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RESEARCH PAPER

The inhibitory effect of ABA on floral transition is mediated by ABI5 in *Arabidopsis*

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Abstract

Seed germination and flowering initiation are both transitions responding to similar seasonal cues. This study shows that ABSCISIC ACID-INSENSITIVE MUTANT 5 (ABI5), a bZIP transcription factor, which plays an important role in the abscisic acid (ABA)-arrested seed germination, is robustly associated with the floral transition in *Arabidopsis*. Under long-day conditions, overexpression of *ABI5* could delay floral transition through upregulating *FLOWERING LOCUS C* (*FLC*) expression. In contrast, ectopically overexpressing *FLC* in an *abi5* mutant reversed the earlier flowering phenotype. Further analysis indicated that transactivation of *FLC* could be promoted by ABI5 and/or other abscisic acid-responsive element (ABRE)-binding factors (ABFs). The expression of *FLC* that was promoted by ABI5 and/or other ABFs could be blocked in a triple SNF1-related protein kinase (SnRK) mutant, *snrk2.2/2.3/2.6*, despite the presence of ABA. In sharp contrast, when SnRK2.6 was coexpressed, the reduction of transactivity of *FLC* was reverted in mesophyll protoplasts of *snrk2.2/2.3/2.6*. Additional results from analysing transgenic plants carrying mutations of phosphoamino acids (*ABI5*^{S42AS145AT201A}), which are conserved in ABI5, suggested that SnRK2-mediated ABI5 and/or ABF phosphorylation may be crucial for promoting *FLC* expression. The transgenic plants *ABI5*^{S42AS145AT201A} were insensitive to ABA in seed germination, in addition to having an earlier flowering phenotype. Direct binding of ABI5 to the ABRE/G-box promoter elements existing in *FLC* was demonstrated by chromatin immunoprecipitation. Mutations at the ABRE/G-box regions in *FLC* promoter sequences abolished the ABI5-promoted transactivation of *FLC*. In summary, these results may decipher the inhibitory effect of ABA on floral transition in *Arabidopsis*.

Key words: ABA, ABFs, ABI5, chromatin immunoprecipitation, FLC, flowering time, SnRK2s.

Introduction

The transition to flowering initiation is one of the most important decisions in the plant life cycle (Simpson and Dean, 2002; Boss *et al*., 2004). Optimal timing for switching from vegetative growth to reproductive development is crucial to maximize the reproductive accomplishment. Accordingly, plants have evolved mechanisms to regulate the timing of floral initiation (Boss *et al*., 2004; Jack, 2004).

Four classic regulatory pathways involving the photoperiod, vernalization, autonomous pathways, and gibberellic acid (GA) have been identified in *Arabidopsis* (Mouradov *et al*., 2002). The environmental and endogenous signals can be collaboratively sensed by the plant in order to initiate transition of timely flowering (Moon *et al*., 2005; Liu *et al*., 2009). FLOWERING LOCUS C (FLC), one of the repressor integrators, tightly controls flowering signals (Michaels and Amasino, 1999, 2001). *FLC* expression can be repressed by vernalization and autonomous pathways via modulating the chromatin structure (Michaels, 2009); thus, flowering can

be promoted through revoking the inhibitory effect of FLC on the expression of *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Helliwell *et al.*, 2006; Searle *et al.*, 2006).

Phytohormones have diverse effects in the growth and development of plants, which regulate multiple physiological, metabolic, and cellular processes. Regulations of phytohormones in the floral transition have been reported (Gray, 2004; Davis, 2009; Domagalska *et al.*, 2010). The involvement of GA in the control of flowering in *Arabidopsis* has been demonstrated by applying GAs to a plant to stimulate bolting (Lang, 1956, 1957). Mutants deficient in GA biosynthesis exhibit dramatic delays in flowering under short-day conditions (Hisamatsu *et al.*, 2000; Moon *et al.*, 2003). The inhibitory effect of ABA in floral transition has been documented in an ABA-deficient mutant (Martinez-Zapater *et al.*, 1994). Modulation of DELLA protein activity can instigate the inhibitory effect of ABA on flowering (Achard *et al.*, 2006). Thus, ABA is considered as a floral repressor.

However, the mechanism of delayed flowering time by ABA in *Arabidopsis* is poorly understood, and whether ABA could undertake a convergent approach to sustain negative regulation on FLC is elusive. A study has shown that *FLC*-mediated seed germination proceeds through genes such as *FT*, *SOC1*, and *APETALA1*, making *FLC* a promising regulator of temperature-dependent seed germination (Chiang *et al.*, 2009). In *Arabidopsis*, most abscisic acid-responsive element (ABRE)-binding factors (ABFs), which are classified as bZIP transcription factors, are involved in ABA signal transduction during seed germination and/or in vegetative growth (Choi *et al.*, 2000; Jakoby *et al.*, 2002). FD, another bZIP protein, mediates signals from FT at the shoot apex in *Arabidopsis* (Abe *et al.*, 2005). ABF1, ABF3, and ABF4 are mainly expressed in vegetative tissues whereas ABSCISIC ACID-INSENSITIVE MUTANT 5 (ABI5), a bZIP transcription factor, is preferentially expressed during seed maturation and seed germination (Finkelstein and Lynch, 2000; Uno *et al.*, 2000). Information about ABI5 in other developmental aspects is inadequate.

Seed germination and flowering initiation are both transitions that could result from responding to a similar seasonal cue and these two important life transitions might share common regulatory elements in genetic pathways. This study shows that, in addition to controlling seed germination, ABI5 may be functional in floral transition in *Arabidopsis*. The results indicate that overexpression of *ABI5* could actually delay flowering initiation via upregulating *FLC* expression. Phosphorylation of ABI5 by sucrose nonfermenting 1-related protein kinase (SnRK) 2 directly influenced floral transition; without phosphorylation, the inhibitory effect of ABI5 on floral transition was abolished. Direct binding of ABI5 to *FLC* promoter regions could transactivate *FLC* expression. All data suggest a positive regulation by ABI5 on FLC activity for the control of floral transition in *Arabidopsis*.

