Abstract

**Background:** Similar to other eukaryotes, splicing is emerging as an important process affecting development and stress tolerance in plants. Ski-interacting protein (SKIP), a splicing factor, is essential for circadian clock function and abiotic stress tolerance; however, the mechanisms whereby it regulates flowering time are unknown.

**Results:** In this study, we found that SKIP is required for the splicing of *serrated leaves and early flowering* (*SEF*) pre-messenger RNA (mRNA), which encodes a component of the ATP-dependent SWR1 chromatin remodeling complex (SWR1-C). Defects in the splicing of *SEF* pre-mRNA reduced H2A.Z enrichment at *FLC*, *MAF4*, and *MAF5*, suppressed the expression of these genes, and produced an early flowering phenotype in *skip-1* plants.

**Conclusions:** Our findings indicate that SKIP regulates SWR1-C function via alternative splicing to control the floral transition in *Arabidopsis thaliana*.

**Keywords:** SKIP, Flowering time, Splicing factor, SEF, SWR1 complex

Background

Correct timing of the vegetative to reproductive phase transition, or floral transition, is essential for seed setting in higher plants. Studies have shown that factors involved in the floral transition, including the polymerase-associated factor (PAF) and FRIGIDA (FRI) complexes, are integrated into such major pathways as the autonomous, vernalization, and photoperiod pathways [1, 2]. *FLOWERING LOCUS C* (*FLC*) encodes a MCM1, agamous, deficiens and serum response family (MADS)-box transcription factor that acts as a central repressor of the floral transition, and five homologs, *MADS AFFECTING FLOWERING* (*MAF*) 1 to *MAF5*, exist in Arabidopsis (*Arabidopsis thaliana*) [3–5]. The repression of *FLC* by factors involved in the vernalization and autonomous pathways activates the expression of *SUPPRESSOR OF CONSTANS 1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*) to accelerate flowering. Vernalization, the acceleration of flowering by prolonged cold, epigenetically silences *FLC* through the expression of polycomb repressive complex 2, which deposits the repressive histone mark trimethylation of lysine 27 on histone 3 (H3K27me3) [6]. The autonomous pathway includes a series of activities that promote the epigenetic modification and RNA-mediated chromatin silencing of *FLC* [7, 8]. The antisense transcript of *FLC* affects the expression of the sense transcript, thereby influencing flowering time in Arabidopsis [9, 10].

In contrast, the PAF1 complex [11, 12], histone 2B ubiquitination [13, 14], histone 3 K4 and K36 methyltransferase complexes [15–18], the ATP-dependent SWR1 chromatin remodeling complex (SWR1-C) [19–21], and *FRI/FRL*1-like genes (e.g., *FRL1* and *FRL2*) [22, 23] are involved in the activation of *FLC* to suppress the floral transition through chromatin modification or remodeling in Arabidopsis [2, 24]. The SWR1-C exchanges histone H2A for H2A.Z, producing variant nucleosomes. Photoperiod-independent early flowering 1 (*PIE1*), actin-related protein 6 (*ARP6*), and *serrated leaves and early flowering* (*SEF*) are components of the SWR1-C in Arabidopsis [24]. Mutations in *PIE1*, *ARP6*, and *SEF* confer early flowering phenotypes through the silencing of *FLC*, *MAF4*, and *MAF5* expression [19, 20, 25]. The SWR1-C is required for H2A.Z deposition at *FLC*, *MAF4*,
indicates that alternative splicing is involved in flowering time control in Arabidopsis. Alternative splicing of **FLOWERING TIME CONTROL LOCUS A** (**FTC**) pre-mRNA is involved in the control of the floral transition [44, 45]. **RZ-1B** and **RZ-1C**, nuclear-localized RNA-binding proteins, are involved in pre-mRNA splicing and flowering time control via interactions with serine/arginine-rich (SR) proteins [46]. AtSF1, the homolog of mammalian splicing factor 1 in Arabidopsis, interacts with U2 snRNP auxiliary factor 65a/b (U2AF65a/b) and acts as a splicing factor to regulate flowering time and ABA signaling [47].

