ABSCISIC ACID-INSENSITIVE 4 negatively regulates flowering through directly promoting Arabidopsis FLOWERING LOCUS C transcription

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Abstract

During the life cycle of a plant, one of the major biological processes is the transition from the vegetative to the reproductive stage. In Arabidopsis, flowering time is precisely controlled by extensive environmental and internal cues. Gibberellins (GAs) promote flowering, while abscisic acid (ABA) is considered as a flowering suppressor. However, the detailed mechanism through which ABA inhibits the floral transition is poorly understood. Here, we report that ABSCISIC ACID-INSENSITIVE 4 (ABI4), a key component in the ABA signalling pathway, negatively regulates floral transition by directly promoting FLOWERING LOCUS C (FLC) transcription. The abi4 mutant showed the early flowering phenotype whereas ABI4-overexpressing (OE-ABI4) plants had delayed floral transition. Consistently, quantitative reverse transcription–PCR (qRT–PCR) assay revealed that the FLC transcription level was down-regulated in abi4, but up-regulated in OE-ABI4. The change in FT level was consistent with the pattern of FLC expression. Chromatin immunoprecipitation-qPCR (ChIP-qPCR), electrophoretic mobility shift assay (EMSA), and tobacco transient expression analysis showed that ABI4 promotes FLC expression by directly binding to its promoter. Genetic analysis demonstrated that OE-ABI4::flc-3 could not alter the flc-3 phenotype. OE-FLC::abi4 showed a markedly delayed flowering phenotype, which mimicked OE-FLC::WT, and suggested that ABI4 acts upstream of FLC in the same genetic pathway. Taken together, these findings suggest that ABA inhibits the floral transition by activating FLC transcription through ABI4.

Key words: ABA, ABI4, chromatin immunoprecipitation, FLC, flowering, transcription factor

Introduction

As sessile organisms, plants monitor the changes in both external and internal signals, including photoperiod, temperature, and phytohormonal levels, to decide their flowering initiation (Andres and Coupland, 2012; Song et al., 2013). This transition from vegetative to reproductive growth is one of the major developmental phases during the life cycle.
of a plant (Boss et al., 2004; He, 2012). Optimizing flowering time is crucial to reproductive success, and hence is of great agricultural value, particularly when one considers the issues posed by climate change (Craufurd and Wheeler, 2009; Michaels, 2009; Riboni et al., 2013). Subsequently, plants have evolved diverse cryptic mechanisms to regulate the timing of flowering precisely.

The distinctive regulatory mechanisms comprising the photoperiod-, autonomous-, vernalization-, and gibberellic acid (GA)-dependent pathways have already been fully elucidated in Arabidopsis (He, 2012). These four pathways form a regulatory network that enables plants to integrate endogenous developmental signals with responses to environmental inputs, including daylength, light quality, and temperature. In this complicated network, the MADS box-containing transcription factor gene FLOWERING LOCUS C (FLC) is a potent integrator, which negatively regulates flowering initiation (Michaels, 2009; Michaels and Amasino, 1999; Son et al., 2014; Berry and Dean, 2015). Consequently, overexpression of FLC results in a late-flowering phenotype (Hepworth et al., 2002). Generally, FLC expression is silenced by vernalization treatment and the autonomous pathway. This involves histone methylation and change of chromatin structure (Bastow et al., 2004; Michaels, 2009). As a transcription factor, FLC represses the expression of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), FLOWERING LOCUS D (FD), and FLOWERING LOCUS T (FT) through directly binding to the promoter regions of SOC1 and FD or the first intron of FT. Thus these three genes represent further key factors in regulating flowering time (Searle et al., 2006). In particular, FT encodes florigen, a flowering inducer (Corbesier et al., 2007). FLC is a key repressor in the initiation of flowering and links the diverse flowering time regulatory pathways (Searle et al., 2006; He, 2009, 2012; Angel et al., 2015). Although numerous studies have investigated the diverse components downstream of transcription factor FLC, including SOC1 and FD, the key regulators that act upstream of FLC remain elusive. A better understanding of these will improve our understanding of FLC-mediated plant floral transition.

