2 co-2	20,1 ± 1,3	14 ± 2,2
co-10 (Col)	38,6 ± 10,9	
hos1-3 co-10	26 ± 6,9	
cop1-4 (Col)	11,8 ± 0,9	12,8 ± 1,6
hos1-3 cop1-4	5 ± 0,6	5,1 ± 1
cop1-4 co-10	28,9 ± 4,2	
hos1-3 cop1-4 co-10	20,3 ± 3,2	
fkf1-1 (Col)	46,1 ± 6,1	
hos1-3 fkf1-1	17,5 ± 1,8	
Col [35S::*CO*]	4 ± 0	
hos1-3 [35S::*CO*]	4,1 ± 0,3	
ft-1 (L*er*)	17,3 ± 1,9	39,2 ± 4,8
hos1-2 ft-1	16,7 ± 0,8	35,9 ± 2,9
soc1-1 (L*er*)	14 ± 1,9	56,2 ± 5,3
hos1-2 soc1-1	10,2 ± 0,6	30,2 ± 5,4
ft-1 soc1-1	37,2 ± 3,3	
hos1-2 ft-1 soc1-1	32,6 ± 2,9	

Table 1. Total number of leaves at the time of flowering for the different wild type ecotypes and single, double and triple mutants described in this work. Data were scored in approximately 30 plants under LD conditions and 15 plants under SD photoperiods and is represented as mean ± standard deviation.

The E3 SUMO ligase SIZ1 promotes *FLC* expression by repressing the autonomous pathway gene *FLD* (Jin et al., 2008). Besides, SIZ1 stabilizes the ICE1 protein, which has been implicated in the regulation freezing tolerance in Arabidopsis (Miura et al., 2007). Because it had been described that ICE1 was also targeted by HOS1 (Dong et al., 2006a), we checked the genetic relationship existing between *hos1* and *siz1* mutants. Flowering time of *siz1* plants relative to wild type was slightly earlier under LDs, and substantially earlier under SDs (Jin et al., 2008) (Table 1). When we combined *siz1-2* with the *hos1-2* mutation, the double mutant flowering time resembled that of *hos1-2* in LDs but was earlier than any of the parental lines in SDs, suggesting a synergistic genetic interaction between both loci (Table 1).

The early flowering phenotype of *hos1-2* requires a functional CO protein

We also analyzed the phenotype of double mutants carrying *hos1* and mutations in genes representative of the photoperiod pathway, such as *CRY2/FHA*, *GI* and *CO*, that delay flowering mainly under LDs (Koornneef et al., 1998). While *hos1-2 fha-1* and *hos1-2 gi-3* double mutants showed an additive flowering phenotype between *hos1-2* and *fha-1* and *gi-3* late flowering mutants, the genetic interaction observed between *hos1-2* and *co-2* was completely different (Figure 4 and Table 1). Under LDs, the *hos1-2* mutation did not accelerate flowering time when it was combined with *co-2* (Figure 4C and Table 1); indeed, *hos1-2 co-2* plants flowered with the same number of leaves as *co-2* mutant, indicating a strong requirement of a functional CO protein for the early flowering phenotype of *hos1-2*. However, under SD conditions *hos1-2 co-2* flowered as early as *hos1-2* (Table 1), given that *co* mutations do not delay flowering under this photoperiodic condition. These genetic results suggest that *HOS1* is involved in the photoperiodic control of flowering time as a negative regulator of CO under LDs.

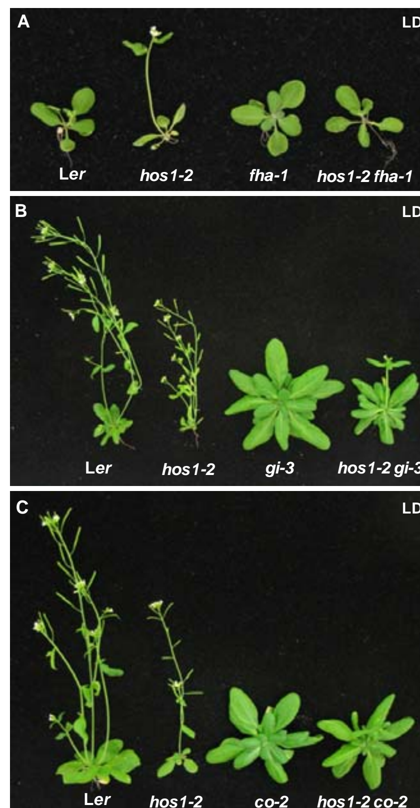


Figure 4. Genetic analyses between *hos1* and mutations in photoperiod pathway genes.
Flowering time phenotype of *hos1-2 fha-1* (A), *hos1-2 gi-3* (B) and *hos1-2 co-2* (C) double mutants grown in LD conditions.

FKF1 is an F-Box protein (Imaizumi et al., 2005) that mediates the cyclic degradation of CDF proteins, which are repressors of *CO* expression (Imaizumi et al., 2005; Fornara et al., 2009). To study if there was any genetic interaction between *FKF1* and *HOS1,* the double mutant *hos1-3 fkf1-1*was analyzed and it showed an additive phenotype between the late flowering time of *fkf1-1* and the early flowering phenotype of *hos1-3* in LDs (Table 1). This result indicates that *HOS1* does not participate in the *FKF1* transcriptional regulation pathway that controls *CO* expression.

***hos1* mutants show an altered pattern of *FT* expression**

FLC acts repressing the expression of the floral integrators *FT* and *SOC1*, while the photoperiod pathway activate *FT* and *SOC1* expression through *CO* (Yoo et al., 2005; Searle et al., 2006, Turck et al., 2008). Because *hos1* mutations showed downregulation of *FLC* expression (Figure 3B) and the *co-2* mutation was epistatic to *hos1-2* under LDs (Figure 4C), we decided to check the genetic relationship between *HOS1* and the floral integrators *FT* and *SOC1*. The *hos1-2 ft-1* double mutant showed a similar flowering phenotype to *ft-1* under LD conditions, suggesting a strong requirement of *FT* by the *hos1* early flowering phenotype (Figure 5A and Table 1). In contrast, *hos1-2 soc1-1* double mutant was additive between both parental lines in both LD and SD conditions (Figure 5A and Table 1). This result is in accordance with the epistasis observed between *co-2* and *hos1-2*, considering that *FT* is the main target of CO under LDs (Yoo et al., 2005). In order to check whether the whole effect of *HOS1* on flowering time was through *FT* and *SOC1*, we generated the triple mutant *hos1-2 ft-1 soc1-1*. Flowering time analysis showed that the triple mutant was slightly earlier than *ft-1soc1-1* (Figure 5A and Table 1). This result indicates that the early flowering phenotype of *hos1* mutation requires functional FT and SOC1 proteins, although we cannot rule out that HOS1 could regulate other protein(s) involved in the control of flowering time.

In order to get a deeper insight into the genetic relationship observed between *HOS1* and the photoperiod pathway, we performed a time course expression analysis over a 24h period in L*er* and *hos1-2* seedlings both in LD and SD. First of all, we demonstrated that *HOS1* transcript did not show a diurnal oscillation in L*er* background (Figure 5B). Subsequently, we analyzed the temporal expression pattern of *CO*, *FT* and *SOC1* genes using semiquantitative RT-PCR and quantitative real-time PCR (Q-PCR) approaches (Fig 5C-E). As previously reported, *CO* transcript level in L*er* background was high at dawn and dusk, and during the night, remaining low for the rest of the light period of the day (Suarez-Lopez et al., 2001) (Fig 5C-E). In *hos1-2*, we observed the same pattern of *CO* expression, indicating that *hos1* mutation did not affect significantly the levels or the expression profile of the *CO* transcript (Figure 5C-E). However, the expression pattern of the floral integrator *FT* was clearly altered in the *hos1-2* mutant compared to the wild-type both in LD and SD conditions (Fig 5C-E). *FT* transcript usually shows a peak of expression at dusk in LDs (around ZT16), following the evening increase observed in *CO* mRNA. In the *hos1-2* mutant, we observed a peak of *FT* expression in the subjective morning, mainly at ZT4, but also at ZT8, when the *CO* transcript levels are barely detectable in the mutant (Figure 5C and D). To check whether this alteration was due to a specific developmental stage of the plant or if it relied on the genetic background, we analysed *FT* transcript levels at ZT4 and ZT8 in Col and *hos1-3* plants harvested 8, 10, 12 and 15 days after germination (DAG). In every single stage tested, *FT* expression was higher in *hos1-3* in relation to Col in the first part of the day (Supplemental Figure 3). In SD, we observed an increased *FT* expression in the *hos1-2* mutant, starting to rise at ZT8 and peaking at ZT12, which may explain the early flowering phenotype displayed by the mutant under non-inductive photoperiods (Figure 5C and E). A small but consistent increase in *SOC1* expression was also detected in *hos1-2* plants grown in LD photoperiods in comparison to the wild type (Figure 5C and D). Thus, we conclude that *CO* transcript levels are not modified substantially by

the *hos1* mutation and that *HOS1* is required to repress the expression of *FT* in the first part of the day in LD. Considering that HOS1 has an E3 ubiquitin ligase activity, we speculated that it may be involved in the degradation of protein(s) that regulate *FT* expression. Both the genetic analysis and the expression assays suggested that this protein could be CO.

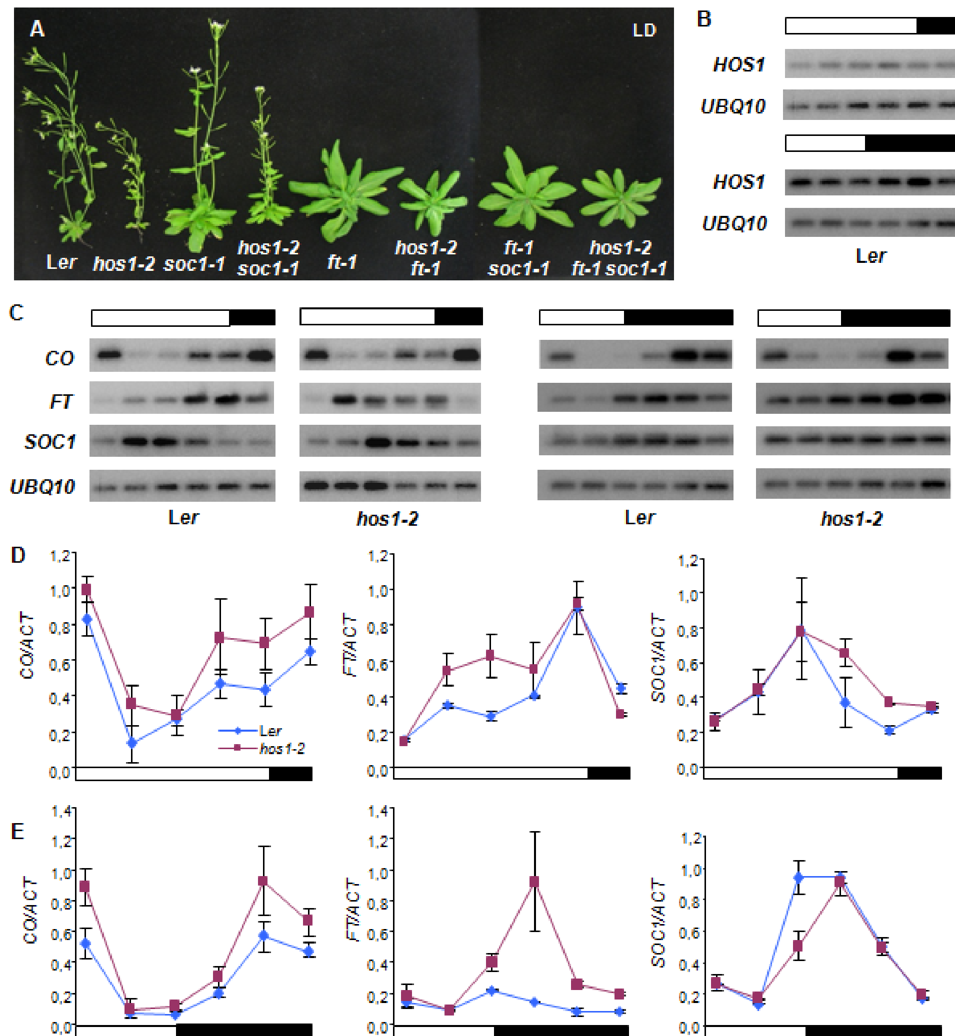


Figure 5. The early flowering phenotype of *hos1* depends on FT and SOC1 functional proteins and *hos1* mutation alters the pattern of expression of *FT*.

(A) Flowering time phenotype of *hos1-2 soc1-1, hos1-2 ft-1* and *hos1-2 ft-1 soc1-1* triple mutant plants grown in LD conditions. (B) *HOS1* expression pattern over a 24h time course in L*er* seedlings grown for 8 days in LDs and for 16 days in SDs. Samples were harvested every 4h after dawn. *HOS1* expression was monitored by semiquantitative RT-PCR analysis over 20 cycles. (C, D, E) Expression analysis of different flowering time genes over a 24h time course in L*er* and *hos1-2* seedlings grown for 8 days in LDs and 16 days in SDs. Samples were harvested every 4h after dawn (C) Semiquantitative RT-PCR analysis comparing *CO* (22 cycles), *FT* (28 cycles) and *SOC1* (24 cycles) expression (D) Quantitative real- time PCR (Q-PCR) analysis of *CO*, *FT* and *SOC1* expression in LD conditions (E) Same as (D) but L*er* and *hos1-2* seedlings grown in SD conditions. Relative expression levels were normalized to *β-ACTIN* expression.

HOS1 interacts *in vitro* and *in vivo* with CO and regulates its abundance

Given the proposed epistatic interaction between *co-2* and *hos1-2* mutants and considering that *CO* transcript levels were not affected in the *hos1-2* mutant, we decided to analyze whether there was a physical interaction between CO and HOS1. For this purpose, *in vitro* pull-down experiments using MBP-HOS1 and *in vitro*-translated CO protein were performed. As shown in Figure 6A, MBP-HOS1, but not MBP alone, was able to interact with CO protein. Whether the interaction between CO and HOS1 also occurred *in vivo* was tested using the bimolecular fluorescence complementation **(**BiFC) technique. For that, the N terminus of YFP was cloned upstream of HOS1 (YFNHOS1) and the C terminus of YFP was fused C-terminally in-frame to CO (COYFC). By *Agrobacterium tumefaciens* coinfiltration, these constructs were transiently expressed in abaxial epidermal cells of tobacco leaves (Voinnet et al., 2003). Reconstitution of YFP fluorescence was examined by confocal microscopy two days after transient coexpression of the protein pairs. Yellow fluorescence in the nucleus was detected for coexpression of COYFC and YFNHOS1, while no yellow fluorescence was observed when COYFC was coexpressed with YFN alone, or when YFC alone was coexpressed with YFNHOS1, as negative controls (Figure 6B). As a positive control, the interaction between amino and carboxy parts of AKINβ and AKIN10 SnRKs proteins (Ferrando et al., 2001), was tested (Figure 6B). CO-HOS1 interaction could be observed in conspicuous nuclear speckles, which have been often associated with foci of proteasome degradation, as previously described for the interaction between CO and COP1 (Jang et al., 2008). These results demonstrate that CO and HOS1 colocalize and physically interact in the nuclei of plant cells.

It has been reported that HOS1 has auto-ubiquitination ligase activity *in vitro* and that it can also mediate the ubiquitination and degradation of ICE1 transcription factor (Dong et al., 2006a). To further analyze whether HOS1 may also regulate CO degradation *in vivo*, we transformed a construct constitutively expressing *CO* fused to *LUC* into wild-type Arabidopsis plants. One representative line, *35S::CO–LUC* 6-2, displaying an early flowering phenotype, was introduced into *hos1*-3 by genetic crossing. We found that the *35S::CO–LUC* 6-2/*hos1-3* plants flowered earlier than either the *hos1-3* mutant or the *35S::CO–LUC* 6-2 plants, indicating that CO–LUC construct was fully functional (Supplemental Table 3). Using luciferase fluorescence *in vivo* imaging, we found that under LDs the CO protein levels were significantly lower in the wild-type than in the *hos1* mutant background three hours after dawn (ZT3) (Figure 6C), suggesting that the degradation of CO that occurs in the wild-type is impaired in the *hos1* mutant. Quantification of luciferase activity corroborated that CO protein accumulated to higher levels in the *hos1* mutant than in the wild type plants (Figure 6D). This accumulation of CO protein observed at ZT3 correlates with the early peak of *FT* expression present in the *hos1* mutant (Figure 5C and D and Supplemental Figure 3). To further assess the role of HOS1 in CO regulation, we performed western blot assays to detect CO protein in nuclear extracts from wild type and *hos1-3* plants grown under LD photoperiods. In these immunoblots, CO protein was present at lower abundance in the wild type than in the *hos1-3* mutant plants, particularly during the daylight period (Figure 6E). From these data, we conclude that HOS1 is involved in the photoperiodic regulation of flowering through the modulation of CO protein levels *in vivo*.

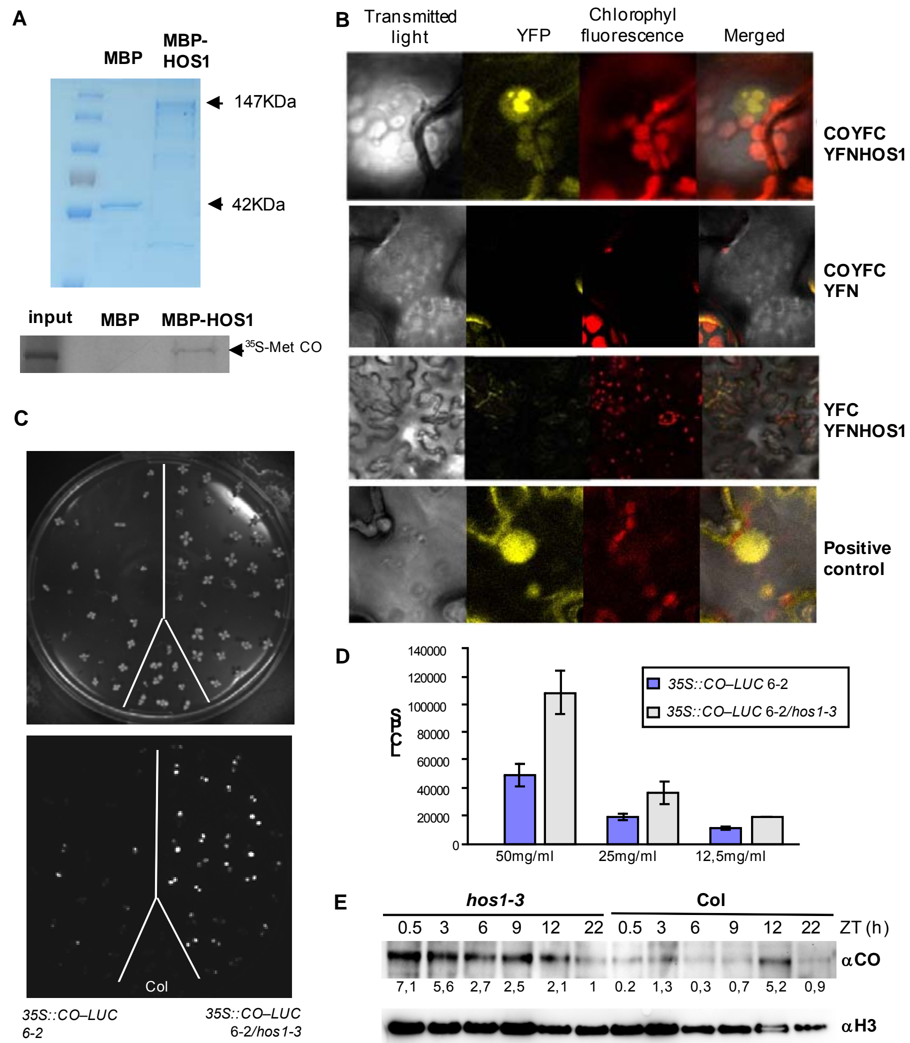


Figure 6. HOS1 interacts with CO and regulates its abundance.

(A) HOS1 and CO interact *in vitro*. A Coomassie stained SDS-PAGE showing MBP (42KDa) and MBP-HOS1 fusion protein (147KDa) expressed in *Escherichia coli BL21* Rosetta strain and purified on amylose resin is shown in the upper panel. The lower panel shows the result of a pull-down assay with MBP and MBP-HOS1 proteins incubated with ^{35}S-Met-labelled CO protein. Retained CO protein was visualized after autoradiography of the dried gel. (B) HOS1 and CO interact *in vivo.* BiFC assay co-expressing the C terminus of YFP fused to CO (COYFC) and the N terminus of YFP to HOS1 (YFNHOS1) in tobacco leaves. Yellow fluorescence in the nucleus was indicative of interaction. Negative (middle panels) and positive (lower panel) controls were included in the assay. (C) Non invasive *in vivo* luciferase imaging of *35S::CO–LUC* 6-2 and *35S::CO–LUC* 6-2/*hos1-3* seedlings. Pictures show 7 day-old seedlings grown in LDs 3 h after the lights are on. At this time, more CO-LUC protein accumulates in *hos1* mutant (below right) than in the wild type (below left). (D) Quantification of the luciferase activity in *35S::CO–LUC* 6-2 (blue bars) and *35S::CO–LUC* 6-2/*hos1-3* (grey bars) seedlings expressed as luciferase counts per second (LCPS) in serial dilutions of fresh tissue in Steadylite Plus Reagent (mg/ml). (E) Immunoblot showing CO protein levels during a 24 h time course in nuclear extracts from Col and *hos1-3* plants grown under LDs. Numbers above each lane represent hours after dawn that the sample was harvested. Histone H3 was used as a loading control. Relative quantification of each band compared to the control is expressed below the upper panel (α-CO).