Materials and methods

Plant materials, growth conditions, and measurement of flowering time

The *abi5* mutant, *abi5-4* (Lopez-Molina and Chua, 2000) and the wild type (Ws-0) were in the Wassilewskija background. Other plants used in this study were in the Columbia (Col-0) background. The *snrk2.2/3/6+* mutant was kindly provided by Dr Jian-Kang Zhu (Purdue University) (Fujii *et al.*, 2007; Fujii and Zhu, 2009). Primer sequences for identifying homozygous *snrk2.2/2.3/2.6* are listed in Supplementary Table S1 (available at *JXB* online). Transgenic plants of *ABI5-5*, $ABI5^{S42A}$, $ABI5^{S145A}$, and $ABI5^{S42AS145AT201A}$ were generated by *Agrobacterium tumefaciens* GV3101-mediated floral infiltration (Clough and Bent, 1998). To overexpress *FLC* in the *abi5-4* mutant background, plasmid p35S::GFP-FLC was transformed into *abi5-4* plants. To assess the expression pattern of *FLC in planta*, the plasmids pFLC::GUS and pFLC(m)::GUS were introduced into *ABI5-5* plants, respectively. Plants were grown in soil or on MS medium (Phyto Technology, USA) containing 1% sucrose and 0.8% (w/v) agar at 23 °C in a growth room under long-day conditions (16h/8h light/dark) or short-day conditions (8h/16h light/dark). To detect the effect of ABA on flowering time, 2-week-old plants were sprayed with 100 µM ABA thrice a week until all plants start flowering. The stock solution of ABA (Sigma-Aldrich, USA) was dissolved in ethanol. The control treatment was performed with an equal amount of the solvent. Flowering time was scored as the number of total rosette leaves at bolting. At least 15 plants were scored in each group.

Plasmid construction

Primer sequences for cloning the constructs in this study are listed in Supplementary Table S2 and detailed information on the plasmids can be found in Supplementary Table S3. In brief, p35S::$ABI5^{S42A}$-GFP, p35S::$ABI5^{S145A}$-GFP, p35S::$ABI5^{S42AS145A}$-GFP and p35S::$ABI5^{S42AS145AT201A}$-GFP were made through site-directed mutagenesis by PCR amplification (Edelheit *et al.*, 2009), based on plasmid p35S::ABI5-GFP. Plasmid p35S::ABI5-HA was constructed by inserting the *ABI5* coding sequence into pBA002 at the *Xho*I/*Sac*II site (Kost *et al.*, 1998).

Transient expression assay

Mesophyll protoplasts were prepared from 4-week-old plants of Col-0 and *snrk2.2/2.3/2.6* according to the methods described previously (Yoo *et al.*, 2007). All plasmids were prepared through the purification with caesium chloride/ethidium bromide (Sambrook *et al.*, 1989). Protein kinase inhibitor K252a (Sigma-Aldrich, USA) was dissolved in DMSO (Sigma-Aldrich, USA) and accordingly added to the culture. The relative activity of LUC/GUS was scored after transformation. The transformed protoplasts were incubated at 23 °C for 12 hours under darkness. Each experiment was repeated at least three times.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay was essentially performed according to methods described in several reports (Lee *et al.*, 2007; Xi *et al.*, 2010). DNA was recovered as described by Wang *et al.* (2002). The precipitated DNA fragments were quantified by quantitative real-time PCR (qRT-PCR) amplification using specific primers (Supplementary Table S4).

Analysis of gene expression

Total RNA was isolated from 5-day-old seedlings. The protocols used for qRT-PCR experiments have been described previously (Wang *et al.*, 2011). Primer sequences are available in Supplementary Table S5.

GUS histochemical staining

GUS signals were detected with the method described by Jefferson *et al*. (1987). Plants were pretreated with or without 100 μM ABA for 3 hours, and then immersed in 90% acetone for 30 minutes. After they were incubated in the GUS staining solution (0.5 mg/ml X-Gluc, 50 mM PBS, pH 7.0; 5.0 mM potassium ferricyanide, 5.0 mM potassium ferrocyanide, 0.1% Triton X-100) at 37 °C overnight, the stained plants were washed with 70% ethanol overnight. Images were taken with an inverted microscope (SMZ1500, Nikon).

Results

Overexpression of ABI5 in Col-0 influences flowering time

To assess the correlation between flowering time and *ABI5* expression, overexpressing transgenic lines carrying plasmid p35S::ABI5-HA in Col-0 background were generated. A total of 43 lines were analysed by examining ABI5-HA protein level in individual transgenic plants (Fig. 1A). Quantifying the rosette leaf numbers indicated that flowering time of transgenic lines *ABI5-5* and *ABI5-18* was delayed under long-day conditions; however, *abi5-4* showed slightly earlier flowering (Fig. 1B, C). Under short-day conditions, the flowering time was not much different in all examined lines (Fig. 1D). Subsequently, the microarray data available in public resources were analysed and the expression pattern of *FLC* was actually similar to that of *ABI5* (Supplementary Fig. S1A; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter *et al*., 2007). Thus, the present study examined the transcriptional correlation between *ABI5* and *FLC* and found that both genes had decreased expression levels during the growth of seedlings (Supplementary Fig. S1B). Under long-day conditions, although the expression level of *FLC* in *abi5-4* plants was downregulated, it was accordingly upregulated in *ABI5-5* and *ABI5-18* plants (Fig. 1C). This phenomenon was not observed in all of the examined lines under short-day conditions (Fig. 1D). To characterize the genetic relationship between *ABI5* and *FLC*, p35S::FLC-GFP was introduced