Besides the effects of environmental cues on plant floral transition, internal phytohormones also play a key role in this process. An early study revealed that GA stimulates bolting in henbane (Hyoscyamus niger) (Lang, 1957). Numerous subsequent studies demonstrated that GA induces flowering through promoting transcription of the floral meristem identity gene LEAFY (LFY) (Blazquez et al., 1998; Achard et al., 2004), which is a key determinant in plant floral decision processes. Overexpression of LFY in transgenic plants rescues the dramatic delayed-flowering phenotype of the GA-deficient mutant gal-3 (Blazquez et al., 1998). The phytohormones abscisic acid (ABA) and GA are the primary endogenous factors which regulate diverse physiological processes antagonistically, including seed germination and plant growth (Seo et al., 2006; Yaish et al., 2010). With regards to floral transition, the antagonistic effect between ABA and GA has also been investigated. The ABA-deficient mutant aba2 shows the early-flowering phenotype (Domagalska et al., 2010), in contrast to the late-flowering phenotype of the GA-deficient mutant gal-3 (Blazquez et al., 1998). In addition, treatment with exogenous ABA delays plant flowering (Wang et al., 2013). Consequently, ABA is considered to be a repressor of plant floral initiation. However, the detailed molecular mechanisms of this are poorly understood.

A pioneering study demonstrated that ABA-delayed flowering may occur in a DELLA-dependent manner (Achard et al., 2006). However, the precise mechanism remains elusive. Recently, an elegant pathway has been described through which ABA affects floral transition negatively (Wang et al., 2013). In this, the transcription factor ABSCISIC ACID-INSENSITIVE MUTANT 5 (ABI5) activates FLC transcription by directly binding to its promoter. Thus, ABI5 is an important factor through which ABA inhibits the plant floral transition (Wang et al., 2013). ABI5 is the only regulator found to date that controls plant floral initiation in the ABA signalling transduction pathway. Whether there are others is not known. To date there is little information about the precise mechanisms which underlie ABA inhibition of flowering. Consequently, a better understanding of this will be valuable.

ABSCISIC ACID-INSENSITIVE 4 (ABI4) is an AP2/ERF domain-containing transcription factor and is an enhancer in the ABA signalling pathway that functions particularly during seed development, seed dormancy, and regulation of germination (Finkelstein, 1994; Finkelstein et al., 1998; Söderman et al., 2000; Shu et al., 2013). Numerous elegant studies have demonstrated that ABI4 is a versatile factor (Wind et al., 2013), which is also involved in many other aspects of plant development, including responses to glucose (Arenas-Huerto et al., 2000), lipid mobilization from the embryo (Penfield et al., 2006), chloroplast and mitochondrial–nucleus retrograde signalling pathways (Koussevitzky et al., 2007; Sun et al., 2011), and plant male sterility (Shu et al., 2014). ABI4 is also involved in the ABA- and jasmonate-dependent signalling cross-talk (Kerchev et al., 2011) and the ABA- and GA cross-talk pathways (Shu et al., 2013). Recently, two independent groups reported that the abi4 mutant has an early-flowering phenotype, and the two different allele mutants of the ABI4 locus result in the same phenotype, strongly suggesting that ABI4 has an important role in floral transition (Foyer et al., 2012; Matsoukas et al., 2013). However, the detailed mechanism through which ABI4 regulates flowering initiation remains elusive.

Here, we perform further investigation of the roles of ABI4 in the initiation of flowering. Consistent with previous reports (Foyer et al., 2012; Matsoukas et al., 2013), the abi4 mutant had the early-flowering phenotype, whereas transgenic overexpression of ABI4 (OE-ABI4) Arabidopsis delays the floral transition. Further, we found that the FLC expression level was down-regulated in abi4, but up-regulated in OE-ABI4. Chromatin immunoprecipitation qPCR (ChiP-qPCR), electrophoretic mobility shift assay (EMSA), and tobacco transient expression analysis showed that ABI4 activates FLC expression by directly binding to its promoter. Consistent with these results, genetic analysis demonstrated that OE-ABI4::flc-3 did not alter the flc-3 phenotype, while OE-FLC::abi4 changed the abi4 early-flowering phenotype.
This indicates that ABI4 acts upstream of FLC in the same genetic pathway. Taken together, the results of this study suggest that ABI4 is a key factor which negatively regulates flowering through activating FLC transcription directly.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 was used as the wild type (WT) in this study. The point mutant abi4-1 (CS8104) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA). This mutant originated from the Finkelstein laboratory (Finkelstein, 1994; Finkelstein et al., 1998). The plasmid pro35S::FLC-GFP was transformed into the abi4 mutant to generate OE-FLC::abi4 and, at the same time, OE-ABI4 was introduced into the flc-3 mutant background by genetic crossing for the generation of OE-ABI4::flc-3. The abi4 point mutant and the functional OE-ABI4 lines (OE1 and OE2) had been generated in our previous study (Shu et al., 2013). Using 10% bleach, Arabidopsis seeds were surface-sterilized and washed four times with sterile water. The sterile seeds were then suspended in 0.2% agarose and sown on half-strength Murashige and Skoog (1/2 MS) medium. The plates were transferred to tissue culture rooms at 22 °C under long-day (16 h light/8 h dark) (LD) or short-day (8 h light/16 h dark) (SD) photoperiod conditions, depending on the needs of the particular experiments. Ten-day-old seedlings were transplanted into soil and placed in a growth chamber, again under LD or SD conditions, at 22 °C and 70% relative humidity.