HOS1 is a nuclear-localized protein

HOS1 protein is ubiquitously expressed in all plant tissues (Lee at al., 2001). Computer analysis of the HOS1 amino acid sequence predicted a nuclear localization signal in the C-terminus of the protein (Supplemental Figure 2). Previous reports localized HOS1 into the cytoplasm of transgenic Arabidopsis seedlings overexpressing a *HOS1-GFP* construct, grown under dark conditions at normal growth temperature. However, in response to low temperature treatments, HOS1-GFP accumulated in the nucleus (Lee at al., 2001). To determine whether the subcellular localization of HOS1 was altered by light/dark conditions, we fused *GFP* C-terminally in frame to *HOS1* and overexpressed this construct in the *hos1-3* mutant. The homozygous line *35S::HOS1-GFP/hos1-3* 4-1-4 showed a delay in flowering time when compared with *hos1*-3, indicating that the fusion protein was functional in the repression of flowering (Supplemental Table 4). Subsequently, we grew *35S::HOS1-GFP/hos1-3* 4-1-4 transgenic plants at 22ºC under both continuous light and dark, and analyzed GFP fluorescence in root cells by confocal microscopy. As shown in Figure 7, HOS1-GFP was clearly targeted to the nucleus, independently of the light growing conditions. The nuclear localization of HOS1-GFP is consistent with the results of the BiFC assay described above (Figure 6B) and with the detection of CO and other HOS1 targets in the nucleus (Valverde et al., 2004; Dong et al., 2006a).

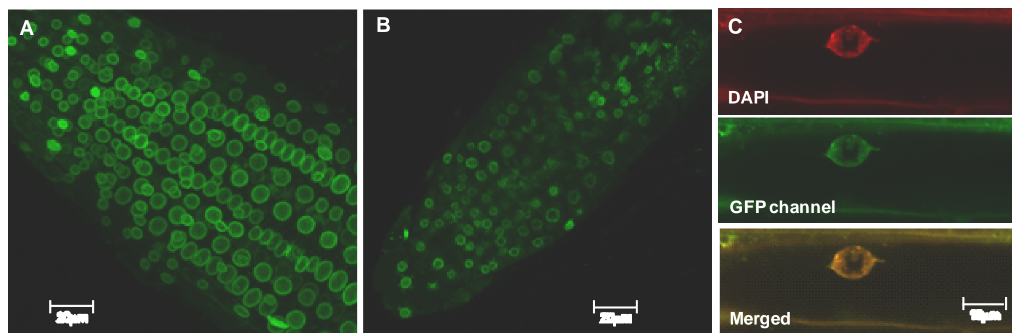


Figure 7. Nuclear localization of HOS1 protein.
Localization of HOS1-GFP in the root cells of 10-day old *35S::HOS1-GFP/hos1-3* plants analysed under confocal microscopy (A) Plants grown under continuous light. (B) Plants grown in darkness. (C) A representative nuclear image of a light-grown seedling showing DAPI staining (upper panel), GFP fluorescence (middle panel) and the merge of both images (lower panel).

HOS1 interacts synergistically with COP1 in the control of flowering time

COP1 E3 ubiquitin ligase has been involved in the degradation of CO protein during the night (Jang et al., 2008; Liu et al., 2008b). However, CO degradation in the morning occurs independently of COP1 (Jang et al., 2008), and for this reason, we speculate that HOS1 may be involved in this process. In our conditions, *cop1-4* mutants flowered dramatically earlier than wild type and *hos1* plants under SDs. However, under LDs *cop1-4* mutants flowered earlier than Col, but later than *hos1* plants (Figure 8A and Table 1). To test the effect of abolishing the activity of both HOS1 and COP1 in the control of flowering time, *hos1* and *cop1* mutations were combined. Interestingly, the *hos1-3 cop1-4* double mutant flowered earlier than both parents in LD and SD, displaying the same number of leaves in both photoperiodic conditions (Figure 8A and Table 1). This result indicates that the combination of both mutations renders a plant with a complete loss of photoperiod sensitivity, and that *HOS1* and *COP1* genes are functionally related in the control of flowering time.

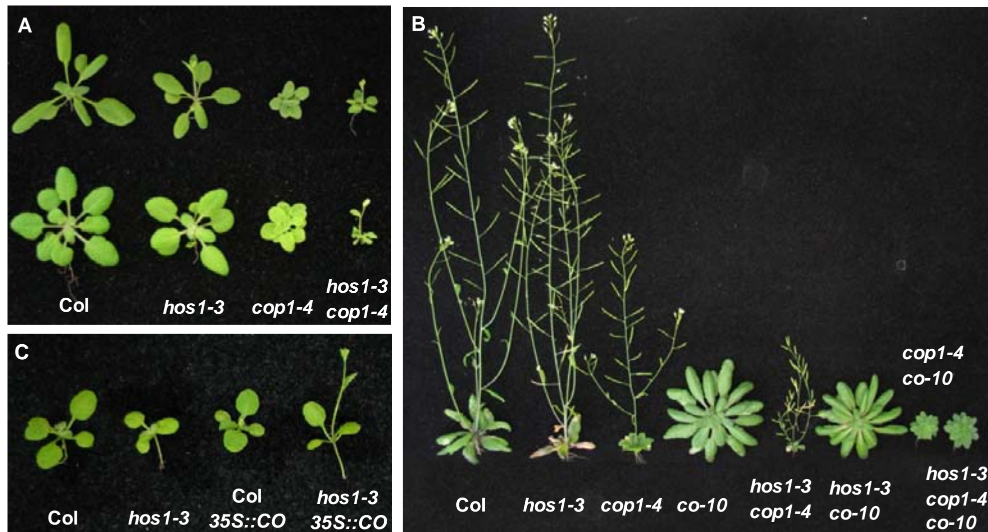


Figure 8. Genetic interaction between *HOS1* and *COP1* in the control of flowering time.
(A) Flowering time phenotype of *hos1-3 cop1-4* double mutant in LD (upper panel) and SD (lower panel) conditions. (B) Flowering time phenotype of double and triple mutant combinations between *hos1-3 co-10 cop1-4* mutants grown in LD conditions. (C) Comparison of flowering time phenotype between LD-grown Col and *hos1-3* plants bearing a *35S::CO* transgene.

To further investigate the genetic interaction between *HOS1*, *COP1* and *CO* genes in controlling flowering time of Arabidopsis, a *hos1 cop1 co* triple mutant was generated and its flowering time was compared with that of *hos1 co* and *cop1 co* double mutants (Figure 8B and Table 1). Under LDs, the *hos1-3 co-10* and the *cop1-4 co-10* plants flowered with 12 and 10 leaves less than *co-10* respectively, indicating that part of the early flowering phenotype of the *hos1-3* and *cop1-4* mutants in Col background occurs independently of *CO*. This result appears to be in contrast with the epistatic genetic relationship observed between *hos1-2* and *co-2* alleles in L*er* background (Figure 4C and Table 1), and can be explained because *hos1* mutation is downregulating *FLC* expression (Figure 3B) and *FLC* is expressed at higher levels in Col than in L*er* (Michaels and Amasino, 1999). Besides, the *hos1 cop1 co* triple mutant flowered with 15 leaves more than the *hos1 cop1* double mutant under LD conditions, demonstrating that *co* mutation notably delays the *hos1 cop1* early flowering phenotype in Col background. Interestingly, the *hos1 cop1 co* triple mutant formed 6 and 9 leaves less than *hos1 co* and *cop1 co* double mutants respectively (Figure 8B and Table 1), confirming the existence of a synergistic genetic interaction between *hos1* and *cop1*, even in the absence of *CO*.

Because *HOS1* seems to exert an effect as a negative regulator of *CO*, we tested whether the extremely early flowering of *35S::CO* plants (Simon et al., 1996) could be further accelerated by the *hos1-3* mutation. To test this hypothesis, the *35S::CO* transgene was introduced into wild type Col and into *hos1-3* mutant plants. Although the number of leaves at flowering for both transgenic plants was very similar, we observed that the *hos1-3 35S::CO* plants bolted consistently earlier than the Col *35S::CO* (Figure 8C and Table 1), supporting a role for *HOS1* in repressing the promotion of flowering mediated by *CO*.

DISCUSSION

In many plants, changes in daylength regulate the transition from vegetative growth to flowering, and plants altered in the daylength-sensing mechanism cannot time flowering properly

in natural environments (Wilczek et al., 2009). In this work, we have demonstrated through both genetic and biochemical approaches that *HOS1* is involved in the photoperiodic control of flowering time. The *esd6/hos1* mutant was identified through a screening devoted to the isolation of early flowering mutants in Arabidopsis. The characterization of these mutants allows unveiling the mechanisms of action of genes involved in the repression of the floral transition and suggests that a large number of genes participate in this process (Pouteau et al., 2004). Besides precocious flowering, the *hos1* mutant showed pleiotropic alterations of leaf, flower and root development, similarly to those displayed by other early flowering mutants (Martin-Trillo et al., 2006; del Olmo et al., 2010).

In Arabidopsis, the flowering response to changes in photoperiod rely on the interaction of light with the circadian clock-regulated rhythmic expression of *CO* (Suarez-Lopez et al., 2001). Besides this transcriptional regulation, a light-dependent regulation of CO protein stability has also been described (Valverde et al., 2004; Kim et al., 2008). We have demonstrated that HOS1 is involved in regulating CO protein abundance *in vivo* (Figure 6), ensuring that CO activation of *FT* only occurs at the appropriate times of the day under inductive photoperiods in Arabidopsis. HOS1 has been reported to work as an E3 ubiquitin ligase that mediates the degradation of ICE1 transcription factor (Dong et al., 2006a), and we have demonstrated that HOS1 interacts *in vitro* and *in vivo* with CO (Figure 6). In addition, *hos1* mutation altered *FT* expression pattern in LD, showing a peak of expression in the subjective morning (Figure 5C and D). Based on these observations, we speculate that HOS1 could mediate CO degradation during the daylight period through a mechanism involving ubiquitination, and that the timing of HOS1 activity is crucial to establish a photoperiodic flowering response (Figure 9). Both the genetic analysis between *CO* and *HOS1* genes and the expression analyses performed involving *CO* transcript and CO protein, support this hypothesis.

Other E3 ubiquitin ligases have been proposed to be involved in the control of flowering time (Cao et al., 2008; Vega-Sanchez et al., 2008; Park et al., 2010). In particular, DAY NEUTRAL FLOWERING (DNF) and COP1 have been demonstrated to regulate the precise pattern of *CO* expression at the transcriptional and the posttranscriptional level respectively (Jang et al., 2008; Liu et al., 2008b; Morris et al., 2010). DNF is an important regulator of the rhythm of *CO* expression, but it is not acting through the GI/FKF1/CDFs regulatory mechanism (Morris et al., 2010). Increased CO transcript in the *dnf* mutant around ZT 4-6 results in an earlier induction of *FT* under SD (Morris et al., 2010). On the other hand, CO protein is degraded in the dark by the SUPPRESSOR OF PHYA-105 1 (SPA1)-COP1 complex (Laubinger et al., 2006; Jang et al., 2008; Liu et al., 2008b). Besides, it has been recently demonstrated that the Arabidopsis CULLIN4 E3 RING ligase bound to Damaged DNA binding protein 1 (DDB1) interacts with SPA1-COP1 complex to regulate flowering time (Chen et al., 2010). We have demonstrated that *HOS1* also interacts genetically with *COP1* in the photoperiodic control of flowering time (Figure 8A). Interestingly, *hos1 cop1* double mutants are completely insensitive to photoperiod, and *co* mutations notably delay the early flowering phenotype of the *hos1 cop1* double mutant (Figure 8B). This can be interpreted as HOS1 and COP1 being functionally related proteins in the control of flowering time, regulating CO abundance during the day and in the night, respectively (Figure 9). This is consistent with the observation that the absence of both E3 ubiquitin ligases renders plants unable to distinguish between LDs and SDs. It has been proposed that a phyB-dependent mechanism occurring early in the day may promote CO degradation as well, but the E3 ubiquitin ligase(s) involved in this process remains to be identified (Valverde et al., 2004; Jang et al., 2008). Our data are consistent with HOS1 playing a crucial role in preventing increased CO protein levels and *FT* expression during early hours of the day. Further analyses will be required to establish the possible participation of HOS1 in the proposed phyB-dependent mechanism of CO proteolysis.

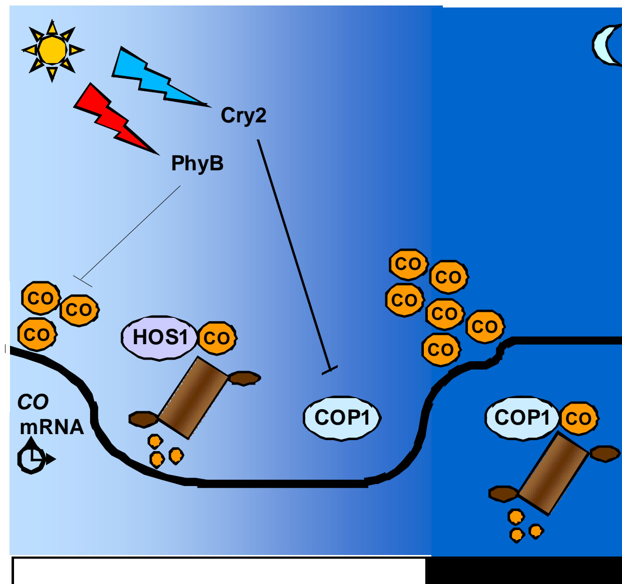


Figure 9. Model for HOS1 function in the photoperiodic control of flowering time.
The transcription of *CO* gene depends primarily on the circadian clock (thick black line). In the evening, the degradation of CDFs by the GI/FKF1 complex allows *CO* transcript levels to increase, and CO protein accumulates due to a photoreceptor-mediated repression of COP1. At this time CO can promote *FT* expression and induce flowering. During the night, COP1 activity causes rapid degradation of CO protein by the ubiquitination/26S proteasome system. In the daylight period HOS1 is required to degrade CO. Additional data will be necessary to establish the possible involvement of HOS1 in the mechanism of CO degradation mediated by PhyB that has been proposed to operate in the morning.

The ability to respond to photoperiod enables plants to anticipate variations in environmental conditions that can be predicted to occur periodically each year. In northern latitudes, shortening daylength in autumn is associated to decreasing cold temperatures while warm temperatures are typical of longer days. In addition to repress the floral transition, HOS1 was previously described as a negative regulator of cold signal transduction (Lee et al., 2001). This suggests that HOS1 might function as an integrative link for both responses, allowing plants to discriminate the duration of the day by regulating CO abundance, and to respond to cold temperatures, by regulating *CBF* (*C-repeat (CRT)-binding factors*) expression through ICE1 degradation (Dong et al., 2006a). Several evidences point to the existence of overlapping pathways for controlling cold stress and flowering time responses in Arabidopsis (Yoo et al., 2007; Seo et al., 2009). The characterization of several mutants altered in cold acclimation responses has uncovered a role of the corresponding genes in flowering time control. *hos9* and *sensitive to freezing 6* (*sf6)* mutants show a late flowering phenotype, while *long vegetative phase 1* (*lov1*) mutant is early flowering (Zhu et al., 2004; Yoo et al., 2007; Knight et al., 2008). These three genes regulate the expression of cold-inducible genes independently of CBFs, and both *LOV1* and *SFR6* control flowering time through the photoperiod pathway. Other mutants, such as *low expression of osmotically responsive genes 4* (*los4*) and *atnup160*, display an early flowering phenotype and altered *CBF* expression levels (Gong et al., 2005; Dong et al., 2006b). On the other hand, *co* and *gi* photoperiod pathway mutants show altered tolerance to freezing temperatures (Cao et al., 2005; Yoo et al., 2007), and *fve* mutant flowers late and shows elevated expression of *FLC* and *CBF* genes (Kim et al., 2004). It has been proposed very recently that in warm late spring SOC1 downregulates *CBFs* expression and promotes flowering, while in cold early spring or fall, induction of *FLC* expression by the CBFs delays flowering and confers cold resistance to the plant (Seo et al., 2009). Besides regulating CO stability, *HOS1* controls *FLC* expression (Figure 3B) (Lee et al., 2001), which is also repressed by

prolonged exposure to cold temperatures (Amasino, 2010). The positive effect of *HOS1* on *FLC* expression appears to be independent of the vernalization pathway (Supplemental Table 2) and awaits to be characterised. Taken together, these results suggest that HOS1, among other genes, may participate in the photoperiod and temperature signal crosstalk, integrating information coming from both pathways and facilitating a proper response to changing environmental conditions. To our knowledge this is the first E3 ubiquitin ligase proposed to integrate both environmental signals, specifically targeting for degradation key factors involved in the regulation of each response. Further studies will be necessary for an in-depth understanding of how these pathways modulate each other´s activity to optimize plant adaptation.

ACKNOWLEDGEMENTS

We thank Dr. I. Ausin (University of California, Los Angeles, USA) for isolating the *esd6* mutant, Dr. R. Catalá (CIB, CSIC, Madrid, Spain) for providing a Gateway-compatible *HOS1* full length cDNA, Dr. J. Salinas (CIB, CSIC, Madrid, Spain) for granting the access to the luciferase imaging system, Dr. P. González-Melendi (CBGP, UPM-INIA, Madrid, Spain) for technical support with confocal microscope and Dr. JC. del Pozo and members of his lab (CBGP, UPM-INIA, Madrid, Spain) for helpful discussions. pYFPN43 and pYFPC43 vectors, and SNF1 protein kinase constructs, used as positive controls in the BiFC assays, were kindly provided by Dr. A. Ferrando (IBMCP, Valencia, Spain). C24 and *hos1-1* mutant seeds, as well as a *HOS1* expression construct in the pMAL vector, were kindly provided by Dr. J.K Zhu (University of California, Riverside, California, USA). The *35S::CO* construct and *co-10 cop1-4* seeds were kindly donated by Dr. G. Coupland (Max Planck Institute, Cologne, Germany), while the *35S::CO-LUC* plasmid was kindly provided by Dr. H-Q Yang (Shanghai Institute for Biological Sciences, Chinese Academy of Science, Shanghai, China). This work was supported by grants BIO2008-00351 to JAJ, BIO2007-61215 to MP, CSD2007-00057 and BIO2010-15589 to JAJ and MP, and BIO2007-61837 and BIO2010-16027 to FV from the Spanish Ministerio de Ciencia e Innovación.

BIBLIOGRAPHY

Amasino, R. (2010). Seasonal and developmental timing of flowering. Plant J **61,** 1001-1013.

An, H., Roussot, C., Suarez-Lopez, P., Corbesier, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., and Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131,** 3615-3626.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., and Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet **36,** 162-166.

Cao, S., Ye, M., and Jiang, S. (2005). Involvement of *GIGANTEA* gene in the regulation of the cold stress response in Arabidopsis. Plant Cell Rep **24,** 683-690.

Cao, Y., Dai, Y., Cui, S., and Ma, L. (2008). Histone H2B monoubiquitination in the chromatin of *FLOWERING LOCUS C* regulates flowering time in Arabidopsis. Plant Cell **20,** 2586-2602.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316,** 1030-1033.

Chen, H., Huang, X., Gusmaroli, G., Terzaghi, W., Lau, O.S., Yanagawa, Y., Zhang, Y., Li, J., Lee, J.H., Zhu, D., and Deng, X.W. (2010). Arabidopsis CULLIN4-Damaged DNA Binding Protein 1 Interacts with CONSTITUTIVELY PHOTOMORPHOGENIC1-SUPPRESSOR OF PHYA Complexes to Regulate Photomorphogenesis and Flowering Time. Plant Cell **22,** 108-123.

Chiang, G.C., Barua, D., Kramer, E.M., Amasino, R.M., and Donohue, K. (2009). Major flowering time gene*, FLOWERING LOCUS C*, regulates seed germination in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **106,** 11661-11666.

de Montaigu, A., Toth, R., and Coupland, G. (2010). Plant development goes like clockwork. Trends Genet **26**, 296-306.

del Olmo, I., Lopez-Gonzalez, L., Martin-Trillo, M.M., Martinez-Zapater, J.M., Pineiro, M., and Jarillo, J.A. (2010). *EARLY IN SHORT DAYS 7* (*ESD7*) encodes the catalytic subunit of DNA polymerase epsilon and is required for flowering repression through a mechanism involving epigenetic gene silencing. Plant J **61,** 623-636.

Deng, X.W., Caspar, T., and Quail, P.H. (1991). *COP1*: a regulatory locus involved in lightcontrolled development and gene expression in Arabidopsis. Genes Dev **5,** 1172-1182.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem **78,** 399-434.

Dong, C.H., Agarwal, M., Zhang, Y., Xie, Q., and Zhu, J.K. (2006a). The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A **103,** 8281-8286.

Dong, C.H., Hu, X., Tang, W., Zheng, X., Kim, Y.S., Lee, B.H., and Zhu, J.K. (2006b). A putative Arabidopsis nucleoporin, AtNUP160, is critical for RNA export and required for plant tolerance to cold stress. Mol Cell Biol **26,** 9533-9543.

Ferrando, A., Koncz-Kalman, Z., Farras, R., Tiburcio, A., Schell, J., and Koncz, C. (2001). Detection of in vivo protein interactions between Snf1-related kinase subunits with intron-tagged epitope-labelling in plants cells. Nucleic Acids Res **29,** 3685-3693.

Fornara, F., de Montaigu, A., and Coupland, G. (2010). SnapShot: Control of flowering in Arabidopsis. Cell **141,** 550, 550 e551-552.

Fornara, F., Panigrahi, K.C., Gissot, L., Sauerbrunn, N., Ruhl, M., Jarillo, J.A., and Coupland, G. (2009). Arabidopsis DOF transcription factors act redundantly to reduce *CONSTANS* expression and are essential for a photoperiodic flowering response. Dev Cell **17,** 75-86.

Gong, Z., Dong, C.H., Lee, H., Zhu, J., Xiong, L., Gong, D., Stevenson, B., and Zhu, J.K. (2005). A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in Arabidopsis. Plant Cell **17,** 256-267.

