Plant Physiology and Biochemistry 139 (2019) 207–214

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

ABSCISIC ACID-INSENSITIVE 3 is involved in brassinosteroid-mediated regulation of flowering in plants

Jeongeui Hong[a,1], Horim Lee[b,1], Jinsu Lee[a], Hyemin Kim[a], Hojin Ryu[a,*]

[a] Department of Biology, Chungbuk National University, Cheongju, 28644, Republic of Korea
[b] Department of Biotechnology, Duksung Women's University, Seoul, 01369, Republic of Korea

ARTICLE INFO

Keywords:
ABI3
BES1
Brassinosteroid
Flowering
RNA-seq

ABSTRACT

ABSCISIC ACID-INSENSITIVE 3 (*ABI3*) is one of the essential transcription factors of ABSCISIC ACID (ABA) signaling, functioning in seed germination, early seedling development, and abiotic stress tolerance. A recent study showed that epigenetic repression of *ABI3* by brassinosteroid (BR)-activated BRI1 EMS SUPPRESSOR1 (BES1)–TOPLESS (TPL)—HISTONE DEACETYLASE 19 (HDA19) repressor complex is a critical event for promoting seed germination and early seedling development. However, other physiological roles of the repression of *ABI3* and ABA responses by BES1-mediated BR signaling pathways remain elusive. Here, we show that BES1-mediated suppression of *ABI3* promotes floral transition and ABI3 acts as a negative regulator for flowering. Ectopic expression of *ABI3* specifically compromised the early flowering phenotype of *bes1-D* and induced severe late-flowering phenotypes in wild-type *Arabidopsis* and *Solanum lycopersicum* plants. Both spatiotemporal expression patterns and global transcriptome analysis of *ABI3*-overexpressing plants supported the biological roles of *ABI3* in the negative regulation of floral transition and reproduction. Finally, we confirmed that the loss of function of *ABI3* induced early-flowering phenotypes in both long- and short-day conditions. In conclusion, our data suggest that BES1-mediated regulation of *ABI3* is important in the reproductive phase transition of plants.

1. Introduction

Flowering marks the phase transition from vegetative to reproductive growth in the plant life cycle. This developmental process is important not only for the reproductive success of plants, but also for maximizing the yields of agriculturally valuable crops. Numerous genetic and physiological studies in *Arabidopsis thaliana* have revealed that optimized flowering occurs through a complicated genetic regulatory program in which diverse internal and external signaling cues such as day length (photoperiod), low temperature (vernalization), developmental age, and endogenous plant hormones are integrated (Bouché et al., 2017; Conti, 2017). Moreover, various floral induction pathways ultimately induce the expression of central flowering integrators, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Lee et al., 2000), which are repressed by *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*) (Hartmann et al., 2000; Lee et al., 2000; Samach et al., 2000). The *FT* gene, which encodes a mobile protein, is expressed in the companion cells of the leaves and transported to the shoot apical meristem (SAM) through the phloem sieve elements to activate early targets including the floral meristem identity genes *LEAFY (LFY) and APETALA1* (*AP1*), and the floral organ-related gene *FRUITFULL* (*FUL*) and *SEPALLATA3 (SEP3)* (Conti, 2017).

Plant hormones play multiple important roles in plant growth and development, including the regulation of flowering (Conti, 2017; Li et al., 2018a). While the essential roles of gibberellin (GA) in the major floral induction pathways have been well characterized (Conti, 2017), the regulation of other hormones, including abscisic acid (ABA), jasmonate (JA), brassinosteroid (BRs), and ethylene (ET), in reproductive phase transition remains elusive. GA signaling is largely mediated

Abbreviations: ABI3, abscisic acid-insensitive 3; ABA, abscisic acid; BES1, bri1 ems suppressor1; TPL, topless; HDA19, histone deacetylase 19; FT, flowering locus t; SOC1, suppressor of overexpression of constans 1; SVP, short vegetative phase; FLC, flowering locus c; LFY, leafy; AP1, apetala 1; FUL, fruitfull; SEP, sepallata 3; BRs, brassinosteroid; BRI1, brassinosteroid insensitive1; BAK1, bri1-associated receptor kinase 1; BZR1, brassinazole resistant1; ABI4, aba insensitive 4; ABI5, aba insensitive 5; MAF5, mads affecting flowering 5; GO, gene ontology; GSEA, gene set enrichment analysis; DEG, differentially expressed genes

* Corresponding author.
E-mail addresses: gns10199@gmail.com (J. Hong), hrlee1375@duksung.ac.kr (H. Lee), jinsulee90@gmail.com (J. Lee), olo53779313@gmail.com (H. Kim), hjryu96@gmail.com (H. Ryu).

[1] These authors are equally contributed to this work.

https://doi.org/10.1016/j.plaphy.2019.03.022
Received 11 September 2018; Received in revised form 28 February 2019; Accepted 12 March 2019
Available online 19 March 2019
0981-9428/

through the degradation of negative regulator DELLA proteins. Interestingly, recent advances have shown the complicated behavior of plant hormones in flowering through crosstalk between the GA/DELLA module and other hormones such as ABA, JA, BR, and ET (Davière and Achard, 2016).

BRs are a group of plant steroid hormones that have a variety of functions, mainly regulating photomorphogenesis, cell growth, and organ formation (Clouse et al., 1996). The involvement of BR in the floral induction network was discovered by observation of the late-flowering phenotype of BR-related loss-of-function mutants (Li et al., 2010). For example, BR biosynthetic mutants, including *det2*, *dwf4*, and *cpd*, and BR-signaling mutant *bri1* showed a delayed flowering time (Azpiroz et al., 1998; Domagalska et al., 2007; Hong et al., 2018), indicating that endogenous BR levels and BR signal transduction affect flowering time. Although BR-signaling-defective *bri1* mutants showed strongly enhanced *FLC* expression with the conditional genetic background of dominant *FRIGIDA* (*FRI*) allele or autonomous pathway gene mutation through increased histone H3 acetylation at the *FLC* chromatin (Domagalska et al., 2007), how BR signaling contributes to the genetic network for floral induction needs to be further defined. *BRASSINOSTEROID INSENSITIVE 1* (BRI1)*/BRI1-ASSOCIATED RECEPTOR KINASE 1* (*BAK1*) co-receptor complex-initiated signaling cues eventually reach the major transcription factors, *BRI1 EMS SUPPRESSOR 1* (*BES1*) and *BRASSINAZOLE RESISTANT 1* (*BZR1*) (Li et al., 2018a). Despite high similarity in amino acid sequence and phosphorylation-dependent regulation of transcriptional activity, the specific DNA binding motifs and different interaction partners of *BES1* and *BZR1* lead to distinct, transcriptional networks during plant growth and development (Sun et al., 2010). Recent studies reported controversial results regarding the physiological roles of BR in flowering. Interestingly, BR signaling activated gain-of-function mutants such as *bzr1-1D* and overexpression of constitutively active *BES1-L* isoform displaying late- and early-flowering phenotypes, respectively (Jiang et al., 2015; Zhang et al., 2013) Because of the positive role of BR levels and BR signaling in the floral induction pathway, as revealed by phenotypic analysis of loss-of-function mutants, it is suggested that BR-signaling-mediated flowering is probably relayed through BES1 in complex BR signaling networks. However, the extremely late-flowering phenotype of gain-of-function *bzr1-1D* was explained by its direct modulation of *FLD* and *FLC* expression (Li et al., 2018b; Zhang et al., 2013). However, constitutively activated BES1-L isoform showed early flowering (Jiang et al., 2015), suggesting the existence of another developmental regulatory layer in BR-mediated floral transition.