Generation of transgenic plants

Transgenic plants carrying constitutively expressed ABI4 which had been generated in a previously reported study (Shu et al., 2013) were also used in this study. In order to produce OE-FLC::abi4 transgenic plants, the FLC coding sequence fragment was amplified by PCR and then cloned into the vector pCanG-HA-GFP, in which FLC was expressed under the control of the Cauliflower mosaic virus (CaMV) 35S promoter. This construct was transformed into the abi4 mutant background by the vacuum infiltration method using the Agrobacterium tumefaciens strain EHA105 (Bechtold and Pelletier, 1998). T2 seeds were germinated on normal 1/2 MS plates containing 50 mg ml\(^{-1}\) kanamycin for vector pCanG-HA-GFP, and then the resistant seedlings were transferred to soil to obtain homozygous T3 seeds. The T3 homozygous lines were employed for detailed pheno-type analysis.

Flowering-time experiment

Plants in a growth chamber (LD or SD conditions) were examined. In this study, flowering time was scored as the days from germination to flowering and the number of total rosette leaves at bolting, according to the protocol of Mai et al. (2011). The plants were checked for flower buds every day. Approximately 15–20 plants were examined for each genotype.

Gene expression analysis

Preparation of total RNA from the 2-week-old seedlings, first-strand cDNA synthesis, and quantitative reverse transcription–PCR (qRT–PCR) were performed as previously described (Cui et al., 2012; Shu et al., 2013). The mRNA was subjected to DNase I treatment, and the double-stranded DNA was obtained through the digestion with BamHI and was ligated into the pMalC2 backbone vector which contains a maltose-binding protein (MBP) tag. Then the ABI4 fusion proteins were expressed in the E. coli BL21 (DE3) cells, and induced by IPTG. The MBP-ABI4 fusion was purified by a resin containing amylose, and the MBP tag was removed and the corresponding probes were carried out according to the protocol provided with the DIG DNA Labeling and Detection Module (Roche). The primer sequence for constructing the MBP–ABI4 fusion

Chromatin immunoprecipitation (ChIP)-qPCR assay

ChIP-qPCR assays were performed as previously described (Shu et al., 2013). Transgenic seedlings containing 35S-ABI4-GFP were harvested (1.5 g) on 1/2 MS medium and then cross-linked with 1% formaldehyde. The seedlings were ground in liquid nitrogen, and then the nuclei were isolated. Immunoprecipitation assays were performed with the anti-green fluorescent protein (GFP) antibody and protein G beads. Immunoprecipitation in the absence of anti-GFP served as the control (CK). The DNA was precipitated by isopropanol, and dissolved in water containing 20 μg ml\(^{-1}\) Nase. The qPCR analysis was performed using specific primers corresponding to the FLC promoter. TUB4 was used as an internal control. The ABI5 promoter fragment was used as a positive control since a previous study demonstrated that ABI5 could directly bind to the promoter of ABI5 (Bossi et al., 2009). The primers used for the ChIP-qPCR assays are shown in Supplementary Table S1 at JXB online.

Analysis of FLC promoter activity by ABI4 in vivo

The transient expression assay was performed in Nicotiana benthamiana leaves as previously described (Yang et al., 2011). The native FLC promoter (Pro-FLC) was amplified from genomic DNA. This promoter fragment was cloned into the pCAMBIA3000-221 vector by replacing the original CaMV 35S promoter, and then pCAMBIA3000-221-ProFLC-GUS was generated. The pCAM-G-ABI4-GFP was the effector construct. The A. tumefaciens-mediated tobacco transient transformation was performed according to our previous protocol (Liu et al., 2010). Agrobacterium cells were cultured at 28 °C overnight, and then collected and re-suspended with infiltration buffer and infiltrated into tobacco leaves. Based on this, the mutated FLC promoters (Supplementary Fig. S4A at JXB online) were also generated, in which some key CCAC motif sequences were changed.