Guo, H., Yang, H., Mockler, T.C., and Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. Science **279,** 1360-1363.

He, Y., Michaels, S.D., and Amasino, R.M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science **302,** 1751-1754.

Imaizumi, T. (2010). Arabidopsis circadian clock and photoperiodism: time to think about location. Curr Opin Plant Biol **13,** 83-89.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A., and Kay, S.A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of *CONSTANS* in Arabidopsis. Science **309,** 293-297.

Jackson, S.D. (2009). Plant responses to photoperiod. New Phytol **181,** 517-531.

Jaeger, K.E., andWigge, P.A. (2007). FT protein acts as a long-range signal in Arabidopsis. Curr Biol **17,** 1050-1054.

Jang, S., Torti, S., and Coupland, G. (2009). Genetic and spatial interactions between *FT*, *TSF* and *SVP* during the early stages of floral induction in Arabidopsis. Plant J **60,** 614-625.

Jang, S., Marchal, V., Panigrahi, K.C., Wenkel, S., Soppe, W., Deng, X.W., Valverde, F., and Coupland, G. (2008). Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J **27**, 1277-1288.

Jin, J.B., Jin, Y.H., Lee, J., Miura, K., Yoo, C.Y., Kim, W.Y., Van Oosten, M., Hyun, Y., Somers, D.E., Lee, I., Yun, D.J., Bressan, R.A., and Hasegawa, P.M. (2008). The SUMO E3 ligase, AtSIZ1, regulates flowering by controlling a salicylic acid-mediated floral promotion pathway and through affects on *FLC* chromatin structure. Plant J **53,** 530-540.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science **290,** 344-347.

Kim, H.J., Hyun, Y., Park, J.Y., Park, M.J., Park, M.K., Kim, M.D., Lee, M.H., Moon, J., Lee, I., and Kim, J. (2004). A genetic link between cold responses and flowering time through FVE in *Arabidopsis thaliana*. Nat Genet **36,** 167-171.

Kim, S.Y., Yu, X., and Michaels, S.D. (2008). Regulation of *CONSTANS* and *FLOWERING LOCUS T* expression in response to changing light quality. Plant Physiol **148,** 269-279.

Knight, H., Thomson, A.J., and McWatters, H.G. (2008). *SENSITIVE TO FREEZING 6* integrates cellular and environmental inputs to the plant circadian clock. Plant Physiol **148,** 293-303.

Kobayashi, Y., and Weigel, D. (2007). Move on up, it's time for change mobile signals controlling photoperiod-dependent flowering. Genes Dev **21,** 2371-2384.

Koornneef, M. (1991). Isolation of higher plant developmental mutants. Symp Soc Exp Biol **45,** 1-19.

Koornneef, M., Alonso-Blanco, C., Peeters, A.J., and Soppe, W. (1998). Genetic Control of Flowering Time in Arabidopsis. Annu Rev Plant Physiol Plant Mol Biol **49,** 345-370.

Kumimoto, R.W., Zhang, Y., Siefers, N., and Holt, B.F., 3rd. (2010). NF-YC3, NF-YC4 and NF-YC9 are required for CONSTANS-mediated, photoperiod-dependent flowering in *Arabidopsis thaliana*. Plant J **63**, 379-391.

Laubinger, S., Marchal, V., Le Gourrierec, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G., and Hoecker, U. (2006). Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development **133,** 3213-3222.

Lazaro, A., Gomez-Zambrano, A., Lopez-Gonzalez, L., Pineiro, M., and Jarillo, J.A. (2008). Mutations in the Arabidopsis *SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development. J Exp Bot **59**, 653-666.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu, J.K. (2001). The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo-cytoplasmic partitioning. Genes Dev **15,** 912-924.

Lee, I., and Amasino, R.M. (1995). Effect of Vernalization, Photoperiod, and Light Quality on the Flowering Phenotype of Arabidopsis Plants Containing the *FRIGIDA* Gene. Plant Physiol **108,** 157-162.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002). Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science **297,** 243-246.

Liu, H., Yu, X., Li, K., Klejnot, J., Yang, H., Lisiero, D., and Lin, C. (2008a). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science **322,** 1535-1539.

Liu, L.J., Zhang, Y.C., Li, Q.H., Sang, Y., Mao, J., Lian, H.L., Wang, L., and Yang, H.Q. (2008b). COP1-Mediated Ubiquitination of CONSTANS Is Implicated in Cryptochrome Regulation of Flowering in Arabidopsis. Plant Cell **20**, 292-306.

Martin-Trillo, M., Lazaro, A., Poethig, R.S., Gomez-Mena, C., Pineiro, M.A., Martinez-Zapater, J.M. and Jarillo, J.A. (2006). *EARLY IN SHORT DAYS 1* (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in Arabidopsis. Development **133,** 1241-1252.

Michaels, S.D. (2009). Flowering time regulation produces much fruit. Curr Opin Plant Biol **12,** 75-80.

Michaels, S.D., and Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.

Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.J., and Hasegawa, P.M. (2007). SIZ1-Mediated Sumoylation of ICE1 Controls *CBF3/DREB1A* Expression and Freezing Tolerance in Arabidopsis. Plant Cell **19,** 1403-1414.

Miura, K., Rus, A., Sharkhuu, A., Yokoi, S., Karthikeyan, A.S., Raghothama, K.G., Baek, D., Koo, Y.D., Jin, J.B., Bressan, R.A., Yun, D.J., and Hasegawa, P.M. (2005). The Arabidopsis SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proc Natl Acad Sci U S A **102,** 7760-7765.

Moran, R. (1982). Formulae for determination of chlorophyllous pigments extracted with n,ndimethylformamide. Plant Physiol **69,** 1376-1381.

Morris, K., Thornber, S., Codrai, L., Richardson, C., Craig, A., Sadanandom, A., Thomas, B., and Jackson, S. (2010). DAY NEUTRAL FLOWERING represses *CONSTANS* to prevent Arabidopsis flowering early in short days. Plant Cell **22,** 1118-1128.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). *FKF1*, a clock controlled gene that regulates the transition to flowering in Arabidopsis. Cell **101,** 331-340.

Park, B.S., Eo, H.J., Jang, I.C., Kang, H.G., Song, J.T., and Seo, H.S. (2010). Ubiquitination of LHY by SINAT5 regulates flowering time and is inhibited by DET1. Biochem Biophys Res Commun **398**, 242-246.

Pineiro, M., Gomez-Mena, C., Schaffer, R., Martinez-Zapater, J.M., and Coupland, G. (2003). EARLY BOLTING IN SHORT DAYS is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. Plant Cell **15,** 1552-1562.

Pouteau, S., Ferret, V., Gaudin, V., Lefebvre, D., Sabar, M., Zhao, G., and Prunus, F. (2004). Extensive phenotypic variation in early flowering mutants of Arabidopsis. Plant Physiol **135,** 201-211.

Reed, J.W., Nagpal, P., Poole, D.S., Furuya, M., and Chory, J. (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. Plant Cell **5,** 147-157.

Roux, F., Touzet, P., Cuguen, J., and Le Corre, V. (2006). How to be early flowering: an evolutionary perspective. Trends Plant Sci **11,** 375-381.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

Sawa, M., Nusinow, D.A., Kay, S.A., and Imaizumi, T. (2007). FKF1 and GIGANTEA Complex Formation is Required for Day-Length Measurement in Arabidopsis. Science **318**, 261-265.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R.A., and Coupland, G. (2006). The transcription factor *FLC* confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev **20,** 898-912.

Seo, E., Lee, H., Jeon, J., Park, H., Kim, J., Noh, Y.S., and Lee, I. (2009). Crosstalk between Cold Response and Flowering in Arabidopsis Is Mediated through the Flowering-Time Gene *SOC1* and Its Upstream Negative Regulator *FLC*. Plant Cell **21**, 3185-3197.

Simon, R., Igeno, M.I., and Coupland, G. (1996). Activation of floral meristem identity genes in Arabidopsis. Nature **384,** 59-62.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., and Coupland, G. (2001). *CONSTANS* mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410,** 1116-1120.

Sung, S., and Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature **427,** 159-164.

Telfer, A., Bollman, K.M., and Poethig, R.S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. Development **124,** 645-654.

Tiwari, S.B., Shen, Y., Chang, H.C., Hou, Y., Harris, A., Ma, S.F., McPartland, M., Hymus, G.J., Adam, L., Marion, C., Belachew, A., Repetti, P.P., Reuber, T.L., and Ratcliffe, O.J. (2010). The flowering time regulator CONSTANS is recruited to the *FLOWERING LOCUS T* promoter via a unique cis-element. New Phytol **187,** 57-66.

Turck, F., Fornara, F., and Coupland, G. (2008). Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol **59,** 573-594.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., and Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science **303,** 1003-1006.

Vega-Sanchez, M.E., Zeng, L., Chen, S., Leung, H., and Wang, G.L. (2008). SPIN1, a K homology domain protein negatively regulated and ubiquitinated by the E3 ubiquitin ligase SPL11, is involved in flowering time control in rice. Plant Cell **20,** 1456-1469.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J **33,** 949-956.

Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A., and Coupland, G. (2006). CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. Plant Cell **18,** 2971-2984.

Wilczek, A.M., Roe, J.L., Knapp, M.C., Cooper, M.D., Lopez-Gallego, C., Martin, L.J., Muir, C.D., Sim, S., Walker, A., Anderson, J., Egan, J.F., Moyers, B.T., Petipas, R., Giakountis, A., Charbit, E., Coupland, G., Welch, S.M., and Schmitt, J. (2009). Effects of genetic perturbation on seasonal life history plasticity. Science **323,** 930-934.

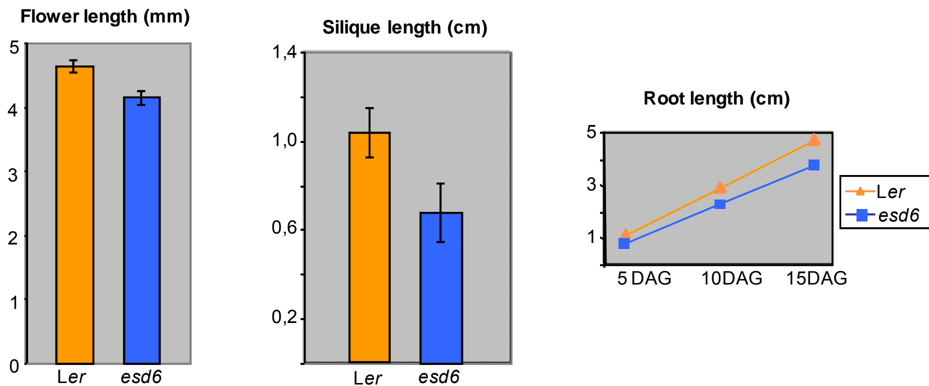
Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., and Ahn, J.H. (2005). *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in Arabidopsis. Plant Physiol **139,** 770-778.

Yoo, S.Y., Kim, Y., Kim, S.Y., Lee, J.S., and Ahn, J.H. (2007). Control of flowering time and cold response by a NAC-domain protein in Arabidopsis. PLoS ONE **2,** e642.

Yu, J.W., Rubio, V., Lee, N.Y., Bai, S., Lee, S.Y., Kim, S.S., Liu, L., Zhang, Y., Irigoyen, M.L., Sullivan, J.A., Zhang, Y., Lee, I., Xie, Q., Paek, N.C., and Deng, X.W. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. Mol Cell **32,** 617-630.

Zhu, J., Shi, H., Lee, B.H., Damsz, B., Cheng, S., Stirm, V., Zhu, J.K., Hasegawa, P.M., and Bressan, R.A. (2004). An Arabidopsis homeodomain transcription factor gene, *HOS9*, mediates cold tolerance through a CBF-independent pathway. Proc Natl Acad Sci U S A **101,** 9873-9878.

SUPPLEMENTAL FIGURES

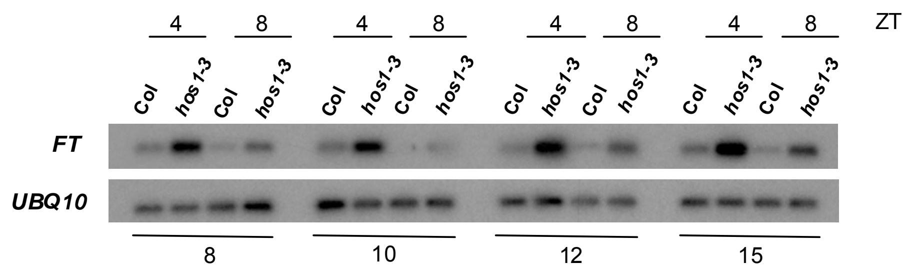


Supplemental Figure 1. Flower, silique and root length measurement in L*er* and *esd6*. Data were scored on an average of 10 plants grown under LD conditions.

```
AtHOS1 : MDTREING---------FASAARSISL-PTQPNYSSKPVQEALKHLASINLRELCNEAKVERCRATRDLASCGRFVNYVLNPCGHASLCTECCQRCDVCPICRSTLPKFGDRLRLRLYYECVEAGLISRTHEEASQDSDEDEHQLAADVHRLYSLFDVAMNNN : 153
RcHOS1 : MERNAVNGRISPSTSADRGKTSRSTTMHIQ-PNYTSRAVQEALEHLASIDLIELCSEAKVERCRAIRDLRSCGRYVQSVLVSCGHASLCSECSQRCDLCPICRVPIPKNSNRLRLRLYYECIEAGLISKKYDERFQEKDDGDNQLTADVQRLYSLFDVSMENN : 162
PtHOS1 : -----MNGRVSPSSSTDCGGTARFTAS-LSQPNYSSRAVQEALEHLASIDLIELCSEAKVERCRATRDLRSCGRYVQYVLNSCSHASLCSECSQRCDICPICRIPIPKTGIRLRPRLYYECIESGLVSKRCDERFQEKEDADNELTTDVQRLYSLFDVALENN : 157
CtHOS1 : MDRSEINGPNLSSISSDTGVSARSPP---P-PNYNSRAVQEALEHLASIDLCELRYEAKVEHCRATRDLRSCGRYVQYVLNSCGHASLCAECSQRCDFCPICRIPVPKNRNSITLRLYDECVEAGLILKRCEEGYHDFEDAENQITADVQRLYSLFDTALENN : 159
VvHOS1 : MERTRFSGSPVSSDSTGIAAAARSVSSHLPQPNYGSRVVQEALEHLASIDLIELCNEAKVERCRATRDLSSCGRYVQHVLNSCGHASLCAECSQRCDVCPICRMPIPKNGNKLRCRLYYECIEAGLISKRYDDRFQEKDDSEKQQTADVQRLYSLFDVAMENN : 163
OsHOS1 : MNALSI---------------FREIQSGIHFGNLHTYPTN-ALEQLASIDLIELCKEARIEHCRATRDLSSCGRYVQHVLNSCGHASLCAECSQRCDVCPICRSPIPDTGNRVRLRLYYKCLEAGLISKQHDERFQEKEDHSDPVNLDVQRLHSLFDVALQNN : 147
PpHOS1 : MADKFSNF----AQSFQVGPVDTPAAF-----SGSGPKREAALERLALLEPRELSAEAKTETCRATRDLRSCGRAVHHVVSTCGHACLCVECLQRTISCPICRTPTGSSGQH-QLRLYDECVDAGLIQKNSDLLSE---------GADVGRLYSFFDVALDNS : 144

AtHOS1 : LISVVCHY--------------------------ITNVCMDETAVSSDPVIAFLLDEVVVKDWVKRTFRSTLAELQEIYNLETKEMQAWLDKLLRCSKQVAGICSVLEVMESAFKGS-VSPQLQDVQTLRENIGK---------------------------- : 261
RcHOS1 : LVSLICHY--------------------------VTDVCMDETAVSSDPVVAILLDEVVVKDWCKQTFRNIVLELQGIYNLEAEEMKTRLNFLVKFSVRLAGLSDVLEVLESSFKGN-LSARLHDLQLLQESILK---------------------------- : 270
PtHOS1 : LVSLICHY--------------------------VTDVCMDESAVSSDPVIAFLLDEVVVKDWCKRTFKNIIAELQGIYNLETEEMKTRLSLLLKLSVHLVGISNVLEVLELSFKDS-LSAQLHDLQLLQENILK---------------------------- : 265
CtHOS1 : LISLICHY--------------------------VRDVCMDEAAVSSDPVVAFLLDEVVVKDWCKRAFKNIIAELKLIYNLEVEVIKTRLSLLLKFQMKLRDISSVIEVLASSFKDD-LSAQVHDLHHFQESILK---------------------------- : 267
VvHOS1 : LVCLICHY--------------------------VTDVCMDESAVSSDPVIAFLLDEVVVKDWCKRTFRNIITELQGIYNLEVEEMKTRLSLLLKFSVQLAGVASVLEVLESSFKGT-ISSQLHDLHQLQESILK---------------------------- : 271
OsHOS1 : LASLICHY--------------------------TTDVCLDENAVSSDPLLAFLLDEVVIKEWCKKAVNALISEINMIYRSGLEMMSKLSQLQKFAVQLAGISSVVEVMITSFSEA-VSAHVNDLHQLMESTLK---------------------------- : 255
PpHOS1 : LVSLICHCILFTSLSCASLLIMSDKFYLDTGESDVAEVCMDEGAVSSNAMVSILLD-----------GDNLSPRQMIFKLDTIEWKGR---------KLGGVIHVLEALEAPLVDQPLSPTLYELRQLLESIRKVVQIVTPVRAFDGLSFKFFVVVTYILRC : 286

AtHOS1 : TKQHLDIMVWCIRHGFLDDVRSRYSNFTSWNALVGERKSNAVKRAWPDAVDQS-SDCSVQSASLFIEDALENLER-EPEYSQEIGAD--LEVGRLQKD----K--RSFLRSKIEGTS-GSYPFENLRTAADMLFLHGGSDLVVAKQAIFLYYLFDRHWTTPEK : 413
RcHOS1 : TKQHMEIMKWCIKHQFLENIKSRHANFSSWRSIVRERKSAAITRSWPDIINQS-ADSSMQTGSLFIEDALSNLEI-EQGYLQDIRED--LELASLQKD----R--GSFFRSKIEGVA-GCYPFESLRAAVDVLFLHGSSDLVVAKQAILLYFLFDRYWTMPDE : 422
PtHOS1 : AKQHMEIIAWCVRHHFLENVGSRYSNLSSWRSVVLERKSAAIKRSWPDVPNQS-AESSMQAGSLFIEDALANLEI-DQGHMQEKGEE--SELALLLKD----G--RLFFRSKIEGLA-VCYPFENLRAAADVLFLHGSSDLLLAKQAIFLYYLFDRHWAMPDE : 417
CtHOS1 : TKQHLEIMMWCAKHQFLENVRSRHASSTSWHSLVRQRKSAATERAWYDPVNYS-AESTKQDGSLFIEDALANLEI-EQEFTQGRGEK--LDITSLHKD----DEGSSFVRSKIEGVS-GCYPFENLRAAVDILFLHGSSDLVLAKQAIFLYYLFDRHWTMPDE : 421
VvHOS1 : TKQHMEIMIWCIRHQFLENVRSRYSKFSSWRSLVRERKSAAIQRSWPDSVDHT-AEPTKECGTLFIEDALLNLEI-DQGRAQEMGEE--SEVASLQKD----G-GSTFFRSKIEGLA-GCYPFENMRAAADILFLSGSSDLVVAKQAIFLYYLFDRHWTMPDE : 424
OsHOS1 : AKQHLEAMMWCIRHKFLEDIPSRHTNLASWSSDVIKRKADAKERKWPEFSDKSSAYNEANQGILFIEQALQNLGI-QESNS-GSEEG--VEIVCLQSE----Q-SSSMFCSTIDQFSVDKYPFKDLREAVDVLFLHGSSDMVIAKQAIFLYYLFDRHWTRPDS : 409
PpHOS1 : ALQHLEVMAWCARHQFLETIRSSYESISQWHTAVKERKLVAQDRAWSVTDKKS-KLGDSQPATLFIEDALANLGLLDDDNEDEMGSEDLPELGWLRQGPTAHTPASLRFRCDEPMQSTSPYPPESVRTAVDLLFLEGACDLVLAKKAIFLYFLFDRHWGLSDE : 448

AtHOS1 : YWKHTIDDFAATFGITRHSLLESFVFYLLDDHSEEALQEACRILPEICGPETYPKVAQVLLERDNPETALMVLRWSGRDG-VS-------ELVSIGEAVTALRVRVECGLLSEAFTYQRTLCLKVKENNLKNGAVKHASDDL--DIWSWTEWMEILVNEFCCL : 566
RcHOS1 : TWRHLIDDFAATFGITRHALLESLAFYLLDDHTDETLKEACHLLPEIGGQTTHPKIAQVLLEREAPEVALMVLRWSGRD--GS-------QMVSLSEAVTAIRVRVECGLLTEAFMHQRMLCTKVKEKKRKDGLPEDASAELKGDCKTWEDWVEVLVTEICCL : 576
PtHOS1 : SWRHIADDFSATFGITRHSLLESLTFYLLDDHTEAALQEACNLLPEISGPSTHPKIAQVLLERKNPETALMVLRWSGHD--GS-------QMVSLNDAVTAVRIRVQCALLTEAFMHQRMLCTKVRENKFKARPPRDASDDLKGECRTWENWVEILVNEICYL : 571
CtHOS1 : NWRHIVDDFAATFSITRHSLLESLTFYLLDDQTDEALQEACHLLPEISGPTTHPKIAQVLLERENPEAALMVLRWSGRDG-GS-------LLVSLSEAVTAVRVRVECALLTEAFTYQRMLCTKVREKKLKFGTIGETFDDLKGGFKTWEQWLEVLVTEICCL : 576
VvHOS1 : KWRHIVDDFAATFSITRHSLLESFTFYLLDDHTDEALQEACLLLPEISGPGTHPKIAQVLLERQNPDAALMVLRWSGHDG-GS-------QLVSLGEAVNAARVRVECALVTEAFMYQRLLCTKIKEKQLRDGLASNVPEVSKGESRTWMDWMETLVTEICCL : 579
OsHOS1 : EWRYLVDDFAATFGISRRTLLECLVFCLLDDHSSHALEEACSLLPKISCKETHPKIAQVLLERHKPDVALVVLKCTGRDTFSSAANIEKDDTESLSEAVTAVRVRIEYGHLTEAFMYHRSYCSKVKEQRSADMSHVEDANSYK---SSWMYHVEVMMTEFCNI : 569
PpHOS1 : KWREVVDDYASIFSIPRHLMLESLVFYLLDDSSDHALEEACQLLPEIASHNTHPKVATVLLERRKPEVALAVLRASGLHGLGSG-------IVPLSDALTTVRVWLQCGLLTEGYSYQRAYVDGVKREE----------------GDWLTSTEVLVSEISRL : 587

AtHOS1 : SIRRNLVDRIIELPWNPDEEKYLHRCLLDSATDDPSSAVGSLLVVFYLQRYRYIQAYQVDLRLQKIEEAFVSDNQIGEEVMFRMRSQSHWRKELVDRAIDILPVIQQQQVRS------GQFS-----EMEDASEGAKKSDLP--DAPDMITSSVPFATTNSVF : 716
RcHOS1 : CIKSKLVDRMIELPWSSDEENYIHKCLLECATHDPSSTTGSLLVVFYLQRYRYAEAYQVDLQLQNVEQDFLSKNSDNEEVLSRMRSASNWRTGLVAKSIELLPQAQQPQAKT------GKLLPQIYNVWREQVEIPAKSEPM-VQQLKSSSLLIPPSDNSSLL : 732
PtHOS1 : CIKNNLVDRMISLPWNLDEEKYLHNCLLDYAFHDPSTTIGSLLVVFYLQRYRYVEAYHVHSKLQGVEQEFISKNSLSEEVLSRMRSASHHRGELAVQSIKLLPKIQQEQLKT------GKLSPEIRNTSGEEVEIQERADLPLAQEPKSSSLLISLPADSSLV : 728
CtHOS1 : CIRRDLVDRMIELPWNSDEEKYLHKCLLDSATDDPSTTVGSLLVVFYIQRYRYAEAYQVNLKLQSVEQDFISKNPVSEEVLSRMQSQIHWRTKFIDTSIELLPEVQRQLVKN------GKLPLNAVNS-SEEVEIPEKSDLHGSQEPKSITLLIPTTADSSHL : 732
VvHOS1 : CIRRGLVDRMIELPWNFDEEKCLHKCLLEYAIDDPSTIVGSLLVVFYLQ----------------GIDGQFRSFSFLFVVIYGLM---------------VTLWCYVQ---LGK------GLLY----NTA------------LCTYAPLLKKIAILQPEGNNFW : 688
OsHOS1 : CIERNFVDRMIDLPWSDEEGKHKGLLDCAREMMEPCGSLLVVFYLQRYRYLAYEVDRSIQSFQNALE--TASEEKASTRTIAQWQSLVTKCLEMLEAEREDMRTIGSAERNQFAVQTMQNSSPANHIVKSPNPV----IAFSLSATPIPQKKSSP : 726
PpHOS1 : CMGMSLLDKMLGLPWRKEEKYVRKLLNRAEEDPECTAGNLVVFYVQRCRYLEARDVHKRLSEVENMVLRCTDGAKN-SVRMASEQSLRIVEKCLELYEVERQQASSA----------IVESREPNVESP-KSRMLAVDQQRFPPLASPLFGSAAGN : 737

AtHOS1 : L-QSANNARAREPVANNGS-PFQ-----SGH-----------------------MIGNASH-DLSHGRLFTNANRGQKSEVRSVTKNLKFGEMSTP------------FKDLNRAR----GNSQLQGKRTEESSPE------------------VNVSRYIE : 813
RcHOS1 : L-QSNHITPFKSSVTETSI-RSG-SVNKSHF-----------------------GLGDNGPPSVLHERLFTNAGKGLKPQV-NTHKSVNYDGTPNHVIPCVSPMSATRLKDVSKTSFNVLSDSHLHHGQLDEFSPE------------------MEQSGFSE : 849
PtHOS1 : S-QSNNNVTVKPAALKTPP-RFGASIKSHL-----------------------EMGNCDSSSVLHQRLFRTPERTQKYQV-SFNKNFKFDGISTPGIHQG----------------KVLPNSNLHHSLFDEISPE------------------REQSGFPK : 830
CtHOS1 : L-PSSNVTPANSSVFESPT-GPGRSIKSHF-----------------------EVGHYGP-SILHERLFMNK-EGSTYDF-GVSKEFEVDGFSTPGVCQSGLMNQTPLKGRNFSS-KTLSNSHRRDKVSDKISPE------------------PEQSGFLS : 847
VvHOS1 : NIHSNHSGAF---SLSAPG-KLKARMKDRG-------------------------------------------------------------------------------------------------------------------------------- : 714
OsHOS1 : L-QSRNINVLNDSVGLNSSARSEFGRKVSSILQCRPVPLSSPISNVRSTAGGLFPSMGQNGEGPYLKGTKELSFTKGESGLK----KGTRPAGYDSLPMYFNMGSVDTPMKEYRSSLLKTEVNKTTPFQVKDSVGKGEFDFGSRAEKPFILSGTGAGQSGHSK : 884
PpHOS1 : L--IREPVAFEENNVPSTS--AWASSSQSSFAQER-LGLRSP-APPSPIFSNMF-PKTNGPTASREFATLGDSPVQGRLGY--EAEQPRESHYPTVDLGNSLRCDGMDAAGPGSWASGLDKRMPVKMNGIDHSGNELF-----------LTM----ESGFRT : 876

AtHOS1 : N--NMSSPYLRRITANNPVTVKSSSNHLNG-----SSQKPESTFFGTRMQPDKDNFVDLDDPMDMSSSLKDNNNNVLATESR--NNSGGLRWRSDE-----------------------------------------------------------TSDDEDELTSF : 911
RcHOS1 : QFQNTSLHYVHKVKT-PIAMSGGSRGFLND-----SSRSSTKRVHSYRPDDGSWNVTSEADPMDIGIS---SREKGFTVDE--GNVNGGLRWRSDE-----------------------------------------------------------SSDEEGEHNLE : 945
PtHOS1 : QLRNTTPPYSHRITANPVAMSGSNNGLPND------------------KNDRSRNKGSIGDPKDIAWR-------------------------------------------------------------------------------------------- : 880
CtHOS1 : QHLNTIHHYSQRMTTNPASTPVSNRGVHNDLAGDLRSNLSSKRVHSDR-EDGPWYMISSEDPMDVSWS---NGKNGLAVEDRQANAGGGLRWRSDE-----------------------------------------------------------TSDEEEQSPE : 950
VvHOS1 : ------------------------------------------------------------------------------------------------------------------------------------------------------------------- : -
OsHOS1 : ISDNAGFHEVHIQKT---------------------------------------KVPPKENVLR-------------------------------------------------------------------------------------------------- : 909
PpHOS1 : DELLTGNGESMEMDAGGTSSWPSNGKRHPS---DRSWLQKDVEKEASKDFTFKGTNGGNELLQDTGVLPRESSRRNAEADEN-----GGSRWRSDEGDEPVSSPPPSTRLKALNQSQARSRNVIEIRLSWSRAFAAHFCGACCEDLIAFRKETAGGNFEDVEE : 1031

AtHOS1 : GSM-------PVKGRRRRRFAAR------------------- :  927
RcHOS1 : RAVGVASFTTPGRGIRRSRFAKR------------------- :  968
PtHOS1 : ------------------------------------------ :    -
CtHOS1 : SAMGVASYTTPRRGIRRSRFARR------------------- :  973
VvHOS1 : ------------------------------------------ :    -
OsHOS1 : ------------------------------------------ :    -
PpHOS1 : GAFGSFGWFVRRHREGRAEAGRRKEPQIRVAWPPRRLKLLP : 1072
```

