The EMBO Journal (2007) 26, 1934–1941 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
www.embojournal.org

THE EMBO JOURNAL

SKB1-mediated symmetric dimethylation of histone H4R3 controls flowering time in *Arabidopsis*

Xin Wang[1,4], Ya Zhang[1,4], Qibin Ma[2], Zhaoliang Zhang[1], Yongbiao Xue[1,3], Shilai Bao[1,*] and Kang Chong[2,3,*]

[1]Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, [2]Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing, China and [3]National Plant Gene Research Centre, Beijing, China

Plant flowering is a crucial developmental transition from the vegetative to reproductive phase and is properly timed by a number of intrinsic and environmental cues. Genetic studies have identified that chromatin modification influences the expression of *FLOWERING LOCUS C* (*FLC*), a MADS-box transcription factor that controls flowering time. Histone deacetylation and methylation at H3K9 and H3K27 are associated with repression of *FLC*; in contrast, methylation at H3K4 and H3K36 activates *FLC* expression. However, little is known about the functions of histone arginine methylation in plants. Here, we report that *Arabidopsis* Shk1 binding protein 1 (SKB1) catalyzes histone H4R3 symmetric dimethylation (H4R3sme2). *SKB1* lesion results in upregulation of *FLC* and late flowering under both long and short days, but late flowering is reversed by vernalization and gibberellin treatments. An *skb1-1flc-3* double mutant blocks late-flowering phenotype, which suggests that *SKB1* promotes flowering by suppressing *FLC* transcription. SKB1 binds to the *FLC* promoter, and disruption of *SKB1* results in reduced H4R3sme2, especially in the promoter of *FLC* chromatin. Thus, SKB1-mediated H4R3sme2 is a novel histone mark required for repression of *FLC* expression and flowering time control.

The EMBO Journal (2007) **26,** 1934–1941. doi:10.1038/sj.emboj.7601647; Published online 15 March 2007
Subject Categories: chromatin & transcription; plant biology
Keywords: FLC; flowering time; histone arginine methylation; PRMT5/SKB1

Introduction

The lifespan in flowering plants is highly controlled by a developmental switch for the vegetative to reproductive transition. Investigations in *Arabidopsis* have identified four major pathways (photoperiod, vernalization, gibberellin (GA) and autonomous) involved in regulating flowering time (Komeda, 2004; He and Amasino, 2005). The photoperiod and vernalization pathways render *Arabidopsis* able to flower in response to external cues. The vernalization pathway accelerates flowering, and at least three genes (*VRN1*, *VRN2* and *VIN3*) are involved (Gendall *et al*, 2001; Levy *et al*, 2002; Sung and Amasino, 2004). *vrn1*, *vrn2* and *vin3* mutants reduce the vernalization response. *VRN1* and *VRN2* are expressed constitutively, but *VIN3* expression is cold induced. The photoperiod flowering pathway is controlled by *CONSTANS*, *CRY2*, *FHA*, *GIGANTEA*, *FT* and *FWA*, and mutants for all are weakly or not at all sensitive to cold treatment (Martinez-Zapater and Somerville, 1990; Koornneef *et al*, 1991; Guo *et al*, 1998; Searle and Coupland, 2004).

The autonomous pathway is defined by a group of late-flowering mutants *fca*, *fpa*, *fve*, *fld*, *ld*, *fy* and *flk*, whose expression does not depend on photoperiod (Koornneef *et al*, 1991; Lee *et al*, 1994; Macknight *et al*, 1997; Schomburg *et al*, 2001; He *et al*, 2003; Simpson *et al*, 2003; Ausin *et al*, 2004; Lim *et al*, 2004; Mockler *et al*, 2004). In contrast to photoperiod mutant plants, the autonomous mutant plants exhibit a marked reduction in flowering time under vernalization treatment (Koornneef *et al*, 1991). Thus, vernalization can overcome the requirement for the autonomous pathway.

In promoting flowering, GA increases the expression of *SOC1* and *LEAFY*, whereas photoperiod controls flowering time through regulation of *CO* (Komeda, 2004; He and Amasino, 2005). Autonomous and vernalization pathways both control flowering by decreasing of *FLC* mRNA level (Komeda, 2004; He and Amasino, 2005). FLC is a MADS-box transcription factor that plays a central role in blocking developmental transition from the vegetative to flowering stage (Michaels and Amasino, 1999a; Sheldon *et al*, 1999). Loss of *FLC* function elevates the expression of *SOC1* and *FT*, two flowering-time integrator genes, and leads to earlier flowering (Hepworth *et al*, 2002). The late-flowering phenotype in autonomous pathway mutant plants was blocked by introducing *flc* (Michaels and Amasino, 2001; He *et al*, 2003). Vernalization accelerates flowering by a permanent epigenetic repression of *FLC* expression despite the presence of autonomous pathway mutations (Komeda, 2004).

Recent studies have revealed that *FLC*-mediated transition of flowering time is associated with histone covalent modification, including acetylation and methylation (He and Amasino, 2005). The autonomous pathway repressors *FLD* and *FVE*, as components of an HDAC complex, inhibit the expression of *FLC* by increasing deacetylation of *FLC* chromatin (He *et al*, 2003; Ausin *et al*, 2004). Vernalization also elevates deacetylation of histone tails of *FLC* chromatin and at the same time, increases methylation in H3K27 and H3K9, and decreases methylation of H3K4 (Bastow *et al*, 2004; He *et al*, 2004; Sung and Amasino, 2004; Mylne *et al*, 2006; Greb

*Corresponding authors. S Bao, Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Datun Road, Chaoyang District, Beijing 100101, China. Tel.: +86 10 64889350; Fax: +86 10 64889350; E-mail: slbao@genetics.ac.cn or K Chong, Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Xiangshan, Beijing 100093, China. Tel.: +86 10 62836517; Fax: +86 10 82594821.
E-mail: chongk@ibcas.ac.cn
[4]These authors contributed equally to this work

Received: 3 November 2006; accepted: 20 February 2007; published online: 15 March 2007

et al, 2007). Recently, methylation of H3K36 was found to be a histone mark required for increasing *FLC* expression in *Arabidopsis* (Zhao *et al*, 2005).