β-Glucuronidase (GUS) activity was detected 3 d after infiltration. Leaves were sampled using a hole punch. The total protein was quantified using the Bradford protein assay kit method (Bio-Rad Company, USA). GUS activity for each combination was determined using the protocol described previously with 4-methylumbelliferyl-β-d-glucuronide (Sigma-Aldrich Company, USA) as a substrate (Jefferson et al., 1987). Histochemical staining for GUS was performed according to the method of Stulberg et al. (1993). The plant leaves harvested by hole punch were immersed in GUS staining buffer at 37 °C for 16 h, and then immersed in 95% (v/v) ethanol to remove the chlorophyll. A Leica MZ16 FA stereomicroscope was used for photography (Leica Company, Germany).

Electrophoretic mobility shift assay (EMSA)

EMSA were performed by the Chemiluminescent Nucleic Acid Detection Module (Thermo Company, Product No. 89880), according to a previously published protocol (Wei et al., 2015). The coding sequence of ABI4 was inserted into the BamHI/XbaI sites in the pMalC2 backbone vector which contains a maltose-binding protein (MBP) tag. Then the ABI4 fusion proteins were expressed in E. coli expression vector pET28a (E. coli BL21 (DE3) cells) and their expression was confirmed using Western blotting. The corresponding proteins were purified by affinity chromatography with amylose. The purified MBP–ABI4 fusion proteins were incubated with the 35S-FLC-GUS promoter fragment in a binding buffer at 37 °C for 1 h, then immunoprecipitated by the anti-GFP antibody, and then analyzed by EMSA. Electrophoretic mobility shift assay (EMSA) was performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Company, Product No. 89880), and the EMSA was performed as previously described (Shu et al., 2013). Transgenic seedlings containing 35S-ABI4-GFP were harvested (1.5 g) on 1/2 MS medium and then cross-linked with 1% formaldehyde. The seedlings were ground in liquid nitrogen, and then the nuclei were isolated. Immunoprecipitation assays were performed with the anti-green fluorescent protein (GFP) antibody and protein G beads. Immunoprecipitation in the absence of anti-GFP served as the control (CK). The DNA was precipitated by isopropanol, and dissolved in water containing 20 μg ml\(^{-1}\) Nase. The qPCR analysis was performed using specific primers corresponding to the FLC promoter. TUB4 was used as an internal control. The ABI5 promoter fragment was used as a positive control since a previous study demonstrated that ABI5 could directly bind to the promoter of ABI5 (Bossi et al., 2009). The primers used for the ChIP-qPCR assays are shown in Supplementary Table S1 at JXB online.

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vector and the probe for the EMSA are given in Supplementary Table S1 at JXB online.

Results

The early flowering phenotype of the abi4 mutant

In order to assess the effect of ABI4 on floral initiation control, the flowering time phenotype of abi4 mutant and transgenic OE-ABI4 plants was analysed in both LD and SD photoperiods illuminated by white light. abi4 mutant plants flowered earlier than the WT in both LD (Fig. 1A–C) and SD conditions (Fig. 1D–F), as indicated by the days to flowering (Fig. 1B, E) and the number of rosette leaves (Fig. 1C, F). In contrast, OE-ABI4 transgenic plants had a late-flowering phenotype when compared with the WT under both LD (Fig. 1B, C; Supplementary Fig. S1 at JXB online) and SD (Fig. 1D, E) growth conditions in terms of the days to flowering. However, with regards to the number of rosette leaves, the OE-ABI4 lines did not show a pronounced late-flowering phenotype (Fig. 1C, F). Altogether, the early-flowering phenotype of the abi4 mutant is consistent with other reports (Foyer et al., 2012; Matsoukas et al., 2013).

Flowering time regulation-related gene expression analysis

To explore further the molecular mechanisms through which ABI4 controls floral transition, the transcription levels of some flowering time regulation-related genes were investigated in abi4 mutant and OE-ABI4 plants. It has been demonstrated that transcription factor FLC is the key repressor in floral transition and links the diverse flowering time regulation pathways (He, 2012). In order to explore whether the flowering phenotypes observed for the abi4 mutant and OE-ABI4 are correlated with the change of the expression of FLC, we examined the FLC transcript levels in these genotypes by qRT–PCR. The level of FLC transcript in the abi4 mutant plants was significantly lower, while in OE-ABI4 plants it was significantly higher than that in the WT plants (Fig. 2A).

