

RESEARCH ARTICLE

NO FLOWERING IN SHORT DAY (NFL) is a bHLH transcription factor that promotes flowering specifically under short-day conditions in *Arabidopsis*

Nidhi Sharma^{1,*‡}, Ruijiao Xin^{1,‡}, Dong-Hwan Kim¹, Sibum Sung¹, Theo Lange² and Enamul Huq^{1,§}

ABSTRACT

Flowering in plants is a dynamic and synchronized process where various cues including age, day length, temperature and endogenous hormones fine-tune the timing of flowering for reproductive success. *Arabidopsis thaliana* is a facultative long day (LD) plant where LD photoperiod promotes flowering. *Arabidopsis* still flowers under short-day (SD) conditions, albeit much later than in LD conditions. Although factors regulating the inductive LD pathway have been extensively investigated, the non-inductive SD pathway is much less understood. Here, we identified a key basic helix-loop-helix transcription factor called NFL (NO FLOWERING IN SHORT DAY) that is essential to induce flowering specifically under SD conditions in *Arabidopsis*. *nfl* mutants do not flower under SD conditions, but flower similar to the wild type under LD conditions. The no-flowering phenotype in SD is rescued either by exogenous application of gibberellin (GA) or by introducing *della* quadruple mutants in the *nfl* background, suggesting that NFL acts upstream of GA to promote flowering. NFL is expressed at the meristematic regions and NFL is localized to the nucleus. Quantitative RT-PCR assays using apical tissues showed that GA biosynthetic genes are downregulated and the GA catabolic and receptor genes are upregulated in the *nfl* mutant compared with the wild type, consistent with the perturbation of the endogenous GA biosynthetic and catabolic intermediates in the mutant. Taken together, these data suggest that NFL is a key transcription factor necessary for promotion of flowering under non-inductive SD conditions through the GA signaling pathway.

KEY WORDS: *Arabidopsis*, BHLH transcription factor, Flowering time, GA signaling pathway, Photoperiod pathway

INTRODUCTION

Flowering is a transition from the vegetative to reproductive phase and is one of the crucial developmental transitions in the plant life cycle. The time of flowering in plants is synchronized by various endogenous and environmental cues to produce flowers only under optimal conditions. Flowering time in the model plant *Arabidopsis thaliana* has been extensively studied. Four major genetic pathways, namely, vernalization (long exposure to cold), autonomous (genetic makeup), hormonal, and photoperiod (day-length) pathways

regulate flowering time in *Arabidopsis*. These four genetic pathways regulate the expression of floral integrator genes, such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) that activate the downstream floral identity genes [e.g. *APETALA1* (*API*) and *LEAFY* (*LFY*)] to promote flowering (Andrés and Coupland, 2012; Kim et al., 2009; Song et al., 2015).

The vernalization pathway controls flowering time through the floral repressors *FLC* and other *FLC* clade members (Kim et al., 2009). The *FLC* clade consists of MADS-box transcription factors *FLOWERING LOCUS M/MADS AFFECTING FACTOR 1* (*MAF1*) and *MAF2-MAF5* (Kim and Sung, 2010). Winter annual accessions of *Arabidopsis* containing *FRIGIDA* (*FRI*) and *FLC* require vernalization treatment to overcome repression of flowering (Michaels and Amasino, 1999a). In non-vernalized plants, *FLC* represses expression of *FT* and *SOC1* in phloem and in the meristem and *FD* in the meristem (Searle et al., 2006). After vernalization, *FLC* expression is strongly repressed through histone modifications (Kim et al., 2009). Repression of *FLC* leads to activation of downstream floral integrators, *FT* and *SOC1* that allow plants to flower after a long duration of cold exposure. Autonomous pathway also represses *FLC* through *LD*, *FCA*, *FY*, *FPA*, *FLD*, *FVE*, *FLK* and *REF6* (Noh et al., 2004; Simpson, 2004). *FCA*, *FY*, *FPA* and *FLK* proteins are predicted to be involved in RNA metabolism (Lim et al., 2004; Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003). *FCA* and *FPA* are RNA-binding proteins involved in repression of *FLC* and other genes. *FY* interacts with the *FCA* WW domain to promote flowering (Simpson et al., 2003). *FVE*, *FLD* and *REF6* have domains similar to chromatin-modifying components, and *FLD* and *REF6* are predicted to encode histone demethylases (He et al., 2003; Jiang et al., 2007; Noh et al., 2004). In summary, both vernalization and autonomous pathways converge on *FLC*, which regulates downstream floral integrator genes to regulate flowering time.

Several phytohormones, such as gibberellin (GA), brassinosteroid (BR), nitric oxide (NO) and salicylic acid (SA) crosstalk to fine-tune the timing of flowering in *Arabidopsis* (Davis, 2009; Domagalska et al., 2010). Among all the hormones, the roles of GA in controlling flowering time have been best understood. Under non-inductive short-day (SD) photoperiod, *ga requiring 1* (*gal*) mutant fails to flower, suggesting an absolute requirement of GA signaling in SD conditions (Wilson et al., 1992). GA directly promotes *SOC1* and *LFY* expression under SD conditions (Moon et al., 2003; Mutasa-Göttgens and Hedden, 2009). Increased levels of *SOC1*, in turn, activate the downstream floral meristem identity genes, *LFY* and *API* to promote flowering. This relay of information from GA to *SOC1* occurs through degradation of the DELLA proteins RGA and RGL2 with a partial contribution from RGL1 (Cheng et al., 2004). In addition, GA has been shown to regulate flowering by interacting

¹Department of Molecular Biosciences and The Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, USA. ²Institute of Plant Biology, Department of Plant Physiology and Biochemistry, Technical University of Braunschweig, Braunschweig D-38106, Germany.

*Present address: Department of Plant Biology, Carnegie Institute of Science, 260 Panama Street, Stanford, CA 94305, USA.

[‡]These authors contributed equally to this work

[§]Author for correspondence (huq@austin.utexas.edu)

with photoperiod, ambient temperature and vernalization pathways (Galvão et al., 2015; Jung et al., 2012; Li et al., 2015; Osnato et al., 2012). The effect of GA is also spatially regulated. Under inductive LD conditions, GA acts in the leaves and vascular tissues to induce *FT* and *TWIN SISTER OF FT (TSF)* expression, while also activating expression of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL)* genes in both the leaves and meristem (Galvão et al., 2012; Porri et al., 2012). Thus, GA optimizes reproductive success by functioning under both LD and SD conditions.