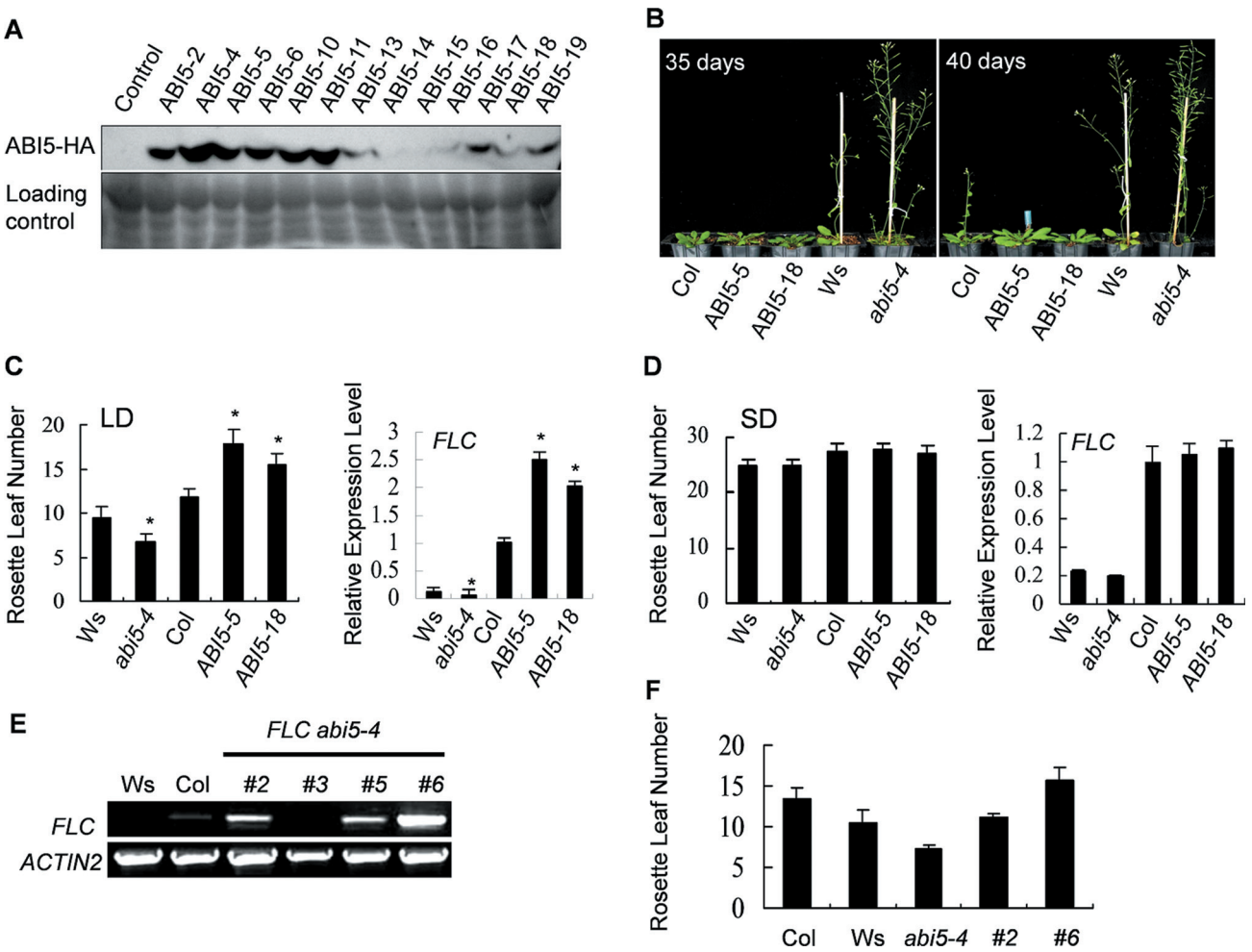

Fig. 1. Overexpression of ABI5 altered flowering time. (A) The expression level of ABI5-HA fusion protein in individual transgenic lines of p35S::ABI5-HA. (B) Flowering-time phenotypes in *abi5-4* (Ws), *ABI5-5* (Col) and *ABI5-18* (Col) under long-day conditions. (C and D) Comparisons of total rosette leaf numbers ($n > 15$ for each experiment) and *FLC* expression under long-day (LD) (C) and short-day (SD) conditions (D) (*$P < 0.05$). (E) *FLC* expression in *FLC abi5-4* transgenic lines (T2, homozygous) (#2, #3, #5, and #6 are individual lines). Total RNA was extracted from 10-day-old seedlings. *ACTIN2* represents the loading control. (F) Comparisons of total rosette leaf numbers. Data are mean ± standard errors of three replicated experiments ($n > 15$ for each experiment).

into *abi5-4* plants. The expression level of *FLC* in transgenic hybrid *FLC abi5-4* plants (T2) was then analysed (Fig. 1E) and the earlier flowering phenotype of *abi5-4* was reverted (Fig. 1F). Thus, these results imply a positive role of ABI5 in regulating *FLC* expression under long-day conditions, in terms of disturbing floral transition in *Arabidopsis*.

ABI5 activates FLC transcription in an ABA-dependent manner

A *FLC* promoter fragment (2.8 kb upstream of ATG) was cloned and fused to the luciferase (LUC) coding sequence (Fig. 2A), the resulting plasmid, pFLC::LUC, was used as the reporter construct. Plasmid pUBQ::GUS was used as the internal control (Wang *et al.*, 2011). Plasmid p35S::ABI5-GFP (ABI5) and pFLC::LUC and pUBQ::GUS were cotransformed into mesophyll protoplasts of Col and *abi5-7* (Col) respectively. The relative activity of LUC/GUS was significantly increased in coexpressing p35S::ABI5-GFP with ABA treatment (Fig. 2B). The fold-change of relative activity (LUC/GUS) was apparently greater in Col than that in *abi5-7* (Fig. 2B). The relative activity (LUC/GUS) of FLC::LUC was further analysed under a range of ABA concentrations (0–100 µM). The relative activity (LUC/GUS) of FLC::LUC was enhanced simultaneously with increasing ABA concentrations when transformed with the same amount (5 µg) of p35S::ABI5-GFP plasmid DNA (Fig. 2C). Hence, the relative activity (LUC/GUS) of FLC::LUC with 50 µM ABA was compared with a gradient dose of p35S::ABI5-GFP plasmid DNA (0–3 µg). FLC::LUC activity gradually increased and was tightly linked to the amount of p35S::ABI5-GFP DNA (Fig. 2C). As ABI5 activity can be modulated by SnRK2s (Nakashima *et al.*, 2009), the transfected protoplasts were treated with 50 nM protein kinase inhibitor K252a. Application of K252a could completely abolish the activity of FLC::LUC despite of presence of ABA (Fig. 2D). In summary, the results suggest an effect of ABA on modulating flowering signalling, which may be through interactive communication between ABI5 and FLC.