**SKIP**, an SNW domain-containing protein, is evolutionarily conserved, with close homologs in yeast and mammals. In mammals, SKIP (also termed SNW1 and NCOA62) plays important roles as a transcriptional co- and splicing factor [48, 49]. SKIP specifically regulates the alternative splicing and expression of **p21** by interacting with the 3’ss recognition factor U2AF65 and recruiting it to **p21** mRNA in vivo [49]. In yeast, the SKIP homolog **Prp45** is a component of the Prp19-related complex (or nineteen complex), which functions as a splicing factor [50, 51]. Weak mutations in **prp45** cause defects in the splicing of **ACTIN** and other genes, resulting in temperature-sensitive growth, while strong **prp45** alleles are lethal [50, 52]. OsSKIPa, which was able to rescue the defects in **prp45** mutant yeast, regulates cell viability and stress tolerance in rice (**Oryza sativa**) [53]. In Arabidopsis, **SKIP** regulates cytokinin-associated leaf growth [54]. **SKIP** expression is induced by salt, mannitol, and ABA. Overexpression of **SKIP** confers tolerance to abiotic stress. By contrast, the down-regulation of **SKIP** causes reduced tolerance to abiotic stress during germination. **SKIP** activated the transcription of a reporter gene in yeast, suggesting that it regulates gene expression as a transcription factor [55]. **SKIP** is a splicing factor that interacts physically with the plant-specific SR protein **SR45** to regulate circadian clock function. The **skip-1** mutation disrupts this clock, creating a lengthened clock period phenotype by altering the alternative splicing of **PSEUDO-RESPONSE REGULATOR 7** (**PRR7**) and **PRR9**, two genes in the morning loop of the oscillator [40]. Genome-wide defects in splicing have been observed in **skip-1** plants through RNA high-throughput sequencing, suggesting that **SKIP** is a splicing factor [40]. In addition to defects in the circadian clock, **skip-1** plants show pleiotropic phenotypes, including early flowering. However, the molecular and biochemical mechanisms whereby **SKIP** represses the floral transition remain obscure.

In this study, we revealed that **SKIP** plays an essential role in regulating flowering time of Arabidopsis. **SKIP** was found to regulate the splicing of **SEF** pre-mRNA and suppress flowering by activating the expression of **FLC**. **FLC** expression was reduced in **skip-1**, leading to an early...
flowering phenotype under long-day (16 h of light/8 h of darkness, LD) and short-day (8 h of light/16 h of darkness, SD) conditions. SKIP was also required for the normal splicing of SEF pre-mRNA (which encodes a component of the SWR1-C) through direct binding. Splicing defects in SEF were found to contribute to the early flowering phenotype of skip-1. Further, H2A.Z enrichment at FLC chromatin was reduced in skip-1. Our findings indicate a role for alternative splicing in regulating SWR1-C function to control the floral transition in Arabidopsis.

Results

SKIP is required for the floral transition and normal development in Arabidopsis

Previous studies showed that the skip-1 mutation lengthened the period of the circadian clock by impairing the alternative splicing of PRR7 and PRR9 in Arabidopsis [40]. The skip-1 mutation confers an early flowering phenotype under LD and SD conditions [40]; however, the underlying molecular mechanisms are unknown. A complementation test using SKIP genomic DNA and skip-1 plants revealed that the skip-1 mutation is responsible for the observed early flowering phenotype of the mutant (Fig. 1a–d; Additional file 1: Table S1 and Additional file 2: Table S2).

Pleiotropic phenotypes were observed in skip-1 compared to the wild type (WT), including reduced root growth (Fig. 1e and f; Additional file 3: Table S3); smaller sepals, petals, and flowers (Fig. 1h–j); shorter stigmas, stamens, and siliques (Fig. 1k–m). In the present study, we focused on dissecting the mechanisms whereby SKIP controls flowering time in Arabidopsis.

SKIP activates the expression of FLC and its homologs to repress flowering

Because the skip-1 mutation is insensitive to photoperiod, we examined the expression of CONSTANS (CO), a key regulator of photoperiodic flowering, in the skip-1 mutant [56]. CO expression in the skip-1 mutant was reduced or similar to that in WT Columbia (Col-0) plants under LD and SD conditions (Additional file 4: Figure S1a and b). To explore the molecular mechanisms whereby SKIP controls flowering time, we analyzed the expression of FLC and its homologs MAF1 to MAF5, which are major suppressors of flowering, in skip-1. The expression of FLC, MAF1, MAF4, and MAF5 was significantly suppressed by the skip-1 mutation, leading to an early flowering phenotype under LD and SD conditions. Genomic DNA corresponding to the SKIP gene was able to recover the suppression of FLC by the skip-1 mutation (Fig. 2a–d).

Down-regulation of FLC is usually accompanied by the up-regulation of downstream genes to accelerate flowering. SOC1 and FT are known to accelerate the vegetative to reproductive phase transition in Arabidopsis downstream of FLC [2]. Twin sister of FT (TSF), an FT homolog, promotes the floral transition [57, 58]. FD, a transcription factor, interacts with FT to promote the floral transition in the shoot apical meristem [59]. Both FT and FD are required for floral meristem formation [59, 60]. As expected, the skip-1 mutation activated the expression of SOC1, FT, and TSF, but not of FD, and it promoted flowering under LD and SD conditions (Fig. 2e–j; Additional file 4: Figure S1c). These results suggest that skip-1 represses the expression of FLC and activates the expression of downstream flowering time integrators (e.g., SOC1, FT, and TSF) to accelerate flowering.