Photoperiod (day length) plays a crucial role in controlling flowering time in *Arabidopsis* (Andrés and Coupland, 2012; Song et al., 2015). The photoperiod signal is perceived in the leaves and this signal, often called a florigen, moves to the shoot apical meristem (SAM) where flowers are produced. *Arabidopsis* is a facultative long day (LD, 16 h light:8 h dark) plant where the long day acts as an inductive photoperiod to promote flowering, and flowering is delayed under a non-inductive SD (8 h light:16 h dark) photoperiod. The biochemical basis for the difference in flowering time under LD and SD is very well documented through an external coincidence model (Song et al., 2015). According to this model, light plays two major roles: resetting the circadian clock that generates daily oscillation of *CO* and regulating *CO* protein stability. The daily oscillation of *CO* is regulated, in part, by two antagonistic groups of genes: activators *FLAVIN-BINDING*, *KELCH REPEAT AND F-BOX 1 (FKF1)*, *GIGANTEA (GI)* and repressors *ELF3*, *CYCLING DOF FACTOR 1 (CDF1)* and *RED AND FAR-RED INSENSITIVE 2 (RFI2)* (Chen and Ni, 2006; Covington et al., 2001; Fowler et al., 1999; Imaizumi et al., 2005; Nelson et al., 2000; Suárez-López et al., 2001). In the dark, CDF1 is present at the *CO* promoter, repressing *CO* expression. However, after light is perceived in the leaves, GI interacts with the F-box protein FKF1 and the GI-FKF1 complex degrades CDF1 through ubiquitin-mediated proteolysis (Sawa et al., 2007). This leads to the de-repression of *CO* transcription. By contrast, *CO* stability is regulated post-translationally, where photoreceptors such as phytochrome A (phyA) and cryptochromes (CRY1 and CRY2) have been shown to prevent *CO* protein degradation, whereas phyB promotes *CO* degradation (Valverde et al., 2004). In this process, PHYTOCHROME-DEPENDENT LATE FLOWERING (PHL) interacts with both phyB and *CO*, and prevents phyB-mediated degradation of *CO* (Endo et al., 2013). HOS1, a ring domain-containing E3 ligase destabilizes during the day (Lazaro et al., 2012). ZTL and LKP2 promote degradation of *CO* in the morning, whereas FKF1 stabilizes *CO* by direct interaction (Song et al., 2014, 2012). TOE1, in turn, prevents FKF1-mediated stabilization of *CO* in the afternoon (Zhang et al., 2015). Thus, *CO* mRNA and protein level peaks at 12 h after dawn, which coincides with light in LD, but dark in SD. Therefore, increased *CO* protein promotes expression of *FT* only under LD. *FT* moves through phloem to the meristem, where it associates with FD, and the FT-FD complex activates expression of *SOC1* and downstream floral identity genes, such as *API* and *LFY*, to promote flowering (Abe et al., 2005; Corbesier et al., 2007; Michaels et al., 2005; Wigge et al., 2005; Yoo et al., 2005). However, in SD, *CO* protein peaks in the dark, where it is degraded through the COP1-SPA complex, resulting in a delayed flowering response.

Although the LD photoperiod pathway is well characterized, recent evidence suggests the presence of a non-inductive SD pathway for promotion of flowering time. For example, plant homeodomain finger-containing proteins such as VIN3-LIKE 1 and 2 [VIL1 (also known as VRN5) and VIL2] have been shown to promote flowering through epigenetic repression of *MAF1* and *MAF5* genes, respectively, under SD conditions (Kim and Sung,

2010; Sung et al., 2006). *vil1* and *vil2* mutants flower later only under SD conditions, but do eventually flower. However, *spal*, *cop1* and *cul4cs* mutants display early flowering only under SD conditions (Chen et al., 2010; Ishikawa et al., 2006; Jang et al., 2008; Laubinger et al., 2006; McNellis et al., 1994; Ranjan et al., 2011). SPA1 physically interacts with COP1, and COP1-SPA1 associates with CUL4, forming an E3 ubiquitin ligase, which promotes *CO* degradation through ubiquitin-mediated proteolysis (Chen et al., 2010; Jang et al., 2008; Laubinger et al., 2006). In response to blue light, CRY1 and CRY2 interact with SPA1 and reorganize the COP1-SPA complex to stabilize *CO* (Liu et al., 2011; Zuo et al., 2011). It appears that these factors are regulating components that control flowering time typically under the LD pathway and mutants activate the LD photoperiod pathway regardless of photoperiods. Nonetheless, the non-inductive SD pathway is still poorly understood. In addition, the factors necessary for plants such as *Arabidopsis thaliana* to evolve as a facultative LD plant is still unknown. Here we describe a basic-helix-loop-helix (bHLH) transcription factor (AT5g65640), NO FLOWERING IN SD (NFL), which functions as a crucial component of the SD pathway. *nfl* mutants fail to flower only under SD conditions, but flower like the wild type (wt) under LD conditions. Therefore, NFL might represent a pivotal transcription factor necessary for *Arabidopsis thaliana* to evolve as a facultative LD plant.

RESULTS

Isolation and characterization of the *nfl* mutant

During genome-wide analyses of T-DNA insertion lines for *Arabidopsis bHLH* transcription factor genes (Toledo-Ortiz et al., 2003), we identified two independent alleles of homozygous T-DNA insertion mutants in the *NFL* (Fig. S1A). Both alleles have T-DNA insertions in the first exon of the *NFL* gene. To investigate whether *NFL* is expressed in these mutant lines, we performed a semi-quantitative RT-PCR analysis on wt and *nfl* mutant alleles. Neither of the alleles showed any detectable expression of *NFL* compared with the wt, suggesting that they are null mutants (Fig. S1B). During growth of these mutants and wt plants under continuous light conditions in a growth room, we observed visible growth defects in the mutant plants compared with wt plants. *nfl* mutants displayed shorter stature, curly and darker green leaves and reduced fertility compared with wt plants (Fig. S2A,B). Measurement of internode lengths showed that internodes 1, 3 and 4 were significantly shorter in mutant plants than in wt plants (Fig. S2B). These data suggest that NFL regulates plant stature, chlorophyll content and fertility at the adult stage.

NFL regulates flowering time specifically under short-day conditions

To investigate adult phenotypes of *nfl* mutant lines, we grew wt and two *nfl* mutants (*nfl-1* and *nfl-2*) in different photoperiod conditions. Under LD (16 h light:8 h dark) conditions, *nfl* mutants flowered similar to wt plants (Fig. 1A). However, under SD (8 h light:16 h dark) conditions, *nfl* mutants failed to flower from the primary meristem (Fig. 1B). Although we observed occasional bolting from axillary meristem at a low frequency (10–20%), most plants underwent senescence without flowering from the primary meristem. We quantified the flowering phenotype using both the number of rosette leaves formed at the time of flowering and days taken to flower. Results showed that the number of rosette leaves and days to flower were similar for both mutant and wt plants grown under LD conditions (Fig. 1C,E), whereas the *nfl* mutants failed to