Supplemental Figure 2. Sequence comparison of Arabidopsis HOS1 (AtHOS1) with the HOS1 orthologues from *Ricinus communis* (RcHOS1), *Populus trichocarpa* (PtHOS1), *Citrus trifoliate* (CtHOS1), *Vitis vinifera* (VvHOS1), *Oryza sativa* (OsHOS1) and *Physcomytrella patents* (PpHOS1).

Amino acid residues in black are identical, and those in grey are functionally similar in the sequences. The conserved cysteine residues in the RING finger domain are indicated with black triangles. The putative nuclear localization signal present in the C terminal region of HOS1 is underlined. Genbank accession numbers are NP_181511 for AtHOS1, XP_002531460 for RcHOS1, XP_002304293 for PtHOS1, ACY92092 for CtHOS1, XP_002264751 for VvHOS1, AAR07079 for OsHOS1 and XP_001780235 for PpHOS1.



Supplemental Figure 3. *FT* expression analysis in LD grown Col and *hos1-3* seedlings harvested 4 and 8 h after dawn at 8, 10, 12 and 15 DAG.

SUPPLEMENTAL TABLES

Supplemental Table 1. Polymorphic molecular markers used for *esd6* mapping

Marker	Type	BAC clone	Primer 1	Primer 2
nga168	SSLP	T7F6	TCGTCTACTGCACTGCCG	GAGGACATGTATAGGAGCCTCG
T16B24	SSLP	T16B24	GCTATTGGTGATGAACGGAG	CATTTGACACTTTCGCGTAG
F12L6	SSLP	F12L6	CCCTGAACTTCACATCTGCTGCAAC	GGTTTCAGTAGTGGTTCTCGTTTAGG
C005	SSLP	T5I7	AATTATCGCACCTAGTTGAGG	TTGTATGAAGGATCATCTGCC
T5I7	SSLP	T5I7	GCGTCTGCACTATGAAATGTTTCG	CCGGAACTTGACCCATATCG
T28M21	SSLP	T28M21	CCGCCACCGAAGCTAAGAATCG	CGACACATCTAAGCAAACACATTCTTATC
T7D17	dCAP	T7D17	GCCATAAGGAACTTTTTGTC	GAGGACATCTTTATCAAACC
T3K9	SSNP	T3K9	GACGAACTTCCAATGGCGGAGGT	CGCTGCCGCCGGGCTTTTGGCTCG

Supplemental Table 2. Flowering time of *hos1* double mutants

	1 week 4°C4 weeks 4°C	
Ler	9,4 ± 0,9	10,2 ± 1
hos1-2	8,5 ± 1	9,6 ± 0,9
fca-1	23,4 ± 1,3	11,2 ± 0,8
hos1-2 fca-1	13 ± 1,3	10,4 ± 1,9
vrn1-2 fca-1	42,5 ± 6,2	31,2 ± 4,7
hos1-2 vrn1-2 fca-1	16,1 ± 2	14,7 ± 3,2
Col	12 ± 1	9,1 ± 0,9
hos1-3	8,1 ± 0,6	6,7 ± 0,5
Col *FRI Sf-2*	66,7 ± 11,6	18,6 ± 2,1
hos1-3 FRI Sf-2	31,6 ± 5,8	9 ± 1,2
vin3-4 FRI Sf-2	76,5 ± 11,4	80,1 ± 10,1
hos1-3 vin3-4 FRI Sf-2	39,8 ± 7,2	43,9 ± 8,8

Number of leaves was scored in approximately 12 plants under LD conditions and is represented as mean ± standard deviation.

Supplemental Table 3. Flowering time of transgenic plants bearing a *CO-LUC* construct

Col	13,2 ± 0,9
hos1-3	7,5 ± 0,6
35S::CO-LUC 6-2/Col	5,9 ± 0,3
35S::CO-LUC 6-2/*hos1-3*	4,6 ± 0,4

Number of leaves was scored in approximately 10 plants under LD conditions and is represented as mean ± standard deviation.

Supplemental Table 4. Flowering time of transgenic plants bearing a *HOS1-GFP* construct

Col	12,9 ± 0,8
hos1-3	7 ± 0,5
*35S::HOS1-GFP*4-1-4/*hos1-3*	9,4 ± 0,4

Number of leaves was scored in approximately 10 plants under LD conditions and is represented as mean ± standard deviation.

DISCUSIÓN

El momento en el que se produce la transición floral está determinado por el balance que existe entre señales promotoras y represoras de la floración. La caracterización de mutantes tempranos en Arabidopsis y el estudio de la interacción que existe entre los genes afectados en dichos mutantes y las rutas inductoras de la floración, ha permitido profundizar en el conocimiento de los distintos mecanismos implicados en la represión floral (Pouteau y col., 2004; Pouteau y col., 2008; Yant y col., 2009). Entre dichos mecanismos se encuentran aquellos dependientes de la acción de factores de transcripción, los procesos de modificación y remodelación de cromatina, los mecanismos de regulación mediados por microRNAs o los circuitos reguladores que dependen de la degradación selectiva de proteínas. Es frecuente que estos mutantes de floración temprana presenten alteraciones pleiotrópicas del fenotipo puesto que los represores florales generalmente afectan a varios procesos del desarrollo. Como ejemplo, se puede citar el caso de los mutantes de floración temprana que hemos descrito a lo largo de este trabajo, *esd1/arp6*, *swc6* y *hos1* (Choi y col., 2005; Deal y col., 2005; Martin-Trillo y col., 2006; Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008; Lazaro y col., 2011 en revisión), u otros mutantes tempranos con los que hemos trabajado en el laboratorio, como *ebs* o *esd7* (Pineiro y col., 2003; del Olmo y col., 2010).

A lo largo de esta Tesis Doctoral se ha profundizado en el papel que desempeñan los mecanismos de remodelación de la cromatina, a través del intercambio de la variante histónica H2A.Z, y la degradación selectiva de proteínas en el control de la transición floral en Arabidopsis.

1. Papel de la variante histónica H2A.Z en la regulación del tiempo de floración

Como hemos visto en este trabajo, las propiedades dinámicas de la cromatina están mediadas por complejos multiproteicos que participan en la incorporación de marcas epigenéticas con distintas funciones sobre el ADN y las histonas (Loidl, 2004). Esclarecer los mecanismos moleculares por los que distintas modificaciones epigenéticas modulan patrones de expresión génica es fundamental para comprender los procesos de desarrollo y diferenciación en organismos eucarióticos. Durante los últimos años, diversos estudios han puesto de manifiesto el papel esencial que desempeña el intercambio de la histona canónica H2A por la variante histónica H2A.Z en la regulación de la expresión génica y del desarrollo en distintos organismos (Draker y Cheung, 2009; Svotelis y col., 2009; Marques y col., 2010). Sin embargo, la base molecular de dicho intercambio y su funcionalidad permanecen en gran medida desconocidas, tanto en sistemas biológicos animales como vegetales. La

secuencia de H2A.Z está muy conservada en diversos organismos y, al contrario de lo que ocurre en levaduras, es esencial para la viabilidad de especies como *Drosophila melanogaster* o ratón (Clarkson y col., 1999; Faast y col., 2001). La función que desempeña la variante histónica H2A.Z en la actividad transcripcional de un gen no está clara, y así, en *D. melanogaster* se ha descrito que se localiza tanto en regiones de heterocromatina como de eucromatina (Leach y col., 2000). A pesar de esto, se ha propuesto que la incorporación de H2A.Z en las zonas promotoras de los genes marca estos loci para su activación y es necesaria para la adecuada regulación de su expresión (Draker y Cheung, 2009). En levaduras, los promotores de distintos genes presentan una región "libre de nucleosomas" 200 pb aguas arriba del codón de inicio de la traducción que está flanqueada por nucleosomas que contienen H2A.Z (Raisner y col., 2005; Yuan y col., 2005). Diversos estudios han descrito que este patrón de H2A.Z está relacionado con la capacidad de inducción de la transcripción de genes que se encuentran en estado reprimido o que presentan un nivel basal de expresión (Guillemette y col., 2005; Li y col., 2005; Millar y col., 2006). De este modo, se ha propuesto que la presencia de H2A.Z prepara la estructura de la cromatina para que la maquinaria de transcripción pueda activar la expresión génica (Guillemette y col., 2005). En células de mamífero la disposición de nucleosomas que contienen H2A.Z afecta de forma más general a la zona de inicio de la transcripción, y también se observa en promotores de genes inducibles que pierden esta variante histónica cuando se activan (Zhang y col., 2005; Schones y Zhao, 2008). De hecho, en humanos, se ha descrito una correlación entre la presencia de H2A.Z en las zonas promotoras y la marca activadora de la transcripción H3K4me3 (Schones y Zhao, 2008). Por otro lado, también se ha propuesto un papel de la H2A.Z como "barrera" a la extensión de la heterocromatina a zonas adyacentes de eucromatina (Meneghini y col., 2003).

La incorporación de la variante histónica H2A.Z a los nucleosomas se produce mediante un mecanismo independiente de la replicación del ADN (Altaf y col., 2009). En levaduras, se ha demostrado que el complejo Swr1 (Swr1-C) es el responsable del intercambio de los dímeros H2A-H2B por H2A.Z-H2B (Krogan y col., 2003; Kobor y col., 2004; Mizuguchi y col., 2004). Este proceso está catalizado por la subunidad Swr1, que es miembro de la familia de ATPasas tipo SWI/SNF2. En experimentos de inmunoprecipitación en levaduras, Swr1 copurifica con 13 proteínas y con el dímero H2A.Z-H2B (Mizuguchi y col., 2004; Wu y col., 2005). Las subunidades Swr1, Swc2, Bdf1, Swc3, Arp6, Swc5, Yaf9, Swc6 y Swc7 están codificadas por genes no esenciales, mientras que Rvb1, Rvb2, Arp4, Swc4 y la actina son indispensables para la viabilidad celular (Wu y col., 2009b). Algunas subunidades que están presentes en el complejo Swr1 también forman parte de otros complejos de remodelación de

cromatina. Es el caso de las helicasas Rvb1 y 2, de la actina y de Arp4, que están presentes en el complejo INO80 (Shen y col., 2000), y de Yaf 9, Swc4 y, de nuevo, de la actina y de Arp4, que también forman parte del complejo con actividad histona acetiltransferasa NuA4 (Galarneau y col., 2000; Zhang y col., 2004; Auger y col., 2008). La deposición de H2A.Z *in vivo* depende únicamente de algunas de las subunidades del complejo, como Swr1, Swc2, Arp6, Swc6 y Yaf9 (Krogan y col., 2003; Zhang y col., 2004). La subunidad Swc2 es la responsable de la interacción entre el Swr1-C y la histona H2A.Z, mientras que Arp6 y Swc6 son necesarias para la unión de Swc2 al dominio ATPasa de Swr1 (Figura 1) (Mizuguchi y col., 2004; Wu y col., 2005; Wu y col., 2009b). Las cepas de levaduras que presentan defectos en la subunidad Swr1 o en otros componentes del Swr1-C muestran una disminución de la variante histónica H2A.Z en las zonas de eucromatina que bordean regiones genómicas silenciadas (Krogan y col., 2003; Kobor y col., 2004). Además, existe una gran similitud entre los perfiles transcriptómicos de los mutantes *swr1* y *h2a.z* (*htz1*) en levaduras (Krogan y col., 2003; Kobor y col., 2004; Mizuguchi y col., 2004).

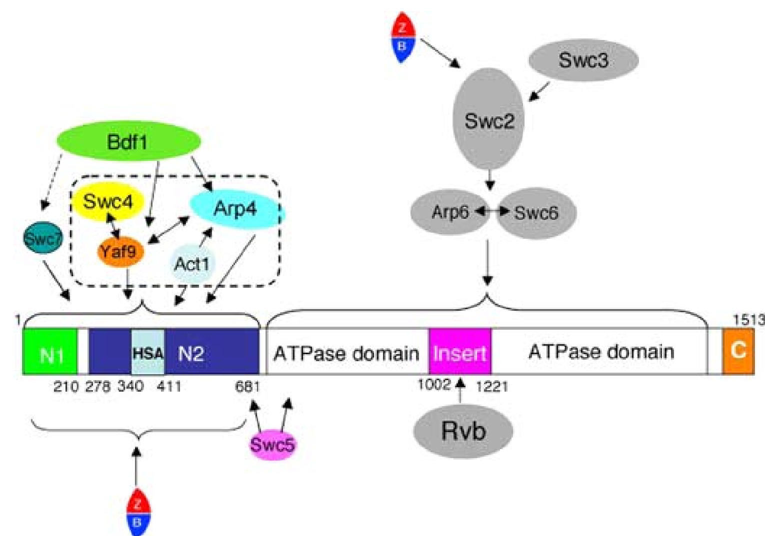


Figura 1. Modelo teórico que representa la asociación de las diferentes subunidades del complejo Swr1 en levaduras (adaptado de Wu y col., 2009).

Además, la subunidad Bdf1 (Bromodomain factor 1) posee la capacidad de unirse a las colas acetiladas de las histonas H3 y H4 *in vitro*, por lo que se ha propuesto que podría ser el componente encargado de dirigir el complejo Swr1 a zonas específicas de la cromatina (Krogan y col., 2003; Matangkasombut y Buratowski, 2003; Kobor y col., 2004). Los ortólogos de la subunidad Swr1 en animales serían las proteínas SRCAP y p400/Tip60 (Wu y col., 2005; Cai y col., 2006). Se ha demostrado que los complejos a los que pertenecen ambas ATPasas son capaces de realizar el intercambio de la variante histónica H2A.Z *in vitro* (Ruhl y col., 2006; Gevry y col.,

2007). En levaduras se ha sugerido que la deposición de H2A.Z mediada por Swr1-C necesita la previa acetilación de las histonas de esa región de la cromatina por el complejo NuA4 y, curiosamente, el complejo Tip60 presente en *D. melanogaster* y humanos parece ser una fusión de los complejos Swr1 y NuA4 de levaduras (Doyon y col., 2004; Auger y col., 2008).