Phosphorylation of ABI5 is crucial in regulating flowering time

Four conserved phosphoamino acids (S41, S42, S145, T201) in ABI5 sequences have been identified (Lopez-Molina *et al.*, 2002). To analyse the importance of ABI5 phosphorylation in the regulation of flowering time, three phosphoamino acids (S42, S145, and T201) were replaced by alanine, and transgenic plants expressing the mutated forms ($ABI5^{S42A}$, $ABI5^{S145A}$, and $ABI5^{S42AS145AT201A}$) were then generated. The expression level of *ABI5* in the transgenic plants were evaluated by reverse-transcription PCR (Fig. 3A). Unlike *ABI5-5* plants, flowering time was evidently earlier in the transgenic plants (Fig. 3B and Supplementary Fig. S2A, B). Moreover, the retarded flowering time with 50 µM ABA was also observed in plants carrying the triple mutation $ABI5^{S42AS145AT201A}$ (Fig. 3C). To further untangle the correlation between the flowering time and ABA response, the ABA response of the $ABI5^{S42AS145AT201A}$ line in seed germination was analysed. Higher germination rates were scored with the $ABI5^{S42AS145AT201A}$ line on the plate containing 1 µM ABA (Supplementary Fig. 2C, D). Therefore, the data demonstrate that failure of ABI5 phosphorylation may lead to dysfunction of ABA signalling and suggest the importance of phosphorylation modification of ABI5 in the regulation of flowering time in *Arabidopsis*.

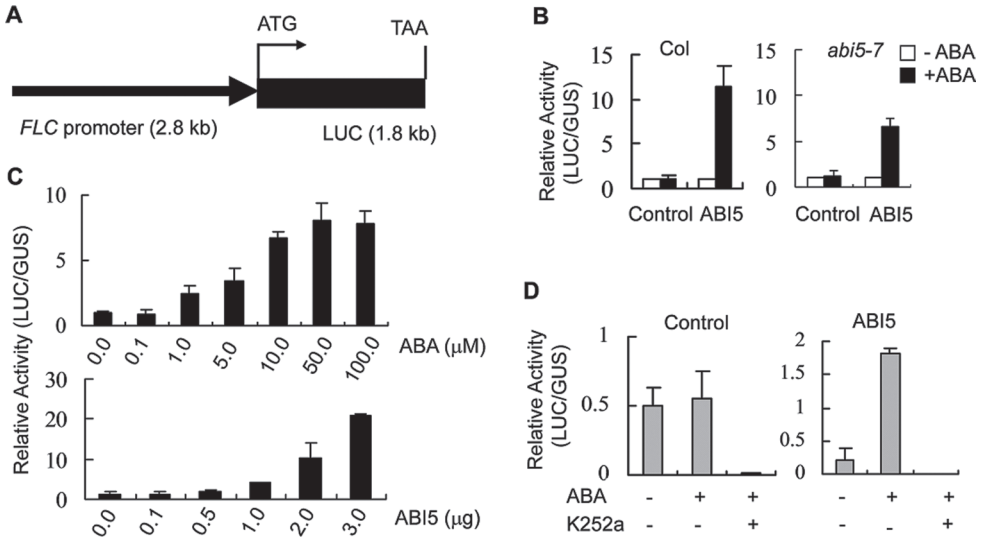

Fig. 2. ABI5-triggered *FLC* promoter activity is associated with abscisic acid (ABA) treatment. (A) Schematic diagram of pFLC::LUC construct. (B) Relative activity (LUC/GUS) of pFLC::LUC in coexpression with ABI5 with and without 50 µM ABA for 12 hours. (C) Relative activity (LUC/GUS) of pFLC::LUC with various concentrations of ABA, and various doses of p35S::ABI5-GFP DNA. (D) Relative activity (LUC/GUS) of pFLC::LUC was abolished when treated with 50 nM protein kinase inhibitor (K252a) for 12 hours. Control, p35S::GFP; ABI5, p35S::ABI5-GFP. Data are mean ± standard errors of three replicated experiments ($n > 5$ for each experiment).