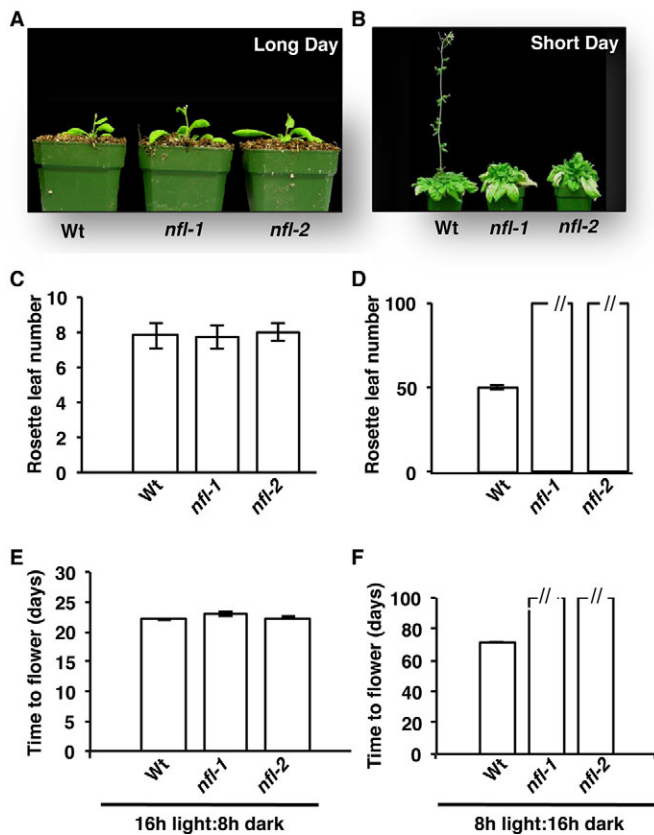


Fig. 1. *nfl* does not flower specifically under short-day conditions.

Flowering time was quantified using both number of rosette leaves formed at the time of flowering and number of days to flower. Photographs of wt and two alleles of *nfl* mutant plants grown under long-day (LD; 16 h light:8 h dark) (A) or short-day (SD; 8 h light:16 h dark) (B) conditions. Rosette leaf numbers for wt and two alleles of *nfl* mutant plants grown under LD (C) or SD (D) conditions. Number of days taken to flower for wt and two alleles of *nfl* mutant plants grown under LD (E) or SD (F) conditions. Error bars indicate s.e.m. ($n \geq 3$).

flower even after producing ~100 leaves under SD conditions (Fig. 1D,E). These data suggest that NFL regulates flowering time specifically under SD conditions.

NFL can complement the *nfl* mutant phenotypes

Although two independent T-DNA insertion alleles of the *nfl* mutants displayed the late-flowering phenotype under SD conditions, we transformed the *pNFL:NFL* transgene with a 2 kb promoter along with the entire coding region into *nfl-1* mutant background for complementation analyses. We selected independent transgenic plants and examined their flowering-time phenotype. Results showed that the native *NFL* gene rescued the *nfl* mutant phenotype under SD (Fig. S3A,B). These data confirmed that the mutant phenotype was indeed due to a disruption in the *NFL* gene.

nfl-1 and *nfl-2* are recessive mutants, because the heterozygous plants flowered like wt plants (data not shown). The heterozygous plants also did not display the other morphological phenotypes such as short stature, curly and darker leaves, and reduced fertility. Ectopic expression of *NFL* (*p35S:NFL-LUC* and *pNFL:NFL-GUS*) in the wt background did not result in any observable difference compared with the wt, including flowering time under either SD or LD conditions (Fig. S4, data not shown). However,

expression of *NFL* fusion proteins either from the 35S promoter or native promoter (*p35S:NFL-LUC* and *pNFL:NFL-GUS*) in the *nfl* mutant background rescued the no-flowering phenotype of *nfl* mutants under SD conditions (Fig. S4), suggesting that these fusion proteins retain normal function of the protein. The lack of overexpression phenotypes for the *p35S:NFL-LUC* and *pNFL:NFL-GUS* might indicate a reduced function protein from these transgenes and/or low expression. Overall, the failure to flower only under SD in the *nfl* mutants and complementation of this phenotype using fusion proteins suggest that *NFL* is essential to induce flowering under SD.

Day-length is crucial for NFL function

Because the *nfl* mutants never flowered under SD, but flowered similar to the wt under LD conditions, we examined the requirement of different lengths of daytime for *nfl* mutants to flower. We grew wt and *nfl* plants under 16 h light:8 h dark (LD), 14 h light:10 h dark, 12 h light:12 h dark, 10 h light:14 h dark, 8 h light:16 h dark (SD) conditions. The wt plants displayed a late-flowering phenotype under 14 h light:10 h dark photoperiod conditions compared with LD conditions (Fig. 2). However, wt plants still flowered earlier under this condition compared with the SD conditions. In addition, the flowering time for wt plants was similar under 12 h light:12 h dark, 10 h light:14 h dark and SD conditions, suggesting that the LD photoperiod pathway is not functional when the day length is shortened to 12 h light or less. Under 14 h light:10 h dark conditions, *nfl* mutants flowered; however, they displayed significantly later flowering compared with wt plants. Strikingly, *nfl* mutants failed to flower when the day length was shortened to 12 h light or less, indicating that the threshold day length for *nfl* plants to flower is 14 h of light – a condition that delays but does not completely block flowering of wt plants. Overall, these data suggest that the no-flowering phenotype of the *nfl* mutants is strictly SD specific.

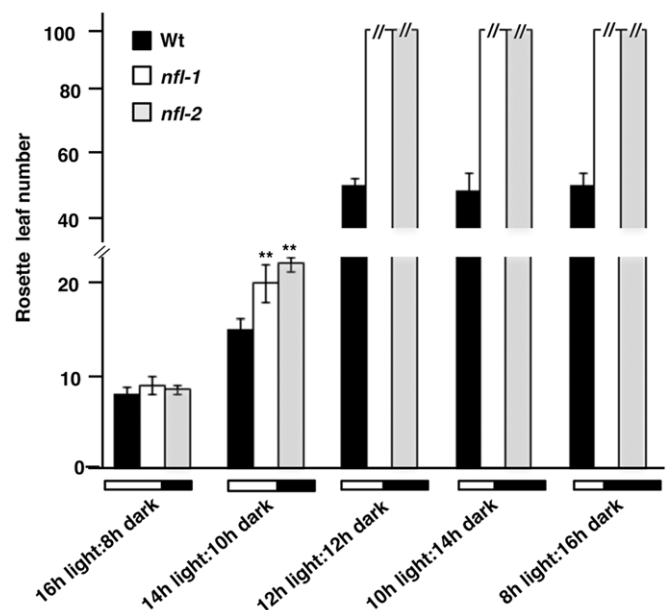


Fig. 2. Effect of day length on the flowering time of the *nfl* mutants. Wild type and *nfl* mutants were grown under different photoperiods as indicated, and flowering time was measured using the number of rosette leaves at the time of flowering. Error bars indicate s.e.m. ($n \geq 3$). ** $P \leq 0.01$, compared with wt.