Los resultados presentados en este trabajo han contribuido a la identificación en Arabidopsis de dos ortólogos de los componentes del complejo Swr1 de levaduras, *ESD1/ARP6* y *SWC6*. El aislamiento de estas subunidades, junto con la identificación de *PIE1,* el homólogo en Arabidopsis de la subunidad catalítica del complejo Swr1 de levaduras (Noh y Amasino, 2003), ha dado pie a la caracterización de un posible complejo homólogo a Swr1 en plantas. El genoma de Arabidopsis presenta ortólogos de la mayor parte de los componentes del Swr1-C. Las células eucariotas poseen al menos ocho proteínas relacionadas con actina (ARPs) de las cuales, ARP4, 5, 6, 7 y 8 se localizan en el núcleo y están implicadas en mecanismos de remodelación de la cromatina (Blessing y col., 2004; Kandasamy y col., 2004). La mayoría de los complejos de remodelación de cromatina que poseen ARPs también presentan una molécula de actina. Por su parte, SWC6 es una proteína que presenta un dedo de Zinc tipo HIT (Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008). Tanto *ESD1/ARP6* como *SWC6* son genes únicos en Arabidopsis, homólogos de los que codifican las subunidades Arp6 y Swc6 de los complejos Swr1 de levaduras y SRCAP de humanos. Además, se han descrito tres genes de la familia H2A denominados HTA8, 9 y 11, que codifican para homólogos de H2A.Z en Arabidopsis (Yi y col., 2006).

Además, la caracterización de ESD1/ARP6 y SWC6 ha permitido desvelar el papel que tiene el intercambio de variantes histónicas en la regulación de la expresión de *FLC*, así como avanzar en el conocimiento de los procesos de remodelación de cromatina involucrados en la regulación de la transición floral en esta especie modelo. Los trabajos publicados a lo largo de los últimos años han demostrado la función de diversas modificaciones histónicas en la regulación de *FLC* a través de *FRI*, la ruta autónoma, la ruta de la vernalización y el complejo PAF1 (He y col., 2003; Ausin y col., 2004; Bastow y col., 2004; He y col., 2004; Sung y Amasino, 2004; He y Amasino, 2005; Kim y col., 2005; Choi y col., 2011). Asimismo, se ha observado que el primer exón y el primer intrón de *FLC* son las regiones genómicas en las que se depositan principalmente las marcas epigenéticas que participan en la regulación transcripcional de este locus (Sheldon y col., 2002).

Las alteraciones pleiotrópicas del fenotipo de los mutantes *esd1/arp6* y *swc6*, así como el patrón de expresión ubicuo de ambos loci, sugiere que estos genes participan en diversos procesos del desarrollo vegetativo y reproductivo de las plantas (Choi y col., 2005; Deal y col., 2005; Martin-Trillo y col., 2006; Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008). El fenotipo descrito para las mutaciones en otros homólogos del complejo SWR1 en Arabidopsis presenta claras similitudes con los observados en *esd1/arp6* y *swc6*. Las plantas que presentan defectos en *PIE1* también muestran un fenotipo de floración temprana, los margenes de las hojas aserrados, pérdida de la dominancia apical y la presencia de pétalos extra en las flores (Noh y Amasino, 2003). De la misma forma, las mutaciones *pie1*, *esd1/arp6* y *swc6* son capaces de suprimir el fenotipo de floración tardío causado por los mutantes de la ruta autónoma o por un alelo funcional del locus *FRI*, gracias a la disminución de los niveles de *FLC* (Noh y Amasino, 2003; Choi y col., 2005; Deal y col., 2005; Martin-Trillo y col., 2006; Choi y col., 2007; Lazaro y col., 2008). A pesar de las semejanzas que se observan entre *pie1*, *esd1/arp6* y *swc6,* algunos de los fenotipos son más dramáticos en el mutante *pie1*, por ejemplo la reducción en la elongación de la inflorescencia primaria es mayor y las hojas son más pequeñas y deformadas (Noh y Amasino, 2003; March-Diaz y col., 2007). Por otra parte, la reducción de los niveles del mensajero de *AtARP4* por aproximaciones de ARNi también provoca una aceleración del tiempo de floración en Arabidopsis, además de alteraciones en la organización de los órganos florales, retraso en el proceso de senescencia y altos niveles de esterilidad (Kandasamy y col., 2005). Estas diferencias con los mutantes *pie1*, *esd1/arp6* y *swc6* sugieren, como ocurre en levaduras, la participación de AtARP4 en distintos complejos de remodelación de cromatina en plantas.

Como se ha descrito con anterioridad, Swc6 y Arp6 son dos componentes íntimamente asociados en el Swr1-C de levaduras (Figura 1) (Wu y col., 2005). De acuerdo con esto, en Arabidopsis el fenotipo del doble mutante *esd1 swc6* es indistinguible de cualquiera de los dos mutantes simples (Choi y col., 2007; Lazaro y col., 2008). Además de interaccionar genéticamente, también se ha demostrado que ESD1/ARP6 y SWC6 interaccionan físicamente en Arabidopsis, así como que ambas proteínas son capaces de interaccionar con PIE1, lo que sugiere que estas tres proteínas actúan en el mismo complejo (Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008). Por otro lado, se ha demostrado la presencia de H2A.Z en la zona de inicio y final de la transcripción del gen *FLC* y de sus homólogos de la familia *MAF, MAF4* y *MAF5* (Deal y col., 2007). En los mutantes *esd1/arp6* y *pie1* se ha observado una disminución en la acumulación de esta variante histónica en la cromatina de *FLC*, que además está relacionada con una reducción de la expresión de este represor floral y con una aceleración del tiempo de floración (Deal y col., 2007).

Igualmente, se ha descrito que tanto ESD1/ARP6 como SWC6 se unen al promotor de *FLC* mediante ensayos de inmunoprecipitación de cromatina (Choi y col., 2007). De forma adicional, se ha demostrado que H2A.Z interacciona físicamente con PIE1, AtSWC2 y ESD1/ARP6, y que la reducción de la expresión de los genes que codifican para H2A.Z provocan un fenotipo similar al que se observa en los mutantes e*sd1/arp6*, *swc6* y *pie1* (Choi y col., 2007; Deal y col., 2007; March-Diaz y col., 2008). También se ha descrito mediante el análisis de perfiles transcriptómicos globales que existe un 65% de solapamiento entre los genes desregulados en el mutante *pie1* y en el doble mutante para dos de los homólogos de *H2A.Z*, *hta9 hta11* (March-Diaz y col., 2008). Este resultado indica que PIE1 y H2A.Z comparten funciones en la regulación de la expresión génica, aunque también presentan un grado de independencia funcional, y de hecho, no es posible descartar que PIE1 tenga funciones independientes del complejo SWR1 en el control de la expresión génica. En levaduras, la mutación de Arp6 o Swc6 da como resultado la pérdida de la otra subunidad en el complejo, así como la pérdida de otras dos proteínas, Swc2 y Swc3, lo que sugiere que Arp6, Swc6, Swc2 y Swc3 forman un subcomplejo que se asocia a Swr1 (Wu y col., 2005). De forma similar, parece que en Arabidopsis ESD1/ARP6 y SWC6, junto con SWC2, pueden formar un subcomplejo que se asocia a PIE1 (Choi y col., 2007; Deal y col., 2007; March-Diaz y Reyes, 2009).

Todos los datos anteriores sugieren la existencia de un complejo de remodelación de cromatina tipo SWR1 en Arabidopsis que cataliza el intercambio de la histona H2A por la variante histónica H2A.Z, y que ESD1/ARP6 y SWC6 forman parte de dicho complejo. Además, se ha observado que este hipotético complejo SWR1 es necesario para el correcto desarrollo de Arabidopsis, ya que parece estar implicado en la regulación de diversos procesos de diferenciación como la transición floral, el desarrollo de los órganos florales y la morfología y tamaño de la hoja (Choi y col., 2005; Deal y col., 2005; Martin-Trillo y col., 2006; Choi y col., 2007; March-Diaz y col., 2007; Lazaro y col., 2008). En concreto, se ha demostrado que el complejo SWR1 participa en el control del tiempo de floración a través de la activación de la expresión del represor floral *FLC* y de los genes *MAF4* y *MAF5* y, por tanto, parece que la presencia de H2A.Z puede ser necesaria para activar la expresión de determinados loci en plantas.

Curiosamente, los niveles de H2A.Z presentes en la cromatina de *FLC* no están relacionados con la tasa de transcripción de este locus en distintos tejidos que presentan una variación de la expresión de *FLC* de hasta diez veces (Deal y col., 2007). La distribución espacial de H2A.Z sobre la cromatina de *FLC* se mantiene constante en todos los tejidos analizados, de modo que se observa un pico en la zona

de inicio y otro en la de terminación de la transcripción. En cambio, no hay una correlación entre la expresión de *FLC* y la cantidad de H2A.Z presente a lo largo de la región codificante del gen en los distintos tejidos, lo que sugiere que la mera presencia de esta variante histónica no es capaz de activar la transcripción (Deal y col., 2007). Además, se ha descrito que durante la vernalización, aunque la expresión de *FLC* está reprimida, se produce un aumento de la presencia de H2A.Z en este locus (Brickner y col., 2007). Ambos resultados sugieren que la función de H2A.Z puede ser la de "marcar" o disponer a los genes en un estado competente para su activación por otros factores que permitan la expresión génica cuando las condiciones sean las adecuadas (Deal y col., 2007). Como se ha descrito en el presente trabajo, ESD1/ARP6 y SWC6 median la regulación de la expresión de *FLC* a través de dos marcas epigenéticas relacionadas con la activación transcripcional: la acetilación de las Lys 9 y 14 de la H3 y la trimetilación de la Lys 4 de la H3. Las mutaciones en los loci *ESD1/ARP6* y *SWC6* provocan una disminución de los niveles de estas dos modificaciones histónicas activadoras en la zona promotora y en el primer intrón de *FLC*. Sin embargo, la posible implicación directa de ESD1/ARP6 y SWC6, y, por tanto del complejo SWR1, en el establecimiento de estas dos marcas epigenéticas no está clara. Los complejos Swr1 y NuA4 de levaduras comparten varias subunidades y las mutaciones en algunos de sus componentes presentan fenotipos similares (Auger y col., 2008), de modo que es posible especular que podrían trabajar de forma conjunta en la acetilación de histonas y en el intercambio de H2A.Z en la cromatina. Además, en *D. melanogaster* y humanos, se ha descrito que el complejo Tip60 correspondería a una fusión de los complejos Swr1 y NuA4 de levaduras, puesto que presenta ortólogos de las subunidades que forman parte de los dos complejos (Doyon y col., 2004; Auger y col., 2008). Por otro lado, se ha descrito que componentes del complejo PAF1 de levaduras muestran una interacción genética con subunidades del Swr1-C (Krogan y col., 2003), lo que sugiere que ambos complejos podrían tener dianas comunes y podría explicar el papel que juegan ESD1/ARP6 y SWC6 en la trimetilación de la H3K4 en la cromatina de *FLC* (Mueller y Jaehning, 2002; Squazzo y col., 2002; Krogan y col., 2003; Krogan y col., 2004). De forma análoga a lo que ocurre en levaduras, el complejo PAF1 de Arabidopsis recluta a la metiltransferasa EFS/SDG8, y así regula los niveles de expresión de *FLC* (Kim y col., 2005). De hecho, las mutaciones que afectan a los componentes del complejo PAF1 presentan alteraciones fenotípicas similares a las de las de los mutantes *esd1/arp6* y *swc6*, además de una disminución de los niveles de H3K4me3 en la cromatina de *FLC* (He y col., 2004), datos que apoyan que los complejos SWR1 y PAF1 puedan actuar de forma conjunta en la remodelación de la cromatina de determinados genes diana en plantas.

Estudios previos han establecido el papel de H2A.Z en el reclutamiento de la ARN polimerasa II (Adam y col., 2001) y la interrelación con complejos de remodelación de nucleosomas (Santisteban y col., 2000). Como se ha descrito recientemente, FRI y otras proteínas relacionadas forman un complejo (FRI-C) que actúa como un activador transcripcional de *FLC* a través del reclutamiento de complejos remodeladores de la cromatina (Choi y col., 2011). Entre los factores que recluta el FRI-C al promotor de *FLC* se encuentra el complejo SWR1, la metiltransferasa EFS y un factor general de la transcripción, TAF14 (Choi y col., 2011). El genoma de Arabidopsis contiene dos proteínas, YAF9 y TAF14, con un dominio YEATS, asociado a la remodelación de la cromatina y a la transcripción génica (Schulze y col., 2010). YAF9 es un componente del SWR1-C, homólogo a la subunidad Yaf9 de los complejos Swr1-C y NuA4 de levaduras (Zhang y col., 2004). Por su parte, la proteína TAF14 forma parte de los factores de transcripción generales TFIID y TFIIF, de los complejos de remodelación de cromatina INO80 y SWI/SNF2, y del complejo NuA3 de acetilación de la histona H3 de levaduras y humanos (Kabani y col., 2005). Por otro lado, EFS/SDG8 cataliza la trimetilación de K4 y K36, que son marcas que promueven el inicio de la transcripción y la elongación del mensajero (Ko y col., 2010). El reciente descubrimiento de la relación que existe entre el complejo de FRI, el SWR1-C, EFS/SDG8 y TAF14 nos da idea de la complicada secuencia de eventos que requiere la transcripción génica en eucariotas y podría ligar la presencia de H2A.Z y de la marca H3K4me3 sobre la cromatina de *FLC* (Choi y col., 2011).

Todavía no se conoce el mecanismo por el cual el complejo Swr1 es reclutado a determinados genes y no a otros. Una de las hipótesis aceptadas en la actualidad se basa en la capacidad de la proteína Bdf1 para reconocer patrones de acetilación de histonas, ya que los mutantes en esta subunidad del complejo presentan una disminución de la deposición de H2A.Z en las zonas promotoras (Raisner y col., 2005; Zhang y col., 2005). Sin embargo, en Arabidopsis no se ha identificado un ortólogo de Bdf1 puesto que en su genoma no se han descrito proteínas que contengan dos dominios de tipo bromodomain. Por otra parte, se ha propuesto que pueda existir una señal en el ADN para la incorporación de H2A.Z, ya que en levaduras se ha observado que el sitio de unión de la proteína tipo Myb Reb1 es suficiente para la formación de una zona "libre de nucleosomas" y para la incorporación de H2A.Z en los nucleosomas que flanquean dicha región (Raisner y col., 2005; Raisner y Madhani, 2006). Por otro lado, se ha propuesto recientemente que la presencia de H2A.Z y la metilación de ADN son marcas de la cromatina mutuamente antagónicas en Arabidopsis (Zilberman y col., 2008). Así, mutaciones en la metiltransferasa de ADN *MET1*, que provocan tanto pérdida como ganancia de metilación del ADN, originan cambios opuestos en los niveles de incorporación de H2A.Z, mientras que mutaciones en *PIE1* conllevan una

moderada hipermetilación general a lo largo del genoma. Por tanto, parece que la presencia de H2A.Z previene el silenciamiento génico mediado por la metilación del ADN (Kobor y Lorincz, 2009).

En Arabidopsis también existe controversia acerca del papel que juega la H2A.Z en la regulación de la transcripción génica. Las mutaciones en componentes del SWR1-C liberan la represión sobre ciertos genes de respuesta a estrés biótico y abiótico, como genes de resistencia a enfermedades, genes inducidos por choque térmico y genes de respuesta a deficiencia de fosfato, lo que apoyaría un efecto negativo de H2A.Z sobre la expresión génica (March-Diaz y col., 2008; Kumar y Wigge, 2010; Smith y col., 2010). En cambio, como ya hemos descrito, la presencia de H2A.Z en el promotor de *FLC* está relacionada con el aumento de la expresión de este locus (Deal y col., 2007). El papel dual que juega H2A.Z en la regulación de la transcripción desde levaduras a humanos puede depender del contexto de la cromatina en el cual se localiza o de su disposición, en uno o dos nucleosomas en las zonas promotoras, o en numerosos nucleosomas contiguos en las zonas teloméricas silenciadas (Guillemette y Gaudreau, 2006). La incorporación de variantes histónicas en ciertos loci confiere a estas zonas del genoma propiedades estructurales y funcionales específicas que afectan a una variedad de procesos biológicos en distintos organismos. Como hemos descrito, la presencia de H2A.Z está relacionada con el control de la expresión de ciertos genes, pero, además, también participa en la progresión del ciclo celular, la estabilidad del genoma, la supresión de ARNs antisentido o la adaptación a altas temperaturas en las plantas (Ahmad y col., 2010). Recientemente, en un ensayo dirigido a la búsqueda de mutantes que presentaban alteraciones en la respuesta a la temperatura de crecimiento, se aisló un nuevo alelo del mutante *arp6* (Kumar y Wigge, 2010). Se ha demostrado que a temperaturas bajas de crecimiento H2A.Z reprime la expresión de una serie de genes, entre los que se encuentra *FT*, pero que un aumento en la temperatura ambiental provoca el desplazamiento de esta variante histónica de dichos genes y la activación de su transcripción. Así, se ha propuesto que la presencia o ausencia de H2A.Z puede ser la base del mecanismo de medida de los cambios de la temperatura ambiental en Arabidopsis (Franklin, 2010; Kumar y Wigge, 2010). Al igual que ocurre en Arabidopsis, la variante H2A.Z se elimina del ADN durante la activación de multitud de genes en levaduras y en humanos (Santisteban y col., 2000; Farris y col., 2005; Zhang y col., 2005), de manera que se ha propuesto que esta variante histónica podría favorecer la transcripción génica al facilitar la remodelación o la liberación de los nucleosomas de las zonas promotoras (Guillemette y Gaudreau, 2006).

La caracterización bioquímica de otros ortólogos de los componentes del Swr1-C, junto con estudios transcriptómicos globales y análisis de distribución genómica (ChIP-chip o ChiP-seq), permitirán en el futuro identificar nuevos loci regulados por este complejo en plantas. Los defectos pleiotrópicos de los mutantes descritos hasta el momento en Arabidopsis indican que los procesos de remodelación de cromatina mediados por SWR1 afectan a gran variedad de procesos de desarrollo, tanto vegetativo como reproductivo, en las plantas.

2. Función de HOS1 en la regulación fotoperiódica de la floración

Debido a que los resultados obtenidos en este trabajo acerca del papel de HOS1 como represor floral ya se han discutido en relación a los últimos avances realizados en el control fotoperíodico del tiempo de floración en Arabidopsis, en este apartado sólo vamos a recoger aquellos aspectos que no se pudieron desarrollar exhaustivamente en la discusión del manuscrito presentado en el Capítulo 3 debido a una limitación de espacio.

Los cambios en el fotoperiodo regulan la transición floral en una gran variedad de plantas, de modo que aquellas que presentan defectos en los mecanismos de respuesta a la duración del día no florecen de forma adecuada en la naturaleza (Wilczek y col., 2009). En el presente trabajo, hemos demostrado mediante el uso aproximaciones genéticas y moleculares, que el locus *HOS1* participa en el control fotoperiódico del tiempo de floración. El mutante *esd6/hos1* se aisló en un programa de búsqueda de mutantes de floración temprana en Arabidopsis. Además de presentar una aceleración de la floración, el mutante *hos1* muestra alteraciones pleiotrópicas del fenotipo que afectan al desarrollo de las hojas, las flores y la raíz, lo que sugiere la implicación de este locus en la regulación del desarrollo de estos órganos (Lazaro y col., 2011 en revisión).

Como hemos expuesto con anterioridad, *CO* está regulado tanto a nivel transcripcional, como a nivel post-traduccional por distintas calidades de luz (Valverde y col., 2004; Kim y col., 2008). Los análisis genéticos llevados a cabo en este trabajo con el mutante *hos1* demuestran que este locus participa en la ruta del fotoperiodo, ya que las mutaciones en *CO* suprimen en gran medida el fenotipo temprano de *hos1*, particularmente en fondo genético L*er*. Por otro lado, los ensayos de expresión mostraron que el patrón del mensajero de *CO* no estaba afectado en el mutante *hos1*. En cambio, sí que observamos una alteración en el patrón de expresión de *FT* en DL en el mutante *hos1*, en el que destaca un pico de expresión en las primeras horas del día. Además, los datos obtenidos en este trabajo indican que la proteína HOS1 interacciona físicamente con CO *in vitro* e *in vivo*, y que los niveles de la proteína CO

son más elevados en el mutante *hos1* que en plantas silvestres durante el día. Por otro lado, se ha descrito que HOS1 es una proteína con un dominio RING que tiene actividad como enzima E3 ligasa de ubiquitina (Dong y col., 2006). En base a todos estos resultados, proponemos que HOS1 participa en la degradación de CO durante el periodo de luz del día mediante un mecanismo de ubiquitinación, y que la actividad de HOS1 en las primeras horas del día es crucial para una correcta regulación fotoperiódica de la floración (Lazaro y col., 2011 en revisión).

Se han descrito otras ligasas de ubiquitina que participan en el control fotoperiódico del tiempo de floración, de las cuales sólo COP1 está implicada de forma directa en la regulación de la estabilidad de la proteína CO (Jang y col., 2008; Liu y col., 2008c; Morris y col., 2010). La proteína CO se degrada en el periodo de oscuridad a través de la actividad de los complejos SPA-COP1 (Laubinger y col., 2006; Jang y col., 2008; Liu y col., 2008c). Además, se ha demostrado recientemente que la E3 ligasa de tipo CUL4-DDB1 interacciona con los complejos SPA-COP1 en el control del tiempo de floración (Chen y col., 2010). Queda por determinar si HOS1 actúa como una E3 ligasa de ubiquitina individual o si forma parte de un complejo multimérico como parece que ocurre con COP1.