In addition to acetylation and methylation at lysines, methylation at histone arginine is a covalent modification that results in monomethylarginines, asymmetric dimethylarginines or symmetric dimethylarginines (Bedford and Richard, 2005; Wysocka *et al*, 2006). In humans, of nine protein arginine methyltransferases (PRMT1–9), three (PRMT1, PRMT4 and PRMT5) have been identified as methylating arginines of histones H2A, H3 and H4 (Bedford and Richard, 2005; Cook *et al*, 2006). PRMT5, also called JBP1 or Skb1, is the only enzyme that methylates histone arginines in symmetric dimethylation (Gilbreth *et al*, 1996; Pollack *et al*, 1999; Bao *et al*, 2001; Bedford and Richard, 2005; Wysocka *et al*, 2006). Histone arginine methylation has been shown to regulate chromatin remodelling, gene transcription, and cell proliferation and differentiation (Bedford and Richard, 2005; Ancelin *et al*, 2006; Wysocka *et al*, 2006; Dacwag *et al*, 2007; Liu *et al*, 2007). In plants, however, the biological functions of histone arginine methylation have never been elucidated.

In this report, we present the identification and characterization of a protein arginine methyltransferase SKB1 involved in controlling flowering time. The late-flowering phenotype of T-DNA insertional *skb1* mutant plants is due to upregulation of *FLC* expression. SKB1 binds to *FLC* chromatin and catalyzes H4R3 symmetric dimethylation (H4R3sme2). H4R3sme2 is a novel histone mark associated with *FLC* expression.

Results and discussion

Loss or gain of SKB1 function alters flowering time in Arabidopsis

The *Arabidopsis* genome contains a single-copy *SKB1* gene (*At4g31120*; see www.arabidopsis.org) of 23 exons (Figure 1A). *Arabidopsis* SKB1, encoding a 642-amino acid-protein of approximately 72 kDa (Figure 1B and C), shows high homology with the human PRMT5 (Pollack *et al*, 1999). SKB1 is about 58% identical to PRMT5 over a motif for the *S*-adenosylmethionine binding site and the C-terminal region of 338 amino acids, and the similarity is about 74% over the same region (see Supplementary Figure S1). SKB1 contains consensus methyltransferase regions I, post I, II, III, and a THW loop in the C-terminus (Pollack *et al*, 1999; Zhang *et al*, 2000; Zhang and Reinberg, 2001; Bedford and Richard, 2005) (Figure 1A), which is an evolutionarily highly conserved core region found in homologous proteins of diverse organisms (see Supplementary Figure S2).

To characterize the function of *SKB1*, we identified two T-DNA insertional mutants (*skb1-1* and *skb1-2*) at the At4g31120 region of *Arabidopsis* in a Columbia (Col) genetic background from the Salk T-DNA collection (Alonso *et al*, 2003). Polymerase chain reaction (PCR) confirmed that the T-DNA in *skb1-1* and *skb1-2* was inserted in exon 21 and intron 22, respectively (Figure 1A) and appeared not to affect the expression of the four genes flanking *SKB1* (Figure 1D). The full-length *SKB1* mRNA was undetectable in the *skb1* mutants, and neither mutants expressed the SKB1 protein (Figure 1E). Both *skb1-1* and *skb1-2* plants flowered much later than wild-type plants under a long-day photoperiod (Figure 2A and B). In addition, as compared with wild-type

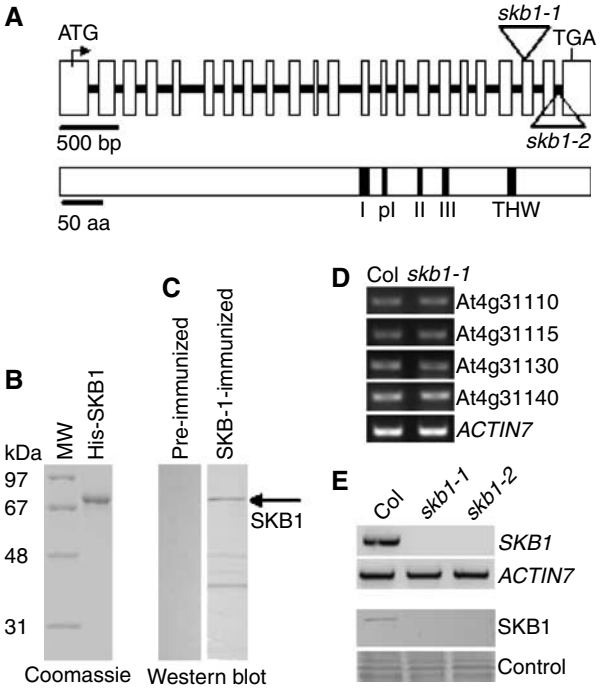

Figure 1 Structure of the *SKB1* gene and identification of *skb1* mutants. (**A**) Structure of the *SKB1* gene and a diagram of the SKB1 protein. Exons are indicated as boxes and introns as lines. T-DNA insertions in *skb1* mutants are indicated by arrowheads. The SKB1 methyltransferase regions I, post I, II, III, and THW loop are shown in black. (**B**) A 72-kDa 6 × His-tagged full-length SKB1 protein purified from *Escherichia coli*. (**C**) Western blot detection of *Arabidopsis* endogenous SKB1 protein with the use of polyclonal anti-SKB1 antiserum. (**D**) RT–PCR analysis of the expression of genes upstream and downstream of *skb1-1*. (**E**) RT–PCR (upper panel) and Western blot (lower panel) analyses of *SKB1* expression in wild-type Col and *skb1-1* and *skb1-2* plants 20 days after sowing; *ACTIN7* serves as an internal control; total proteins stained with Coomassie blue showed equal loading.

plants, *skb1* plants had an increased number of rosette leaves (26 leaves as compared with 12 for wild-type plants at bolting) (Figure 2B and C), and displayed severe developmental retardation 14 days after germination (DAG) (Figure 2D), which is often associated with late-flowering mutants (Lee *et al*, 1994; Schomburg *et al*, 2001; He *et al*, 2003; Simpson *et al*, 2003; Ausin *et al*, 2004; Kim *et al*, 2004; Lim *et al*, 2004; Mockler *et al*, 2004; Henderson *et al*, 2005). The *skb1* mutant plants also formed leaves slightly more curled and darker than wild-type plants and showed slightly reduced fertility (~85% of wild-type seed set when self-pollinated), although floral organs were normal (data not shown). The late-flowering phenotype observed only in homozygous plants revealed that the *skb1* mutation is recessive.