Exogenous application of GA₄ rescues the late-flowering phenotype of the *nfl* mutants under SD conditions

GA has been shown to be essential to promote flowering predominantly under SD conditions (Wilson et al., 1992). To examine whether the flowering phenotype of *nfl* mutants can be rescued by exogenous application of GA, we externally applied biologically active GA₄ on the meristem of wt, *nfl* and *phyB* mutants grown under SD conditions. The *nfl* mutants flowered at a similar time as the wt under SD conditions with exogenous GA application, suggesting that GA rescues the mutant phenotype under SD (Fig. 3A, Fig. S5A). Interestingly, exogenous application of GA also rescued the other phenotypes, such as curly and twisted leaves of *nfl* plants under SD conditions (Fig. S5B). These data suggest either that *NFL* is involved in GA biosynthesis and/or signaling or that GA is acting further downstream from *NFL* in regulating flowering time under SD conditions.

della mutants are epistatic to *nfl* under SD conditions

To provide genetic evidence that *NFL* functions through the GA pathway, we created *rga gai rgl1 rgl2 nfl* quintuple mutants between the quadruple mutant *della* (*rga gai rgl1 rgl2*) and *nfl-1*. Examination of the flowering-time phenotype showed that *della* quadruple mutants are epistatic to *nfl*, resulting in complete rescue of the no-flowering phenotype of the *nfl* mutants under SD conditions (Fig. 3B). *della* quadruple mutants also displayed early flowering compared with wt controls under these conditions, as

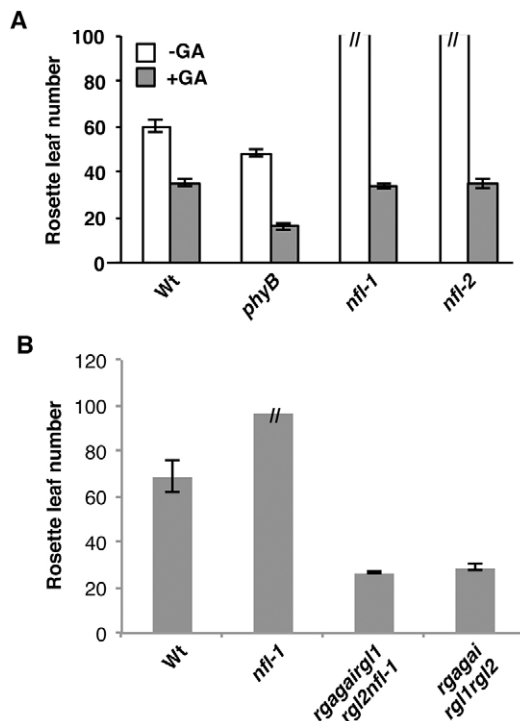


Fig. 3. *NFL* acts upstream of the GA pathway. (A) Exogenous application of gibberellin (GA₄) rescues the late-flowering phenotypes of *nfl* mutants under SD conditions. Bar graph showing the number of rosette leaves produced by various genotypes at the time of flowering. Plants were grown under SD (8 h light:16 h dark) conditions with and without application of GA. (B) *rga gai rgl1 rgl2* quadruple mutant is epistatic to *nfl* under SD conditions. Plants were grown under SD conditions and the rosette leaves were counted. The bar graph shows the average of three independent biological replicates. Error bars indicate s.e.m. ($n \geq 3$).

previously observed. Thus, *NFL* regulates flowering time mainly through the GA pathway under SD conditions.

Prolonged vernalization did not rescue the no-flowering phenotype of the *nfl* mutants under SD conditions

To investigate whether *nfl* is defective in the vernalization pathway, we vernalized wt and *nfl* mutant plants for 0, 6, 8 and 10 weeks at 4°C and then transferred to SD conditions to examine flowering time. Even the extended vernalization treatment did not induce flowering of *nfl* mutant plants, whereas the wt plants flowered earlier than the non-vernalized plants under these conditions (Fig. S6). The non-vernalization response observed in *nfl* mutants under SD is similar to that observed in *gai* mutants under SD (Michaels and Amasino, 1999b). Therefore, these data support the idea that *NFL* acts through the GA pathway to regulate flowering time under SD.

NFL is expressed in a tissue-specific and developmental stage-dependent manner

To investigate the spatial and temporal expression patterns of *NFL* under SD and LD conditions, we produced transgenic plants expressing *NFL* fused to β -glucuronidase (*GUS*) from the native *NFL* promoter (*pNFL:NFL-GUS*). The construct was transformed into the wt background and homozygous single-copy transgenic lines were selected. We performed histochemical *GUS* assays at different stages of development (2-, 4-, 8- and 10-day-old seedlings) using *pNFL:NFL-GUS* plants grown under different photoperiods. Under SD conditions, these seedlings displayed *GUS* activity mainly in root tips and SAM, with weak expression in cotyledons throughout development (Fig. 4, Fig. S7A). *GUS* activity was observed in the hypocotyl only at day 2, with reduced or no activity at older ages (Fig. 4A-D). *GUS* activity was also observed in the whole meristem at day 2 (Fig. S7A); however, the expression was reduced at the tip, with visible expression at the base of the meristem (Fig. S7A,B). Under LD conditions, strong *GUS* activity was observed in the hypocotyl at day 2 and in root tips and SAM throughout the developmental stages (Fig. 4E-H). The *GUS* activity was reduced from cotyledons during development with the strongest activity in 2-day-old seedlings (Fig. 4E) and almost no activity in 10-day-old seedlings (Fig. 4H) under LD conditions. However, 8- and 10-day-old seedlings displayed strong *GUS* activity in the primary leaves (Fig. 4G-H). The expression pattern in the hypocotyls was similar under both SD and LD, with the strongest activity at day 2 and a gradual reduction in activity from day 4 to 10 during development (Fig. 4A-H). A common feature of both SD- and LD-grown plants is the strong *GUS* expression in the root tips (Fig. 4A-H). These data are largely consistent with digital expression data on publicly available websites (see Fig. S8), and suggest that *NFL* is expressed in a tissue-specific and developmental stage-dependent manner and might function in specific tissues and/or at specific developmental stages.

We also examined *NFL* expression in 4-day-old seedlings and leaves and meristem tissues from 45-day-old adult plants grown under 12 h dark:12 h light conditions using qRT-PCR assays. Results show that *NFL* is more highly expressed in meristematic tissues than in leaves and seedlings (Fig. 4I, left). Strikingly, the lower expression in seedlings and leaves was also observed in transgenic plants expressing *35S::NFL-GFP* from a constitutively active 35S promoter (Fig. 4I, right). These data suggest that *NFL* is expressed at a very low level in a tissue-specific manner and that *NFL* might be under post-transcriptional regulation in seedlings and leaves.