To test whether FLC is genetically necessary for repression of the floral transition by SKIP, FLC-dependent late flowering mutants, including FRI (Col-0 background), fve, and flowering locus k (flk), were crossed with skip-1 [22, 61–63]. The late flowering phenotypes of FRI, fve, and flk were dramatically suppressed by the skip-1 mutation (Fig. 3a; Additional file 5: Table S4).

To confirm that FLC repression is the major mechanism that promotes flowering in skip-1, skip-1 plants were transformed with a 3SS:FLC construct. Flowering time in the 3SS:FLC transgenic lines was similar to that in WT plants because of the overexpression of FLC, indicating that FLC was able to recover the early flowering phenotype of skip-1 (Fig. 3b and c; Additional file 6: Table S5 and Additional file 7: Table S6).

Together, these genetic and molecular data demonstrate that SKIP suppresses the floral transition in Arabidopsis in an FLC-dependent manner.

SKIP promotes FLC expression by controlling the alternative splicing of SEF pre-mRNA

We next investigated the mechanisms whereby SKIP regulates FLC expression in detail. There are three possible explanations for the silencing of FLC by skip-1. First, the skip-1 mutation may cause the abnormal splicing of FLC pre-mRNA and decrease the accumulation of functional FLC mRNA. Second, the skip-1 mutation may cause the overexpression of genes in the autonomous or vernalization pathway to reduce FLC expression. Third, the skip-1 mutation may repress the expression of flowering time suppressors, including genes encoding members of the PAF1 complex, the SWR1-C, and so forth.

To explore the silencing mechanisms of FLC, MAF1, MAF4, and MAF5 in the skip-1 mutant, we first tested for defects in the splicing of FLC, MAF1, MAF4, and MAF5 pre-mRNA in the skip-1 mutant; except for MAF1, no splicing defects were detected (Additional file 8: Figure S2a; Additional file 9: Figure S6b, d, and f). Next, we assessed the mRNA expression of genes in both the autonomous and vernalization pathways, including FCA, FY, FLK, FLD, FPA, FVE, LD, VRN1, VRN2, and VIN3 [24]. The expression
Fig. 1 Pleiotropic phenotypes of the skip-1 mutant. a-d Early flowering phenotype of skip-1 under LD (a, c) and SD (b, d) conditions. e, f Inhibition of root growth in skip-1 plants. g Shoot and root weights in skip-1 mutant plants. h-j Comparison of sepal (h), petal (i), and flower (j) sizes in wild-type (WT) and skip-1 plants. k-m Comparison of stigma (k), stamen (l), and silique (m) lengths in WT and skip-1 plants. L12-9, L29-11, L30-2, and L18-5 are skip-1 transgenic lines harboring the pSKIP:SKIP genomic DNA construct. All data are given as the mean ± standard deviation (s.d.) (n = 31–36 in c, n = 11–12 in d, and n = 34–40 in f). Six pools of five seedlings are represented in g. Scale bars are indicated in the images.

16L/8D: 16 h of light/8 h of darkness, 8L/16D: 8 h of light/16 h of darkness
Fig. 2 (See legend on next page.)
levels of these genes in skip-1 were similar to those in WT plants, suggesting that the skip-1 mutation does not silence FLC by enhancing the expression of these genes (Additional file 8: Figure S2b and c). Finally, we analyzed the expression of floral suppressors, which were chosen according to a review [24]. Our results revealed aberrant alternative splicing and reduced levels of mature SEF mRNA, which encodes a component of the SWR1-C (Additional file 8: Figure S2d).

The expression and abnormal alternative splicing of SEF caused by the skip-1 mutation were confirmed by reverse transcription-PCR (RT-PCR) and quantitative reverse transcription-PCR (qRT-PCR), respectively (Fig. 4; Additional file 10: Figure S3). Elevated accumulation of alternatively spliced SEF isoforms, including those showing intron retention, compared to the WT was detected in skip-1 (Fig. 4). However, the level of mature SEF mRNA encoding functional SEF protein was reduced by
skip-1 (Fig. 4; Additional file 10: Figure S3). SEF has been characterized as a suppressor of flowering time and an SWR1-C component [20]. Therefore, we hypothesized that the down-regulation of FLC in skip-1 mutants is caused by the abnormal splicing of SEF pre-mRNA.

To test this hypothesis, we generated three SEF complementary DNA (cDNA) constructs: one carrying the full-length SEF coding sequence (CDS), the second carrying the CDS plus the first intron (WT SEF IR, wtSEFIR), and the third carrying the CDS plus a mutated version of the first intron (mutated SEF IR, mSEFIR), driven by the 35S promoter. The 35S:wtSEFIR and 35S:mSEFIR constructs were identical except for the 5’ss and 3’ss in the first intron, which were mutated in mSEFIR to produce a transcript that included the first intron (Fig. 5a). All three constructs were transformed into sef-2 plants [20].