Recent findings have shown that BES1–TPL–HDA19 co-repressor complex-mediated epigenetic repression of *ABI3* is one of the critical mechanisms of antagonistic crosstalk between BR and ABA in early seedling development (Ryu et al., 2014). *ABI3*, a B3 domain transcription factor, plays essential roles in the ABA-mediated regulation of seed dormancy and young seedling development. However, the expression patterns of *ABI3* in vegetative stages and the early-flowering phenotype of the *abi3-4* mutant suggest other developmental roles beyond seed dormancy and early seedling development (Kurup et al., 2000). Consistent with this, other ABA-related transcription factors, *ABA INSENSITIVE 4* (*ABI4*) and *ABA INSENSITIVE 5* (*ABI5*), have also been reported to act as negative regulators of flowering (Shu et al., 2015). These findings imply that the BES1-mediated antagonistic interactions of ABA and BR signaling pathways play diverse roles in plant growth and development.

We here demonstrate that ABI3 attenuates the BES1-induced early-flowering phenotype. Importantly, a BES1-mediated BR signaling pathway was shown to be positively involved in the floral transition, but its functions were diminished by the overexpression of *ABI3*, but not *ABI5*. In addition, global genome-wide transcriptome analysis of plants overexpressing *ABI3* showed the increased expression of floral repressor *FLC* clade members such as *FLC* and *MADS AFFECTING FLOWERING 5* (*MAF5*) and decreased expression of floral activators/integrators such as *FT* and *SOC1*. Therefore, our data reveal that the positive roles of BRs in the reproductive phase transition occur via BES1-mediated repression of *ABI3*.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0, Ler, and *Solanum lycopersicum* cultivars Micro-Tom (MT) were used as wild-type controls as the genetic backgrounds of transgenic lines. *Arabidopsis* seeds were germinated on solid media (pH 5.7–5.8) containing 1/2 Gamborg B5 salts (Duchefa, Netherlands), 1% sucrose and 0.8% plant-agar and all plants were grown in a greenhouse under long-day conditions (16-h light/8-h dark cycles) and short-day conditions (14-h light/10-h dark cycles) at 20–22 °C. The mutant seeds were ordered from ABRC stock center. All plants were grown in a greenhouse at 20–22 °C under a long-day conditions with 60% humidity.

2.2. Transgenic plants and immunoblotting assay

To generate transgenic plants overexpressing HA-tagged *ABI3* in the Col-0 background, genomic DNA fragment was cloned into *pCB302ES* containing the 35S promoter and HA tag sequences as described previously (Ryu et al., 2014). Electroporation was used to introduce the *gABI3-PCB302ES* vector into *Agrobacterium tumefaciens* strain GV3101. All transgenic lines were generated by Agrobacterium-mediated floral dipping methods. For *ABI3*-overexpressing plants in Micro-tom (MT), genomic DNA fragment was cloned into *pBI121* containing the 35S promoter and FLAG tag sequences. A single, well-formed colony from the bacterial selection plate was transferred to 4 ml of LB selective medium containing 50 mg/L kanamycin and cultured in a shaking incubator at 28 °C for 16 h to reach an OD600 of 0.6–0.7. Cotyledon segments of tomato seedlings grown for 10 days were transformed by dipping into *Agrobacterium* suspension containing 100 μM of acetosyringone. The transformed cells were selected and regenerated by previously reported (Ryu et al., 2014). Transgene expression was verified by immunoblotting. Total proteins from seedlings were extracted with protein extraction buffer (50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1 × protease inhibitor cocktail (Roche), and 1% Triton X-100). Total protein (3–20 μg) was separated by SDS–PAGE (10% polyacrylamide), transferred to a polyvinylidene difluoride membrane and immunodetected using 1/2000 dilution of peroxidase-conjugated high-affinity anti-HA (Roche), anti-FLAG (Sigma) and anti-actin (MP Biomedicals, catalogue number 69100) antibodies.

2.3. RNA extraction and qRT-PCR analysis

Total RNAs were extracted from seedlings using a Total RNA extraction kit (Intron Biotechnology, Korea) according to the manufacturer's instructions. Total RNA concentration and quality were measured using a K5600 Micro-spectrophotometer (Shanghai Biotechnol Co., China). A first-strand synthesis kit (Enzynomics, Korea) with oligo (dT) primers was used for cDNA synthesis from 1 μg of total RNA. The cDNA was then used for real-time quantitative PCR with a Quant Studio 3 (Applied Biosystems, USA) instrument using SYBR Green Real-time PCR Master Mix (Applied Biosystems) or semi-quantitative RT-PCR for *GUS* and *ABI3* expression. Primers are listed in Supplementary Table 3. Threshold cycle (Ct) values were used to calculate 2-ΔΔCt for expression analysis, where ΔΔCt for treated plants was determined as follows: (Ct target gene - Ct actin gene) - control plant (Ct target gene - Ct actin gene) (Livak and Schmittgen, 2001).

2.4. Flowering-time experiment

Plants in a growth greenhouse and chamber under 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). At maturity, the fluorescence internode number and height were determined. More than 10 plants were examined for each genotype.

2.5. Histochemical GUS staining and paraffin sections

To investigate GUS expression patterns during vegetative growth, 3, 6, 9, and 18-day-old *pABI3-GUS* seedlings were used. The seedlings were stained with GUS-staining buffer (100 mM Sodium Dihydrogen Phosphate Monohydrate, 10 mM Ethylenediaminetetraacetic acid disodium salt dehydrate, 0.5 mM Potassium ferricyanide, 0.5 mM Potassium ferrocyanide, 0.1% Triton X-100 and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronidase)) for 16 h at 37 °C. The hand cut section of around SAM from the GUS stained seedlings were prepared. For paraffin sectioning, tissues were dehydrated and embedded in paraffin. The samples were prepared into 10-15 μm-thick sections and mounted onto slides.

2.6. RNA-seq transcriptome and data analysis

All transcriptomic analyses were based on RNA-seq data (NCBI accession number is SRP142568). Differential gene expression analysis was based on previously published protocols (Trapnell et al., 2012). Functional hierarchical enrichment of DEGs was analysed by gene set enrichment toolkit (Yi et al., 2013) Plant GSEA, http://structuralbiology.cau.edu.cn/PlantGSEA/), GO Analysis Toolkit, and AgriGo (Du et al., 2010) (http://bioinfo.cau.edu.cn/agriGO/) were used in further analysis of DEGs. The lists of GO terms were clustering by REVIGO (Supek et al., 2011) (http://revigo.irb.hr/). Data are expressed as means ± standard error of mean. The expression values were calculated by log2 (FPKM) and were presented in heat map using XLSTAT software. Statistical differences were assessed by student's t-test or one-way ANOVA. The significance of differences between the means was assessed using a p-value of < 0.05. All analyses were performed using PRISM 6 software (ver. 6.01, GraphPad Software Inc.).