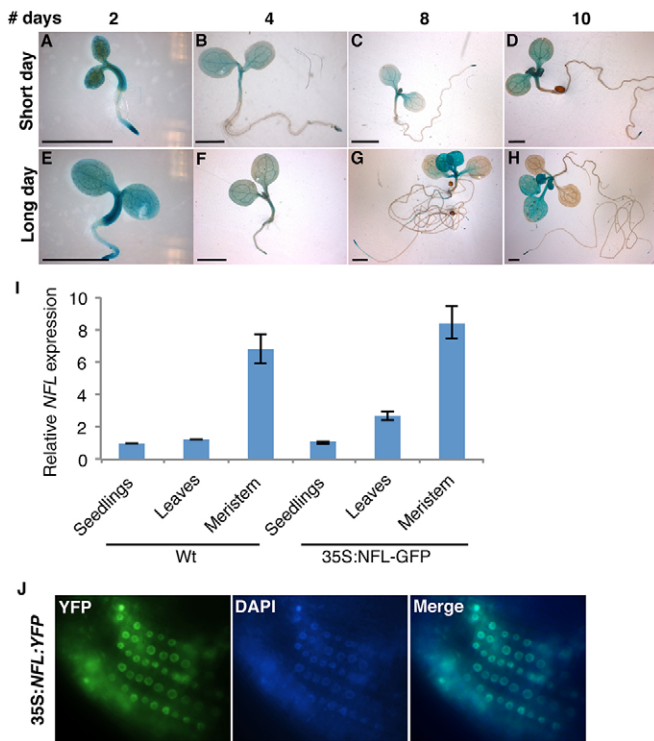


Fig. 4. Tissue-specific expression of *NFL* and subcellular localization of *NFL*. (A-H) Tissue-specific expression of *NFL* under SD (A-D) and LD (E-H) conditions. Transgenic plants express the *pNFL:NFL-GUS:NOS* construct in the wt Col-0 background. Histochemical GUS staining was performed on young seedlings grown for 2 to 10 days under SD and LD conditions as indicated. Scale bars: 1 mm. (I) qRT-PCR analysis shows more robust *NFL* expression in meristem tissue compared with seedlings and leaves. RNA was isolated from 4-day-old seedlings or leaves and meristem tissue from 45-day-old adult plants grown under 12 h:12 h dark:light cycles. Error bars indicate s.e.m. ($n \geq 3$). (J) *NFL* localizes to the nucleus. Subcellular localization of *NFL*-YFP fusion protein in transgenic plants. Left panel shows the YFP fluorescence, the middle panel shows DAPI staining of the nuclei and the right panel shows the merge of YFP and DAPI signals.

The expression pattern of *NFL* was monitored during diurnal growth conditions using publicly available data (<http://diurnal.mocklerlab.org/>) (Michael et al., 2008). *NFL* expression is regulated under diurnal conditions with a peak at 12-16 h after dawn under SD conditions (Fig. S9A). Under LD conditions, *NFL* is expressed constitutively without much variation (Fig. S9B). A more robust diurnal regulation of *NFL* expression under SD conditions is consistent with the role of *NFL* in regulation of flowering time specifically under SD conditions.

***NFL* is localized to the nucleus**

To determine the subcellular localization of *NFL*, we introduced the *p35S:NFL-YFP* transgene into the wt background and selected single-copy homozygous transgenic plants. These plants expressing *NFL*-YFP fusion protein were stained with DAPI and examined under a fluorescence microscope. Strong YFP fluorescence was observed in an organelle that was also stained with DAPI (Fig. 4J), suggesting that *NFL*-YFP is localized in the nucleus in these stable transgenic plants. We also examined whether *NFL* can homodimerize using yeast two-hybrid assays. Results showed that *NFL* can homodimerize (Fig. S10), suggesting that *NFL* might function as a transcriptional regulator in the nucleus, controlling gene expression as expected like other bHLH proteins.

***NFL* regulates several genes involved in GA and other flowering pathways at the adult stage**

To address transcriptional regulation by *NFL*, we investigated the molecular phenotype of *nfl* mutant compared with the wt control. During growth of the *nfl* mutant and wt plants under SD conditions, the phenotypes of the *nfl* mutants (e.g. defective meristem, dark green and twisted leaves) become more evident at later stages of development (Fig. S11). Therefore, we performed quantitative RT-PCR assays for selected GA pathway and flowering-time genes. Total RNA was isolated from the apical tissues including the meristem from 60-day-old wt and *nfl* mutant plants prior to the floral transition as indicated by the equal expression of *API* between wt and *nfl* mutants (Fig. 5). Strikingly, the results show that expression of GA biosynthetic genes (*GA3ox1*, *GA3ox2* and *GA20ox1*) was downregulated and GA catabolic genes (*GA2ox2* and *GA2ox7*) were upregulated in the *nfl* mutants compared with the wt (Fig. 5A). Expression of GA signaling genes (*RGA*, *GAI* and *RGL1*) was reduced, whereas the expression of GA receptor genes (*GID1A*, *GID1B* and *GID1C*) was upregulated in the *nfl* mutants compared with the wt (Fig. 5B), suggesting feedback regulation of these genes. Analyses of vernalization and other flowering-time genes also show that *FLC* and *MAF5* were slightly upregulated, whereas *LFY* was downregulated in the *nfl* mutants compared with wt controls (Fig. 5C). These data strongly suggest that the various developmental defects (e.g. short stature, twisted and dark color leaves, no flowering under SD) of the *nfl* mutants are mainly due to a reduced level of GA. However, *NFL* might also regulate other pathways including, *FLC*- and *MAF5*-mediated pathways, that might contribute to the no-flowering phenotype of the *nfl* mutants compared with the wt under SD conditions.

GA biosynthesis and metabolism is altered in *nfl* mutants compared with the wild type

Because application of GA rescued the *nfl* mutant phenotype similar to the *della* quadruple mutants under SD conditions, we measured GA biosynthetic and catabolic intermediates from 55-day-old wild-type and *nfl* apical tissue. Our results show that the levels of particularly GA_{12} were lower in the mutant compared with wt plants (Table 1), indicating either reduced GA biosynthetic activity in the early steps of the pathway or increased activity of an early catabolic step, as confirmed by qRT-PCR for *GA2ox7*. Levels of other GAs of the biosynthetic pathway, including GA_{24} and GA_{19} , are also lower in the mutant compared with the wt indicating lower expression of GA 20-oxidases, as has been shown by qRT-PCR (Fig. 5). GA 20-oxidases catalyze several successive oxidation steps at C20 of the GA molecule. Low GA 20-oxidase activity frequently leads to accumulation of intermediate precursors (Lange et al., 1993). Therefore, in the mutants, the slightly higher levels of GA_{15} might be due to reduced activity of GA 20-oxidase, and the slightly higher levels of GA_9 might be due to reduced GA 3-oxidase activity (Table 1, Fig. 5A). These data suggest a broad perturbation in the GA biosynthetic and catabolic pathways in the mutant including 20-oxidation, 3-oxidation and early 2-oxidation steps that contributes to the phenotype under SD conditions.