As expected, RT-PCR showed that, unlike wtSEFIR, which was efficiently spliced, mSEFIR plants accumulated unspliced SEF IR transcripts, which mimicked the large transcript in skip-1 mutant plants (Fig. 5b–d).

Transgenic plants expressing full-length SEF cDNA and wtSEFIR, but not mSEFIR, partially rescued the early flowering phenotype of sef-2, indicating that wtSEFIR has a similar function to full-length SEF cDNA. In contrast, the unspliced form (mSEFIR) could not activate FLC expression and hence failed to delay flowering under LD and SD conditions (Fig. 5b and e; Fig. 6; Additional file 11: Table S7; Additional file 12: Table S8).

These results reveal that the derepression of FLC in skip-1 was partially caused by a deficiency in the splicing of SEF, which is the major target through which SKIP regulates FLC expression (Figs. 5 and 6).

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**Fig. 4** SKIP is essential for the splicing of SEF pre-mRNA. a Locations of the primer pairs used to amplify mature SEF mRNA and its alternatively spliced isoforms. SEF-Aa, SEF-Ab, SEF-Ac, SEF-Ad (SEF-A), and SEF represent the mature mRNA, SEF mRNA containing the first intron, SEF mRNA containing the second intron, alternatively spliced mRNA containing the first and second introns from the SEF-A fragment, and all isoforms of SEF. UTR represents untranslated region. I1, I2, and I3 represent introns 1, 2, and 3. E1, E2, E3, and E4 represent exons 1, 2, 3, and 4. The black arrows indicate the location of primers. b, c, e, f The levels of mature SEF mRNA and its alternatively spliced isoforms in WT plants, skip-1, and the skip-1 complemented transgenic lines described in Fig. 1 under LD (b, e) and SD (c, f) conditions as determined by semi-RT-PCR (b, c) or qRT-PCR (e, f). d The levels of mature SEF mRNA and its alternatively spliced isoforms in WT and skip-1 plants at different times across the circadian cycle as determined by semi-RT-PCR. ZT indicates zeitgeber time or the time under continuous light. The values in e and f are the mean ± s.d.
Fig. 5 Splicing of SEF pre-mRNA regulates FLC expression. a Schematic representation of the constructs carrying the full-length CDS of SEF (SEFc), WT CDS with the first intron of SEF (wtSEF\(_{IR}\)), and mutated SEF sequence with the first intron (mSEF\(_{IR}\)) driven by the 35S promoter. In the mSEF\(_{IR}\) construct, the 5'ss and 3'ss of the first intron were mutated, as shown in red. UTR represents untranslated region. I1, I2, and I3 represent introns 1, 2, and 3. E1, E2, E3, and E4 represent exons 1, 2, 3, and 4. The black arrows indicate the location of primers for RT-PCR. b SEF and FLC expression in WT plants, sef-2, and sef-2 transgenic lines carrying 35S:SEFc, 35S:wtSEF\(_{IR}\), or 35S:mSEF\(_{IR}\) under LD and SD conditions as determined by RT-PCR. c, d FLC expression in WT plants, sef-2, and sef-2 transgenic lines carrying 35S:SEFc, 35S:wtSEF\(_{IR}\), or 35S:mSEF\(_{IR}\) under LD and SD conditions as determined by qRT-PCR. e FLC expression in WT plants, sef-2, and sef-2 transgenic lines carrying 35S:SEFc, 35S:wtSEF\(_{IR}\), or 35S:mSEF\(_{IR}\) under LD and SD conditions as determined by qRT-PCR. ACT2 was used as an endogenous control. The data are the mean ± s.d. (n = 3) in c–e. SEF-Aa and SEF-Ab represent the mature mRNA and transcript containing the first and second introns of the SEF-A fragment (as shown in Fig. 5a). C5-15, C13-5, and C22-8 are sef-2 transgenic lines carrying the 35S:SEFc construct; W2-2, W23-7, and W33-2 are sef-2 transgenic lines carrying the 35S:wtSEF\(_{IR}\) construct; and M2-13, M33-13, and M39-8 are sef-2 transgenic lines carrying the 35S:mSEF\(_{IR}\) construct.
SKIP is associated with SEF pre-mRNA

SKIP is localized to the nucleus [40, 55] (Additional file 13: Figure S4). During pre-mRNA splicing, the spliceosome is recruited to pre-mRNAs. Therefore, we examined whether the SKIP-containing spliceosome is associated with SEF pre-mRNA in vivo by RNA immunoprecipitation (RNA-IP).

C12-1, a transgenic line expressing a green fluorescent protein (GFP)-SKIP fusion protein (from SKIP:GFP-SKIP), recovered the early flowering phenotype of the skip-1, indicating that SKIP was functional in this line (Fig. 7a). We next used a commercial antibody against GFP to immunoprecipitate GFP-SKIP. The GFP-SKIP immunoprecipitates were then reverse-transcribed into cDNA and amplified by PCR with SEF-specific primers. Four sets of samples were prepared: no immunoprecipitation (Input; positive control), immunoprecipitation without anti-GFP antibodies (NA-IP; negative control), and PCR following RNA-IP against GFP with (RT+) or without (RT–) reverse transcription (Fig. 7b).