Se ha descrito que COP1 presenta mayor actividad durante la oscuridad que en el periodo de luz (Jang y col., 2008). Se ha propuesto que uno de los mecanismos que podría explicar esta regulación diferencial es la translocación de la proteína COP1 del núcleo al citoplasma en presencia de luz, puesto que la mayor parte de sus dianas son factores de transcripción (von Arnim y Deng, 1994). Sin embargo, resultados recientes cuestionan la exclusión de COP1 del núcleo en presencia de luz, ya que demuestran que los niveles de COP1 no varían entre la fracción citoplásmica y la fracción nuclear de plántulas etioladas o tratadas con luz roja (Jang y col., 2010). Los datos de localización subcelular de HOS1 presentados en esta Tesis tampoco coinciden con las observaciones previas que describen la presencia de HOS1-GFP en el citoplasma cuando las plántulas se cultivan en oscuridad y a temperatura normal de crecimiento (Lee y col., 2001). En este trabajo, hemos demostrado que en plántulas de Arabidopsis que sobreexpresan la proteína de fusión de HOS1-GFP se observa un patrón de localización nuclear tanto en condiciones de luz contínua como en plántulas etioladas, lo que sugiere que HOS1 se localiza en el núcleo independientemente de las condiciones lumínicas (Lazaro y col., 2011 en revisión). Esta discrepancia puede deberse a que la construcción *35S::HOS1-GFP* usada previamente por Lee y col. no contenía la secuencia codificante completa de HOS1, que hemos reanotado en este trabajo (Lazaro y col., 2011 en revisión). Futuros estudios serán necesarios para comprender en profundidad cómo la luz es capaz de controlar la actividad de COP1, y

para comprobar si distintas calidades de luz también afectan a la actividad de otras ligasas de ubiquitina involucradas en el control de la transición floral, como HOS1.

Mientras que la transcripción de *CO* está regulada por el reloj circadiano, los niveles de proteína CO son regulados de manera diferencial por la luz. Valverde y colaboradores describieron cómo la estabilidad de CO se veía afectada por distintas calidades de luz mediante ensayos de western blot con un anticuerpo específico anti-CO y estudios de localización subcelular con una fusión de la proteína CO a GFP (Valverde y col., 2004). Estos experimentos demostraron que las plántulas de Arabidopsis expuestas a luz roja o mantenidas en oscuridad presentaban unos niveles de CO muy inferiores a los que se observaban en las plántulas tratadas con luz azul, luz roja lejana y luz blanca. En plantas sobreexpresoras de CO, esta proteína se detectaba por western blot a las 8, 12 y 16 horas después del amanecer en condiciones de DL, mientras que sus niveles eran casi indetectables a ZT4. Además, se detectaba un aumento transitorio de los niveles de CO a los 30 minutos después del amanecer tanto en DL como en DC (Valverde y col., 2004). El efecto de los distintos fotoreceptores sobre la cantidad de CO fue analizado en las plantas que contenían la construcción *35S::CO*. En el mutante *phyB* se observa un aumento de la estabilidad de CO durante la mañana; en cambio, en el doble mutante *cry1 cry2* y en el mutante *phyA* desaparecen los picos de la proteína CO que se observan a primera y última hora del día en las plantas sobreexpresoras de CO. Estos resultados indican que al comienzo del día PhyB participa en la degradación de CO, mientras que en la tarde, los criptocromos y PhyA antagonizan dicha degradación y estabilizan a la proteína CO, lo que permite que se induzca la expresión de *FT* en condiciones de DL (Valverde y col., 2004). Hasta el momento se desconoce el mecanismo que regula el cambio de actividad de los fotorreceptores entre la mañana y la tarde, puesto que PhyB es un fitocromo estable en luz y los criptocromos están presentes de forma constante durante las condiciones fotoperiódicas de DL (El-Din El-Assal y col., 2001; Mockler y col., 2003). Datos recientes indican que los criptocromos regulan negativamente la actividad de COP1 en la luz, lo cual estabiliza a CO e induce la floración en Arabidopsis (Liu y col., 2008c). Además, también se ha demostrado que COP1 regula a CO a nivel transcripcional a través de GI (Yu y col., 2008). COP1 y ELF3, una de las proteínas asociadas al reloj circadiano, median la señal transmitida por Cry2 y participan en la degradación de GI (Yu y col., 2008). Posteriormente, se describió el papel de COP1 en la degradación de CO en la última parte del día y en la noche, y se estableció que la degradación de esta proteína en luz roja no estaba afectada por la mutación *cop1* (Jang y col., 2008; Liu y col., 2008c). Estos resultados apuntan que otra ligasa de ubiquitina podría ser la responsable de la degradación de CO dependiente de PhyB que tiene lugar en las primeras horas del día (Jang y col.,

2008). Nuestros datos demuestran que HOS1 juega un papel relevante en el ajuste de los niveles de la proteína CO durante el periodo de luz y que, de este modo, previene el aumento prematuro de *FT* en las primeras horas del día, si bien se necesitan más datos experimentales que permitan establecer la posible participación de HOS1 en la proteólisis de CO mediada por PhyB.

En plantas se han descrito varios ejemplos de proteínas que, al igual que CO, se degradan en un proceso dependiente de los fitocromos. Así, PHYTOCHROME-INTERACTING FACTOR 1 (PIF1), PIF3, PIF4 y PIF5 son factores de transcripción tipo basic helix-loop-helix que participan en la ruta de señalización de la luz roja y la luz roja lejana (Ni y col., 1999; Huq y Quail, 2002; Fujimori y col., 2004; Huq y col., 2004). Se ha observado que los fitocromos, cuando se activan por la luz, sufren una translocación al núcleo e interaccionan con los PIFs. La unión entre el Phy fotoactivado y las proteínas PIF1, PIF3 y PIF5 induce la fosforilación de éstas últimas y su degradación por el proteosoma en menos de cinco minutos (Al-Sady y col., 2006; Shen y col., 2007; Shen y col., 2008). Recientemente, se ha demostrado que la señalización de la luz roja mediada por PhyB conlleva un mecanismo de terminación de la respuesta que implica a COP1. Cuando el PhyB es activado y se transloca al núcleo, COP1 promueve su degradación mediante un mecanismo en el que participan los PIFs (Jang y col., 2010). De esta manera, se ha propuesto que PhyB y los PIFs se regulan mutuamente de forma negativa (Henriques y col., 2009; Jang y col., 2010). Igualmente, COL3, un homologo de CO que reprime la floración, se ha identificado como un nuevo interactor de COP1 que participa como regulador positivo de la respuesta a la luz roja (Datta y col., 2006).

Nuevas aproximaciones experimentales serán necesarias para comprender en profundidad los mecanismos moleculares que median el papel de HOS1 en la regulación fotoperiódica de la floración en Arabidopsis, y en particular para abordar el estudio de la posible implicación de esta E3 ligasa de ubquitina en la degradación de CO dependiente de luz roja. Nuestra hipótesis de trabajo contempla que PhyB, una vez activo tras exposición a luz roja, podría translocarse al núcleo y activar a HOS1 en las primeras horas del día. Un posible mecanismo de activación podría conllevar la modificación post-traduccional tanto de HOS1 como de sus posibles sustratos, incluyendo a CO. El esclarecimiento de estos mecanismos arrojará luz sobre el control de la inducción de la floración en respuesta a fotoperiodo, un proceso complejo cuya regulación empezamos a comprender gracias a estudios realizados en la especie modelo Arabidopsis, pero cuyos componentes esenciales están altamente conservados en otras especies.

Por último, aunque en este trabajo hemos demostrado que HOS1 está implicado en la degradación de CO, no descartamos que existan otras proteínas que participen en el control del tiempo de floración y cuya estabilidad pueda estar regulada por esta E3 ligasa. De hecho, HOS1 regula de forma positiva la expresión de *FLC* (Lee y col., 2001), que a su vez, está reprimida por la exposición prolongada a bajas temperaturas (Amasino, 2010). Es posible, por tanto, que HOS1 pueda estar involucrado en la degradación de un regulador negativo de la expresión de *FLC*, aunque los análisis genéticos realizados no han permitido identificar por el momento cuál puede ser esta diana.

CONCLUSIONES

En el presente trabajo hemos abordado la caracterización genética y molecular de los loci *ESD1/ARP6*, *SWC6* y *ESD6/HOS1* para establecer su papel en el control del tiempo de floración en Arabidopsis, y hemos profundizado en los mecanismos de represión floral mediados por la remodelación de la estructura de la cromatina y la degradación selectiva de proteínas.

1. Los mutantes de floración temprana de Arabidopsis *esd1/arp6* y *swc6* están afectados en genes que codifican proteínas ortólogas de las subunidades Arp6 y Swc6 del complejo de remodelación de cromatina Swr1 de levaduras, implicado en el intercambio de la variante histónica H2A.Z. La identificación de estas proteínas apoya la existencia de un complejo SWR1 en Arabidopsis.
2. Además de una aceleración del tiempo de floración, los mutantes *esd1/arp6* y *swc6* presentan otras alteraciones de su fenotipo, que afectan al desarrollo de los órganos florales y a la morfología y tamaño de la hoja, lo que sugiere la posible participación del complejo SWR1 en la regulación de esos procesos de desarrollo. De acuerdo con estas observaciones, el patrón de expresión de *ESD1/ARP6* y *SWC6* es ubicuo.
3. Las mutaciones *esd1/arp6* y *swc6* interaccionan de forma epistática con mutaciones en genes de la ruta autónoma y suprimen parcialmente el fenotipo de floración tardio conferido por alelos activos de *FRI.*
4. *ESD1/ARP6* y *SWC6* son necesarios para la activación del represor floral *FLC.* Además, ambos loci desempeñan un papel independiente de *FLC* en el control del tiempo de floración, posiblemente mediado por la activación de los genes *MAF4* y *MAF5*.
5. *ESD1/ARP6* y *SWC6* se requieren para mantener los niveles de dos marcas epigenéticas activadoras sobre la cromatina de *FLC*, la acetilación de la histona H3 y la trimetilación de la lisina 4 de la histona H3.
6. ESD1/ARP6 y SWC6 interaccionan físicamente, lo que sugiere que forman parte de un mismo subcomplejo proteico dentro de SWR1-C.
7. El mutante de floración temprana *esd6/hos1* está afectado en el locus *HOS1*, que codifica una enzima E3 ligasa de ubiquitina identificada previamente por su participación en la respuesta de aclimatación a bajas temperaturas en Arabidopsis. Además de participar en el control del tiempo de floración, *ESD6/HOS1* está implicado en la regulación del desarrollo de las hojas, flores y raíces.
8. *ESD6/HOS1* regula de forma positiva la expression de *FLC*, aunque también tiene un efecto independiente de *FLC* en el control del tiempo de floración.

9. *HOS1* interacciona genéticamente con *CO,* si bien la mutación *esd6/hos1* no altera el patrón ni los niveles de expresión de *CO* a lo largo del día.
10. HOS1 se localiza en el núcleo e interacciona con CO, participando en la modulación de los niveles de dicha proteína durante las primeras horas del día en fotoperiodos de DL.
11. *HOS1* interacciona genéticamente con *COP1*, un gen que codifica otra E3 ligasa de ubiquitina involucrada en la regulación de los niveles de la proteína CO durante la noche, lo que sugiere que HOS1 y COP1 ajustan de manera precisa la acumulación de CO a lo largo del ciclo diario.
12. A través de su papel en la modulación de los niveles de CO, HOS1 regula el patrón de expresión diario de *FT* durante el periodo de luz, impidiendo su activación durante las primeras horas del día, y permitiendo así una correcta regulación fotoperiódica del tiempo de floración en Arabidopsis.

BIBLIOGRAFÍA

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., y Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309**, 1052-1056.

Adam, M., Robert, F., Larochelle, M., y Gaudreau, L. (2001). H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions. Mol Cell Biol **21**, 6270-6279.

Adrian, J., Farrona, S., Reimer, J.J., Albani, M.C., Coupland, G., y Turck, F. (2010). *cis*-Regulatory elements and chromatin state coordinately control temporal and spatial expression of *FLOWERING LOCUS T* in Arabidopsis. Plant Cell **22**, 1425-1440.

Ahmad, A., Zhang, Y., y Cao, X.F. (2010). Decoding the epigenetic language of plant development. Mol Plant **3**, 719-728.

Al-Sady, B., Ni, W., Kircher, S., Schafer, E., y Quail, P.H. (2006). Photoactivated phytochrome induces rapid PIF3 phosphorylation prior to proteasome-mediated degradation. Mol Cell **23**, 439-446.

Altaf, M., Auger, A., Covic, M., y Cote, J. (2009). Connection between histone H2A variants and chromatin remodeling complexes. Biochem Cell Biol **87**, 35-50.

Amasino, R. (2010). Seasonal and developmental timing of flowering. Plant J **61**, 1001-1013.

Amasino, R.M., y Michaels, S.D. (2010). The timing of flowering. Plant Physiol **154**, 516-520.

An, H., Roussot, C., Suarez-Lopez, P., Corbesier, L., Vincent, C., Pineiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., y Coupland, G. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development **131**, 3615-3626.

Auger, A., Galarneau, L., Altaf, M., Nourani, A., Doyon, Y., Utley, R.T., Cronier, D., Allard, S., y Cote, J. (2008). Eaf1 is the platform for NuA4 molecular assembly that evolutionarily links chromatin acetylation to ATP-dependent exchange of histone H2A variants. Mol Cell Biol **28**, 2257-2270.

Aukerman, M.J., y Sakai, H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its *APETALA2*-like target genes. Plant Cell **15**, 2730-2741.

Aukerman, M.J., Hirschfeld, M., Wester, L., Weaver, M., Clack, T., Amasino, R.M., y Sharrock, R.A. (1997). A deletion in the *PHYD* gene of the Arabidopsis Wassilewskija ecotype defines a role for phytochrome D in red/far-red light sensing. Plant Cell **9**, 1317-1326.

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L., y Martinez-Zapater, J.M. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. Nat Genet **36**, 162-166.

Balasubramanian, S., Sureshkumar, S., Lempe, J., y Weigel, D. (2006a). Potent induction of *Arabidopsis thaliana* flowering by elevated growth temperature. PLoS Genet **2**, e106.

Balasubramanian, S., Sureshkumar, S., Agrawal, M., Michael, T.P., Wessinger, C., Maloof, J.N., Clark, R., Warthmann, N., Chory, J., y Weigel, D. (2006b). The PHYTOCHROME C photoreceptor gene mediates natural variation in flowering and growth responses of *Arabidopsis thaliana*. Nat Genet **38**, 711-715.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., y Dean, C. (2004). Vernalization requires epigenetic silencing of *FLC* by histone methylation. Nature **427**, 164-167.

Ben-Naim, O., Eshed, R., Parnis, A., Teper-Bamnolker, P., Shalit, A., Coupland, G., Samach, A., y Lifschitz, E. (2006). The CCAAT binding factor can mediate interactions between CONSTANS-like proteins and DNA. Plant J **46**, 462-476.

Blazquez, M.A., Ahn, J.H., y Weigel, D. (2003). A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. Nat Genet **33**, 168-171.

Blazquez, M.A., Soowal, L.N., Lee, I., y Weigel, D. (1997). *LEAFY* expression and flower initiation in Arabidopsis. Development **124,** 3835-3844.

Blazquez, M.A., Green, R., Nilsson, O., Sussman, M.R., y Weigel, D. (1998). Gibberellins promote flowering of arabidopsis by activating the LEAFY promoter. Plant Cell **10,** 791-800.

Blessing, C.A., Ugrinova, G.T., y Goodson, H.V. (2004). Actin and ARPs: action in the nucleus. Trends Cell Biol **14,** 435-442.

Brickner, D.G., Cajigas, I., Fondufe-Mittendorf, Y., Ahmed, S., Lee, P.C., Widom, J., y Brickner, J.H. (2007). H2A.Z-mediated localization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. PLoS Biol **5,** e81.

Cai, X., Ballif, J., Endo, S., Davis, E., Liang, M., Chen, D., DeWald, D., Kreps, J., Zhu, T., y Wu, Y. (2007). A putative CCAAT-binding transcription factor is a regulator of flowering timing in Arabidopsis. Plant Physiol **145,** 98-105.

Cai, Y., Jin, J., Gottschalk, A.J., Yao, T., Conaway, J.W., y Conaway, R.C. (2006). Purification and assay of the human INO80 and SRCAP chromatin remodeling complexes. Methods **40,** 312-317.

Cao, Y., Dai, Y., Cui, S., y Ma, L. (2008). Histone H2B monoubiquitination in the chromatin of *FLOWERING LOCUS C* regulates flowering time in Arabidopsis. Plant Cell **20,** 2586-2602.

Carrozza, M.J., Utley, R.T., Workman, J.L., y Cote, J. (2003). The diverse functions of histone acetyltransferase complexes. Trends Genet **19,** 321-329.

Castillejo, C., y Pelaz, S. (2008). The balance between CONSTANS and TEMPRANILLO activities determines *FT* expression to trigger flowering. Curr Biol **18,** 1338-1343.

Clarkson, M.J., Wells, J.R., Gibson, F., Saint, R., y Tremethick, D.J. (1999). Regions of variant histone His2AvD required for Drosophila development. Nature **399,** 694-697.

Coen, E.S., y Meyerowitz, E.M. (1991). The war of the whorls: genetic interactions controlling flower development. Nature **353,** 31-37.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., y Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316,** 1030-1033.

Crevillen, P., y Dean, C. (2011). Regulation of the floral repressor gene *FLC*: the complexity of transcription in a chromatin context. Curr Opin Plant Biol **14,** 38-44.

Chen, H., Huang, X., Gusmaroli, G., Terzaghi, W., Lau, O.S., Yanagawa, Y., Zhang, Y., Li, J., Lee, J.H., Zhu, D., y Deng, X.W. (2010). Arabidopsis CULLIN4-Damaged DNA Binding Protein 1 Interacts with CONSTITUTIVELY PHOTOMORPHOGENIC1-SUPPRESSOR OF PHYA Complexes to Regulate Photomorphogenesis and Flowering Time. Plant Cell **22,** 108-123.

Chen, M., y Ni, M. (2006a). RED AND FAR-RED INSENSITIVE 2, a RING-domain zinc finger protein, mediates phytochrome-controlled seedling deetiolation responses. Plant Physiol **140,** 457-465.

Chen, M., y Ni, M. (2006b). RFI2, a RING-domain zinc finger protein, negatively regulates *CONSTANS* expression and photoperiodic flowering. Plant J **46,** 823-833.

Cheng, X.F., y Wang, Z.Y. (2005). Overexpression of *COL9,* a *CONSTANS-LIKE* gene, delays flowering by reducing expression of *CO* and *FT* in *Arabidopsis thaliana*. Plant J **43,** 758-768.

Choi, K., Park, C., Lee, J., Oh, M., Noh, B., y Lee, I. (2007). Arabidopsis homologs of components of the SWR1 complex regulate flowering and plant development. Development **134,** 1931-1941.

Choi, K., Kim, J., Hwang, H.J., Kim, S., Park, C., Kim, S.Y., y Lee, I. (2011). The FRIGIDA Complex Activates Transcription of *FLC*, a Strong Flowering Repressor

in Arabidopsis, by Recruiting Chromatin Modification Factors. Plant Cell **23,** 289-303.

Choi, K., Kim, S., Kim, S.Y., Kim, M., Hyun, Y., Lee, H., Choe, S., Kim, S.G., Michaels, S., y Lee, I. (2005). *SUPPRESSOR OF FRIGIDA3* encodes a nuclear ACTIN-RELATED PROTEIN6 required for floral repression in Arabidopsis. Plant Cell **17,** 2647-2660.

Datta, S., Hettiarachchi, G.H., Deng, X.W., y Holm, M. (2006). Arabidopsis CONSTANS-LIKE3 is a positive regulator of red light signaling and root growth. Plant Cell **18,** 70-84.

De Lucia, F., Crevillen, P., Jones, A.M., Greb, T., y Dean, C. (2008). A PHD-polycomb repressive complex 2 triggers the epigenetic silencing of *FLC* during vernalization. Proc Natl Acad Sci U S A **105,** 16831-16836.

de Montaigu, A., Toth, R., y Coupland, G. (2010). Plant development goes like clockwork. Trends Genet **26,** 296-306.

Deal, R.B., y Henikoff, S. (2010). Histone variants and modifications in plant gene regulation. Curr Opin Plant Biol, doi:10.1016/j.pbi.2010.1011.1005.

Deal, R.B., Kandasamy, M.K., McKinney, E.C., y Meagher, R.B. (2005). The nuclear actin-related protein ARP6 is a pleiotropic developmental regulator required for the maintenance of *FLOWERING LOCUS C* expression and repression of flowering in Arabidopsis. Plant Cell **17,** 2633-2646.

Deal, R.B., Topp, C.N., McKinney, E.C., y Meagher, R.B. (2007). Repression of flowering in Arabidopsis requires activation of *FLOWERING LOCUS C* expression by the histone variant H2A.Z. Plant Cell **19,** 74-83.

del Olmo, I., Lopez-Gonzalez, L., Martin-Trillo, M.M., Martinez-Zapater, J.M., Pineiro, M., y Jarillo, J.A. (2010). *EARLY IN SHORT DAYS 7* (*ESD7*) encodes the catalytic subunit of DNA polymerase epsilon and is required for flowering repression through a mechanism involving epigenetic gene silencing. Plant J **61,** 623-636.

Demarsy, E., y Fankhauser, C. (2009). Higher plants use LOV to perceive blue light. Curr Opin Plant Biol **12,** 69-74.

Deshaies, R.J., y Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem **78,** 399-434.

Dong, C.H., Agarwal, M., Zhang, Y., Xie, Q., y Zhu, J.K. (2006). The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. Proc Natl Acad Sci U S A **103,** 8281-8286.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognar, L., Nagy, F., Millar, A.J., y Amasino, R.M. (2002). The *ELF4* gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. Nature **419,** 74-77.

Doyon, Y., Selleck, W., Lane, W.S., Tan, S., y Cote, J. (2004). Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. Mol Cell Biol **24,** 1884-1896.

Draker, R., y Cheung, P. (2009). Transcriptional and epigenetic functions of histone variant H2A.Z. Biochem Cell Biol **87,** 19-25.