3. Results

3.1. BES1-mediated BR signaling is involved in the promotion of flowering

To investigate the relationship between BR signaling and floral transition, we first compared flowering time phenotypes of BR biosynthetic or signaling mutants, including, *det2*, *dwf1*, and *bri1-116* (Fig. 1A and B). Our recent study revealed that an activation tagging mutant *dwf4-D* containing high levels of endogenous BR showed a longer petiole and a slightly early-flowering phenotype, but a *bri1-301* mutant displayed a late flowering (Hong et al., 2018). Consistently, both BR-biosynthesis-defective mutants, such as *det2* and *dwf1*, and a BR-signaling defective *bri1-116* mutant showed dwarfism, a shorter petiole, and a late flowering time (Fig. 1A and B). Oddly, the BR biosynthetic mutants including *det2* and *dwf1* showed fewer rosette leaves at flowering than Col-0, which suggests that non-canonical BR signaling pathways may be involved in the regulation of leaf development (Fig. 1B). To test whether canonical BR signaling pathways are directly involved in the promotion of floral transition, we determined the flowering time of BR-activated gain-of-function mutants *bes1-D* (En-2 background) and *bzr1-D* (Col-0 background) compared with that of wild-type (WT) plants such as En-2 and Col-0, respectively. Consistent with previous reports (Zhang et al., 2013; Jiang et al., 2015), *bzr1-1D* showed delayed floral transition, but *bes1-D* showed the early-flowering phenotype under long-day (LD) conditions (Fig. 1C).

3.2. Overexpression of ABI3 but not ABI5 restores the early-flowering phenotype of bes1-D

Previously, we reported that BR signaling is integrated into the inhibitory mechanism of ABA-mediated early seedling development by epigenetically suppressing *ABI3* and *ABI5* via the BES1–TPL–HDA19 repressor complex (Ryu et al., 2014). For instance, we observed that the overexpression of *ABI3* or *ABI5* completely restored the suppressed ABA responses of *bes1-D* in seed germination and root growth inhibition (Ryu et al., 2014). There was also evidence that *ABI3* might be involved in flowering (Kurup et al., 2000), and previous studies showed that FUS3, a B3 domain transcription factor family member having similar roles to *ABI3*, acts on flowering (Duong et al., 2017). To elucidate whether BR signaling promotes flowering time via BES1-mediated downstream regulators such as ABI3 and ABI5 through decreasing ABA responses (Lopez-Molina et al., 2002), we retested *35S:ABI3-HA* and *35S:ABI5-HA* transgenic lines in the *bes1-D* mutant background, which were generated in our previous work (Ryu et al., 2014). Intriguingly, we found that most lines overexpressing *ABI3* but not *ABI5* complemented the early-flowering phenotype of *bes1-D*, indicated by both bolting date and rosette leaf number (referred to as *bes1-D/35S:ABI3-HA* and *bes1-D/35S:ABI5-HA*, respectively) (Fig. 2A–C). The *bes1-D/35S:ABI3-HA #5* T3 homozygote line, which showed the highest level of ABI3 protein as previously shown by Ryu et al., (2014), did not induce any floral organs (Fig. 2A). Despite the roles of *ABI3* and *ABI5* in BR-mediated ABA responses in early seedling development (Ryu et al., 2014), only *ABI3* exhibited an effect in regulating flowering time in the *bes1-D* mutant background (Fig. 2B and C). Furthermore, similar effects of exogenous ABA to repression of flowering in both *bes1-D* and *bes1-D/35S:ABI5-HA* lines were observed (Supplementary Fig. 1A). Since *ABI5* acts as a downstream target of ABI3 to promote ABA responses (Lopez-Molina et al., 2002), we reconfirmed that the *ABI5* expression level was highly enriched in both *bes1-D/35S:ABI3-HA* #3 and *bes1-D/35S:ABI5-HA* #18 lines (Supplementary Fig. 1B), suggesting that ABI3, but not ABI5, plays important negative roles in flowering. Taken together, these results indicate that BR-signaling-mediated regulation of floral induction is presumably divergent from the ABI5-mediated ABA response.

3.3. Ectopic expression of ABI3 produces the late-flowering phenotype

Studies on the biological roles of *ABI3* in plants have mainly focused on the regulation of seed desiccation, early seedling development, and stress responses. However, our results clearly indicated that the high expression level of *ABI3* rescued the early-flowering phenotype of *bes1-D* in the reproductive phase (Fig. 2), suggesting that *ABI3* plays additional roles beyond the early developmental processes in plants. To test this, we examined the spatiotemporal expression patterns of *ABI3* during various developmental stages of *Arabidopsis* seedlings using a *pABI3-GUS* transgenic line (Rohde et al., 1999) (Supplementary Fig. 2). Similar to the findings in a previous report (Rohde et al., 1999), expression was observed throughout early stage seedlings (3 DAG, days after germination) and its expression was continuously detected in several limited tissues, such as hypocotyl, cotyledons, and shoot apex, over 18 DAG (Supplementary Fig. 2A). *GUS* and endogenous *ABI3* expression level were further confirmed by a semi-quantitative reverse transcriptase PCR analysis (Supplementary Fig. 2B). To further analyze the expression patterns of *ABI3* in the shoot apical meristem (SAM) region in more detail, we performed paraffin sectioning and whole-tissue clearing using *pABI3-GUS* seedlings (Supplementary Fig. 2C). *ABI3* promoter-driven GUS activity was found in the SAM at 3 DAG but not after 8 DAG (Supplementary Fig. 2C). These results suggest that the biological functions of *ABI3* are not restricted to early development in plants.

To determine whether *ABI3* directly regulates flowering regardless of BR signaling, we generated *35S:ABI3-HA* transgenic plants in the wild-type Col-0 background. Interestingly, we observed significantly