SEF pre-mRNA was detected in the Input and RT+ samples from line C12-1 and confirmed by sequencing (Fig. 7b). These results suggest a role for the SKIP-containing spliceosome in the splicing of SEF pre-mRNA.

SKIP activates FLC by regulating the splicing of SEF pre-mRNA by replacing H2A with H2A.Z at FLC chromatin

SEF is a component of the SWR1-C, which is involved in the deposition of H2A.Z to target chromatin to activate gene expression [20]. To determine whether the reduced expression of SEF in skip-1 plants influences the deposition of H2A.Z on a global scale, nucleoproteins were extracted from WT, skip-1, sef-2, and arp6-5 plants and Western blot analyses were performed using anti-H2A.Z antibodies. Compared to the WT, the H2A.Z enrichment on a global scale was significantly reduced by skip-1, sef-2, and arp6-5 mutations, indicating that SKIP may be involved in chromatin remodeling through the splicing of SEF in vivo (Fig. 7c).
Fig. 7 (See legend on next page.)
To verify whether the reduced transcript level of SEF in skip-1 affects the deposition of H2A.Z at FLC, we performed a chromatin immunoprecipitation (ChIP) assay to determine the level of H2A.Z across the entire FLC locus (Fig. 7d and e). In the WT, H2A.Z was predominantly enriched around the translation start site and first intron (regions B5, B6, and B9), stop site, and 3’ untranslated region (regions B13, B14, and B15), consistent with previous data [21] (Fig. 7d). The abundance of H2A.Z across FLC chromatin was lower in skip-1 than in the WT, except in regions B8, B11, and B12 (Fig. 7d and e).

To analyze whether the reduced level of SEF in skip-1 was responsible for the down-regulation of MAF4 and MAF5, the abundance of H2A.Z across MAF4 and MAF5 was examined in WT, skip-1, sef-2, and arp6-5 plants. The H2A.Z levels across most regions of MAF4 and MAF5 were obviously decreased in skip-1 (Fig. 7f and g).

These results indicate that the reduced expression of SEF in skip-1 decreased the deposition of H2A.Z at FLC, MAF4, and MAF5 in vivo to suppress their expression and promote flowering (Fig. 7d and f).

Discussion

RNA splicing is an essential posttranscriptional process that controls gene expression and increases transcriptomic and proteomic diversity in eukaryotes. The accuracy and efficiency of pre-mRNA splicing, controlled by cis-acting elements and trans-acting factors, play important roles in regulating the expression and normal cellular function of genes [26]. The identification of splicing factors is a key step in dissecting the posttranscriptional regulation of plant development. SKIP, an SNW domain-containing protein, has been reported to be a splicing factor [40]. In this study, the mechanisms whereby SKIP controls flowering time through the alternative splicing were investigated.

**SKIP is essential for normal plant development**

According to the present and previous studies, SKIP is a nuclear splicing factor that controls the circadian clock through alternative splicing in Arabidopsis [40, 55] (Additional file 13: Figure S4). Mutations in SKIP globally affect pre-mRNA splicing under normal growth conditions [40]. This finding is consistent with the pleiotropic phenotypes of skip-1 observed in this study. The loss-of-function skip-1 mutant confers early flowering phenotype under LD and SD conditions, which is consistent with the previous results [40, 64] (Fig. 1a–d, Additional files 1 and 2: Tables S1 and S2). In addition, the skip-1 mutant exhibits multiple developmental defects, including short roots, stamens, pistils, and siliques, and small flowers, sepals, and petals, indicating that SKIP plays essential roles in plant development (Fig. 1e–m; Additional file 3: Table S3). However, the mechanisms of SKIP that regulate these biological processes remain unknown.

**SKIP is involved in the transcriptional activation of FLC and its homologs**

The mutation in skip-1 suppressed FLC, MAF1, MAF4, and MAF5 expression, accelerated flowering by activating the expression of downstream flowering time integrators (including SOC1, FT, and TSF), and promoted early flowering (Fig. 2; Additional file 4: Figure S1). Consistent with this, the inactivation of FLC in skip-1 partially recovered the late flowering phenotype of the FLC-activated mutants FRI, fve, and flk (Fig. 3a; Additional file 5: Table S4). Overexpression of FLC rescued the early flowering phenotype of skip-1 (Fig. 3b and c; Additional file 6: Table S5 and Additional file 7: Table S6). These results provide solid evidence that the suppression of FLC expression is responsible for the early flowering phenotype of skip-1, suggesting that SKIP is required for the activation of FLC expression. However, the results of our pre-mRNA splicing assay and the previous report show that SKIP did not affect the splicing of FLC pre-mRNA, implying that SKIP indirectly regulates FLC expression to control flowering [64] (Additional file 8: Figure S2a).