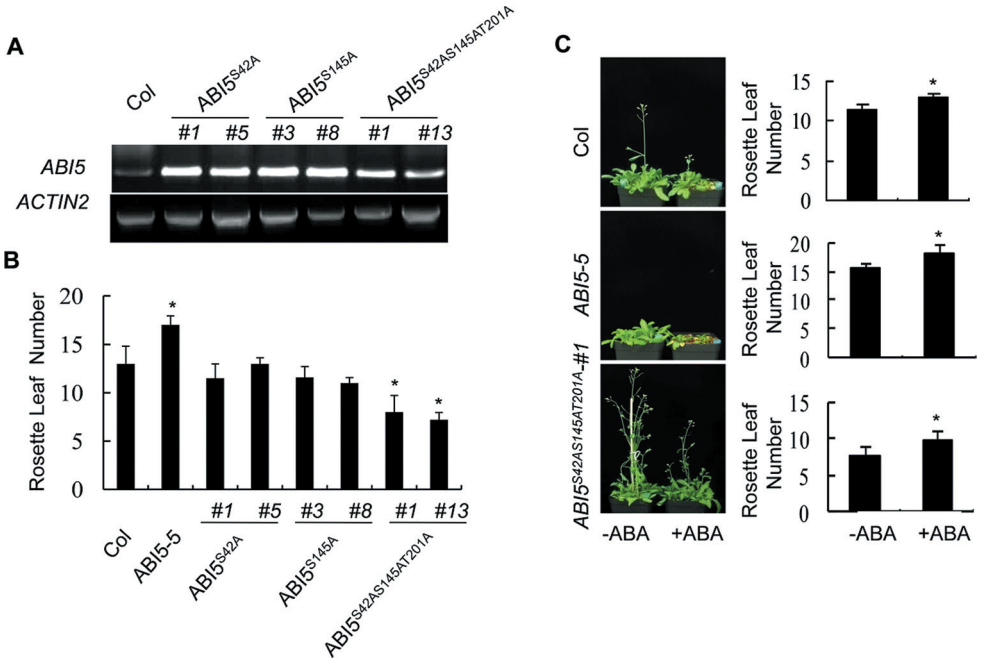

Fig. 3. ABI5 phosphorylation is essential for flowering time regulation. (A) *ABI5* expression was evaluated in independent transgenic lines, *ABI5*^{S42A}, *ABI5*^{S145A}, and *ABI5*^{S42AS145AT201A}. *ACTIN2* represents the loading control. (B) Comparative analysis of total rosette leaf numbers. Data represent the means ± SEs of three replicated experiments ($n > 20$ for each experiment). (C) Application of exogenous abscisic acid (ABA) significantly delays flowering time in Col and *ABI5-5* plants, but a partial effect is observed in *ABI5*^{S42AS145AT201A} plants. Data are mean ± standard errors of three independent experiments ($n > 15$ for each experiment).

Downstream targets in the flowering pathways can be altered by ABI5

Several downstream targets whose expression can be regulated by FLC are key players in flowering signal transduction pathways. This study scored the expression of *FLC* in *ABI5-5* plants under 50 μM ABA treatment (Fig. 4). In contrast, the level of *FLC* expression was reduced in *abi5-7* and *ABI5*^{S42AS145AT201A} plants, which may explain their earlier flowering phenotypes (Figs. 1B and C and Supplementary Fig. S2A). Notably, the expression of *FCA* in *ABI5-5* plants was inhibited; however, it was slightly increased in *abi5-7* and *ABI5*^{S42AS145AT201A} plants after ABA treatment. Without ABA, *FT* expression was significantly upregulated in *abi5-7* plants. Contrarily, with ABA treatment the level of *FT* expression was drastically decreased in *abi5-7* but not in Col or *ABI5*^{S42AS145AT201A} plants. In addition, *FT* expression was sustained at lower level with or without ABA treatment in

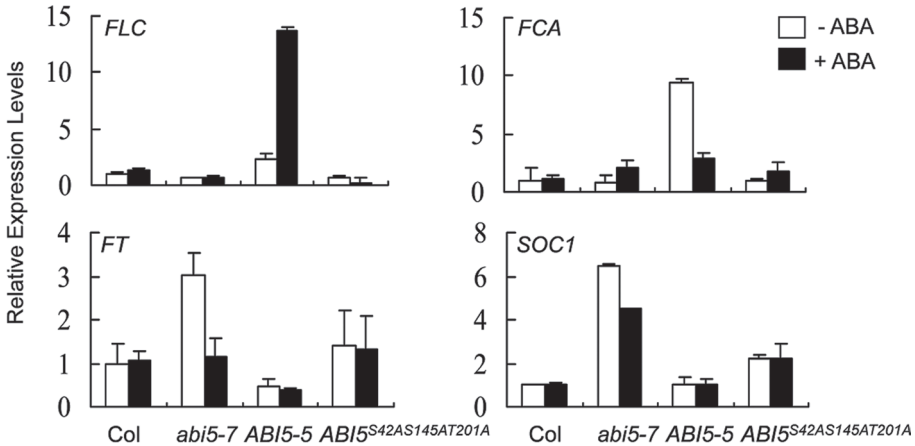

Fig. 4. Relative expression levels of flowering-related genes. 10-day-old seedlings were treated with or without 50 μM abscisic acid (ABA) for 3 hours. Gene expression in Col without ABA treatment were taken as the reference level; then fold-changes in *abi5-7* (Col), *ABI5-5*, and *ABI5*^{S42AS145AT201A} plants were normalized relative to the reference. Data are mean ± standard errors of three independent experiments ($n > 4$ for each experiment).

ABI5-5 plants. Similar expression patterns of *SOC1* were also obtained (Fig. 4). Taken together, these data extend the suggestion that the negative effect of ABI5 on activation of *FT* and *SOC1* may be associated with the ABI5 activity that is dependent on ABA signalling.

SnRK2s are required for ABI5-promoted FLC::LUC activity

The relative activity (LUC/GUS) of FLC::LUC was abolished despite ABA treatment when p35S::ABI5^{S42A}-GFP was coexpressed with pFLC::LUC. Similar results were obtained in coexpressions of p35S::ABI5^{S145A}-GFP, p35S::ABI5^{S42AS145A}-GFP, and p35S::ABI5^{S42AS145AT201A}-GFP (Fig. 5A). Changing the phosphoamino acids to alanine did not affect the stability of ABI5s (Fig. 5A). A transient expression assay was conducted in mesophyll protoplasts isolated from homozygous plants of the triple mutant *snrk2.2/2.3/2.6*. ABA treatment failed to promote activation of FLC::LUC, even when *ABI5* was coexpressed (Fig. 5B). When *SnRK2.6* and *ABI5* were coexpressed in Col protoplasts, the relative activity (LUC/GUS) of FLC::LUC could be detected; in addition, it was enhanced by 50 μM ABA (Fig. 5C). These data are consistent with the earlier flowering phenotype observed in heterozygous triple mutant *snrk2.2/3/6+* plants (Supplementary Fig. S3A). Subsequently, the roles of other ABA-responsive ABFs in regulating *FLC* expression were analysed. With 50 μM ABA, all three tested ABFs (ABF1, ABF3, and ABF4) could trigger the relative activity of FLC::LUC in Col but not in *snrk2.2/2.3/2.6* (Supplementary Fig. S3B). These data demonstrate that SnRK2s may play an important role in the ABI5- and/or ABFs-modulated activation of *FLC* at the transcriptional level.