We introduced a *35S*::*SKB1* construct that expressed *SKB1* cDNA constitutively into *skb1-1* mutant plants. *35S*::*SKB1* could rescue the s*kb1-1* mutant, resulting in transgenic plants with a wild-type phenotype (Figure 2C). Further, by introducing *35S*::*SKB1* into wild-type plants, plants overexpressing *SKB1* had early-flowering features (Figure 2E and G), and *SKB1*-promoted flowering time showed a stoichiometric relation with SKB1 protein level (Figure 2F and G). As loss of SKB1 function induced late flowering and overexpression of SKB1 resulted in early flowering, we concluded that SKB1

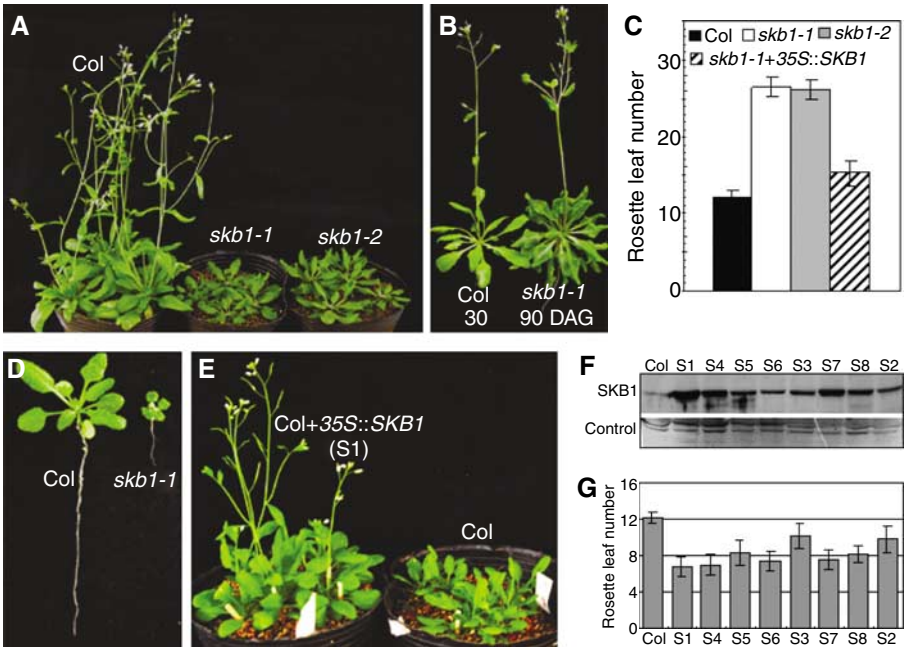

Figure 2 Phenotype of skb1 mutants and SKB1 overexpression. (**A**) Wild-type Col (Col) and *skb1* mutations grown under long-day (LD) conditions for 35 days after germination (DAG). (**B**) Col and *skb1-1* mutant at the flowering stage grown under LD. (**C**) Flowering times of Col, *skb1* mutants and transgenic plants under LD. (**D**) Col and *skb1-1* mutant at 14 DAG under LD. (**E**) Col- and SKB1-overexpressing (S1) plants at 24 DAG under LD. (**F**) Abundance of SKB1 protein levels in homozygous transgenic seedlings (12 DAG under LD) determined by Western blot with anti SKB1 antibody. (**G**) Flowering times of the transgenic plants in (F) under LD. Bars represent means ± s.d. of rosette leaf number at bolting. For each line, 20 plants were scored.

is a positive regulator of floral initiation. Gain-of-function *35S*::*SKB1* transgenic plants flowering early resembled those from a previous report showing that overexpression of *FLK*, a gene of the autonomous pathway, accelerates the flowering process (Mockler *et al*, 2004), but differed from *FVE*-over-expressing plants, which did not show altered flowering time (Ausin *et al*, 2004).

SKB1 is a new member of the autonomous pathway repressing FLC expression

As four major pathways (photoperiod, vernalization, GA and autonomous) have been found to regulate flowering time in *Arabidopsis thaliana* (Komeda, 2004), we sought to identify the pathway that *SKB1* was involved in by treating *skb1* mutant plants with the different physiologic conditions. Under long- or short-day photoperiods, both *skb1-1* and *skb1-2* mutant plants displayed later flowering than wild-type plants, but flowered later in short day than in long day (Figure 3A and C), which indicates that *skb1* mutants were sensitive to photoperiod. After exposure to vernalization, *skb1* mutant plants flowered rapidly (Figure 3A and B), which is similar to results for several autonomous pathway mutants (Schomburg *et al*, 2001; He *et al*, 2003; Simpson *et al*, 2003; Ausin *et al*, 2004; Lim *et al*, 2004; Mockler *et al*, 2004; Henderson *et al*, 2005). GA treatment reversed in part the delayed flowering of *skb1* mutant plants (Figure 3A and D), an observation consistent with that of the autonomous pathway mutants *flk* and *fca*, which respond to both vernalization and GA (Michaels and Amasino, 1999b; Mouradov *et al*, 2002; Lim *et al*, 2004). Taken together, these results suggest that *SKB1* is a new member of the autonomous pathway and prevents late flowering.