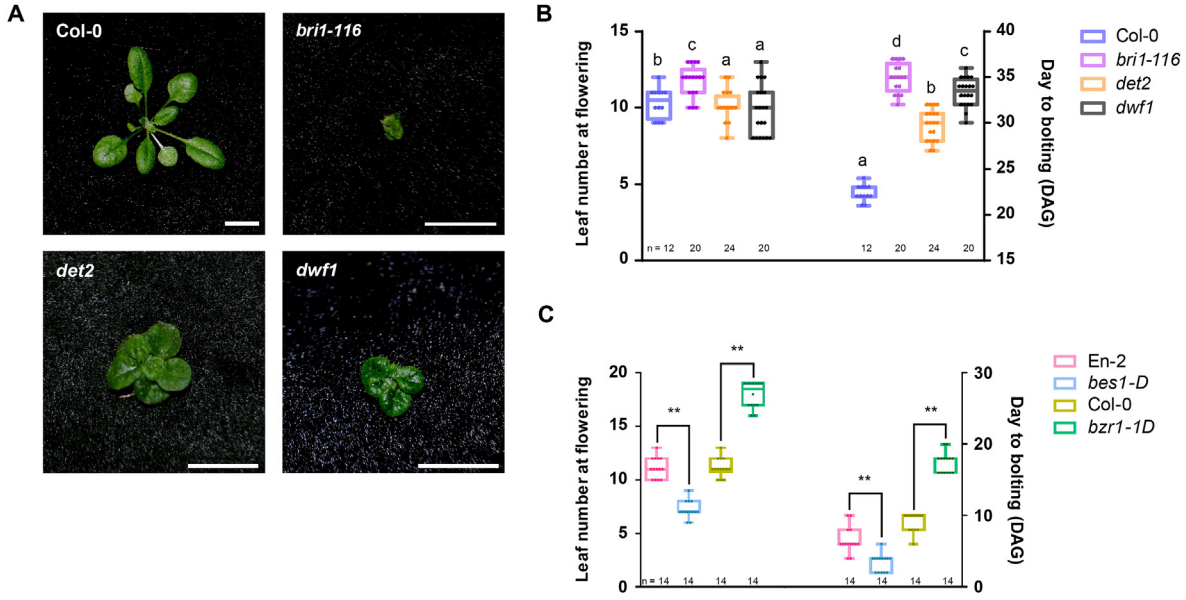

Fig. 1. BR signaling pathway positively affects flowering time via BES1
(A) Flowering phenotypes of Col-0, *bri1-116*, *det2*, and *dwf1* plants under LD at 20 DAG. Bar indicates 1 cm. (B) Days to bolting and leaf number at flowering of Col-0, *bri1-116*, *det2*, and *dwf1* plants grown under LD (n ≥ 10). Each box is located between the upper and the lower quartiles and the whiskers represent the lowest or highest data point within the 1.5 interquartile range of the lower or upper quartile. The thick horizontal lines in the boxes represent the mean and dots represent all value ($P < 0.05$; one-way ANOVA). (C) Days to bolting and leaf number at flowering of En-2, *bes1-D*, Col-0, and *bzr1-1D* plants grown under LD (n = 14). The details of the box plots are the same as those in Fig. 1B (*$P < 0.05$, **$P < 0.01$, Student's *t*-test).

shifted distributions of late-flowering phenotypes of *35S:ABI3-HA* T1 transgenic lines (Supplementary Figs. 3A and B). In addition, about 12% of them did not flower until 80 DAG. We selected three independent homozygous *35S:ABI3-HA* lines (Fig. 3A) showing late-flowering phenotypes with ectopic expression levels of ABI3-HA proteins (Fig. 3 and Supplementary Fig. 3C). For example, the *35S:ABI3-HA* #4 line containing the highest accumulation of ABI3 proteins displayed the most severe late-flowering phenotypes in terms of bolting date (51.0 ± 0.8 DAG in *35S:ABI3-HA* #4, 21.4 ± 0.4 DAG in Col-0, $P < 0.01$) and rosette leaf number (39.4 ± 1.4 DAG in *35S:ABI3-HA* #4, 8.3 ± 0.3 DAG in Col-0, $P < 0.01$), compared with those of WT Col-0 (Fig. 3 and Supplementary Fig. 3C). Moreover, the number of inflorescence internodes (cauline leaf), which is another indicator of flowering time (Pouteau and Albertini, 2011), was also increased in all *35S:ABI3-HA* lines (Supplementary Fig. 3D), exhibiting delay of the phase transition in reproductive development. Oddly, a loss-of-function *abi3-8* mutant allele in the Col-0 background rarely differed in flowering time from WT plants (Fig. 3C and Supplementary Fig. 3C). However, the *abi3-8* mutant showed significant reductions in both the number of inflorescence internodes and height, but all *35S:ABI3-HA* lines showed an increased number of internodes relative to WT plants (Supplementary Fig. 3D). Consistently, a loss-of-function *abi3-4* strong

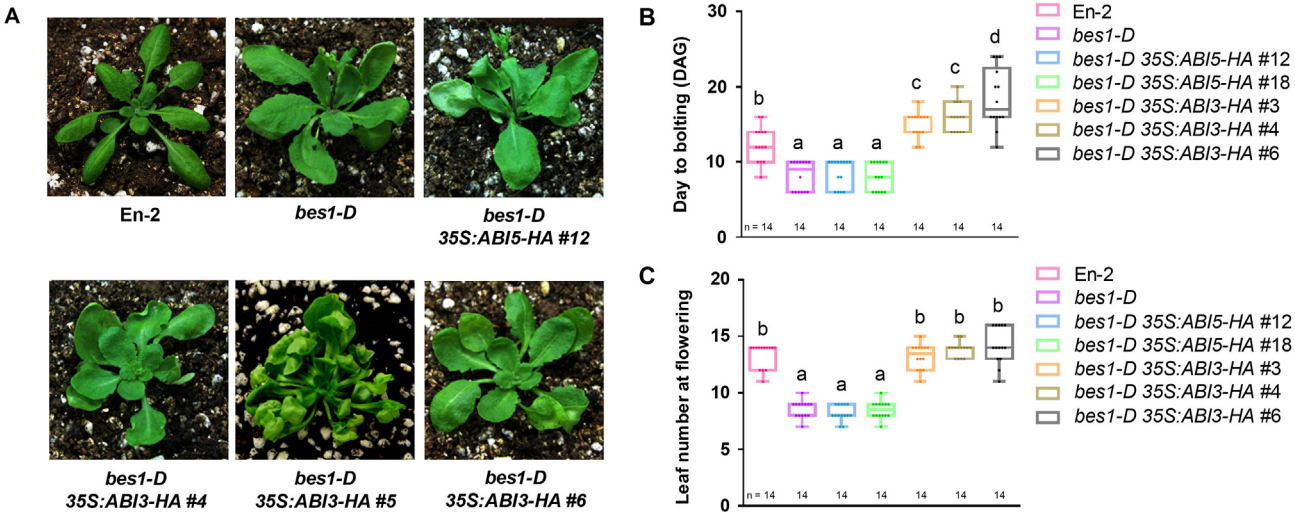

Fig. 2. Overexpression of ABI3, but not *ABI5*, restored early-flowering phenotypes of *bes1*-D.
(A) Flowering phenotypes of *bes1-D* and representative transgenic complementation lines; *bes1-D 35S-ABI5-HA* #12, *bes1-D 35S-ABI3-HA* #4, #5, and #6 plants under LD. (B, C) Flowering times of En-2, *bes1-D bes1-D 35S-ABI5-HA* (#12, #18), and *bes1-D 35S-ABI3-HA* (#3, #4, #6) plants under LD (n = 14). Days to bolting (B) and leaf number at flowering (C) in En-2, *bes1-D*, and *bes1-D ABI3-* or *ABI5*-overexpressing lines. Each box is located between the upper and the lower quartiles and the whiskers represent the lowest or highest data point within the 1.5 interquartile range of the lower or upper quartile. The thick horizontal lines in the boxes represent the mean and dots represent all value ($P < 0.05$; one-way ANOVA).