A previous study revealed that FLC directly represses the flowering identity gene FT (Searle et al., 2006); thus, the FT expression level was also determined. Consistent with the trends in FLC transcription levels, the expression of FT also showed significant changes (Fig. 2B). In the abi4 mutant, FLC expression was decreased compared with the WT (Fig. 2A), and, accordingly, FT transcription was significantly increased. There was no obvious change in its level in OE-ABI4 (Fig. 2B). Furthermore, GA promotes flowering through inducing transcription of another floral identity gene, LFY (Blazquez et al., 1998; Aachard et al., 2004). In a previous study, we found that ABI4 negatively regulates GA biogenesis (Shu et al., 2013) and thus the LFY mRNA level in abi4 and OE-ABI4 was examined. Our result revealed that the LFY expression level in the abi4 mutant was significantly increased compared with the WT (Fig. 2C). Together, the changes in the transcription levels of these three key genes were consistent with the phenotype analysis. They suggest that the flowering phenotype of both genotypes (abi4 and OE-ABI4) may result from changes in the transcription levels of FLC, FT, and LFY.
We next investigated whether ABI4 directly binds to the promoters of FLC, FT, and/or LFY in vivo (ChIP-qPCR) and in vitro (EMSA).

We initially explored the promoters of these three genes, as shown in Fig. 3A. Seven CCAC elements were detected in the FLC promoter fragment. We then performed ChIP-qPCR assays with the OE-ABI4 lines to test whether ABI4 directly binds to this promoter in vivo. The results revealed that the DNA fragments of the FLC promoter were enriched in the chromatin-immunoprecipitated DNA using the anti-GFP antibody, particularly the P4 and P5 regions, which are far from the FLC start codon (Fig. 3B). In addition, because a previous study demonstrated that ABI4 binds to the ABI5 promoter directly (Bossi et al., 2009), a DNA fragment of the ABI5 promoter was used as a positive control. The sequences of the ABI5 promoter were dissected, and the fragment used is highlighted in Supplementary Fig. S3 at JXB online and the primer sequences are shown in Supplementary Table S1. During this analysis, two independent transgenic lines (OE1 and OE2) were employed, and similar results were obtained. However, we did not detect the enrichment of the FT and LFY promoter fragments, although there were four and five CCAC cis-elements in their promoters, respectively.

Based on the ChIP-qPCR results, an EMSA was employed to confirm further the interaction between the transcription factor ABI4 and the FLC promoter in vitro. The recombinant ABI4 fusion protein was expressed and purified from E. coli, and the P4 fragment (Fig. 3A) was chosen as a probe for this assay. The results revealed that the mobility rate of the P4 fragment was significantly delayed in the presence of ABI4 protein (Fig. 3C, lane 2). Further, the cold-competitor probes (excess of unlabelled fragments) were sufficient to compete for the ABI4 binding activity (Fig. 3C, lanes 3–4). Taken together, these EMSA results demonstrated that ABI4 indeed directly binds to the FLC promoter fragments, which is consistent with the ChIP-qPCR evidence.

**ABI4 directly binds to the FLC promoter in vivo and in vitro**

Previous studies have demonstrated that ABI4 binds to the CCAC motifs in some promoters to regulate the transcription of its targets genes directly (Koussevitzky et al., 2007; Bossi et al., 2009). We next investigated whether ABI4 directly binds to the promoters of FLC, FT, and/or LFY in vivo (ChIP-qPCR) and in vitro (EMSA).

![Fig. 2. Expression analysis of the flowering-time-related genes FLC, FT, and LFY in abi4, WT, and OE-ABI4 plants. Gene expression was detected by qRT-PCR, and three replications were performed. Primers used in the qRT-PCR assay are listed in Supplementary Table S1 at JXB online. (A) The FLC transcription level in abi4 is decreased but is increased in OE-ABI4 plants. (B) The FT expression level in abi4 is increased. (C) The LFY transcription level in abi4 is increased but is decreased in OE-ABI4 plants.](https://academic.oup.com/jxb/article-abstract/67/1/195/2885147)

**ABI4 activates FLC transcription in vivo**

Combined with the qRT-PCR data (Fig. 2A), the ChIP-qPCR (Fig. 3B) and EMSA (Fig. 3C) results indicate that ABI4 may activate FLC transcription by directly binding to its promoter. To explore the effect of ABI4 on FLC expression directly, we made use of the transient expression system to investigate whether ABI4 activates the expression of FLC in vivo.