To investigate the molecular mechanism underlying the late-flowering phenotype of *skb1*, we examined the transcript levels of critical genes in the different flowering pathways (Komeda, 2004; He and Amasino, 2005). Consistent with their showing a normal photoperiod-responsive phenotype, *skb1* mutants and wild-type plants did not show a significant difference in transcript levels of *GI* and *CO* genes, two key components of the photoperiod pathway (Samach *et al*, 2000; Suarez-Lopez *et al*, 2001); *SPY* in the GA pathway (Tseng *et al*, 2004); *VRN1* and *VRN2* in the vernalization pathway (Gendall *et al*, 2001; Levy *et al*, 2002) (Figure 3E). The expression of *LD*, in the autonomous pathway upstream of *FLC* (He and Amasino, 2005), did not differ between *skb1-1* and wild-type plants. The expression of the flowering repressor gene *FLC*, however, was significantly upregulated in *skb1-1* mutant plants (Figure 3E). Increased *FLC* expression is a characteristic of other autonomous mutants such as *fca, ld, fy, fld, flk, fpa* and *fve* (Schomburg *et al*, 2001; He *et al*, 2003; Simpson *et al*, 2003; Ausin *et al*, 2004; Mockler *et al*, 2004; Henderson *et al*, 2005). Loss of *SKB1* downregulated *LFY*, *FT* and *SOC1*, and upregulated *EMF1*, *EMF2* and *TFL1* (Figure 3E). *LFY*, *FT* and *SOC1* are all downstream of *FLC* and are expressed in an *FLC*-dependent manner (He and Amasino, 2005). The function of *EMF1*, *EMF2* and *TFL1* genes may not influence the four flowering pathways, but their reduced expression induces early flowering (Shannon and Meeks-Wagner, 1991; Moon *et al*, 2003; Komeda, 2004).

To examine whether accumulation of *FLC* mRNA directly results in late flowering in *skb1* mutant plants, we introduced *skb1-1* into an *FLC*-null mutant, *flc-3* (Michaels and Amasino, 1999a). The late-flowering phenotype of *skb1-1* was suppressed by *flc-3* (Figure 3F and G). Thus, the flowering time

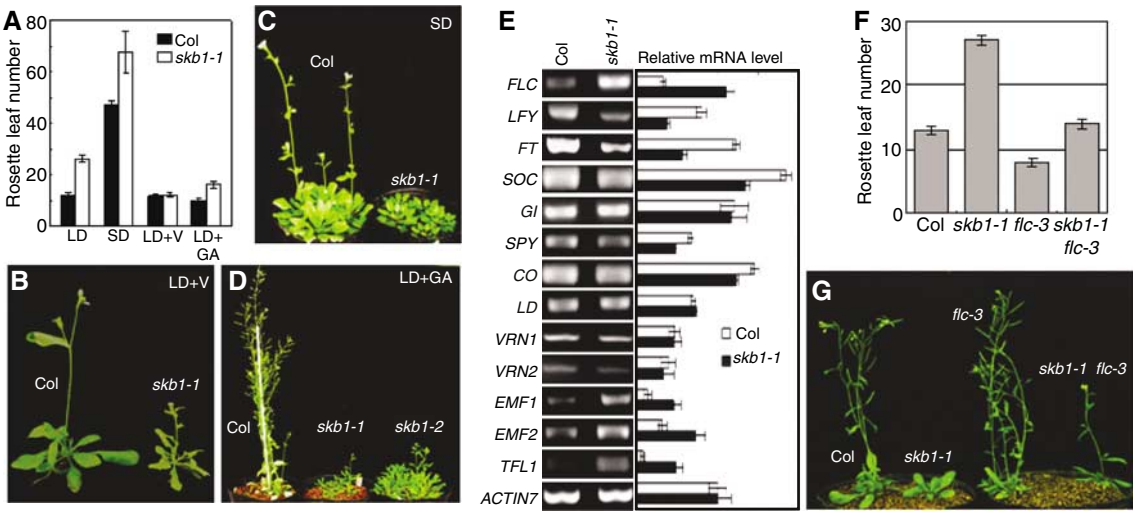

Figure 3 Analysis of *skb1* mutant response to environmental cues and target genes. (**A**) Flowering times of *skb1-1* under LD, short-day (SD), vernalization (V) and GA treatment. Bars represent means ± s.d. For each line, 30–50 plants were scored. (**B**) Col and *skb1-1* mutant plants germinated and grown at 2–4°C for 6 weeks and moved to LD conditions for 26 days. (**C**) Plants grown at 98 DAG in SD. (**D**) Plants treated with GA_3 and grown at 37 DAG under LD. (**E**) RT–PCR analysis of flowering control genes expression in wild-type and *skb1-1* mutant plants. RNA was isolated from 20-day-old seedlings grown under LD. ACTIN7 was used as a loading control. Signal intensities were normalized relative to ACTIN7 with the use of ImageQuant and shown by bar graphs at the right from three independent experiments. (**F**) Flowering time of Col, *skb1-1*, *flc-3* and *skb1-1 flc-3*. Ten plants were scored and grown under LD. (**G**) The phenotypes of wild-type Col, *skb1-1*, *flc-3* and *skb1-1 flc-3* mutant grown under LD.

control by *SKB1* is targeted towards *FLC* expression. The *skb1-1 flc-3* double-mutant plants (14.8 rosette leaves) flowered later than *flc-3* (9 rosette leaves) or wild-type Col plants (12.7 rosette leaves) but much earlier than *skb1-1* mutant plants (27 rosette leaves). It is likely that *FLC*-independent factors also contribute to the later flowering phenotype of the *skb1* mutants. This assumption is consistent with the previous observation of upregulated expression of *EMF1*, *EMF2* and *TFL1* (Figure 3E).

Expression pattern of SKB1 protein and mRNA

The temporal and spatial expression patterns of the SKB1 protein was analyzed with the use of a specific antibody against SKB1 (Figure 1C). Western blotting results showed that SKB1 was highly expressed in flowers, roots and siliques, but less so in stems and mature leaves (Figure 4A). The SKB1 protein was maintained at high levels during vegetative development (from 5 to 20 days after germination) (Figure 4B). Consistent with the protein expression pattern, *SKB1* RNA was more abundant in shoot apex, young leaves and leaf primordia, floral and inflorescence meristems (Figure 4C–E), and was also expressed in gynoecium, stamens, sepals and young siliques (especially ovules), but not as much in older leaves, petals and vascular tissues (Supplementary Figure S3). This expression pattern was similar to that of genes affecting flowering transition (Lee *et al*, 1994; Michaels and Amasino, 1999a; Schomburg *et al*, 2001; He *et al*, 2003, 2004; Simpson *et al*, 2003; Ausin *et al*, 2004; Bastow *et al*, 2004; Mockler *et al*, 2004; Henderson *et al*, 2005; Zhao *et al*, 2005).