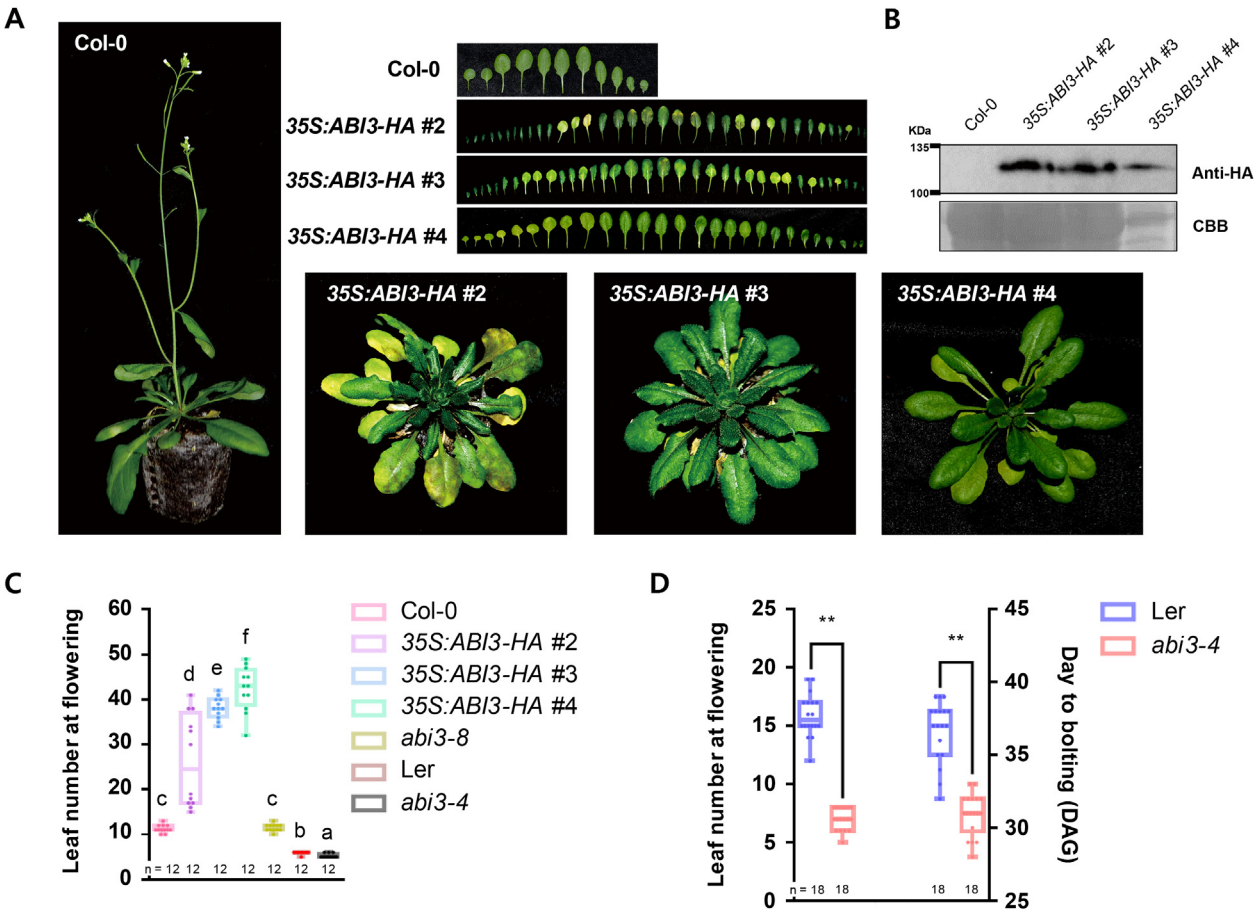

Fig. 3. Ectopic expression of ABI3 resulted in late-flowering phenotype
(A) Flowering phenotype and rosette leaf numbers at flowering of Col-0 and *35S:ABI3-HA* lines growing under LD at 35 DAG. (B) Western blot showing HA-tagged ABI3 proteins from three independent *35S:ABI3-HA* lines. Col-0, negative control; CBB staining indicates loading control. (C) Leaf number at flowering of Col-0, *35S:ABI3-HA* lines, *abi3-8*, Ler, and *abi3-4* plants under LD conditions (n = 12). Each box is located between the upper and the lower quartiles and the whiskers represent the lowest or highest data point within the 1.5 interquartile range of the lower or upper quartile. The thick horizontal lines in the boxes represent the mean and dots represent all value ($P < 0.05$; one-way ANOVA). (D) Day to bolting and leaf number at flowering of Ler and *abi3-4* plants grown under SD (n = 18). The details of the box plots are the same as those in Fig. 1B (*$P < 0.05$, **$P < 0.01$, Student's t-test).

mutant allele in the Ler background displayed early-flowering phenotypes under both LD and SD condition as previously reported (Fig. 3C and D and Supplementary Fig. 3C; Kurup et al., 2000). To further confirm the biological role of *ABI3* in floral induction using other plant species, we overexpressed the *Arabidopsis ABI3* (*AtABI3*) gene in micro-tom (MT) tomato (Martí et al., 2006). Similar to *Arabidopsis*, MT plants overexpressing *AtABI3* exhibited significant delay in the transition from vegetative to reproductive phase, producing more vegetative leaves before the first inflorescence emerged (Vicente et al., 2015) (Supplementary Fig. 4).

3.4. Ectopic expression of ABI3 induces both ABA-responsive genes and negative regulators of flowering

To evaluate transcriptional regulatory networks mediated by ABI3 encoding a B3 domain TF in plant development, genome-wide transcriptome analyses using WT Col-0 and *ABI3*-overexpressing seedlings (referred to as *35S:ABI3-HA* #4) were carried out by an RNA-seq method (Fig. 4 and Supplementary Fig. 5A). We found 693 differentially expressed genes (DEGs, p-value < 0.05 and fold change ≥ 1.5) in *35S:ABI3-HA* #4 compared with the levels in Col-0 from the RNA-seq results (Supplementary Fig. 5A). Gene Ontology (GO) analysis was performed with the Plant GSEA database (Yi et al., 2013) to predict the major biological functions of isolated DEGs (Supplementary Figs. 5B and 6). As expected, most DEGs were significantly enriched in ABA-related terms including responses to stress, response to ABA, lipid metabolism, seed germination, and seed development (Supplementary Figs. 6 and 7). Moreover, flowering-related GO terms including reproduction, reproductive process, and reproductive development were also revealed to be significantly enriched in *35S:ABI3-HA* #4 plants (Supplementary Fig. 6C, and Supplementary Table 2). To investigate the regulatory effects of *ABI3* on floral induction in more detail, we visualized the expression patterns of the reproduction-related genes (Fig. 4B, Supplementary Fig. 7 and Supplementary Table 3). Interestingly, major flowering-time-related regulatory genes such as *FLC*, *COL1*, *MAF5*, and *SOC1* were differentially enriched in reproduction-related GO terms and these expression patterns were correlated with the late-flowering phenotype of overexpression of *ABI4* (Fig. 4B). Interestingly, *ABI4* and *ABI5* functioning as negative regulators of flowering through the induction of *FLC* (Shu et al., 2015) were highly expressed in *35S:ABI3-HA* #4 plants (Fig. 4B). The different expression patterns of these flowering-time-related genes were subjected to quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 4C and D). Consistent with the RNA-seq results, the expression of flowering-promoting genes, including *SOC1*, *FT*, *CO*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL15*), *AP1*, and *PIF4*, was significantly reduced in the *35S:ABI3-HA* #4 plants (Fig. 4C and D), whereas we found the induced expression of floral repressors such as *FLC* and *MAF5* (Fig. 4C). These results strongly suggest that *ABI3* plays critical roles in not only early seedling development and stress