DISCUSSION

Arabidopsis thaliana is a facultative LD plant where transition from vegetative to reproductive phase is accelerated under LD conditions compared with SD conditions. *Arabidopsis* still flowers under SD conditions, but much later than under LD conditions. The molecular basis for the facultative flowering behavior is still unknown. Here, we provide genetic evidence that a transcription factor, called *NFL*, is

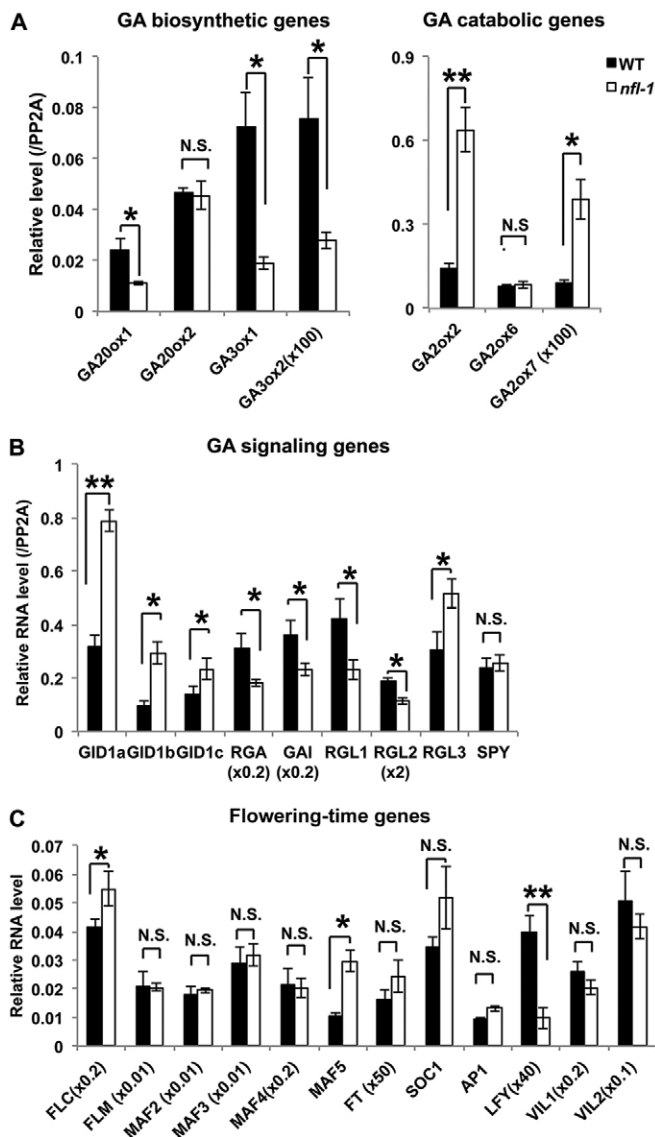


Fig. 5. Quantitative RT-PCR analyses of various genes in *nfl* mutant and wt apical tissues at the adult stage. qRT-PCR analyses of GA biosynthetic and catabolic genes (A), GA signaling genes (B) and flowering-time genes including vernalization pathway genes (C). Total RNA was isolated from meristem tissues of 60-day-old *nfl* and wt plants grown under SD conditions. Numbers in parentheses on x-axes were used to multiply the expression level to plot the data on the same graph. Error bars indicate s.e.m. ($n \geq 3$). * $P \leq 0.05$ and ** $P < 0.005$; N.S., not significant.

essential to induce flowering specifically under SD conditions in *Arabidopsis*. Some autonomous pathway mutants also fail to flower under SD. However, they flower very late under LD as well (Kim et al., 2009). Unlike known late-flowering mutants, *nfl* mutants failed to flower only under SD conditions, but not under LD conditions (Figs 2 and 3). Thus, NFL is absolutely required for the transition from vegetative to reproductive phase under SD conditions.

Previously, flowering-time mutants that are affected only under SD conditions have been reported. For example, *phyB*, *dnf*, *spa1* and *cop1* flower early, whereas *vill1*, *vill2* and a gain-of-function mutation in *IAA7/AXR2* (*axr2-1*) flower late specifically under SD conditions (Ishikawa et al., 2006; Kim and Sung, 2010; Laubinger et al., 2006; Mai et al., 2011; McNellis et al., 1994; Morris et al., 2010; Reed et al., 1993; Sung et al., 2006). However, *vill1*, *vill2* and *axr2-1* mutants display quantitative differences in flowering time

Table 1. Endogenous gibberellin (GA) levels in meristems of wt and *nfl-1* plants (13-H and 13-OH GAs)

	wt	<i>nfl-1</i> mutant
13-H GAs		
GA ₁₂	308.0±11.3	167.9±8.4*
GA ₁₅	17.6±0.4	25.4±0.3*
GA ₂₄	168.4±9.1	109.6±13.0*
GA ₉	0.9±0.0	1.1±0.0*
GA ₄	4.2±0.5	3.6±0.2 ^a
GA ₃₄	8.3±0.0	6.4±0.3*
GA ₅₁	3.9±0.0	4.2±0.1 ^a
13-OH GAs		
GA ₅₃	28.5±1.4	27.8±0.4*
GA ₄₄	0.5±0.1	0.3±0.0*
GA ₁₉	21.0±1.5	13.8±1.6*
GA ₂₀	0.1±0.0	0*
GA ₁	0	0*
GA ₈	0.3±0.0 ^a	0.2±0.0*
GA ₂₉	0.1±0.1	0*

GA analysis was performed three times with individual plant samples, except for ^awhere only two samples were analyzed. Means (ng g^{-1} dry weight) with s.e.m. are shown. Asterisks indicate significant differences between wt and *nfl-1* mutant plants ($P < 0.05$, Student's *t*-test).

compared with the wt and still flower under SD conditions (Kim and Sung, 2010; Mai et al., 2011; Sung et al., 2006). Although the *gal* mutant also failed to flower under SD, *gal* flowers significantly later under LD as well (Michaels and Amasino, 1999b; Wilson et al., 1992). However, the *nfl* mutants failed to flower in SD without any discernible delay in flowering under LD conditions. Therefore, NFL represents a novel positive regulator of floral transition functioning specifically under SD conditions.

Phenotypic characterizations and gene expression assays strongly suggest that *nfl* is defective in the GA pathway (Figs 3 and 5, Fig. S5, Table 1). Several lines of evidence support this hypothesis. First, although the rosette size of *nfl* mutant plants was similar to wt plants (Fig. 3, Fig. S5), the stunted growth, reduced fertility, curly and darker leaves suggest that *nfl* plants might have defects in the GA pathway. Second, exogenous application of GA completely rescued all the phenotypes of *nfl* mutants, including the flowering-time phenotype under SD conditions (Fig. 3A, Fig. S5). Third, the *della* quadruple mutant completely suppressed the late-flowering phenotype of *nfl* plants under SD conditions (Fig. 3B). Fourth, the qRT-PCR assays using the apical tissues from 60-day-old plants displayed strong difference in expression of GA biosynthetic and catabolic intermediates showed broad alteration in this pathway in the mutants (Table 1). In addition, downregulation of GA biosynthetic genes (e.g. *GA3ox1*, *GA3ox2* and *GA20ox1*), upregulation of GA catabolic genes (e.g. *GA2ox2* and *GA2ox7*) and upregulation of the GA receptor genes (e.g. *GID1A*, *GIF1B* and *GID1C*) at the apical tissue of *nfl* mutants compared with wt plants support this perturbation of the GA biosynthetic or catabolic pathway. Because the GA pathway has strong feedback regulation, as previously shown (Griffiths et al., 2006), these data strongly suggest that *nfl* is defective in the GA pathway. This behavior is similar to the *svp* mutant, although *svp* flowers late under both LD and SD conditions (Andres et al., 2014).