Arginine methyltransferase SKB1 methylates H4R3sme2 both in vitro and in vivo

PRMT5, the human homologue of SKB1, methylates histones H2A, H3 and H4 (Pollack *et al*, 1999; Ancelin *et al*, 2006; Dacwag *et al*, 2007). To examine whether *Arabidopsis* SKB1 methylates histone arginines, we assayed *in vitro* a GST-SKB1 fusion protein, purified by affinity chromatography from *Escherichia coli*, for methylation activity, with histones and myelin basic protein (MBP) used as substrates. Both histone and MBP were methylated, but of the five histones (H1, H2A, H2B, H3 and H4), only H4 was methylated (Figure 4F). We then used specific antibodies to examine whether SKB1 methylates H4R3. GST-SKB1 catalyzed H4R3 symmetric dimethylation (H4R3sme2) but not asymmetric dimethylation (Figure 4G), which suggests that SKB1 can symmetrically methylate H4R3 as does PRMT5 (Pollack *et al*, 1999; Ancelin *et al*, 2006).

We then examined whether SKB1 methylates H4R3 *in vivo*. Figure 4H shows the s*kb1* mutants, with no difference from wild type in the level of asymmetric dimethylated H4R3, but markedly reduced level of symmetric dimethylated H4R3, which indicates that H4R3 contains symmetric and asymmetric forms of dimethylation, and that SKB1 is a major enzyme controlling H4R3 symmetric dimethylation in *Arabidopsis*. Further investigation revealed that impaired H4R3 symmetric dimethylation had little effect on dimethylated H3K4, H3K9 and acetylated H4K5 and H4K8 (Figure 4H).

SKB1 and the histone mark H4R3sme2 are associated with FLC expression

The level of H4R3sme2 was also examined during developmental stages of the wild-type Col line. Plants grown under short- and long-day conditions at 5, 9, 14 and 20 days after germination were collected for protein isolation. SKB1 protein levels were weakly increased with plant age under short-day condition (Figure 4I) and slightly decreased with age under long-day conditions (Figure 4B). Consistent with SKB1 expression, H4R3sme2 was maintained at high levels during vegetative development under both short- and long-day photoperiods, but showed a weak increase under short-day

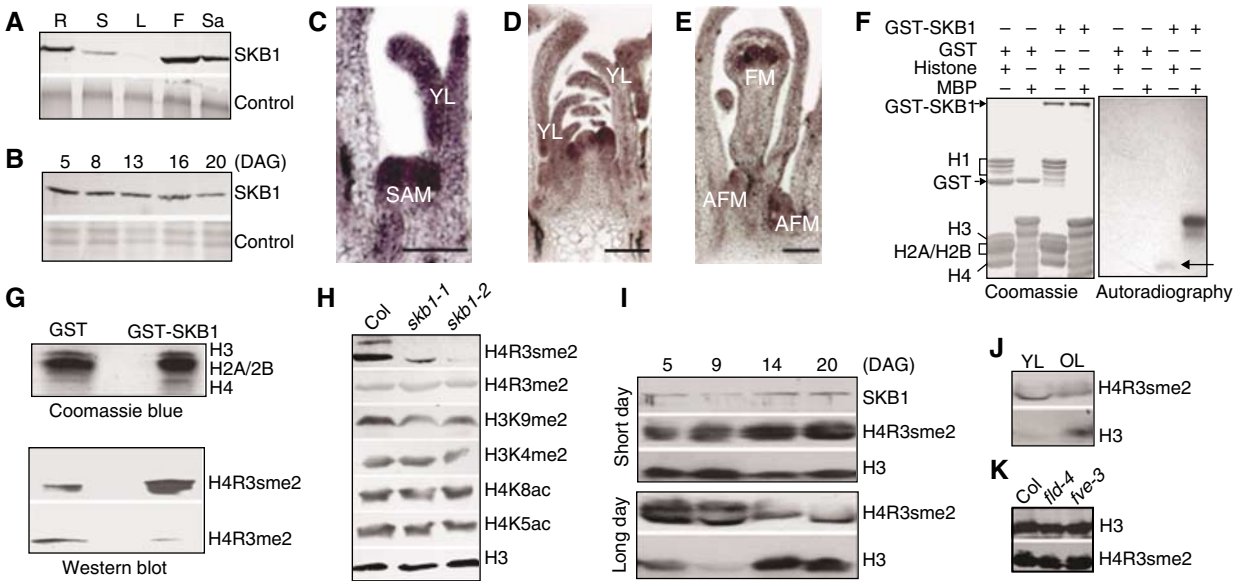

Figure 4 Analysis of SKB1 expression pattern and methyltransferase activity. (**A, B**) Immunoblot analyses of SKB1 with the use of polyclonal anti-SKB1 antiserum. (A) Total protein extracts from different tissues of the wild type at 38 days. R, root; S, stem; L, leaf; F, flower; Sa, silique. Control shows equal loading. (B) Expression of SKB1 protein at different developmental stages. Col was grown under LD and whole parts were harvested at the indicated growth stages for total protein isolation. (**C–E**) RNA *in situ* hybridization analysis of SKB1 expression in Col grown under LD for 10 (C), 21 (D) and 26 (E) days. AFM: axillary flower meristem; SAM, shoot apical meristem; FM, floral meristem; YL, young leaf. Scale bar, 100 μm. (**F**) *In vitro* methylation of histones (10 μg) and myelin basic protein (MBP, 10 μg) by GST-SKB1 (2 μg) purified from *E. coli*. GST (3 μg) was a negative control; left, Coomassie blue-stained gel; right, autoradiograph of ^{3}H-labelled proteins produced by *in vitro* methylation; methylated H4 is indicated by an arrow. (**G**) SKB1-mediated H4R3sme2 was analyzed by Western blot with specific antibodies. (**H**) Western blot analysis of modification of histone H3 and H4. Histone-enriched protein extracts from 20-day-old Col and *skb1* mutant plants grown under LD were probed with antibodies that specifically recognize the indicated forms of histone H3 and H4. (**I**) Levels of SKB1 and H4R3sme2 at different developmental stages in Col. Total soluble proteins isolated from seedlings grown in SD were exposed to antibody against SKB1. Histone-enriched proteins were extracted from plants grown under SD or LD and immunoblotted with antibodies against H4R3sme2 and H3. (**J**) The level of H4R3sme2 in specific tissues of Col grown under LD. YL: young leaves plus apex from seedlings at 5 DAG; OL: old leaves from plants at 35 DAG. (**K**) Abundance of H4R3sme2 in Col, *fld-4* and *fve-3*. Proteins were isolated from seedlings grown at 20 DAG under LD.