El-Din El-Assal, S., Alonso-Blanco, C., Peeters, A.J., Raz, V., y Koornneef, M. (2001). A QTL for flowering time in Arabidopsis reveals a novel allele of *CRY2*. Nat Genet **29,** 435-440.

Faast, R., Thonglairoam, V., Schulz, T.C., Beall, J., Wells, J.R., Taylor, H., Matthaei, K., Rathjen, P.D., Tremethick, D.J., y Lyons, I. (2001). Histone variant H2A.Z is required for early mammalian development. Curr Biol **11,** 1183-1187.

Farris, S.D., Rubio, E.D., Moon, J.J., Gombert, W.M., Nelson, B.H., y Krumm, A. (2005). Transcription-induced chromatin remodeling at the *c-myc* gene involves the local exchange of histone H2A.Z. J Biol Chem **280,** 25298-25303.

Farrona, S., Coupland, G., y Turck, F. (2008). The impact of chromatin regulation on the floral transition. Semin Cell Dev Biol **19,** 560-573.

Farrona, S., Hurtado, L., Bowman, J.L., y Reyes, J.C. (2004). The *Arabidopsis thaliana* SNF2 homolog AtBRM controls shoot development and flowering. Development **131,** 4965-4975.

Finnegan, E.J., y Dennis, E.S. (2007). Vernalization-induced trimethylation of histone H3 lysine 27 at *FLC* is not maintained in mitotically quiescent cells. Curr Biol **17,** 1978-1983.

Fornara, F., y Coupland, G. (2009). Plant phase transitions make a SPLash. Cell **138,** 625-627.

Fornara, F., de Montaigu, A., y Coupland, G. (2010). SnapShot: Control of flowering in Arabidopsis. Cell **141,** 550 e551-552.

Fornara, F., Panigrahi, K.C., Gissot, L., Sauerbrunn, N., Ruhl, M., Jarillo, J.A., y Coupland, G. (2009). Arabidopsis DOF transcription factors act redundantly to reduce *CONSTANS* expression and are essential for a photoperiodic flowering response. Dev Cell **17,** 75-86.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G., y Putterill, J. (1999). *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in Arabidopsis and encodes a protein with several possible membrane-spanning domains. EMBO J **18,** 4679-4688.

Franklin, K.A. (2010). Plant chromatin feels the heat. Cell **140,** 26-28.

Fujimori, T., Yamashino, T., Kato, T., y Mizuno, T. (2004). Circadian-controlled basic/helix-loop-helix factor, PIL6, implicated in light-signal transduction in *Arabidopsis thaliana*. Plant Cell Physiol **45,** 1078-1086.

Galarneau, L., Nourani, A., Boudreault, A.A., Zhang, Y., Heliot, L., Allard, S., Savard, J., Lane, W.S., Stillman, D.J., y Cote, J. (2000). Multiple links between the NuA4 histone acetyltransferase complex and epigenetic control of transcription. Mol Cell **5,** 927-937.

Gazzani, S., Gendall, A.R., Lister, C., y Dean, C. (2003). Analysis of the molecular basis of flowering time variation in Arabidopsis accessions. Plant Physiol **132,** 1107-1114.

Gendall, A.R., Levy, Y.Y., Wilson, A., y Dean, C. (2001). The *VERNALIZATION 2* gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell **107,** 525-535.

Geraldo, N., Baurle, I., Kidou, S.I., Hu, X., y Dean, C. (2009). FRIGIDA delays flowering in Arabidopsis via a co-transcriptional mechanism involving direct interaction with the nuclear cap binding complex. Plant Physiol **150,** 1611-1618.

Gevry, N., Chan, H.M., Laflamme, L., Livingston, D.M., y Gaudreau, L. (2007). *p21* transcription is regulated by differential localization of histone H2A.Z. Genes Dev **21,** 1869-1881.

Greb, T., Mylne, J.S., Crevillen, P., Geraldo, N., An, H., Gendall, A.R., y Dean, C. (2007). The PHD finger protein VRN5 functions in the epigenetic silencing of Arabidopsis *FLC*. Curr Biol **17,** 73-78.

Grennan, A.K. (2006). Variations on a theme. Regulation of flowering time in Arabidopsis. Plant Physiol **140,** 399-400.

Gu, X., Jiang, D., Wang, Y., Bachmair, A., y He, Y. (2009). Repression of the floral transition via histone H2B monoubiquitination. Plant J **57,** 522-533.

Guillemette, B., y Gaudreau, L. (2006). Reuniting the contrasting functions of H2A.Z. Biochem Cell Biol **84,** 528-535.

Guillemette, B., Bataille, A.R., Gevry, N., Adam, M., Blanchette, M., Robert, F., y Gaudreau, L. (2005). Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning. PLoS Biol **3,** e384.

Guo, H., Yang, H., Mockler, T.C., y Lin, C. (1998). Regulation of flowering time by Arabidopsis photoreceptors. Science **279,** 1360-1363.

Hazen, S.P., Schultz, T.F., Pruneda-Paz, J.L., Borevitz, J.O., Ecker, J.R., y Kay, S.A. (2005). *LUX ARRHYTHMO* encodes a Myb domain protein essential for circadian rhythms. Proc Natl Acad Sci U S A **102,** 10387-10392.

He, Y., y Amasino, R.M. (2005). Role of chromatin modification in flowering-time control. Trends Plant Sci **10,** 30-35.

He, Y., Michaels, S.D., y Amasino, R.M. (2003). Regulation of flowering time by histone acetylation in Arabidopsis. Science **302,** 1751-1754.

He, Y., Doyle, M.R., y Amasino, R.M. (2004). PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev **18,** 2774-2784.

Hennig, L., y Derkacheva, M. (2009). Diversity of Polycomb group complexes in plants: same rules, different players? Trends Genet **25,** 414-423.

Henriques, R., Jang, I.C., y Chua, N.H. (2009). Regulated proteolysis in light-related signaling pathways. Curr Opin Plant Biol **12,** 49-56.

Heo, J.B., y Sung, S. (2011). Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science **331,** 76-79.

Huq, E., y Quail, P.H. (2002). PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in Arabidopsis. EMBO J **21,** 2441-2450.

Huq, E., Al-Sady, B., Hudson, M., Kim, C., Apel, K., y Quail, P.H. (2004). Phytochrome-interacting factor 1 is a critical bHLH regulator of chlorophyll biosynthesis. Science **305,** 1937-1941.

Imaizumi, T. (2010). Arabidopsis circadian clock and photoperiodism: time to think about location. Curr Opin Plant Biol **13,** 83-89.

Imaizumi, T., Tran, H.G., Swartz, T.E., Briggs, W.R., y Kay, S.A. (2003). FKF1 is essential for photoperiodic-specific light signalling in Arabidopsis. Nature **426,** 302-306.

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A., y Kay, S.A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of *CONSTANS* in Arabidopsis. Science **309,** 293-297.

Jacobsen, S.E., y Olszewski, N.E. (1993). Mutations at the *SPINDLY* locus of Arabidopsis alter gibberellin signal transduction. Plant Cell **5,** 887-896.

Jaeger, K.E., Graf, A., y Wigge, P.A. (2006). The control of flowering in time and space. J Exp Bot **57,** 3415-3418.

Jang, I.C., Henriques, R., Seo, H.S., Nagatani, A., y Chua, N.H. (2010). Arabidopsis PHYTOCHROME INTERACTING FACTOR Proteins Promote Phytochrome B Polyubiquitination by COP1 E3 Ligase in the Nucleus. Plant Cell **22,** 2370-2383.

Jang, S., Marchal, V., Panigrahi, K.C., Wenkel, S., Soppe, W., Deng, X.W., Valverde, F., y Coupland, G. (2008). Arabidopsis COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. EMBO J **27,** 1277-1288.

Jarillo, J.A., Pineiro, M., Cubas, P., y Martinez-Zapater, J.M. (2009). Chromatin remodeling in plant development. Int J Dev Biol **53,** 1581-1596.

Jarillo, J.A., Capel, J., Tang, R.H., Yang, H.Q., Alonso, J.M., Ecker, J.R., y Cashmore, A.R. (2001). An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature **410,** 487-490.

Jarillo, J.A., Del Olmo, I., Gómez-Zambrano, A., Lázaro, A., López-González, L., Miguel., E., Narro-Diego, L., Sáez, D., y Piñeiro, M. (2008). Photoperiodic control of flowering time. Spanish Journal of Agricultural Research **6,** 221-244.

Jiang, D., Wang, Y., y He, Y. (2008). Repression of *FLOWERING LOCUS C* and *FLOWERING LOCUS T* by the Arabidopsis Polycomb repressive complex 2 components. PLoS One **3,** e3404.

Jiang, D., Yang, W., He, Y., y Amasino, R.M. (2007). Arabidopsis relatives of the human lysine-specific Demethylase1 repress the expression of *FWA* and

FLOWERING LOCUS C and thus promote the floral transition. Plant Cell **19,** 2975-2987.

Jin, J.B., y Hasegawa, P.M. (2008). Flowering time regulation by the SUMO E3 ligase SIZ1. Plant Signal Behav **3,** 891-892.

Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., y Dean, C. (2000). Molecular analysis of *FRIGIDA*, a major determinant of natural variation in Arabidopsis flowering time. Science **290,** 344-347.

Jung, J.H., Seo, Y.H., Seo, P.J., Reyes, J.L., Yun, J., Chua, N.H., y Park, C.M. (2007). The *GIGANTEA*-regulated microRNA172 mediates photoperiodic flowering independent of *CONSTANS* in Arabidopsis. Plant Cell **19,** 2736-2748.

Kabani, M., Michot, K., Boschiero, C., y Werner, M. (2005). Anc1 interacts with the catalytic subunits of the general transcription factors TFIID and TFIIF, the chromatin remodeling complexes RSC and INO80, and the histone acetyltransferase complex NuA3. Biochem Biophys Res Commun **332,** 398-403.

Kandasamy, M.K., Deal, R.B., McKinney, E.C., y Meagher, R.B. (2004). Plant actin-related proteins. Trends Plant Sci **9,** 196-202.

Kandasamy, M.K., Deal, R.B., McKinney, E.C., y Meagher, R.B. (2005). Silencing the nuclear actin-related protein AtARP4 in Arabidopsis has multiple effects on plant development, including early flowering and delayed floral senescence. Plant J **41,** 845-858.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., y Weigel, D. (1999). Activation tagging of the floral inducer *FT*. Science **286,** 1962-1965.

Kim, D.H., Doyle, M.R., Sung, S., y Amasino, R.M. (2009). Vernalization: winter and the timing of flowering in plants. Annu Rev Cell Dev Biol **25,** 277-299.

Kim, S.Y., Yu, X., y Michaels, S.D. (2008). Regulation of *CONSTANS* and *FLOWERING LOCUS T* expression in response to changing light quality. Plant Physiol **148,** 269-279.

Kim, S.Y., He, Y., Jacob, Y., Noh, Y.S., Michaels, S., y Amasino, R. (2005). Establishment of the vernalization-responsive, winter-annual habit in Arabidopsis requires a putative histone H3 methyl transferase. Plant Cell **17,** 3301-3310.

Klejnot, J., y Lin, C. (2004). A *CONSTANS* experience brought to light. Science **303,** 965-966.

Knight, H., Thomson, A.J., y McWatters, H.G. (2008). SENSITIVE TO FREEZING 6 integrates cellular and environmental inputs to the plant circadian clock. Plant Physiol **148,** 293-303.

Ko, J.H., Mitina, I., Tamada, Y., Hyun, Y., Choi, Y., Amasino, R.M., Noh, B., y Noh, Y.S. (2010). Growth habit determination by the balance of histone methylation activities in Arabidopsis. EMBO J **29,** 3208-3215.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., y Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science **286,** 1960-1962.

Kobor, M.S., y Lorincz, M.C. (2009). H2A.Z and DNA methylation: irreconcilable differences. Trends Biochem Sci **34,** 158-161.

Kobor, M.S., Venkatasubrahmanyam, S., Meneghini, M.D., Gin, J.W., Jennings, J.L., Link, A.J., Madhani, H.D., y Rine, J. (2004). A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin. PLoS Biol **2,** E131.

Koornneef, M., Alonso-Blanco, C., y Vreugdenhil, D. (2004). Naturally occurring genetic variation in *Arabidopsis thaliana*. Annu Rev Plant Biol **55,** 141-172.

Kotake, T., Takada, S., Nakahigashi, K., Ohto, M., y Goto, K. (2003). Arabidopsis *TERMINAL FLOWER 2* gene encodes a heterochromatin protein 1 homolog and represses both *FLOWERING LOCUS T* to regulate flowering time and several floral homeotic genes. Plant Cell Physiol **44,** 555-564.

Krogan, N.J., Baetz, K., Keogh, M.C., Datta, N., Sawa, C., Kwok, T.C., Thompson, N.J., Davey, M.G., Pootoolal, J., Hughes, T.R., Emili, A., Buratowski, S., Hieter, P., y Greenblatt, J.F. (2004). Regulation of chromosome stability by the histone H2A variant Htz1, the Swr1 chromatin remodeling complex, and the histone acetyltransferase NuA4. Proc Natl Acad Sci U S A **101,** 13513-13518.

Krogan, N.J., Keogh, M.C., Datta, N., Sawa, C., Ryan, O.W., Ding, H., Haw, R.A., Pootoolal, J., Tong, A., Canadien, V., Richards, D.P., Wu, X., Emili, A., Hughes, T.R., Buratowski, S., y Greenblatt, J.F. (2003). A Snf2 family ATPase complex required for recruitment of the histone H2A variant Htz1. Mol Cell **12,** 1565-1576.

Kumar, S.V., y Wigge, P.A. (2010). H2A.Z-Containing Nucleosomes Mediate the Thermosensory Response in Arabidopsis. Cell **140,** 136-147.

Laubinger, S., Marchal, V., Le Gourrierec, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G., y Hoecker, U. (2006). Arabidopsis SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. Development **133,** 3213-3222.

Lazaro, A., Valverde, F., Pineiro, M., y Jarillo, J.A. (2011 en revisión).

Lazaro, A., Gomez-Zambrano, A., Lopez-Gonzalez, L., Pineiro, M., y Jarillo, J.A. (2008). Mutations in the Arabidopsis *SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development. J Exp Bot **59,** 653-666.

Leach, T.J., Mazzeo, M., Chotkowski, H.L., Madigan, J.P., Wotring, M.G., y Glaser, R.L. (2000). Histone H2A.Z is widely but nonrandomly distributed in chromosomes of *Drosophila melanogaster*. J Biol Chem **275,** 23267-23272.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., y Zhu, J.K. (2001). The Arabidopsis *HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleo--cytoplasmic partitioning. Genes Dev **15,** 912-924.

Lee, I., Aukerman, M.J., Gore, S.L., Lohman, K.N., Michaels, S.D., Weaver, L.M., John, M.C., Feldmann, K.A., y Amasino, R.M. (1994). Isolation of *LUMINIDEPENDENS*: a gene involved in the control of flowering time in Arabidopsis. Plant Cell **6,** 75-83.

Lee, J., y Lee, I. (2010). Regulation and function of SOC1, a flowering pathway integrator. J Exp Bot **61,** 2247-2254.

Lee, J., Oh, M., Park, H., y Lee, I. (2008). SOC1 translocated to the nucleus by interaction with AGL24 directly regulates *LEAFY*. Plant J **55,** 832-843.

Lee, J.H., Yoo, S.J., Park, S.H., Hwang, I., Lee, J.S., y Ahn, J.H. (2007). Role of SVP in the control of flowering time by ambient temperature in Arabidopsis. Genes Dev **21,** 397-402.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., y Dean, C. (2002). Multiple roles of Arabidopsis *VRN1* in vernalization and flowering time control. Science **297,** 243-246.

Li, B., Pattenden, S.G., Lee, D., Gutierrez, J., Chen, J., Seidel, C., Gerton, J., y Workman, J.L. (2005). Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. Proc Natl Acad Sci U S A **102,** 18385-18390.

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C.A., Ito, T., Meyerowitz, E., y Yu, H. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. Dev Cell **15,** 110-120.

Lim, M.H., Kim, J., Kim, Y.S., Chung, K.S., Seo, Y.H., Lee, I., Hong, C.B., Kim, H.J., y Park, C.M. (2004). A new Arabidopsis gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via *FLOWERING LOCUS C*. Plant Cell **16,** 731-740.

Liu, C., Chen, H., Er, H.L., Soo, H.M., Kumar, P.P., Han, J.H., Liou, Y.C., y Yu, H. (2008a). Direct interaction of *AGL24* and *SOC1* integrates flowering signals in Arabidopsis. Development **135,** 1481-1491.

Liu, H., Yu, X., Li, K., Klejnot, J., Yang, H., Lisiero, D., y Lin, C. (2008b). Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral initiation in Arabidopsis. Science **322,** 1535-1539.

Liu, L.J., Zhang, Y.C., Li, Q.H., Sang, Y., Mao, J., Lian, H.L., Wang, L., y Yang, H.Q. (2008c). COP1-Mediated Ubiquitination of CONSTANS Is Implicated in Cryptochrome Regulation of Flowering in Arabidopsis. Plant Cell **20,** 292-306.

Liu, Y., Wang, F., Zhang, H., He, H., Ma, L., y Deng, X.W. (2008d). Functional characterization of the Arabidopsis ubiquitin-specific protease gene family reveals specific role and redundancy of individual members in development. Plant J **55,** 844-856.

Loidl, P. (2004). A plant dialect of the histone language. Trends Plant Sci **9,** 84-90.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., y Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature **389,** 251-260.

Ma, H. (1998). To be, or not to be, a flower-control of floral meristem identity. Trends Genet **14,** 26-32.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., y Dean, C. (1997). *FCA*, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. Cell **89,** 737-745.

Manzano, D., Marquardt, S., Jones, A.M., Baurle, I., Liu, F., y Dean, C. (2009). Altered interactions within FY/AtCPSF complexes required for Arabidopsis FCA-mediated chromatin silencing. Proc Natl Acad Sci U S A **106,** 8772-8777.

March-Diaz, R., y Reyes, J.C. (2009). The Beauty of Being a Variant H2AZ and the SWR complex in plants. Molecular Plant **2,** 565-577.

March-Diaz, R., Garcia-Dominguez, M., Florencio, F.J., y Reyes, J.C. (2007). SEF, a new protein required for flowering repression in Arabidopsis, interacts with PIE1 and ARP6. Plant Physiol **143,** 893-901.

March-Diaz, R., Garcia-Dominguez, M., Lozano-Juste, J., Leon, J., Florencio, F.J., y Reyes, J.C. (2008). Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in Arabidopsis. Plant J **53,** 475-487.

Marques, M., Laflamme, L., Gervais, A.L., y Gaudreau, L. (2010). Reconciling the positive and negative roles of histone H2A.Z in gene transcription. Epigenetics **5,** 267-272.

Martin-Trillo, M., Lazaro, A., Poethig, R.S., Gomez-Mena, C., Pineiro, M.A., Martinez-Zapater, J.M., Jarillo, J.A., (Print), -., Article, J., y Research Support, N.-U.S.G.t. (2006). *EARLY IN SHORT DAYS 1* (*ESD1*) encodes ACTIN-RELATED PROTEIN 6 (AtARP6), a putative component of chromatin remodelling complexes that positively regulates *FLC* accumulation in Arabidopsis. Development **133,** 1241-1252.

Martínez-Zapater, J.M., Coupland, G., Dean, C., y Koornneef, M. (1994). The transition to flowering in Arabidopsis. Arabidopsis 403-433. Cold Spring Harbour Laboratory Press. New York.

Matangkasombut, O., y Buratowski, S. (2003). Different sensitivities of bromodomain factors 1 and 2 to histone H4 acetylation. Mol Cell **11,** 353-363.

Mathieu, J., Yant, L.J., Murdter, F., Kuttner, F., y Schmid, M. (2009). Repression of flowering by the miR172 target SMZ. PLoS Biol **7,** e1000148.

Meneghini, M.D., Wu, M., y Madhani, H.D. (2003). Conserved histone variant H2A.Z protects euchromatin from the ectopic spread of silent heterochromatin. Cell **112,** 725-736.

Michaels, S.D. (2009). Flowering time regulation produces much fruit. Curr Opin Plant Biol **12,** 75-80.

Michaels, S.D., y Amasino, R.M. (1999). *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.

Michaels, S.D., y Amasino, R.M. (2001). Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. Plant Cell **13,** 935-941.

Michaels, S.D., He, Y., Scortecci, K.C., y Amasino, R.M. (2003). Attenuation of FLOWERING LOCUS C activity as a mechanism for the evolution of summer-annual flowering behavior in Arabidopsis. Proc Natl Acad Sci U S A **100,** 10102-10107.

Millar, C.B., Xu, F., Zhang, K., y Grunstein, M. (2006). Acetylation of H2AZ Lys 14 is associated with genome-wide gene activity in yeast. Genes Dev **20,** 711-722.

Miura, K., y Hasegawa, P.M. (2010). Sumoylation and other ubiquitin-like post-translational modifications in plants. Trends Cell Biol **20,** 223-232.

Miura, K., Jin, J.B., y Hasegawa, P.M. (2007a). Sumoylation, a post-translational regulatory process in plants. Curr Opin Plant Biol **10,** 495-502.

Miura, K., Jin, J.B., Lee, J., Yoo, C.Y., Stirm, V., Miura, T., Ashworth, E.N., Bressan, R.A., Yun, D.J., y Hasegawa, P.M. (2007b). SIZ1-Mediated Sumoylation of ICE1 Controls *CBF3/DREB1A* Expression and Freezing Tolerance in Arabidopsis. Plant Cell **19,** 1403-1414.

Mizuguchi, G., Shen, X., Landry, J., Wu, W.H., Sen, S., y Wu, C. (2004). ATP-driven exchange of histone H2AZ variant catalyzed by SWR1 chromatin remodeling complex. Science **303,** 343-348.

Mockler, T., Yang, H., Yu, X., Parikh, D., Cheng, Y.C., Dolan, S., y Lin, C. (2003). Regulation of photoperiodic flowering by Arabidopsis photoreceptors. Proc Natl Acad Sci U S A **100,** 2140-2145.