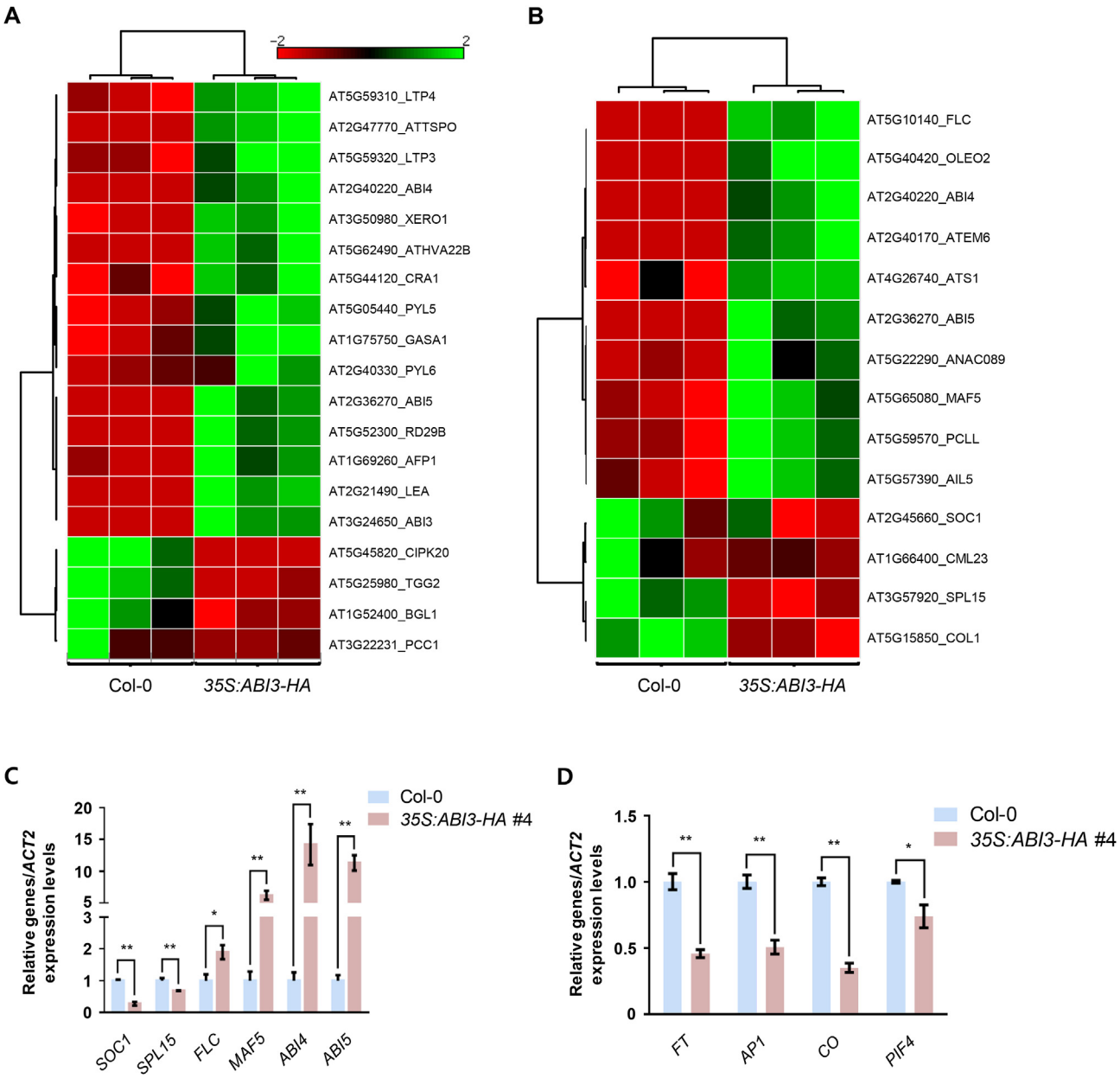

Fig. 4. Ectopic expression of ABI3-induced ABA signaling and flowering-related genes
(A, B) Heat map of ABA response- (A) and flowering-related DEGs (B). Green, upregulation; red, downregulation; black, intermediate. (C, D) Expression levels of flowering-related genes were determined by real-time qRT-PCR. Validation of the expression levels of the flowering-related DEGs presented in Fig. 4C (C) and the expression levels of positive regulators of flowering, including *FT*, *AP1*, *CO*, and *PIF4* (D), in Col-0 and *35S:ABI3-HA* #4 plants at 8 DAG (n = 3). Error bars indicate S.E.M. (*P < 0.05, **P < 0.01, Student's *t*-test). . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tolerance, but also modulation of spatiotemporal flowering-time gene regulatory networks through negative regulation of the phase transition from vegetative to reproductive stages.

4. Discussion

The control of flowering time to ensure its optimization is essential for the success of plant reproduction. Numerous studies have revealed the central regulatory networks that are key for the developmental transition from vegetative to reproductive phases. Plant hormones play important roles in various stages of development and various physiological responses including flowering (Conti, 2017). BRs are well characterized as regulators of growth throughout the entire plant developmental process, and recent studies have shown the possibility that they play biological roles in flowering (Li et al., 2018a). In this study, we revealed that *ABI3* plays a negative role in flowering via BES1-mediated signaling to promote the reproductive phase transition.

Recently, it has been reported that BR plays positive roles in GA responses through the BES1-induced activation of GA biosynthesis genes (Unterholzner et al., 2015). Interestingly, the endogenous levels of bioactive GA were significantly reduced in the BR signaling mutant *bri1-301*, which exhibited broad growth defects in germination, hypocotyl length, and flowering time, whereas overexpression of the GA biosynthetic gene *GA20ox1* enabled recovery from various growth defects including the late-flowering phenotype of the *bri1-301* mutant (Unterholzner et al., 2015). These results suggest that BES1-mediated BR signaling could promote floral induction through increasing GA biosynthesis. Our data also suggest that BR-signaling-mediated

promotion of flowering time probably occurs in a BES1-dependent manner, but not one involving BZR1 (Fig. 1). Unlike the positive role of BES1 in the regulation of floral transition, several recent studies have reported the negative role of BZR1 in floral transition by surveying the extremely late-flowering phenotype of the gain-of-function mutant *bzr1-1D* through the direct regulation of *FLD* and *FLC* (Li et al., 2018b; Zhang et al., 2013). Consistent with this, our investigation of different flowering times of *bes-1D* and *bzr1-1D* clearly supported the opposite roles of the two transcription factors, at least in the reproductive phase transition (Fig. 1C). Because BR-defective and BR signaling mutants show delayed flowering time (Hong et al., 2018; Li et al., 2010) and BES1 is reported to interact with both *EARLY FLOWERING 6* (*ELF6*) and *RELATIVE OF EARLY FLOWERING 6* (*REF6*) regulating H3K27me3 status on the chromatin of the *FLC* locus (Yu et al., 2008), it is suggested that BRI1-initiated BR signaling pathways are integrated into BES1-modulated transcriptional networks for accelerating the floral transition.