ABI5 regulates FLC expression via directly binding to FLC promoter elements

To determine the regulatory rule of ABI5 on *FLC* expression, this study analysed the 2.8-kb promoter sequences of *FLC* and found six putative variants of ABRE-like elements (PyACGTGG/TG) (Fig. 6A). In addition, a G-box (CACGTG) element in the promoter region might be classified as a ubiquitous regulatory DNA element, which might be bound by bZIP proteins. Thus, a chromatin immunoprecipitation assay was performed in ABI5-HA transgenic plants. Except for the DNA4 fragment, all other five DNA fragments (DNA1, DNA2, DNA3, DNA5, and DNA6) (Fig. 6B) were immunoprecipitated by ABI5-HA. The best binding efficiency was obtained with the proximal DNA6 fragment (Fig. 6C). The sequence information conserved in the DNA6 fragment was analysed further (Fig. 6B) and, not surprisingly, a putative G-box and a single ABRE-like motif were contained in

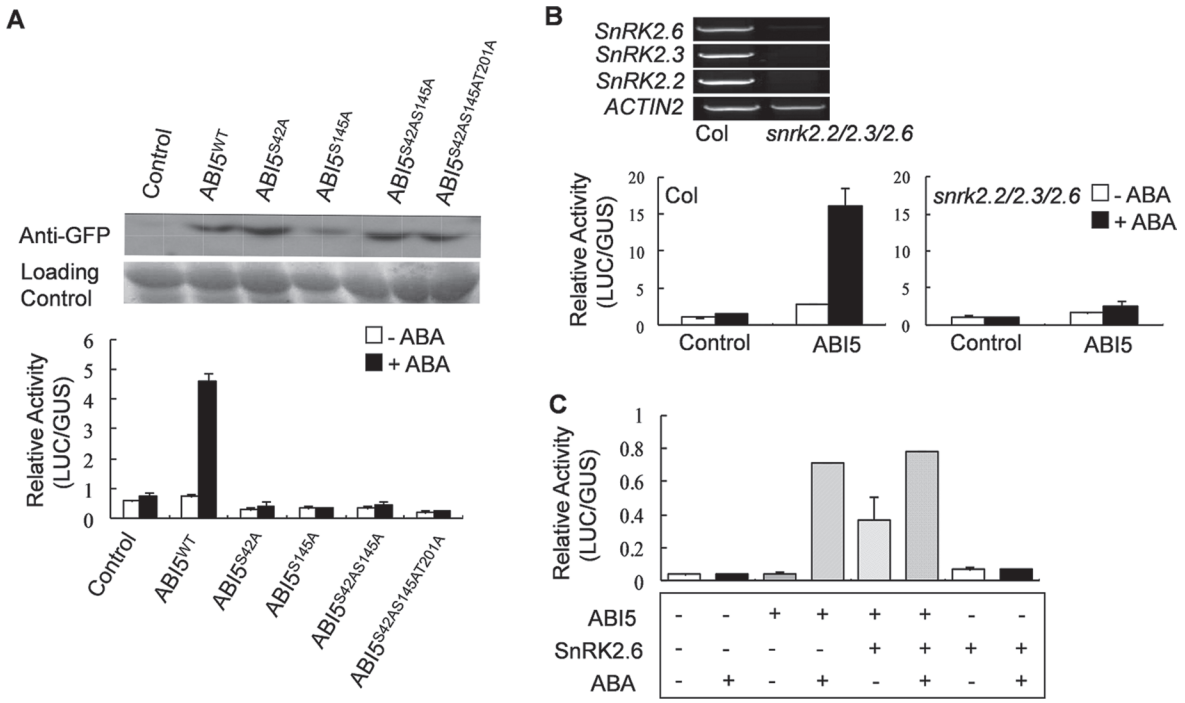

Fig. 5. SNF1-related protein kinases (SnRKs) may be involved in the positive regulation by ABI5 on *FLC* transcription. (A) Phosphoamino acid mutations of ABI5 inhibited the abscisic acid (ABA)-dependent FLC::LUC activity. Western blotting was used to detect ABI5. Data are mean ± standard errors of more than five independent experiments ($n > 4$ for each experiment). (B) The effect of ABI5 on FLC::LUC activity is significantly decreased in protoplasts of *snrk2.2/2.3/2.6* mutants. Homozygous *snrk2.2/2.3/2.6* plants were verified by semi-quantitative reverse-transcription PCR. Data are mean ± standard errors of three replicated experiments ($n > 4$ for each experiment). (C) Transient coexpression of p35S::SnRK2.6 (SnRK2.6) activates *FLC* promoter activity. Data are mean ± standard errors of more than five experiments ($n > 4$ for each experiment).