In addition to defects in the GA pathway, *nfl* mutants also displayed defects in other flowering-time pathways. Prolonged vernalization did not rescue the late-flowering phenotype of *nfl* mutants (Fig. S6), suggesting that the vernalization pathway is defective in this mutant. However, prolonged vernalization of *gal*

mutant also did not result in flowering under SD conditions, similar to results with *nfl* mutants (Fig. 4) (Michaels and Amasino, 1999b), suggesting that the lack of flowering of the *nfl* mutants after vernalization treatment might be due to defects in GA pathway under SD. However, the expression of *FLC* and *MAF5* is upregulated in *nfl* compared with the wt in adult plants, suggesting that NFL is also required for repressions of some FLC clade members. *vil2* mutants flower later only under SD as a result of de-repression of *MAF5* (Kim and Sung, 2010). Thus, NFL might partly contribute to *MAF5*-mediated floral promotion in SD. In addition, variations in day length showed that the *nfl* mutants failed to flower as soon as the LD photoperiod pathway was turned off (i.e. the day length was 12 h or shorter) (Fig. 2). The *nfl* mutants flowered, although later than wt controls, when the day length was increased to 14 h light, a condition where the LD photoperiod pathway was still operative. Taken together, these data suggest that *nfl* is defective not only in GA pathway, but also under the non-inductive SD and vernalization pathways.

In summary, the data presented here suggest that NFL functions mainly in the GA pathway with additional defects in other flowering-time pathways (Fig. 6). Because *nfl* plants never flowered under SD, but flowered like the wt under LD conditions, NFL might be involved in specifying the facultative flowering behavior of *Arabidopsis*. In this case, facultative plants might have evolved with regulators such as NFL that provide an evolutionary advantage for reproductive success of facultative flowering behavior in varying environmental conditions. Studies on the photoperiod-dependent regulation of NFL, as well as identification and characterization of direct targets of NFL, will help us to understand the facultative behavior of flowering plants.

MATERIALS AND METHODS

Plant growth conditions and phenotypic analyses

Plants were grown in Metro-Mix 200 soil (Sun Gro Horticulture, Bellevue, WA, USA) under 24 h light or long day (LD, 16 h light, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 8 h dark), short day (SD, 8 h light, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 16 h dark), 14 h:10 h, 12 h:12 h or 10 h:14 h light:dark photoperiod at $21 \pm 0.5^\circ\text{C}$. Light fluence rates were measured using a spectroradiometer (model EPP2000; StellarNet) as described (Shen et al., 2005). T-DNA-tagged *nfl* seeds from the SALK collection were obtained from the *Arabidopsis* Biological Resource Center (Alonso et al., 2003). Seeds were surface sterilized and plated on Murashige and Skoog growth medium (GM) containing 0.9% agar without Suc (GM-Suc) as described (Shen et al., 2005). After 4 days of stratification at 4°C , seeds were exposed to SD or LD or continuous white light conditions. T-DNA insertion lines were PCR-screened using primers described in Table S1.

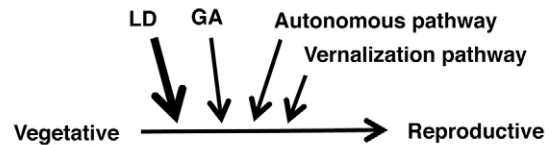
Generation of the quintuple mutant and phenotypic analyses

The *rga-28* (Tyler et al., 2004), *gai-td2* (Plackett et al., 2014) and *rgl1-SK62* (Park et al., 2013) mutants have been described. The *rgl2* mutant was isolated from the *Arabidopsis* Biological Resource Center (SAIL_345). These mutants were crossed sequentially with *nfl-1* to produce the *rga gai rgl1 rgl2 nfl* quintuple mutant. Determination of the flowering time under SD conditions is described above. The experiment was repeated three times and an average of all three experiments is shown.

Complementation analysis

To confirm the role of NFL in controlling flowering time in SD, a genomic DNA fragment containing the entire NFL gene with 1953 bp promoter and 531 bp 3'-untranslated regions (*pNFL:NFL*) was transformed into the *nfl-1* background. Single-locus transgenic plants were selected based on kanamycin resistance. Homozygous transgenic lines were grown in SD and flowering time was quantified using number of days and number of rosette leaves.

Inductive LD photoperiod



Non-inductive SD photoperiod

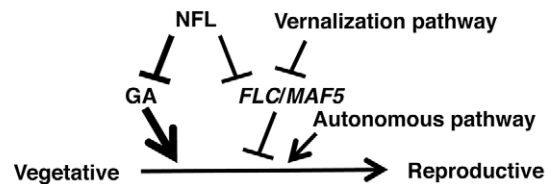


Fig. 6. A simplified model showing the role of NFL in a photoperiod-dependent regulation of flowering time. NFL is not necessary for floral transition under inductive photoperiod (LD) conditions (top), where the LD pathway plays a major role in regulating flowering time. Autonomous, GA and vernalization pathways play minor roles under LD conditions. By contrast, NFL is essential for floral transition under non-inductive photoperiod (SD) conditions (bottom). GA also plays a major role, whereas autonomous and vernalization pathways play minor roles in promoting flowering time under non-inductive SD conditions.

Exogenous GA application and vernalization response assays

GA_4 (100 μM) was applied twice a week directly on the meristem of the wt, *nfl-1*, *nfl-2*, *gai1* and *phyB* mutant plants grown under SD starting at day 6 until the plants flowered (increasing amount of GA_4 starting at 10 μl up to 300 μl). Flowering time was quantified using the number of rosette leaves produced at the time of bolting. For vernalization response assays, seeds were surface sterilized and plated on GM-Suc plates as described above and exposed to SD for germination for 7 days. Then the seedlings were transferred to 4°C for 6, 8 and 10 weeks. Seedlings were transplanted on soil and then grown under SD (8 h light:16 h dark) conditions at 21°C until bolting.

Spatial and temporal analyses of NFL expression

For tissue-specific and developmental expression of NFL, a 3680 bp DNA fragment including the 1953 bp promoter and the complete open reading frame without the stop codon was amplified by PCR using PFU polymerase (Stratagene, La Jolla, CA) and cloned into pBI121 vector to replace the 35S promoter. This construct (*pNFL:NFL-GUS*) was then transformed into the wt using the *Agrobacterium*-mediated transformation protocol as described (Clough and Bent, 1998). Single-locus transgenic plants were selected based on kanamycin resistance. A transgenic plant carrying the *pNFL:NFL-GUS* transgene in the wt background was crossed into the *nfl-1* mutant and homozygous lines were produced for GUS analysis. Homozygous transgenic lines were grown on GM-Suc plates for various time points under SD, LD and continuous light as indicated, and histochemical GUS assays were performed as described (Shen et al., 2007).