and a slight decrease under long-day condition with plant age (Figure 4I). As *FLC* is also highly expressed at these states (Michaels and Amasino, 1999a), SKB1 activity and H4R3sme2 could be associated with *FLC* expression. This association was further confirmed by tissue-specific differences. Both *FLC* and *SKB1* are highly expressed in young leaves and apical meristems, with low levels in old leaves (Michaels and Amasino, 1999a) (Figure 4A–E). Indeed, H4R3sme2 was also detected at high levels in young leaves and apical meristems, but at low levels in old leaves (Figure 4J).

We then examined H4R3sme2 in other late-flowering mutants: *fld-4* and *fve-3*, two autonomous pathway mutants upregulating *FLC* expression by increasing histone acetylation (He *et al*, 2003; Ausin *et al*, 2004; Kim *et al*, 2004). Histone-enriched proteins were isolated from plants grown for 20 days after germination under long-day conditions. The level of H4R3sme2 in both *fld-4* and *fve-3* mutant plants did not significantly differ from that in wild-type plants (Figure 4K), which suggests that increased histone acetylation resulting from loss of *FLD* or *FVE* function may not affect the H4R3sme2 mark on global level.

SKB1 associates with FLC chromatin and is required for symmetric dimethylation of H4R3 in FLC chromatin

The transcription of *FLC* regulated through chromatin remodelling has been well studied (He *et al*, 2003, 2004; Bastow *et al*, 2004; Zhao *et al*, 2005). In addition, the mammalian SKB1 homologue PRMT5 interacts with chromatin and suppresses gene transcription (Bedford and Richard, 2005). We thus asked whether SKB1 binds to the five *FLC* chromatin regions covering its promoter (region A), the first exon (region B) and the first intron (regions C–E), as was previously reported (Bastow *et al*, 2004) (Figure 5A). Chromatin immunoprecipitation (ChIP) assay revealed that SKB1 antibody could specifically pull down the *FLC* promoter (region A) (Figure 5B), a chromatin region that is critical for *FLC* transcription (He *et al*, 2003; Bastow *et al*, 2004). SKB1 association with other regions (B–E) was undetectable (Figure 5B). By contrast, SKB1 antibody could not pull down DNA in regions A–E in the *skb1-1* mutant (Figure 5B). Consistent with SKB1 strongly binding to the *FLC* promoter, the *FLC* promoter also showed a high level of H4R3sme2 in the wild type; in contrast, the *skb1* mutant showed greatly decreased H4R3sme2 (Figure 5B and Supplementary Figure S4).

In addition, consistent with a previous report (Bastow *et al*, 2004), dimethylation in H3K9 was undetectable in all regions in both wild-type and mutant plants (Figure 5C). The asymmetric dimethylated H4R3 was weakly detected at *FLC* chromatin regions A, B, D and E, with no difference between the wild type and *skb1* mutant (Figure 5C). Compared with the wild type, the s*kb1* plants showed no change in the levels of dimethylated H3K4 in all regions (Figure 5C). The acetylation in H3K14 was found in all regions

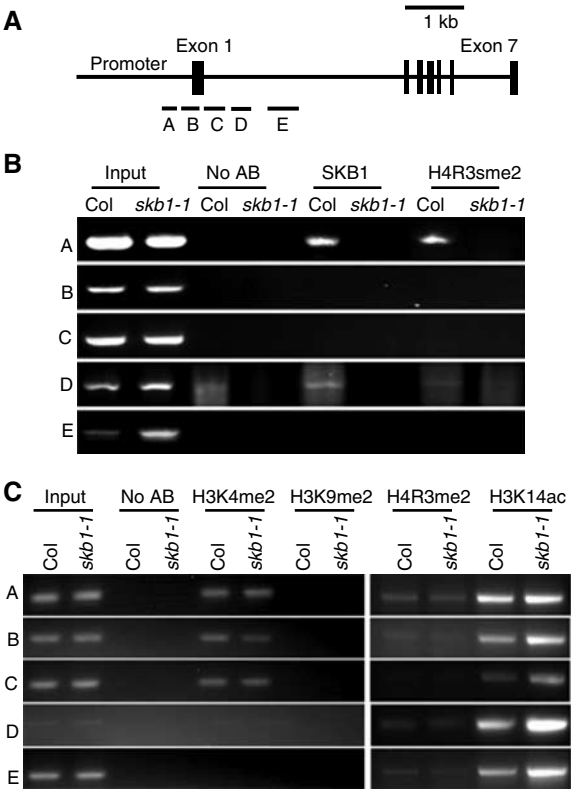

Figure 5 ChIP assays of wild type and *skb1-1* mutant at the *FLC* locus. (**A**) A diagram of the *FLC* gene structure, with bars representing the A–E regions examined by ChIP. (**B, C**) ChIP results with antibodies against SKB1, H4R3sme2, H3K4me2, H3K9me2, H4R3me2 and acetylated H3K14 (H3K14ac). Samples were from wild-type and s*kb1-1* plants in LD for 20 days. The input is chromatin before immunoprecipitation. 'No AB': the control sample lacks an antibody. ChIP assays involved at least three independent experiments; data represent results of one experiment.

(A–E) both in the wild type and s*kb1* mutant, with increased levels in *skb1* mutant in regions A–E (Figure 5C and Supplementary Figure S4), which is consistent with a previous report of hyperacetylation activating *FLC* expression (He *et al*, 2003; Ausin *et al*, 2004). These findings suggest that *SKB1* affects flowering development by altering *FLC* expression via symmetric dimethylation of H4R3 in its promoter.