**SKIP links the posttranscriptional regulation of SWR1-C to activation of FLC and its homologs**

SKIP regulates the splicing of SEF pre-mRNA to control FLC, MAF4, and MAF5 expression and flowering time.
The *skip-1* mutation did not impact the levels of mature mRNA produced from genes encoding *FLC* suppressors and activators of the autonomous and vernalization pathways [24], except for *SEF* (Additional file 8: Figure S2b–d). *SEF* is a component of the SWR1-C, which exchanges histone H2A for H2A.Z, producing variant nucleosomes. The SWR1-C is required for H2A.Z deposition at three loci: *FLC*, *MAF4*, and *MAF5*. The enrichment of H2A.Z deposition at *FLC*, *MAF4*, and *MAF5* chromatin promotes their transcription and delays flowering [21]. However, the regulatory mechanisms (especially post-transcriptional regulation) affecting the functions of the SWR1-C are unknown. In this study, we found that H2A.Z enrichment at *FLC*, *MAF4*, and *MAF5* was decreased in *skip-1* mutant, resulting in the inactivation of their expression and the promotion of flowering in *skip-1* plants (Fig. 7d and f). Our findings reveal a novel mechanism whereby SKIP controls flowering time through pre-mRNA splicing and postranscriptional regulation of the SWR1-C via ATP-dependent chromatin remodeling processes (Fig. 8). SKIP is required for the alternative splicing of the *SEF* pre-mRNA; the splicing of *SEF* pre-mRNA affects the deposition of H2A.Z at *FLC*, *MAF4*, and *MAF5* chromatin; H2A.Z deposition activates the expression of these genes to delay flowering in Arabidopsis.

It should be indicated that the levels of mature *SEF* mRNA and presumably its protein were reduced through alternative splicing but they were not knocked out in *skip-1* (e.g., in the *sef-2* mutant). It is reasonable to expect that the deposition of H2A.Z in the *skip-1* mutant would be intermediate between WT and *sef-2*; however, the level of H2A.Z at some regions of *MAF4* chromatin in *skip-1* was lower than that in *sef-2* mutant. It is possible that *SEF* targets substrates with differential affinities or that other unknown factors regulating the deposition of H2A.Z were inactivated in *skip-1*. This will be confirmed in future experiments.

### Conclusions

Our study reveals the regulatory roles of SKIP in flowering time control of Arabidopsis. SKIP is a splicing factor. The *skip-1* mutant exhibits an early flowering phenotype, indicating that SKIP is a suppressor of flowering time. SKIP promotes the expression of floral repressors, including *FLC*, *MAF1*, *MAF4*, and *MAF5*, and delays flowering time through regulating the pre-mRNA splicing of *SEF*. SKIP binds to the pre-mRNA of *SEF* to control its splicing and activates its expression. The elevated expression of *SEF* by SKIP accelerates the substitution of H2A by H2A.Z at *FLC*, *MAF4*, and *MAF5* expression.
and MAF5 chromatin, leading to increased FLC, MAF4, and MAF5 expression and delayed flowering time in Arabidopsis.

Methods

Plant materials and growth conditions
All plant materials used in this study were of Arabidopsis ecotype Col-0. Seeds were sterilized and placed on Murashige and Skoog (MS) medium with 0.3% agar and 1% sucrose. After stratification in the dark at 4 °C for 2 days, the plates were transferred to white light (70 μmol m⁻² s⁻¹) in a Percival CU36L5 growth chamber (Percival Scientific, Perry, IA, USA). Plants for flowering time determination were grown under various light-dark photocycles with cool white fluorescent light (100 μmol m⁻² s⁻¹) at 22 °C during the day and 18 °C at night.

Root elongation and fresh weight assays
Five-day-old seedlings grown in MS medium were transferred to fresh MS agar medium and allowed to grow for an additional 14 days before being harvested. The root lengths and shoot and root fresh weights were then measured.

Complementation test
The construction of pSKIP:SKIP and pSKIP:GFP-SKIP plasmids were described in our previous report [40]. The resulting pSKIP:SKIP and pSKIP:GFP-SKIP plasmids were introduced into skip-1 by Agrobacterium-mediated transformation [67]. Flowering time was examined in T3 transgenic lines (SKIP:SKIP/skip-1). Complemented T3 lines (SKIP:GFPSKIP/skip-1) were used in RNA-IP.

To produce the 35S:FLC construct, the 591-bp CDS of FLC was amplified and cloned into pCambia1300 under the control of the 35S constitutive promoter. The resulting construct was transformed into skip-1 plants via the Agrobacterium-mediated floral dip method [67]. Transformants were selected on MS medium containing hygromycin. Single insertion lines were selected based on the segregation of antibiotic resistance. Flowering time and FLC mRNA enrichment were examined in T3 transgenic lines.