The reporter plasmid Promoter-FLC-GUS and the effector plasmid pCanG-ABI4-GFP were constructed separately. Normal levels of GUS activity was detected when the Promoter-FLC-GUS construct combined with pCanG-HA-GFP (Fig. 3D, E). Subsequently, when the control vector pCanG-HA-GFP was substituted for an equal amount of the effector pCanG-ABI4-GFP, GUS activity increased significantly (Fig. 3D, E). These results suggest that ABI4 has an activation effect on FLC transcription in vivo. To study further the effect of the CCAC motifs on this activation effect, we mutated the key CCAC elements (altered to CCAA) in
the FLC promoter. The P4 and P5 fragments were chosen as they showed the highest binding activity for ABI4 protein, revealed by ChIP-qPCR and EMSA (Fig. 3). Subsequently, the constructs Pro-FLC (m1)-GUS and Pro-FLC (m2)-GUS were generated (Supplementary Fig. S4A at JXB online). Using the transient expression system, the results revealed that the activation effect of ABI4 on FLC transcription was impaired in the presence of the mutated promoter constructs (Supplementary Fig. S4B–E). Altogether, combined with the ChIP-qPCR and EMSA results, the transient expression system analysis demonstrated that ABI4 directly promotes FLC expression, and this effect is dependent on some key CCAC elements in the FLC promoter.

**ABI4 acts upstream of FLC genetically to regulate flowering time**

The phenotypic analysis, and biochemical and molecular evidence demonstrated that the transcription factor ABI4 negatively regulates flowering time through activation of FLC expression. To confirm this conclusion further, the genetic relationship between ABI4 and FLC was explored.
The *OE-ABI4* construct was introduced into the *flc-3* mutant background by genetic crossing, and the flowering phenotypes of *flc-3* and *OE-ABI4::flc-3* were examined. In our experimental conditions, we detect the early-flowering phenotype of *flc-3* mutant plants, and overexpression of *ABI4* did not change the early-flowering phenotype of *flc-3* in terms of the number of rosette leaves and the days to flowering (Fig. 4A–C), although *OE-ABI4* in the WT background significantly delayed the floral transition (Fig. 1). On the other hand, we also generated transgenic overexpression of *FLC* in the *abi4* mutant background (*OE-FLC::abi4*) by transgenic assay, and examined the floral phenotype of *abi4* and *OE-FLC::abi4*. The results showed that overexpression of *FLC* significantly changed the early-flowering phenotype of *abi4* (Fig. 4D–F), mimicking the clearly late-flowering phenotype of *OE-FLC::WT*. Together, the genetic analysis indicated that *ABI4* acts upstream of *FLC* in the same genetic pathway.

**Discussion**

The activation effect of the phytohormone GA on plant floral transition has been thoroughly investigated in the past decades. In contrast, the mechanisms by which ABA affects plant flowering time are not fully understood. Our study, using phenotypic, biochemical, and genetic analysis, has demonstrated that the transcription factor ABI4, a positive regulator of the ABA signalling pathway, negatively regulates flowering through activating *FLC* expression. However, we believe that this is the tip of an iceberg, and further details of the regulatory mechanisms of ABA on plant floral transition will be discovered in the near future.

**ABI4 negatively regulates flowering time**

During the past few decades, four distinct pathways that affect plant flowering time in response to internal signals and external factors were comprehensively described in the model plant *Arabidopsis* (He, 2012, 2009). In the agronomic research field, the flowering time (heading date) is a critical factor in determining adaptation to different cultivation areas and cropping seasons (Yamamoto et al., 2000). As a consequence, flowering time regulation mechanisms have attracted increasing attention.

A number of studies have demonstrated that some mutants involved in the ABA signalling pathway reveal the flowering phenotype, including *abi5* (Wang et al., 2013), *CmMYB2-RNAi* plants (Shan et al., 2012), and *abi4* (Foyer et al., 2012; Matsoukas et al., 2013). Generally, the ABA-insensitive mutants have the early-flowering phenotype, and the ABA-sensitive mutants have the late-flowering phenotype. Additionally, ABA biogenesis pathway mutants such as *aba2* also reveal the flowering phenotype as a consequence of alteration of endogenous ABA biogenesis (Domagalska et al., 2010). This is in contrast to the GA-deficient mutant *gal-3* which has a drastically late-flowering phenotype as a consequence of a large reduction in the internal GA level (Blazquez et al., 1998). Although the molecular mechanisms by which GA promotes flowering are well described, the mechanism by which ABA regulates flowering has been unclear, especially for the effect of *ABI4* on floral regulation.