The antagonistic functions of BR and GA in ABA responses during plant growth and development have been extensively explored (Conti, 2017; Ryu et al., 2014). For instance, BR-induced BES1–TPL–HDA19 co-repressor complex is specifically involved in the epigenetic repression of *ABI3*, and its regulation is critical for ABA signaling in early seedling development (Ryu et al., 2014). To date, studies of the physiological functions of *ABI3* have mainly focused on seed development, germination, and response to stress. However, *ABI3* expression was not found to be restricted to the period during seed development or stress conditions, but was extended until the late vegetative growth stage (Supplementary Fig. 2). Moreover, the accumulation of ABI3 or its paralogous FUS3 protein in the *aip2-1* mutant, which exhibits impaired E3 ubiquitin ligase-mediated protein degradation of ABI3 and FUS3, resulted in a late-flowering phenotype (Duong et al., 2017). These results support the possibility of *ABI3* playing other biological roles in flowering beyond seed dormancy and early seedling development. Consistent with this, a loss-of-function *abi3-4* mutant displayed early flowering, whereas overexpression of *ABI3* restored the early-flowering phenotype of *bes1-D* and markedly delayed flowering time in wild-type Col-0 (Figs. 2 and 3, and Supplementary Fig. 3). Notably, it has been shown that *ABI3* is required for the expression of downstream *ABI5* for ABA responses (Lopez-Molina et al., 2002). However, the overexpression of *ABI5* in the *bes1-D* background could not rescue the early-flowering phenotype (Fig. 2). Because plants overexpressing *ABI5* decreased the ABA insensitivity of *bes1-D*, it is indicated that ABI3-mediated negative regulation of the floral transition would be independent of ABI5 activity, at least under higher-BR-response conditions. These findings were further supported by a global genome-wide transcriptome analysis using plants overexpressing *ABI3*. GSEA and GO enrichment analysis of DEGs revealed the high correlation of primary stress and ABA responses with the overexpression of *ABI3* (Fig. 4B). In addition, diverse GO terms related to reproduction and flowering were also highly enriched (Fig. 4B, Supplementary Figs. 5, 6 and Supplementary Table 1). We confirmed the upregulation of negative flowering regulators, such as *FLC* and *MAF5*, but the lower expression patterns of *SOC1*, *FT*, and *PIF4*, which act as floral stimulators (Fig. 4B–D and Supplementary Tables 2 and 3). Recent findings have shown that *VIN3-LIKE 2* (*VIL2*) promotes flowering in the noninductive photoperiod through epigenetic repression at *MAF5* chromatin, in which VIL2 maintains the status of H3K9me2 and H3K27me3 through physical interaction with polycomb repression complex 2 (PRC2) (Kim and Sung, 2010). It has also been reported that PRC1 RING-finger protein AtRING1A regulates floral transition through the repression of *MAF4* and *MAF5* expression by affecting H3K27me3 via interaction with the PRC2 complex, which in turn activates the expression of *FT* and *SOC1* to promote flowering (Shen et al., 2014). Though associated with the transition from seed maturation to germination and seedling development, it is clear from the work of Qüesta et al. (2016) that the B3 domain transcriptional repressors VAL1 and VAL2 are also required for the epigenetic repression of the *FLC* locus. It should be noted that the expression of *ABI3*, along with other B3 domain genes *FUS3* and *LEC2* is upregulated in *val1* plants; however, the repression of these genes by VAL1 is, apparently, indirect (Chen et al., 2018). However, it is possible that repression of *ABI3* by VALs-mediated epigenetic repression to promote floral transition during vernalization. Taken together, these findings support the negative roles of *ABI3* in flowering, in which its biological functions are linked to BES1-mediated floral transition.

Recently, the critical roles of BRs in crosstalk with other plant hormone pathways and diverse developmental plasticity such as morphogenic changes in response to light and elevated temperature have been reported (Martins et al., 2017; Wang et al., 2012). For example, prolonged high temperature induced thermomorphogensis including cell elongation and early flowering through the activation of PIF4 and BR signaling pathways (Ibañez et al., 2018). Our data demonstrated the novel function of *ABI3* in BR-mediated floral transition with repressed *PIF4* expression. Therefore, it is supposed that that BES1-TPL-HDA19-mediated repression of *ABI3* expression appears to be involved in the environmentally-responsive regulation of plant developmental transitions such as flowering. More detailed studies of the interactions of BR and ABA through ABI3-controlled transcriptional networks should be performed.

Author's contribution

J.H., H.L., J.L., H.K., and H.R. performed experiments. J.H., H.L., and H.R. designed experiments and analysed the data. J.H., H.L., and H.R. wrote the manuscript.

Acknowledgements

This work was carried out with the support of the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01283704), Rural Development Administration, Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.022.

References

Azpiroz, R., Wu, Y., LoCascio, J.C., Feldmann, K.A., 1998. An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10, 219–230.
Bouché, F., Woods, D.P., Amasino, R.M., 2017. Winter memory throughout the plant kingdom: different paths to flowering. Plant Physiol. 173, 27–35.
Chen, N., Veerappan, V., Abdelmageed, H., Kang, M., Allen, R.D., 2018. HSI2/VAL1 silences AGL15 to regulate the developmental transition from seed maturation to vegetative growth in Arabidopsis. Plant Cell 30, 600–619.
Clouse, S.D., Langford, M., McMorris, T.C., 1996. A brassinosteroid-insensitive mutant in Arabidopsis thaliana exhibits multiple defects in growth and development. Plant Physiol. 111, 671–678.
Conti, L., 2017. Hormonal control of the floral transition: can one catch them all? Dev. Biol. 430, 288–301.
Davière, J.-M., Achard, P., 2016. A pivotal role of DELLAs in regulating multiple hormone signals. Mol. Plant 9, 10–20.
Domagalska, M.A., Schomburg, F.M., Amasino, R.M., Vierstra, R.D., Nagy, F., Davis, S.J., 2007. Attenuation of brassinosteroid signaling enhances FLC expression and delays flowering. Development 134, 2841–2850.
Du, Z., Zhou, X., Ling, Y., Zhang, Z., Su, Z., 2010. agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res. 38, W64–W70.
Duong, S., Vonapartis, E., Li, C.-Y., Patel, S., Gazzarrini, S., 2017. The E3 ligase ABI3-INTERACTING PROTEIN2 negatively regulates FUSCA3 and plays a role in cotyledon development in Arabidopsis thaliana. J. Exp. Bot. 68, 1555–1567.
Hartmann, U., Höhmann, S., Nettesheim, K., Wisman, E., Saedler, H., Huijser, P., 2000. Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant J. 21, 351–360.
Hong, J., Sung, J., Ryu, H., 2018. Brassinosteroids-mediated regulation of *ABI3* is involved in high-temperature induced early flowering in plants. J. Plant Biotechnol. 45, 83–89.