Moon, J., Parry, G., y Estelle, M. (2004). The ubiquitin-proteasome pathway and plant development. Plant Cell **16,** 3181-3195.

Moon, J., Suh, S.S., Lee, H., Choi, K.R., Hong, C.B., Paek, N.C., Kim, S.G., y Lee, I. (2003). The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. Plant J **35,** 613-623.

Morris, K., Thornber, S., Codrai, L., Richardson, C., Craig, A., Sadanandom, A., Thomas, B., y Jackson, S. (2010). *DAY NEUTRAL FLOWERING* represses *CONSTANS* to prevent Arabidopsis flowering early in short days. Plant Cell **22,** 1118-1128.

Mueller, C.L., y Jaehning, J.A. (2002). Ctr9, Rtf1, and Leo1 are components of the Paf1/RNA polymerase II complex. Mol Cell Biol **22,** 1971-1980.

Mutasa-Gottgens, E., y Hedden, P. (2009). Gibberellin as a factor in floral regulatory networks. J Exp Bot **60,** 1979-1989.

Mylne, J.S., Barrett, L., Tessadori, F., Mesnage, S., Johnson, L., Bernatavichute, Y.V., Jacobsen, S.E., Fransz, P., y Dean, C. (2006). LHP1, the Arabidopsis homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of *FLC*. Proc Natl Acad Sci U S A **103,** 5012-5017.

Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., y Bartel, B. (2000). *FKF1*, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. Cell **101,** 331-340.

Ng, H.H., Robert, F., Young, R.A., y Struhl, K. (2003). Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. Mol Cell **11,** 709-719.

Ni, M., Tepperman, J.M., y Quail, P.H. (1999). Binding of phytochrome B to its nuclear signalling partner PIF3 is reversibly induced by light. Nature **400,** 781-784.

Niu, L., Zhang, Y., Pei, Y., Liu, C., y Cao, X. (2008). Redundant requirement for a pair of PROTEIN ARGININE METHYLTRANSFERASE4 homologs for the proper regulation of Arabidopsis flowering time. Plant Physiol **148,** 490-503.

Noh, B., Lee, S.H., Kim, H.J., Yi, G., Shin, E.A., Lee, M., Jung, K.J., Doyle, M.R., Amasino, R.M., y Noh, Y.S. (2004). Divergent roles of a pair of homologous

jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. Plant Cell **16,** 2601-2613.

Noh, Y.S., y Amasino, R.M. (2003). *PIE1*, an ISWI family gene, is required for *FLC* activation and floral repression in Arabidopsis. Plant Cell **15,** 1671-1682.

Park, B.S., Eo, H.J., Jang, I.C., Kang, H.G., Song, J.T., y Seo, H.S. (2010a). Ubiquitination of LHY by SINAT5 regulates flowering time and is inhibited by DET1. Biochem Biophys Res Commun **398,** 242-246.

Park, B.S., Sang, W.G., Yeu, S.Y., Do Choi, Y., Paek, N.C., Kim, M.C., Song, J.T., y Seo, H.S. (2007). Post-translational regulation of FLC is mediated by an E3 ubiquitin ligase activity of SINAT5 in Arabidopsis. Plant Science **173,** 269-275.

Park, S., Oh, S., Ek-Ramos, J., y van Nocker, S. (2010b). *PLANT HOMOLOGOUS TO PARAFIBROMIN* is a component of the PAF1 complex and assists in regulating expression of genes within H3K27ME3-enriched chromatin. Plant Physiol **153,** 821-831.

Pien, S., Fleury, D., Mylne, J.S., Crevillen, P., Inze, D., Avramova, Z., Dean, C., y Grossniklaus, U. (2008). ARABIDOPSIS TRITHORAX1 dynamically regulates *FLOWERING LOCUS C* activation via histone 3 lysine 4 trimethylation. Plant Cell **20,** 580-588.

Pineiro, M., Gomez-Mena, C., Schaffer, R., Martinez-Zapater, J.M., y Coupland, G. (2003). EARLY BOLTING IN SHORT DAYS is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. Plant Cell **15,** 1552-1562.

Poethig, R.S. (1990). Phase change and the regulation of shoot morphogenesis in plants. Science **250,** 923-930.

Poethig, R.S. (2010). The past, present, and future of vegetative phase change. Plant Physiol **154,** 541-544.

Pouteau, S., Carre, I., Gaudin, V., Ferret, V., Lefebvre, D., y Wilson, M. (2008). Diversification of photoperiodic response patterns in a collection of early-flowering mutants of Arabidopsis. Plant Physiol **148,** 1465-1473.

Pouteau, S., Ferret, V., Gaudin, V., Lefebvre, D., Sabar, M., Zhao, G., y Prunus, F. (2004). Extensive phenotypic variation in early flowering mutants of Arabidopsis. Plant Physiol **135,** 201-211.

Putterill, J., Robson, F., Lee, K., Simon, R., y Coupland, G. (1995). The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell **80,** 847-857.

Raisner, R.M., y Madhani, H.D. (2006). Patterning chromatin: form and function for H2A.Z variant nucleosomes. Curr Opin Genet Dev **16,** 119-124.

Raisner, R.M., Hartley, P.D., Meneghini, M.D., Bao, M.Z., Liu, C.L., Schreiber, S.L., Rando, O.J., y Madhani, H.D. (2005). Histone variant H2A.Z marks the 5' ends of both active and inactive genes in euchromatin. Cell **123,** 233-248.

Rando, O.J. (2007). Global patterns of histone modifications. Curr Opin Genet Dev **17,** 94-99.

Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L., y Riechmann, J.L. (2001). Regulation of flowering in Arabidopsis by an *FLC* homologue. Plant Physiol **126,** 122-132.

Ratcliffe, O.J., Kumimoto, R.W., Wong, B.J., y Riechmann, J.L. (2003). Analysis of the Arabidopsis *MADS AFFECTING FLOWERING* gene family: *MAF2* prevents vernalization by short periods of cold. Plant Cell **15,** 1159-1169.

Ringrose, L., y Paro, R. (2007). Polycomb/Trithorax response elements and epigenetic memory of cell identity. Development **134,** 223-232.

Robson, F., Costa, M.M., Hepworth, S.R., Vizir, I., Pineiro, M., Reeves, P.H., Putterill, J., y Coupland, G. (2001). Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. Plant J **28,** 619-631.

Roux, F., Touzet, P., Cuguen, J., y Le Corre, V. (2006). How to be early flowering: an evolutionary perspective. Trends Plant Sci **11,** 375-381.

Rubio, V., y Deng, X.W. (2007). Standing on the shoulders of GIGANTEA. Science **318,** 206-207.

Ruhl, D.D., Jin, J., Cai, Y., Swanson, S., Florens, L., Washburn, M.P., Conaway, R.C., Conaway, J.W., y Chrivia, J.C. (2006). Purification of a human SRCAP complex that remodels chromatin by incorporating the histone variant H2A.Z into nucleosomes. Biochemistry **45,** 5671-5677.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., y Coupland, G. (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288,** 1613-1616.

Santisteban, M.S., Kalashnikova, T., y Smith, M.M. (2000). Histone H2A.Z regulats transcription and is partially redundant with nucleosome remodeling complexes. Cell **103,** 411-422.

Santner, A., y Estelle, M. (2010). The ubiquitin-proteasome system regulates plant hormone signaling. Plant J **61,** 1029-1040.

Sawa, M., Nusinow, D.A., Kay, S.A., y Imaizumi, T. (2007). FKF1 and GIGANTEA Complex Formation is Required for Day-Length Measurement in Arabidopsis. Science **318,** 261-265.

Scortecci, K., Michaels, S.D., y Amasino, R.M. (2003). Genetic interactions between *FLM* and other flowering-time genes in *Arabidopsis thaliana*. Plant Mol Biol **52,** 915-922.

Schmitz, R.J., Sung, S., y Amasino, R.M. (2008). Histone arginine methylation is required for vernalization-induced epigenetic silencing of *FLC* in winter-annual *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **105,** 411-416.

Schmitz, R.J., Tamada, Y., Doyle, M.R., Zhang, X., y Amasino, R.M. (2009). Histone H2B deubiquitination is required for transcriptional activation of *FLOWERING LOCUS C* and for proper control of flowering in Arabidopsis. Plant Physiol **149,** 1196-1204.

Schomburg, F.M., Patton, D.A., Meinke, D.W., y Amasino, R.M. (2001). *FPA*, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. Plant Cell **13,** 1427-1436.

Schones, D.E., y Zhao, K. (2008). Genome-wide approaches to studying chromatin modifications. Nat Rev Genet **9,** 179-191.

Schonrock, N., Bouveret, R., Leroy, O., Borghi, L., Kohler, C., Gruissem, W., y Hennig, L. (2006). Polycomb-group proteins repress the floral activator *AGL19* in the *FLC*-independent vernalization pathway. Genes Dev **20,** 1667-1678.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M., y Kay, S.A. (2001). A role for LKP2 in the circadian clock of Arabidopsis. Plant Cell **13,** 2659-2670.

Schulze, J.M., Wang, A.Y., y Kobor, M.S. (2010). Reading chromatin: Insights from yeast into YEATS domain structure and function. Epigenetics **5,** 573-577.

Searle, I., y Coupland, G. (2004). Induction of flowering by seasonal changes in photoperiod. EMBO J **23,** 1217-1222.

Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R.A., y Coupland, G. (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. Genes Dev **20,** 898-912.

Sheldon, C.C., Conn, A.B., Dennis, E.S., y Peacock, W.J. (2002). Different regulatory regions are required for the vernalization-induced repression of *FLOWERING LOCUS C* and for the epigenetic maintenance of repression. Plant Cell **14,** 2527-2537.

Sheldon, C.C., Finnegan, E.J., Dennis, E.S., y Peacock, W.J. (2006). Quantitative effects of vernalization on *FLC* and *SOC1* expression. Plant J **45,** 871-883.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J., y Dennis, E.S. (2000). The molecular basis of vernalization: the central role of *FLOWERING LOCUS C* (*FLC*). Proc Natl Acad Sci U S A **97,** 3753-3758.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., y Dennis, E.S. (1999). The *FLF* MADS box gene: a repressor of

flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11,** 445-458.

Shen, H., Zhu, L., Castillon, A., Majee, M., Downie, B., y Huq, E. (2008). Light-induced phosphorylation and degradation of the negative regulator PHYTOCHROME-INTERACTING FACTOR1 from Arabidopsis depend upon its direct physical interactions with photoactivated phytochromes. Plant Cell **20,** 1586-1602.

Shen, X., Mizuguchi, G., Hamiche, A., y Wu, C. (2000). A chromatin remodelling complex involved in transcription and DNA processing. Nature **406,** 541-544.

Shen, Y., Khanna, R., Carle, C.M., y Quail, P.H. (2007). Phytochrome induces rapid PIF5 phosphorylation and degradation in response to red-light activation. Plant Physiol **145,** 1043-1051.

Shindo, C., Aranzana, M.J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., y Dean, C. (2005). Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of Arabidopsis. Plant Physiol **138,** 1163-1173.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., y Dean, C. (2003). FY is an RNA 3' end-processing factor that interacts with FCA to control the Arabidopsis floral transition. Cell **113,** 777-787.

Smalle, J., y Vierstra, R.D. (2004). The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol **55,** 555-590.

Smith, A.P., Jain, A., Deal, R.B., Nagarajan, V.K., Poling, M.D., Raghothama, K.G., y Meagher, R.B. (2010). Histone H2A.Z regulates the expression of several classes of phosphate starvation response genes but not as a transcriptional activator. Plant Physiol **152,** 217-225.

Somers, D.E., Schultz, T.F., Milnamow, M., y Kay, S.A. (2000). *ZEITLUPE* encodes a novel clock-associated PAS protein from Arabidopsis. Cell **101,** 319-329.

Squazzo, S.L., Costa, P.J., Lindstrom, D.L., Kumer, K.E., Simic, R., Jennings, J.L., Link, A.J., Arndt, K.M., y Hartzog, G.A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors *in vivo*. EMBO J **21,** 1764-1774.

Stone, S.L., y Callis, J. (2007). Ubiquitin ligases mediate growth and development by promoting protein death. Curr Opin Plant Biol **10,** 624-632.

Suarez-Lopez, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., y Coupland, G. (2001). *CONSTANS* mediates between the circadian clock and the control of flowering in Arabidopsis. Nature **410,** 1116-1120.

Sung, S., y Amasino, R.M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. Nature **427,** 159-164.

Sung, S., Schmitz, R.J., y Amasino, R.M. (2006a). A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. Genes Dev **20,** 3244-3248.

Sung, S., He, Y., Eshoo, T.W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S.E., y Amasino, R.M. (2006b). Epigenetic maintenance of the vernalized state in *Arabidopsis thaliana* requires LIKE HETEROCHROMATIN PROTEIN 1. Nat Genet **38,** 706-710.

Sung, Z.R., Chen, L., Moon, Y.H., y Lertpiriyapong, K. (2003). Mechanisms of floral repression in Arabidopsis. Curr Opin Plant Biol **6,** 29-35.

Svotelis, A., Gevry, N., y Gaudreau, L. (2009). Regulation of gene expression and cellular proliferation by histone H2A.Z. Biochem Cell Biol **87,** 179-188.

Swiezewski, S., Liu, F., Magusin, A., y Dean, C. (2009). Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. Nature **462,** 799-802.

Takada, S., y Goto, K. (2003). TERMINAL FLOWER2, an Arabidopsis Homolog of HETEROCHROMATIN PROTEIN1, Counteracts the Activation of *FLOWERING LOCUS T* by CONSTANS in the Vascular Tissues of Leaves to Regulate Flowering Time. Plant Cell **15,** 2856-2865.

Telfer, A., Bollman, K.M., y Poethig, R.S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. Development **124,** 645-654.

Tiwari, S.B., Shen, Y., Chang, H.C., Hou, Y., Harris, A., Ma, S.F., McPartland, M., Hymus, G.J., Adam, L., Marion, C., Belachew, A., Repetti, P.P., Reuber, T.L., y Ratcliffe, O.J. (2010). The flowering time regulator CONSTANS is recruited to the *FLOWERING LOCUS T* promoter via a unique *cis*-element. New Phytol **187,** 57-66.

Turck, F., y Coupland, G. (2011). When vernalization makes sense. Science **331,** 36-37.

Turck, F., Fornara, F., y Coupland, G. (2008). Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol **59,** 573-594.

Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A., y Coupland, G. (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science **303,** 1003-1006.

von Arnim, A.G., y Deng, X.W. (1994). Light inactivation of Arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. Cell **79,** 1035-1045.

Wang, J.W., Czech, B., y Weigel, D. (2009). miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. Cell **138,** 738-749.

Wang, X., Zhang, Y., Ma, Q., Zhang, Z., Xue, Y., Bao, S., y Chong, K. (2007). SKB1-mediated symmetric dimethylation of histone H4R3 controls flowering time in Arabidopsis. Embo J **26,** 1934-1941.

Weigel, D., y Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature **377,** 495-500.

Wenkel, S., Turck, F., Singer, K., Gissot, L., Le Gourrierec, J., Samach, A., y Coupland, G. (2006). CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of Arabidopsis. Plant Cell **18,** 2971-2984.

Werner, J.D., Borevitz, J.O., Uhlenhaut, N.H., Ecker, J.R., Chory, J., y Weigel, D. (2005). *FRIGIDA*-independent variation in flowering time of natural *Arabidopsis thaliana* accessions. Genetics **170,** 1197-1207.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., y Weigel, D. (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309,** 1056-1059.

Wilczek, A.M., Roe, J.L., Knapp, M.C., Cooper, M.D., Lopez-Gallego, C., Martin, L.J., Muir, C.D., Sim, S., Walker, A., Anderson, J., Egan, J.F., Moyers, B.T., Petipas, R., Giakountis, A., Charbit, E., Coupland, G., Welch, S.M., y Schmitt, J. (2009). Effects of genetic perturbation on seasonal life history plasticity. Science **323,** 930-934.

Wilkinson, K.A., y Henley, J.M. (2010). Mechanisms, regulation and consequences of protein SUMOylation. Biochem J **428,** 133-145.

Wilson, R.N., Heckman, J.W., y Somerville, C.R. (1992). Gibberellin Is Required for Flowering in *Arabidopsis thaliana* under Short Days. Plant Physiol **100,** 403-408.

Wood, A., Schneider, J., Dover, J., Johnston, M., y Shilatifard, A. (2003). The Paf1 complex is essential for histone monoubiquitination by the Rad6-Bre1 complex, which signals for histone methylation by COMPASS and Dot1p. J Biol Chem **278,** 34739-34742.

Wood, C.C., Robertson, M., Tanner, G., Peacock, W.J., Dennis, E.S., y Helliwell, C.A. (2006). The *Arabidopsis thaliana* vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proc Natl Acad Sci U S A **103,** 14631-14636.

Wu, G., y Poethig, R.S. (2006). Temporal regulation of shoot development in *Arabidopsis thaliana* by *miR156* and its target *SPL3*. Development **133,** 3539-3547.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.W., Weigel, D., y Poethig, R.S. (2009a). The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell **138,** 750-759.

Wu, W.H., Alami, S., Luk, E., Wu, C.H., Sen, S., Mizuguchi, G., Wei, D., y Wu, C. (2005). Swc2 is a widely conserved H2AZ-binding module essential for ATP-dependent histone exchange. Nat Struct Mol Biol **12,** 1064-1071.

Wu, W.H., Wu, C.H., Ladurner, A., Mizuguchi, G., Wei, D., Xiao, H., Luk, E., Ranjan, A., y Wu, C. (2009b). N terminus of Swr1 binds to histone H2AZ and provides a platform for subunit assembly in the chromatin remodeling complex. J Biol Chem **284,** 6200-6207.

Xu, L., Zhao, Z., Dong, A., Soubigou-Taconnat, L., Renou, J.P., Steinmetz, A., y Shen, W.H. (2008). Di- and tri- but not monomethylation on histone H3 lysine 36 marks active transcription of genes involved in flowering time regulation and other processes in *Arabidopsis thaliana*. Mol Cell Biol **28,** 1348-1360.

Xu, L., Menard, R., Berr, A., Fuchs, J., Cognat, V., Meyer, D., y Shen, W.H. (2009). The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of *FLC* expression and repression of flowering in *Arabidopsis thaliana*. Plant J **57,** 279-288.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., y Araki, T. (2005). *TWIN SISTER OF FT* (*TSF*) acts as a floral pathway integrator redundantly with *FT*. Plant Cell Physiol **46,** 1175-1189.

Yamaguchi, A., Wu, M.F., Yang, L., Wu, G., Poethig, R.S., y Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of *LEAFY*, *FRUITFULL*, and *APETALA1*. Dev Cell **17,** 268-278.

Yant, L., Mathieu, J., y Schmid, M. (2009). Just say no: floral repressors help Arabidopsis bide the time. Curr Opin Plant Biol **12,** 580-586.

Yi, C., y Deng, X.W. (2005). COP1 - from plant photomorphogenesis to mammalian tumorigenesis. Trends Cell Biol **15,** 618-625.

Yi, H., Sardesai, N., Fujinuma, T., Chan, C.W., Veena, y Gelvin, S.B. (2006). Constitutive expression exposes functional redundancy between the Arabidopsis histone H2A gene *HTA1* and other H2A gene family members. Plant Cell **18,** 1575-1589.

Yoo, S.K., Chung, K.S., Kim, J., Lee, J.H., Hong, S.M., Yoo, S.J., Yoo, S.Y., Lee, J.S., y Ahn, J.H. (2005). *CONSTANS* activates *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* through *FLOWERING LOCUS T* to promote flowering in Arabidopsis. Plant Physiol **139,** 770-778.

Yoo, S.Y., Kim, Y., Kim, S.Y., Lee, J.S., y Ahn, J.H. (2007). Control of flowering time and cold response by a NAC-domain protein in Arabidopsis. PLoS ONE **2,** e642.

Yu, J.W., Rubio, V., Lee, N.Y., Bai, S., Lee, S.Y., Kim, S.S., Liu, L., Zhang, Y., Irigoyen, M.L., Sullivan, J.A., Zhang, Y., Lee, I., Xie, Q., Paek, N.C., y Deng, X.W. (2008). COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. Mol Cell **32,** 617-630.

Yu, X., y Michaels, S.D. (2010). The Arabidopsis Paf1c complex component CDC73 participates in the modification of *FLOWERING LOCUS C* chromatin. Plant Physiol **153,** 1074-1084.

Yuan, G.C., Liu, Y.J., Dion, M.F., Slack, M.D., Wu, L.F., Altschuler, S.J., y Rando, O.J. (2005). Genome-scale identification of nucleosome positions in *S. cerevisiae*. Science **309,** 626-630.

Zhang, H., y van Nocker, S. (2002). The *VERNALIZATION INDEPENDENCE 4* gene encodes a novel regulator of *FLOWERING LOCUS C*. Plant J **31,** 663-673.

Zhang, H., Roberts, D.N., y Cairns, B.R. (2005). Genome-wide dynamics of Htz1, a histone H2A variant that poises repressed/basal promoters for activation through histone loss. Cell **123,** 219-231.

Zhang, H., Ransom, C., Ludwig, P., y van Nocker, S. (2003). Genetic analysis of early flowering mutants in Arabidopsis defines a class of pleiotropic developmental regulator required for expression of the flowering-time switch flowering locus C. Genetics **164,** 347-358.

Zhang, H., Richardson, D.O., Roberts, D.N., Utley, R., Erdjument-Bromage, H., Tempst, P., Cote, J., y Cairns, B.R. (2004). The Yaf9 component of the SWR1 and NuA4 complexes is required for proper gene expression, histone H4 acetylation, and Htz1 replacement near telomeres. Mol Cell Biol **24,** 9424-9436.

Zilberman, D., Coleman-Derr, D., Ballinger, T., y Henikoff, S. (2008). Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. Nature **456,** 125-129.