Based on our findings, we propose that SKB1 methylates H4R3 of the *FLC* chromatin symmetrically, which in turn suppresses *FLC* expression to induce flowering. H4R3 is also asymmetrically dimethylated by PRMT1 in animal cells, which facilitates subsequent acetylation at H3K9, H3K14, H4K5 and H4K12 and is required for transcriptional activation (Huang *et al*, 2005). Interestingly, in plants, reduced H4R3 symmetric dimethylation resulting from SKB1 knockdown has no effect on H4R3 asymmetric dimethylation but, rather, increases H3K14 acetylation in the *FLC* chromatin and activates or maintains its transcription (Figure 5B and C). This finding suggests that symmetric and asymmetric dimethylations in H4R3 have distinct functions in regulation of chromatin status and gene transcription. Further exploration will define the difference in biological function between symmetric and asymmetric dimethylation in H4R3.

Materials and methods

Plant materials and growth conditions

Arabidopsis ecotype Columbia (Col) was used in this work. *skb1-1* and *skb1-2* alleles were isolated from the SALK T-DNA collection (Salk_065814 and Salk_095085). *flc-3*, *fld-4* and *fve-3* were described previously (Michaels and Amasino, 1999a; He *et al*, 2003; Ausin *et al*, 2004). Plants were grown at 22°C under long-day (16 h light and 8 h dark) or short-day (8 h light and 16 h dark) conditions. Vernalization treatment was as described (Bastow *et al*, 2004) and GA treatment was as described (Lim *et al*, 2004). Flowering time was measured as the number of rosette leaves at bolting.

RT–PCR analysis

RNA was isolated from 20-day-old seedlings grown under long-day conditions with use of TRI reagent as recommended by the manufacturer. cDNAs were synthesized from 2.0 μg of total RNA by use of Superscript reverse transcriptase. RT–PCR was performed with gene-specific primers (see Supplementary Table SI) and runs were 18–25 cycles depending on the linear range of products for each gene.

Western blot analysis

Histone-enriched protein extraction from 20-day-old seedlings with or without vernalization treatment was as described (Houben *et al*, 2003). Western blot analysis was performed with Upstate antibodies against histone H4 dimethyl Arg 3 (catalogue no. 07-213), H4 acetyl Lys 5 (catalogue no. 07-327), H4 acetyl Lys 8 (catalogue no. 07-328), H3 dimethyl Lys 4 (catalogue no. 05-790), H3 dimethyl Lys 9 (catalogue no. 05-768), H3 acetyl Lys 14 (catalogue no. 07-353), H3 (catalogue no. 07-690) and with Abcam antibodies against H4 symmetric dimethyl Arg 3 (catalogue no. 5823). Anti-SKB1 polyclonal antibody was generated with 6 × His-tagged full-length SKB1 protein purified from *E. coli*.

In situ hybridization

Tissue preparation of 10-, 21- and 26-day-old seedlings grown under long day, digoxigenin labelling of RNA probes and *in situ* hybridization were performed as described (Wu *et al*, 2006). DNA fragments containing the 330-base-pair (21–350) coding region of *SKB1* were used as sense and antisense probes.

Chromatin immunoprecipitation

ChIP assay involved the usage of 20-day-old seedlings grown under long-day conditions as previously described (Bowler *et al*, 2004). Primers and PCR detection of *FLC* regions were as described (Bastow *et al*, 2004).

Constructs, protein purification and methylation analysis

Full-length *SKB1* cDNA was cloned into *pBI121*, resulting in the binary vector *p35S::SKB1*, which was introduced into *Agrobacterium tumefaciens* to transform *Arabidopsis* plants. Full-length *SKB1* cDNA was cloned into *pGEX4T-1* in-frame. GST-SKB1 expression, purification and methylation assays were as previously described (Bao *et al*, 2001).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank Jiayang Li for his useful comments on the manuscript, Xiaofeng Cao for supplying the greenhouse and Richard Amasino, Jianru Zuo and Ligeng Ma for seeds of the *flc-3*, *fld-4* and *fve-3* mutant lines, respectively. This work was supported by National Basic Research Program of China (2005CB522400), the President's Fund of the Chinese Academy of Sciences to SB; the National Natural Sciences Foundation of China (NSFC) for distinguished Young Scholars (30525026) to KC; and NSFC (30671186) to XW.

References

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301:** 653–657

Ancelin K, Lange UC, Hajkova P, Schneider R, Bannister AJ, Kouzarides T, Surani MA (2006) Blimp1 associates with Prmt5 and directs histone arginine methylation in mouse germ cells. *Nat Cell Biol* **8:** 623–630

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

Bao S, Qyang Y, Yang P, Kim H, Du H, Bartholomeusz G, Henkel J, Pimental R, Verde F, Marcus S (2001) The highly conserved protein methyltransferase, Skb1, is a mediator of hyperosmotic stress response in the fission yeast *Schizosaccharomyces pombe*. *J Biol Chem* **276:** 14549–14552

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

Bedford MT, Richard S (2005) Arginine methylation an emerging regulator of protein function. *Mol Cell* **18:** 263–272

Bowler C, Benvenuto G, Laflamme P, Molino D, Probst AV, Tariq M, Paszkowski J (2004) Chromatin techniques for plant cells. *Plant J* **39:** 776–789

Cook JR, Lee JH, Yang ZH, Krause CD, Herth N, Hoffmann R, Pestka S (2006) FBXO11/PRMT9, a new protein arginine methyltransferase, symmetrically dimethylates arginine residues. *Biochem Biophys Res Commun* **342:** 472–481

Dacwag CS, Ohkawa Y, Pal S, Sif S, Imbalzano AN (2007) The protein arginine methyltransferase Prmt5 is required for myogenesis because it facilitates ATP-dependent chromatin remodeling. *Mol Cell Biol* **27:** 384–394

Gendall AR, Levy YY, Wilson A, Dean C (2001) The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in *Arabidopsis*. *Cell* **107:** 525–535

Gilbreth M, Yang P, Wang D, Frost J, Polverino A, Cobb MH, Marcus S (1996) The highly conserved skb1 gene encodes a protein that interacts with Shk1, a fission yeast Ste20/PAK homolog. *Proc Natl Acad Sci USA* **93:** 13802–13807