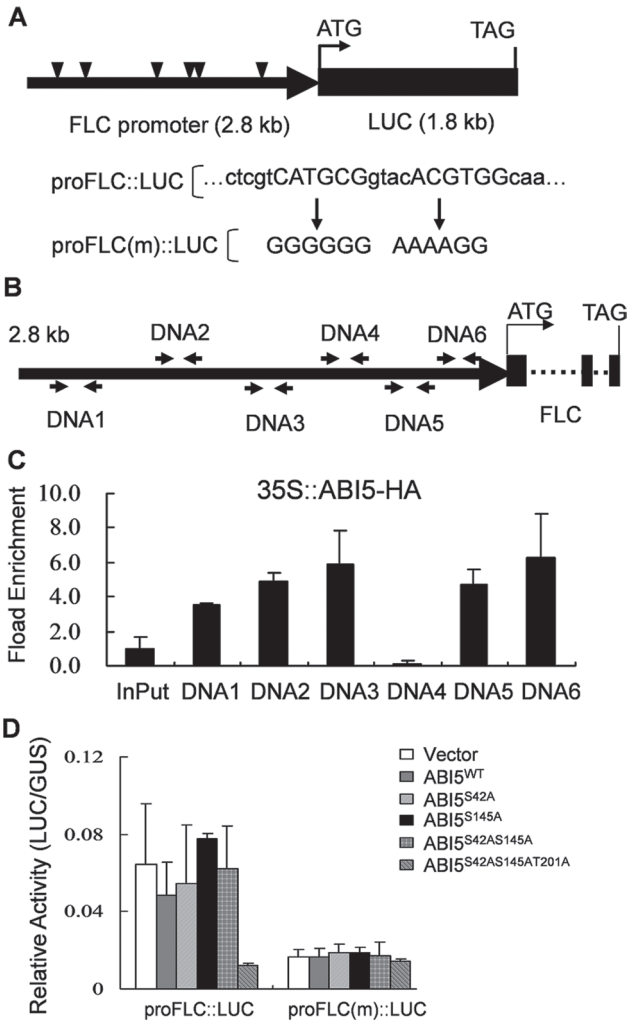

Fig. 6. ABI5 directly binds to DNA elements in the *FLC* promoter. (A) Schematic diagram showing the fusion of reporter constructs of *FLC::LUC*; triangles indicate the positions of putative ABRE motifs (PyACGTGG/TG) located in the 2.8-kb *FLC* promoter segment; arrows indicate mutated sequences of ABRE-like and G-box motifs. (B) Schematic diagram to show *FLC* promoter elements; DNA1–6 indicate the DNA fragments; pairs of arrows represent the DNA fragments amplified in the chromatin immunoprecipitation assay. (C) Chromatin immunoprecipitation enrichment to show the binding ability of ABI5-HA to the DNA fragments of the *FLC* promoter, as shown by qRT-PCR. InPut, total input chromatin DNA. Data are mean ± standard error of one experiment ($n > 3$). Similar results were obtained from four independent experiments. (D) Mutated ABRE-like elements in the *FLC* promoter could partially abolish *FLC* promoter activity in a transient expression assay. Data are mean ± standard errors of three replicated experiments ($n > 3$ for each experiment).

DNA6 (Fig. 6A). Next, the elements of the putative G-box and ABRE-like motifs were mutated to evaluate their functions in modulating *FLC* transcription in a transient expression assay (Fig. 6D). When the ABRE-like motif was mutated alone, there was no obvious effect on *FLC* promoter activity (Supplementary Fig S4A). However, when mutations were created in both motifs, the relative activity of LUC/GUS, representing the expression of proFLC(m)::LUC, was impaired (Fig. 6D). Furthermore, the hybrid lines *pFLC::GUS ABI5-5* and *pFLC(m)::GUS ABI5-5* were generated to confirm the role of the ABRE/G-box elements in ABI5-regulated *FLC* promoter activity. Under 100 µM ABA treatment for 3 hours, a significantly enhanced GUS signal was detectable in all examined tissues of *pFLC::GUS ABI5-5* plants, but no GUS signals were detected in tissues of *pFLC(m)::GUS ABI5-5* plants (Supplementary Fig. S4C). Together with chromatin immunoprecipitation analysis, the results suggest that the ABA inhibitory effect on floral transition is most likely mediated by a bZIP transcription factor, such as ABI5 and/or other ABFs promoting *FLC* expression.

Discussion

Negative effect of ABA on flowering time regulation in Arabidopsis

To control flowering time, plants have evolved a complex genetic network for responding to endogenous cues and environmental factors. The mechanism underlying ABA regulation on flowering time is poorly understood. This study showed that the bZIP transcription factors, including ABI5 and other ABFs (such as ABF1, ABF3, and ABF4) play negative roles in ABA-mediated inhibition of flowering time. Application of ABA may delay the flowering time in *Arabidopsis* (Fig. 3 and Supplementary Fig. S2; Jiang *et al.*, 2012). ABI5 and/or other ABFs could distinctly promote the expression of *FLC* (Figs. 1–6 and Supplementary Figs. S3 and S4). The stimulation of *FLC* expression was dependent upon ABA treatment, which was mediated by SnRK2s regulation on ABI5 or other ABFs activity (Figs. 2 and 5 and Supplementary Fig. S3). Coupled with the genetic analysis of the hybrid lines such as *FLC abi5-4* and *pFLC::GUS ABI5-5* (Fig. 1 and Supplementary Fig. S4), it is most likely that the inhibitory effect of ABA on flowering time is achieved by activation of ABI5 and/or other ABFs and successive transactivation of *FLC*.

ABI5 phosphorylation is essential for promoting FLC expression

Protein kinases involved in ABA signal transduction have been identified, of which the SnRK2s have been widely studied (Anderberg and Walker-Simmons, 1992; Boudsocq *et al.*, 2004). In *Arabidopsis*, there are 10 members in the SnRK2 family (SnRK2.1–SnRK2.10) (Hrabak *et al.*, 2003). The triple mutant *snrk2.2/2.3/2.6* plants show severe impairment in vegetative and reproductive growth, as well as in the control of transpiration water loss (Fujii and Zhu, 2009; Nakashima *et al.*, 2009). The present study shows that the heterozygous triple mutant *snrk2.2/3/6+* has an early flowering phenotype (Supplementary Fig. S3). The stimulation of *FLC* promoter activity by ABI5 and other ABFs was abolished in *snrk2.2/2.3/2.6* plants (Fig. 5 and Supplementary Fig. S3), implying the correlation between SnRKs and ABI5-regulated *FLC* promoter activity (Fig. 5).

On the contrary, coexpressing *SnRK2.6* and *ABI5* enhanced *FLC* promoter activity significantly (Fig. 5). Thus, the data not only pinpoint the role of SnRK2s in activating ABI5 but also indicate the importance of the phosphorylation state of ABI5 for transactivating *FLC* promoter activity. Transgenic plants carrying *ABI5* mutations at phosphorylation sites recovered the early flowering phenotype (Fig. 3 and Supplementary Fig. S2), which clearly demonstrate the importance of ABI5 phosphorylation status in the control of flowering time. By analysing the expression of *FLC in planta*, this study found that *FLC* expression was upregulated by ABA in *ABI5-5* plants and, in contrast, downregulated in *abi5-7* and $ABI5^{S42AS145AT201A}$ plants (Figs. 1 and 4). The role of individual consensus phosphoamino acids (such as S41, S42, S145, T201) in ABI5 activity has been evaluated *in planta* and it was suggested that none are essential for ABA-induced ABI5 function, based on the assessment of seed germination (Lopez-Molina *et al.*, 2002). The present study found that each phosphoamino acid, S41, S42, S145, and T201, was actually critical for ABI5 to promote *FLC* expression (Figs. 3 and 5 and Supplementary Fig. S2). The possible explanation is that each individual consensus phosphoamino acid shares a redundant function in maintaining ABI5 activity during seed germination, because the prominent effect was obtained with the triple mutation $ABI5^{S42AS145AT201A}$ (Figs. 3 and 5). It is speculated that the phosphorylation of ABI5 (including other ABFs) might be a limiting but essential step for facilitating ABI5 to interact with *FLC* promoter elements, which, in turn, triggers *FLC* transcription. Future experimental evidence is needed to clarify the differential roles of these consensus phosphoamino acids of bZIP transcription factors on the regulation of flowering time.