Ibañez, C., Delker, C., Martinez, C., Bürstenbinder, K., Janitza, P., Lippmann, R., Ludwig, W., Sun, H., James, G.V., Klecker, M., 2018. Brassinosteroids dominate hormonal regulation of plant thermomorphogenesis via BZR1. Curr. Biol. 28, 303–310.
Jiang, J., Zhang, C., Wang, X., 2015. A recently evolved isoform of the transcription factor BES1 promotes brassinosteroid signaling and development in Arabidopsis thaliana. The Plant Cell tpc 114, 133678.
Kim, D.-H., Sung, S., 2010. The Plant Homeo Domain finger protein, VIN3-LIKE 2, is necessary for photoperiod-mediated epigenetic regulation of the floral repressor, MAF5. Proc. Natl. Acad. Sci. Unit. States Am. 201010834.
Kurup, S., Jones, H.D., Holdsworth, M.J., 2000. Interactions of the developmental regulator ABI3 with proteins identified from developing Arabidopsis seeds. Plant J. 21, 143–155.
Lee, H., Suh, S.-S., Park, E., Cho, E., Ahn, J.H., Kim, S.-G., Lee, J.S., Kwon, Y.M., Lee, I., 2000. The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes Dev. 14, 2366–2376.
Li, J., Li, Y., Chen, S., An, L., 2010. Involvement of brassinosteroid signals in the floral-induction network of Arabidopsis. J. Exp. Bot. 61, 4221–4230.
Li, Q.-F., Lu, J., Yu, J.-W., Zhang, C.-Q., He, J.-X., Liu, Q.-Q., 2018a. The brassinosteroid-regulated transcription factors BZR1/BES1 function as a coordinator in multisignal-regulated plant growth. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms 1861, 561–571.
Li, Z., Ou, Y., Zhang, Z., Li, J., He, Y., 2018b. Brassinosteroid signaling recruits histone 3 lysine-27 demethylation activity to FLOWERING LOCUS C chromatin to inhibit the floral transition in Arabidopsis. Mol. Plant 11, 1135–1146.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408.
Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T., Chua, N.H., 2002. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J. 32, 317–328.
Mai, Y.X., Wang, L., Yang, H.Q., 2011. A gain-of-function mutation in IAA7/AXR2 confers late flowering under short-day light in Arabidopsis F. J. Integr. Plant Biol. 53, 480–492.
Martí, E., Gisbert, C., Bishop, G.J., Dixon, M.S., García-Martínez, J.L., 2006. Genetic and physiological characterization of tomato cv. Micro-Tom. J. Exp. Bot. 57, 2037–2047.
Martins, S., Montiel-Jorda, A., Cayrel, A., Huguet, S., Paysant-Le Roux, C., Ljung, K., Vert, G., 2017. Brassinosteroid signaling-dependent root responses to prolonged elevated ambient temperature. Nat. Commun. 8, 309.
Pouteau, S., Albertini, C., 2011. An assessment of morphogenetic fluctuation during reproductive phase change in Arabidopsis. Ann. Bot. 107, 1017–1027.
Qüesta, J.I., Song, J., Geraldo, N., An, H., Dean, C., 2016. Arabidopsis transcriptional repressor VAL1 triggers Polycomb silencing at FLC during vernalization. Science 353, 485–488.
Rohde, A., Van Montagu, M., Boerjan, W., 1999. The ABSCISIC ACID-INSENSITIVE 3 (ABI3) gene is expressed during vegetative quiescence processes in Arabidopsis. Plant Cell Environ. 22, 261–270.
Ryu, H., Cho, H., Bae, W., Hwang, I., 2014. Control of early seedling development by BES1/TPL/HDA19-mediated epigenetic regulation of ABI3. Nat. Commun. 5, 4138.
Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., Coupland, G., 2000. Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science 288, 1613–1616.
Shen, L., Thong, Z., Gong, X., Shen, Q., Gan, Y., Yu, H., 2014. The putative PRC1 RING-finger protein AtRING1A regulates flowering through repressing MADS AFFECTING FLOWERING genes in Arabidopsis. Development dev 104513.
Shu, K., Chen, Q., Wu, Y., Liu, R., Zhang, H., Wang, S., Tang, S., Yang, W., Xie, Q., 2015. ABSCISIC ACID-INSENSITIVE 4 negatively regulates flowering through directly promoting Arabidopsis FLOWERING LOCUS C transcription. J. Exp. Bot. 67, 195–205.
Sun, Y., Fan, X.-Y., Cao, D.-M., Tang, W., He, K., Zhu, J.-Y., He, J.-X., Bai, M.-Y., Zhu, S., Oh, E., 2010. Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. Dev. Cell 19, 765–777.
Supek, F., Bošnjak, M., Škunca, N., Šmuc, T., 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. PLoS One 6, e21800.
Trapnell, Cole, Roberts, Adam, Goff, Loyal, Pertea, Geo, Kim, Daehwan, Kelley, David R., Pachter, Lior, 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat. Protoc. 7 (3), 562.
Unterholzner, S.J., Rozhon, W., Papacek, M., Ciomas, J., Lange, T., Kugler, K.G., Mayer, K.F., Sieberer, T., Poppenberger, B., 2015. Brassinosteroids are master regulators of gibberellin biosynthesis in Arabidopsis. Plant Cell 27, 2261–2272.
Vicente, M.H., Zsögön, A., de Sá, A.F.L., Ribeiro, R.V., Peres, L.E., 2015. Semi-determinate growth habit adjusts the vegetative-to-reproductive balance and increases productivity and water-use efficiency in tomato (Solanum lycopersicum). J. Plant Physiol. 177, 11–19.
Wang, Z.-Y., Bai, M.-Y., Oh, E., Zhu, J.-Y., 2012. Brassinosteroid signaling network and regulation of photomorphogenesis. Annu. Rev. Genet. 46, 701–724.
Yi, X., Du, Z., Su, Z., 2013. PlantGSEA: a gene set enrichment analysis toolkit for plant community. Nucleic Acids Res. 41, W98–W103.
Yu, X., Li, L., Li, L., Guo, M., Chory, J., Yin, Y., 2008. Modulation of brassinosteroid-regulated gene expression by Jumonji domain-containing proteins ELF6 and REF6 in Arabidopsis. Proc. Natl. Acad. Sci. Unit. States Am. 105, 7618–7623.
Zhang, Y., Li, B., Xu, Y., Li, H., Li, S., Zhang, D., Mao, Z., Guo, S., Yang, C., Weng, Y., 2013. The cyclophilin CYP20-2 modulates the conformation of BRASSINAZOLE-RESISTANT1, which binds the promoter of FLOWERING LOCUS D to regulate flowering in Arabidopsis. Plant Cell tpc 113, 110296.