To produce 35S:SEFc, 35S:wtSEFIR, and 35S:mSEFIR, the 516-bp CDS of SEF (SEFc), the SEF CDS plus the first intron (594 bp; wtSEFIR), and a mutated version of SEF with the first intron that contained an altered 5'ss and 3'ss (594 bp; mSEFIR) were amplified and cloned into pCambia1300 under the control of the 35S promoter. Next, the constructs were transformed into skip-1 via the Agrobacterium-mediated floral dip method [67].
Transformants were selected on MS medium containing hygromycin. Single insertion lines were selected based on the segregation of antibiotic resistance. Flowering time and the expression of SEF and FLC were examined in T3 transgenic lines.

Gene expression assays
For RT-PCR and qRT-PCR, total RNA was extracted from 10-day-old seedlings using Takara RNAiso Plus (Takara Bio Inc., Otsu, Japan). After RNase-free DNase I (RQ1 RNase-Free DNase; Promega, Madison, WI, USA) treatment, 3 μg of RNA was used for first-strand cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit; Fermentas, Waltham, MA, USA). Takara SYBR Premix Ex Taq (Takara Bio Inc.) and a 7500 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA, USA) were used for qRT-PCR.

Confocal microscopy and subcellular localization analysis
Protoplasts isolated from 3- or 4-week-old Arabidopsis leaves grown under 12 h of light/12 h of darkness as described by Asai et al. [68] and transiently transformed with SKIP-GFP-SKIP (constructed in pCambia1300) and roots from 7-day-old T3 transgenic seedlings harboring SKIP-GFP-SKIP were used for a subcellular localization analysis. Images were collected using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) as described previously [69].

Western blot
For immunoblotting, Arabidopsis seedlings were ground to a powder in liquid nitrogen and then homogenized in extraction buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM MgCl₂, 1% Nonidet P-40, complete protease inhibitor [Roche, Basel, Switzerland], and 1 mM phenylmethylsulfonyl fluoride). The extracts were then centrifuged, the pellet removed, and the supernatant boiled in 6X SDS sample buffer. The proteins in the samples were separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA), and detected using different antibodies. The antibodies used for H2A.Z and tubulin detection were anti-histone H2A.Z antibodies (Abcam Cat. no. ab4174, Bat. No. 185934-1, RRID: AB_304345; Abcam, Cambridge, UK) and anti-α-tubulin (Sigma-Aldrich Cat. No. T5168, Bat. No. 072 M4809, RRID: AB_477579; Sigma-Aldrich, St. Louis, MO, USA). The bound antibodies were visualized using Amersham ECL reagents (GE Healthcare, Little Chalfont, UK). The band intensity was analyzed with Image J software.

RNA-IP
RNA-IP was conducted as described previously [70, 71] with slight modifications. Thirteen-day-old whole seedlings of transgenic complementation lines harboring SKIP-GFP-SKIP in a skip-1 background and grown under LD conditions were harvested to detect the association of SKIP with SEF pre-mRNA. After crosslinking in 1% formaldehyde, pre-immunoprecipitation treatment, and immunoprecipitation with anti-GFP antibodies (Abcam Cat. No. ab290, Bat. No. GR197631-1, RRID: AB_303395), the immunoprecipitation products were eluted with elution buffer. The associated RNAs were quantified by RT-PCR with primer pairs crossing the intron-exon junctions in SEF pre-mRNA after reversal of the crosslinks.

ChIP
ChIP was performed as described by Gendrel et al. [72] using 13-day-old seedlings grown on MS medium under LD conditions. Anti-H2A.Z antibodies were purchased from Abcam (Abcam Cat. No. ab4174, Bat. No. 185934-1, RRID: AB_304345). The primers used for qPCR are provided in Additional file 15: Table S9.

Accession numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: SKIP (At1g77180), FRI (Col-0) (16-bp deletion), fve (SALK_013789.44.25.x), and flk (SALK_001523.29.35.x). These mutants were ordered from the Arabidopsis Biological Resource Center. The sef-2 (CS841940) and arp5-6 (CS872355) mutants were kindly provided by Dr. Ilha Lee in the Laboratory of Plant Developmental Genetics and School of Biological Sciences at Seoul National University in Korea.