FLC transcription is directly activated by bZIP transcription factors

Previous studies have provided experimental evidence to support the notion that bZIP transcription factors, including ABI5 and ABFs, are involved in various developmental processes in plants (Lopez-Molina and Chua, 2000; Kang *et al.*, 2002). The roles of bZIP transcription factors in the transition from vegetative growth to reproductive development are elusive. As for the control of flowering time, specific histone modifications at the *FLC* locus associated with the chromatin structure of the gene segment are indispensable for maintaining a regular expression level of *FLC* under various conditions (Dennis and Peacock, 2007). Many genes involved in the modification of *FLC* chromatin (i.e. methylation and acetylation) have been identified and they usually form a protein complex to collaboratively regulate *FLC* expression (Kim and Michaels, 2006; Kim *et al.*, 2006, Deal *et al.*, 2007). A feedback regulatory loop involving *SOC1* (one of the targets of *FLC*) and many other transcription regulators (including bZIP transcription factors) has been suggested (Tao *et al.*, 2012). This study found an interesting connection between floral transition and ABA signal transduction, which ABI5 and other ABFs can promote *FLC* transactivation *in vivo* (Figs. 2 and 5 and Supplementary Fig. S3). The correlation between ABI5 activity and *FLC* expression was verified by analysing the expression patterns of pFLC::GUS and pFLC(m)::GUS in *ABI5-5* transgenic plants, providing genetic evidence of the requirement of ABRE/G-box elements for ABI5 to activate *FLC* expression (Fig. 4). The data tally the expression patterns of *ABI5* and *FLC* in various tissues of *Arabidopsis* (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), suggesting that *ABI5* and *FLC* may be commonly activated during seed germination and seedling growth.

Each bZIP transcription factor (ABF) possesses its preferential expression property in various tissues during *Arabidopsis* development and while responding to diverse environmental signals (Choi *et al.*, 2000; Yamaguchi-Shinozaki and Shinozaki, 2006). The redundant or synergistic function of ABFs in regulating *FLC* expression may be remarkable during the plant life cycle. It is most likely that ABI5 (and/or other ABFs) is involved in the floral transition of *Arabidopsis* plants while responding to environmental cues. The fact that ABA concentrations more than 50 µM ABA did not increase the effect suggests that regulation of *FLC* expression by ABI5 (and/or other ABFs) is limited under stress conditions. In these analyses, ABI5 could directly bind to the putative ABRE/G-box elements in promoter segments of *FLC in vivo* (Fig. 6). An enriched binding efficiency of ABI5 to the proximal DNA6 element embedded in the *FLC* promoter was identified (Fig. 6), implicating the diversity of *FLC* promoter properties. The ABRE-element and G-box are common coupling partners and are functional as *cis*-elements in ABA signalling.

Conclusion

Overall, the existence of a previously unidentified mechanism underlying the transactivation of *FLC* expression by ABI5 (and/or other ABFs) is suggested in this study (Fig. 7). While plants are exposed to environmental stresses, the increased

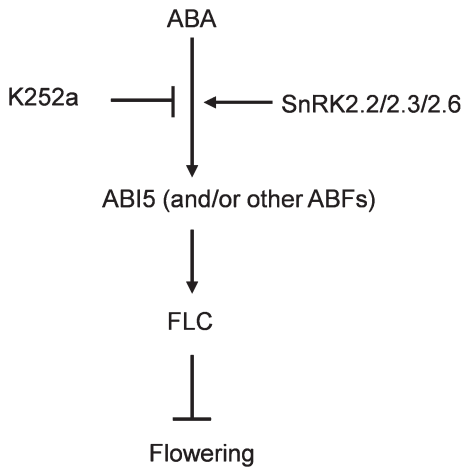

Fig. 7. A working model to suggest the inhibitory effect of abscisic acid (ABA) on flowering time in *Arabidopsis*. Activation of ABI5 (or other ABFs) by ABA stimulation requires SnRK2.2/2.3/2.6. The direct binding of ABI5 and/or ABFs to the promoter elements of *FLC* is a critical step for promoting *FLC* transcription. Thereafter, flowering time may be postponed.

ABA content may stimulate the regulatory flexibility on floral transition through activating bZIP transcription factors whose activities can be modulated by SnRK2s. As such, activated ABI5 (and/or other ABFs) may trigger the expression of *FLC* in time; then the immediate adjustment in plants for responding to ambient changes may be initiated.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Comparisons of *ABFs*, *ABI5*, and *FLC* expression patterns in *Arabidopsis*.

Supplementary Fig. S2. Comparisons of flowering phenotypes, total rosette leaf numbers, and seed germination phenotypes in wild-type and transgenic lines.

Supplementary Fig. S3. SnRK2s are involved in regulation of *FLC* expression.

Supplementary Fig. S4. ABRE-like and G-box motifs in the *FLC* promoter are essential for *FLC* expression in responding to ABA.

Supplementary Table S1. Primer sequences for identification of *snrk2.2/2.3/2.6* homozygous plants.

Supplementary Table S2. Primer sequences for plasmid construction.

Supplementary Table S3. Plasmids used in this study.

Supplementary Table S4. Primer sequences for analysis of enrichment of DNA fragments in chromatin immunoprecipitation assay.

Supplementary Table S5. Primer sequences for qRT-PCR experiments.

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