![Fig. 4](https://academic.oup.com/jxb/article-abstract/67/1/195/2885147)
Prior to our study, two independent groups had reported that mutations at the \textit{ABI4} locus promote plant flowering (Foyer \textit{et al.}, 2012; Matsoukas \textit{et al.}, 2013). The mutant alleles in these studies are different. In the study by Foyer \textit{et al.} (2012), the \textit{abi}4 mutant originated from sugar-insensitive (\textit{sis}) mutant screening, and is a point mutation generated by ethylmethane sulphonate (EMS)-induced mutagenesis. It has also been called sugar-insensitive 5 (\textit{sis}5) (Laby \textit{et al.}, 2000; Kerchev \textit{et al.}, 2011; Foyer \textit{et al.}, 2012). In the study reported by Matsoukas \textit{et al.} (2013), the \textit{abi}4 mutant resulted from a T-DNA insertion line occurring at the \textit{ABI4} locus. Additionally it has been called glucose insensitive 6 (\textit{gin}6) (Arenas-Huerto \textit{et al.}, 2000; Matsoukas \textit{et al.}, 2013). The \textit{abi}4 mutant used in our study arose from a point mutation at the \textit{ABI4} locus and originated from Finkelstein’s group (Finkelstein, 1994; Finkelstein \textit{et al.}, 1998). It is different from the allelic mutants, \textit{sis}5 and \textit{gin}6. The fact that all three allelic mutants show a similar phenotype strongly suggests that mutations in the \textit{ABI4} locus indeed are responsible for the altered flowering phenotypes of \textit{abi}4 and \textit{OE-ABI4} plants (Fig. 1).

As described above, \textit{ABI4} was also identified from the screening of sugar- or glucose-insensitive mutants, and its alternative names are \textit{SIS5} and \textit{GIN6}. Actually, sugar indeed affects flowering time: it promotes floral transition, but high concentrations of sugar remarkably delay flowering (Zhou \textit{et al.}, 1998; Ohto \textit{et al.}, 2001). The detailed mechanisms underlying this delayed effect resulted from the delayed activation of \textit{LFY} transcription (Ohto \textit{et al.}, 2001). However, the more precise mechanisms through which sugar content and/or signalling regulate plant floral transition need further investigation. Furthermore, it is noted that the transgenic \textit{OE-ABI4} had the late flowering phenotype in terms of days to flowering, but not in terms of the number of rosette leaves (Fig. 1). This apparent inconsistency has been reported previously (Mai \textit{et al.}, 2011). It may be a consequence of the very weak growth of \textit{OE-ABI4} plants (Shu \textit{et al.}, 2013). Actually, a previous study has also demonstrated that \textit{OE-ABI4} seedlings are weak in terms of root length and shoot growth (Shkolnik-Inbar and Bar-Zvi, 2010), which is consistent with our investigation.

\textit{ABI4} is a novel factor in the ABA signalling pathway which inhibits floral transition

The promotion effect of GA on plant floral transition has been well documented (Andres and Coupland, 2012; He, 2009, 2012). In contrast, the mechanism by which ABA affects flowering has been elusive (Wang \textit{et al.}, 2013). A recent study demonstrated that application of exogenous ABA delays flowering time, and the bZIP transcription factor genes \textit{ABI5}, \textit{ABF1}, \textit{ABF3}, and \textit{ABF4} play negative roles in ABA-mediated inhibition of floral transition (Wang \textit{et al.}, 2013). These genes are key components in the ABA signalling transduction pathway, indicating that they have important functions through which ABA affects floral transition.

\textit{ABI4} is another versatile factor which promotes ABA signalling, to regulate diverse physiological processes including seed dormancy, seed germination, lateral root initiation, and cross-talk between many hormones including ABA and GA, and ABA and auxin (Finkelstein \textit{et al.}, 1998; Shkolnik-Inbar and Bar-Zvi, 2010; Foyer \textit{et al.}, 2012; Shu \textit{et al.}, 2013). In addition to these, our study suggests other possible roles for \textit{ABI4} in the regulation of plant floral transition. As well as the phenotypic description, and genetic and biochemical analysis, we described the mechanism by which \textit{ABI4} directly activates transcription of the key floral repressor \textit{FLC}, and negatively regulates floral transition. Our findings suggest that just like \textit{bZIP} transcription factors, \textit{ABI4} is a novel regulator involved in the ABA signalling pathway and inhibits plant flowering.