Additional files

Additional file 1: Table S1. Early flowering phenotypes of skip-1 under LD conditions. (DOC 49 kb)
Additional file 2: Table S2. Early flowering phenotypes of skip-1 under SD conditions. (DOC 37 kb)
Additional file 3: Table S3. Root length and fresh weight of skip-1 under LD conditions. (DOC 34 kb)
Additional file 4: Figure S1. SKIP expression. Ten- or 17-day-old seedlings were used for qRT-PCR analysis. a Expression of CO in WT and skip-1 plants under LD and SD conditions. b Diurnal expression of CO in WT and skip-1 plants under SD conditions. c Diurnal expression of FT in WT and skip-1 plants under SD conditions. ACT2 was used as an endogenous control. Three biological replicates were performed with similar results, and the result from one of the experiments is shown. The values are the mean ± s.d. (TIF 996 kb)
Additional file 5: Table S4. skip-1 is able to recover the late flowering phenotypes of FRI and fve, and flk mutants under LD conditions. (DOC 43 kb)
Additional file 6: Table S5. Overexpression of FLC is able to recover the early flowering phenotypes of skip-1 under LD conditions. (DOC 34 kb)
Additional file 7: Table S6. FLC expression as measured by qRT-PCR in the skip-1 transgenic lines under LD conditions. (DOC 32 kb)
Additional file 8: Figure S2. Identification of factors through which SKIP activates FLC expression. a Measurement of FLC splicing defects in WT plants, skip-1, and the three skip-1 complemented transgenic lines described in Fig. 1. The locations of the primer pairs used to amplify mature FLC mRNA and its alternatively spliced isoforms (upper panel). Scale bar, 1 kb. FLC was divided into two fragments to amplify its alternatively spliced isoforms by semi-RT-PCR under LD and SD conditions (lower panel). b and c Expression of genes encoding FLC transcriptional activators in WT and skip-1 plants as detected by semi-RT-PCR and qRT-PCR. Three biological replicates were performed with similar results, and the result from one of the experiments is shown. The values are the mean ± s.d. in c. d The expression of genes encoding FLC transcriptional repressors in WT and skip-1 plants as determined by semi-RT-PCR. ACT2 was used as an endogenous control. IR, alternatively spliced isoforms produced by intron retention. (TIF 7388 kb)

Additional file 9: Figure S6. The expression and alternative splicing of MAF1, MAF4, and MAF5 in skip-1 and sef-2 mutants under LD conditions. a, c, e MAF1, MAF4, and MAF5 expression in WT, skip-1, sef-2, and three of the skip-1 complemented transgenic lines, including L12-9, L29-11, and L30-2, described in Fig. 1 under LD conditions. b, d Expression of genes encoding MAF1, MAF4, and MAF5 in WT, skip-1, sef-2, and three of the skip-1 complemented transgenic lines, including L12-9, L29-11, and L30-2, described in Fig. 1 under LD conditions. ACTIN 2 (ACT2) was used as an endogenous control. Three technical replicates were performed. The values are the mean ± s.d. One-way analysis of variance (ANOVA; Tukey’s multiple comparison test) was performed for data in a, c, and e. Statistically significant differences are indicated by different lowercase letters (P < 0.05). There are statistically significant differences between all non-identical letters. (TIF 3062 kb)

Additional file 10: Figure S3. SKIP, a splicing factor, is essential for the splicing of SEF pre-mRNA. a Locations of the primer pairs used to amplify mature SEF mRNA and its alternatively spliced isoforms. SEF-Aa, SEF-Ba, SEF-Bb, and SEF-Bd represent the mature mRNA, isoform containing the second intron, isoform containing the third intron, and isoform containing the second and third introns of the SEF-B fragment. The black arrows indicate the location of primers. b and c The levels of mature SEF mRNA and alternatively spliced isoforms in WT and skip-1 plants under LD and SD conditions as determined by qRT-PCR. Three technical replicates were performed. The values are the mean ± s.d. in b and c. (TIF 1341 kb)

Additional file 11: Table S7. SEF regulates flowering time through alternative splicing under LD conditions. (DOC 38 kb)

Additional file 12: Table S8. SEF regulates flowering time through alternative splicing under SD conditions. (DOC 40 kb)

Additional file 13: Figure S4. SKIP is localized to the nucleus to perform its function. a A SKIP construct encoding GFP driven by the native SKIP promoter (SKIP:GFP-SKIP) was transiently expressed in Arabidopsis protoplasts. b Roots of 7-day-old seedlings stably transformed with SKIP:GFP-SKIP. The images were produced by laser scanning confocal microscopy. DAPI 4',6-diamidino-2-phenylindole. Scale bar, 10 μm in a and 20 μm in b. (TIF 4964 kb)

Additional file 14: Figure S5. The expression of FLC in WT, skip-1, sef-2, and three of the skip-1 complemented transgenic lines, including L12-9, L29-11, and L30-2, described in Fig. 1 under LD conditions. ACTIN 2 (ACT2) was used as an endogenous control. Three technical replicates were performed. The values are the mean ± s.d. One-way analysis of variance (ANOVA; Tukey’s multiple comparison test) was performed. Statistically significant differences are indicated by different lowercase letters (P < 0.05). There are statistically significant differences between all non-identical letters. (TIF 503 kb)

Additional file 15: Table S9. Primers used in ChIP assays. (DOC 43 kb)
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