Greb T, Mylne JS, Crevillen P, Geraldo N, An H, Gendall AR, Dean C (2007) The PHD finger protein VRN5 functions in the epigenetic silencing of *Arabidopsis* FLC. *Curr Biol* **17:** 73–78

Guo H, Yang H, Mockler TC, Lin C (1998) Regulation of flowering time by *Arabidopsis* photoreceptors. *Science* **279:** 1360–1363

He Y, Amasino RM (2005) Role of chromatin modification in flowering-time control. *Trends Plant Sci* **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 Dev* **18:** 2774–2784

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

Henderson IR, Liu F, Drea S, Simpson GG, Dean C (2005) An allelic series reveals essential roles for FY in plant development in addition to flowering-time control. *Development* **132:** 3597–3607

Hepworth SR, Valverde F, Ravenscroft D, Mouradov A, Coupland G (2002) Antagonistic regulation of flowering-time gene SOC1 by CONSTANS and FLC via separate promoter motifs. *EMBO J* **21:** 4327–4337

Houben A, Demidov D, Gernand D, Meister A, Leach CR, Schubert I (2003) Methylation of histone H3 in euchromatin of plant chromosomes depends on basic nuclear DNA content. *Plant J* **33:** 967–973

Huang S, Litt M, Felsenfeld G (2005) Methylation of histone H4 by arginine methyltransferase PRMT1 is essential *in vivo* for many subsequent histone modifications. *Genes Dev* **19:** 1885–1893

Kim HJ, Hyun Y, Park JY, Park MJ, Park MK, Kim MD, Kim HJ, Lee MH, Moon J, Lee I, Kim J (2004) A genetic link between cold responses and flowering time through FVE in *Arabidopsis thaliana*. *Nat Genet* **36:** 167–171

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

Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol Gen Genet* **229:** 57–66

Lee I, Aukerman MJ, Gore SL, Lohman KN, Michaels SD, Weaver LM, John MC, Feldmann KA, Amasino RM (1994) Isolation of LUMINIDEPENDENS: a gene involved in the control of flowering time in *Arabidopsis*. *Plant Cell* **6:** 75–83

Levy YY, Mesnage S, Mylne JS, Gendall AR, Dean C (2002) Multiple roles of *Arabidopsis* VRN1 in vernalization and flowering time control. *Science* **297:** 243–246

Lim MH, Kim J, Kim YS, Chung KS, Seo YH, Lee I, Kim J, Hong CB, Kim HJ, Park CM (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 Z, Zhou Z, Chen G, Bao S (2007) A putative transcriptional elongation factor hIws1 is essential for mammalian cell proliferation. *Biochem Biophys Res Commun* **353:** 47–53

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

Martinez-Zapater JM, Somerville CR (1990) Effect of light quality and vernalization on late-flowering mutants of *Arabidopsis thaliana*. *Plant Physiol* **92:** 770–776

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

Michaels SD, Amasino RM (1999b) The gibberellic acid biosynthesis mutant ga1–3 of *Arabidopsis thaliana* is responsive to vernalization. *Dev Genet* **25:** 194–198

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. *Plant Cell* **13:** 935–941

Mockler TC, Yu X, Shalitin D, Parikh D, Michael TP, Liou J, Huang J, Smith Z, Alonso JM, Ecker JR, Chory J, Lin C (2004) Regulation of flowering time in *Arabidopsis* by K homology domain proteins. *Proc Natl Acad Sci USA* **101:** 12759–12764

Moon YH, Chen L, Pan RL, Chang HS, Zhu T, Maffeo DM, Sung ZR (2003) EMF genes maintain vegetative development by repressing the flower program in *Arabidopsis*. *Plant Cell* **15:** 681–693

Mouradov A, Cremer F, Coupland G (2002) Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell* **14** (Suppl)**:** S111–S130

Mylne JS, Barrett L, Tessadori F, Mesnage S, Johnson L, Bernatavichute YV, Jacobsen SE, Fransz P, Dean C (2006) LHP1, the *Arabidopsis* homologue of HETEROCHROMATIN PROTEIN1, is required for epigenetic silencing of FLC. *Proc Natl Acad Sci USA* **103:** 5012–5017

Pollack BP, Kotenko SV, He W, Izotova LS, Barnoski BL, Pestka S (1999) The human homologue of the yeast proteins Skb1 and Hsl7p interacts with Jak kinases and contains protein methyltransferase activity. *J Biol Chem* **274:** 31531–31542

Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, Coupland G (2000) Distinct roles of CONSTANS target genes in reproductive development of *Arabidopsis*. *Science* **288:** 1613–1616

Schomburg FM, Patton DA, Meinke DW, Amasino RM (2001) FPA, a gene involved in floral induction in *Arabidopsis*, encodes a protein containing RNA-recognition motifs. *Plant Cell* **13:** 1427–1436

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

Shannon S, Meeks-Wagner DR (1991) A mutation in the *Arabidopsis* TFL1 gene affects inflorescence meristem development. *Plant Cell* **3:** 877–892

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. *Plant Cell* **11:** 445–458

Simpson GG, Dijkwel PP, Quesada V, Henderson I, 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

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

Sung S, Amasino RM (2004) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* **427:** 159–164

Tseng TS, Salome PA, McClung CR, Olszewski NE (2004) SPINDLY and GIGANTEA interact and act in *Arabidopsis thaliana* pathways involved in light responses, flowering, and rhythms in cotyledon movements. *Plant Cell* **16:** 1550–1563

Wu C, Ma Q, Yam KM, Cheung MY, Xu Y, Han T, Lam HM, Chong K (2006) *In situ* expression of the GmNMH7 gene is photoperiod-dependent in a unique soybean (Glycine max [L.] Merr.) flowering reversion system. *Planta* **223:** 725–735

Wysocka J, Allis CD, Coonrod S (2006) Histone arginine methylation and its dynamic regulation. *Front Biosci* **11:** 344–355

Zhang X, Zhou L, Cheng X (2000) Crystal structure of the conserved core of protein arginine methyltransferase PRMT3. *EMBO J* **19:** 3509–3519

Zhang Y, Reinberg D (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev* **15:** 2343–2360

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. *Nat Cell Biol* **7:** 1256–1260