The negative effect of ABA on floral transition has been investigated prior to our studies (Domagalska \textit{et al.}, 2010; Wang \textit{et al.}, 2013). Further, the inducing effect of ABA on \textit{ABI4} transcription was detected in the present study (Supplementary Fig. S2 at JXB online) and in previous studies (Söderman \textit{et al.}, 2000; Bossi \textit{et al.}, 2009). Combined with the present available evidence, we suggest that ABA inhibits the floral transition by activating \textit{FLC} transcription through \textit{ABI4}, at least partially. In addition, it was recently reported that ABA is required for the drought-escape response through positively regulating plant flowering (Riboni \textit{et al.}, 2013). This is logical, as under normal growth conditions the endogenous ABA level will negatively regulates the floral transition, in contrast to the effect of GA. However, the environmental stress of drought elevates ABA levels, promoting flowering, and allowing the plant to complete its life cycle.

\textit{FLC} is the target of both \textit{ABI4} and \textit{ABI5}

Extensive studies demonstrated that \textit{FLC} is the key integrator which links the four flowering regulation pathways and inhibits plant floral transition (Koornneef \textit{et al.}, 1998; Komeda, 2004; Andres and Coupland, 2012; Song \textit{et al.}, 2013). Therefore, the mechanisms regulating \textit{FLC} at the transcription level are of the utmost importance for controlling floral transition. A previous study demonstrated that the pattern of \textit{FLC} expression is associated with epigenetic modification and changes in chromatin structure (Dennis and Peacock, 2007). Many factors are involved in this, including acetylation and methylation, and these modifications usually result in a protein complex to regulate \textit{FLC} transcription collaboratively (Kim and Michaels, 2006; Deal \textit{et al.}, 2007).

The \textit{SUPPRESSOR OF FRIGIDA 4} (\textit{SUF4})-mediated transcription factor complex negatively regulates flowering through promoting \textit{FLC} expression directly; thus, the \textit{suf}4 mutant has the early-flowering phenotype (Choi \textit{et al.}, 2011). We found that \textit{FLC} expression is also directly regulated by the transcription factor \textit{ABI4} (Fig. 3). Another transcription factor, \textit{ABI5}, involved in the ABA signalling pathway has also been reported to regulate \textit{FLC} transcription directly (Wang \textit{et al.}, 2013). Together, the evidence suggests that a number of transcription factors control \textit{FLC} expression. \textit{FLC} appears to be the target of both \textit{ABI4} and \textit{ABI5} concurrently. In line
with this, Reeves et al. (2011) demonstrated that ABI4 and ABI5 share some target genes. Furthermore, a recent study demonstrated that Diacylglycerol acyltransferase1 (DGAT1), encoding the rate-limiting enzyme in the triacylglycerol biosynthesis pathway (Kong et al., 2013), is also regulated by ABI4 and ABI5 concurrently. It seems, therefore, that ABI5 may be an accessory factor with ABI4 in the regulation of a number of genes.

FLC is the target of both the transcription factors ABI4 and ABI5; thus an interesting question arises. How do the plant discriminate between the roles of ABI4 and ABI5 in flowering time regulation? Combined with a previous study, we know that both transcription factors bind to the FLC promoter through different motifs. ABI4 binds the CCAC motif (Fig. 3), while ABI5 binds the FLC promoter through ABRE (abscisic acid-responsive element) or ABRE-like elements (CATGCG) (Wang et al., 2013). Thus we speculated that the flanking sequences of the CCAC and/or CATGCG motifs may possess some cryptic and elusive effects affecting the interaction between the FLC promoter and ABI5/ABI4. Consequently, further bioinformatics analysis is needed to deepen our understanding of the overlap and/or distinct roles of ABI5 and ABI4 in the control of flowering.

Taking our findings together, we propose a working model illustrated in Fig. 5. In the ABA signalling pathway, the transcription factors ABI4 and/or ABI5 regulate FLC expression directly to control plant floral transition precisely. Because ABI4 also binds directly to the ABI5 promoter and activates its transcription (Bossi et al., 2009), ABI4 may also activate FLC transcription through increasing ABI5 expression. Interestingly, it is noted that the model allows for the possibility that other mutants involved in the ABA signalling pathway, such as abi3, may regulate the flowering time, which should be the focus of future investigations.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Late-flowering phenotype of *OE-ABI4* under long-day conditions.

**Figure S2.** ABA induces *ABI4* transcription.

**Figure S3.** The *ABI5* promoter was dissected.

**Figure S4.** ABI4 promotes *FLC* transcription in a CCAC-dependent manner.

**Table S1.** The primers used in this study.

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