Subcellular localization of NFL

For the subcellular localization assay, the open reading frame of NFL without the stop codon was cloned into pENTR_D_TOPO vector (Invitrogen) and recombined with a destination vector pB7WGY2 (Karimi et al., 2005). This construct, named *p35S:NFL-YFP*, was sequenced and then transformed into wt plants using the *Agrobacterium*-mediated transformation protocol as described (Clough and Bent, 1998). Several homozygous transgenic plants containing the transgene were identified based on Basta selection. Four-day-old dark-grown *p35S:NFL-YFP* seedlings were used to investigate the subcellular localization of NFL in stable transgenic plants using a fluorescent microscope.

Quantitative RT-PCR analyses

The qRT-PCR was performed as previously described (Kim and Sung, 2010; Moon et al., 2008). Briefly, apical tissues including meristem were harvested from 60-day-old wt and *nfl* mutant plants grown under SD conditions at T0 (just prior to turning on light at dawn) and total RNA was isolated as described above. Real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems) using the Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems). *PP2A* (At1g13320) was used as a control for normalization of the expression data. The resulting cycle threshold (CT) values were used for calculation of the levels of expression of different genes relative to *PP2A* as follows: $2^{\Delta CT}$ where $\Delta CT = CT(PP2A) - CT(\text{specific gene})$. Primer sequences used for semi-quantitative RT-PCR and qRT-PCR are listed in Table S1.

Measurement of GA pathway intermediates

Apical tissues from 72 plants were harvested from 55-day-old SD-grown plants, in liquid nitrogen, freeze dried and ground and pooled as one replicate; three of these replicates for each Col-0 and *nfl-1* mutant plants were prepared for gibberellin analyses. Plant material (100 mg dry weight) was spiked with 17,17-d2-GA standards (1 ng each; from Prof. L. Mander, Australian National University, College of Physical and Mathematical Sciences, Canberra, Australia). Samples were extracted, purified, derivatized and analyzed by combined gas chromatography-mass spectrometry using selected ion monitoring as described (Lange et al., 2005). Seven successive GAs of the nonhydroxylated pathway (GA₁₂, GA₁₅, GA₂₄, GA₉, GA₅₁, GA₄ and GA₃₄) and seven of the 13-hydroxylated pathway (GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₂₉, GA₁ and GA₈) were further analyzed.

Acknowledgements

We thank Dr Byung-Ho Kang for sectioning and taking GUS pictures of the apical tissue, Anja Liebrandt for preparing GC-MS sample and Dr Tai-ping Sun for sharing the *rag-28* allele.

Competing interests

The authors declare no competing or financial interests.

Author contributions

The project was designed by E.H., N.S. and R.X. Most experiments were conducted by N.S., R.X., D.-H.K. and T.L.; N.S. and E.H. wrote the manuscript; S.S., R.X., D.-H.K. and T.L. commented on the manuscript.

Funding

This work was supported by grants from the National Institutes of Health (NIH) [1R01 GM-114297], National Science Foundation [MCB-1543813 to E.H.], a Human Frontier Science Program grant [HFSP# RGP0025/2013 to E.H.] and a National Science Foundation grant [IOS-0950785 to S.S.]. Deposited in PMC for release after 12 months.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.128595/-DC1>

References

- Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K. and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309**, 1052-1056.
- Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R. et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653-657.
- Andrés, F. and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nat. Rev. Genet.* **13**, 627-639.
- Andres, F., Porri, A., Torti, S., Mateos, J., Romera-Branchat, M., Garcia-Martinez, J. L., Fornara, F., Gregis, V., Kater, M. M. and Coupland, G. (2014). SHORT VEGETATIVE PHASE reduces gibberellin biosynthesis at the *Arabidopsis* shoot apex to regulate the floral transition. *Proc. Natl. Acad. Sci. USA* **111**, E2760-E2769.
- Chen, M. and Ni, M. (2006). RFI2, a RING-domain zinc finger protein, negatively regulates CONSTANS expression and photoperiodic flowering. *Plant J.* **46**, 823-833.
- Chen, H. D., Huang, X., Gusmaroli, G., Terzaghi, W., Lau, O. S., Yanagawa, Y., Zhang, Y., Li, J. G., Lee, J.-H., Zhu, D. M. et al. (2010). *Arabidopsis* CULLIN4-damaged DNA binding protein 1 interacts with CONSTITUTIVELY PHOTOMORPHOGENIC1-SUPPRESSOR OF PHYA complexes to regulate photomorphogenesis and flowering. *Plant Cell* **22**, 108-123.
- Cheng, H., Qin, L. J., Lee, S. C., Fu, X., Richards, D. E., Cao, D., Luo, D., Harberd, N. P. and Peng, J. (2004). Gibberellin regulates *Arabidopsis* floral development via suppression of DELLA protein function. *Development* **131**, 1055-1064.
- Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.
- Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C. et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* **316**, 1030-1033.
- Covington, M. F., Panda, S., Liu, X. L., Strayer, C. A., Wagner, D. R. and Kay, S. A. (2001). ELF3 modulates resetting of the circadian clock in *Arabidopsis*. *Plant Cell* **13**, 1305-1316.
- Davis, S. J. (2009). Integrating hormones into the floral-transition pathway of *Arabidopsis thaliana*. *Plant Cell Environ.* **32**, 1201-1210.
- Domagalska, M. A., Sarnowska, E., Nagy, F. and Davis, S. J. (2010). Genetic analyses of interactions among gibberellin, abscisic acid, and brassinosteroids in the control of flowering time in *Arabidopsis thaliana*. *PLoS ONE* **5**, e14012.
- Endo, M., Tanigawa, Y., Murakami, T., Araki, T. and Nagatani, A. (2013). PHYTOCHROME-DEPENDENT LATE-FLOWERING accelerates flowering through physical interactions with phytochrome B and CONSTANS. *Proc. Natl. Acad. Sci. USA* **110**, 18017-18022.
- Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. and Putterill, J. (1999). GIGANTEA: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *EMBO J.* **18**, 4679-4688.
- Galvão, V. C., Horrer, D., Küttner, F. and Schmid, M. (2012). Spatial control of flowering by DELLA proteins in *Arabidopsis thaliana*. *Development* **139**, 4072-4082.
- Galvão, V. C., Collani, S., Horrer, D. and Schmid, M. (2015). Gibberellic acid signaling is required for ambient temperature-mediated induction of flowering in *Arabidopsis thaliana*. *Plant J.* **84**, 949-962.
- Griffiths, J., Murase, K., Rieu, I., Zentella, R., Zhang, Z.-L., Powers, S. J., Gong, F., Phillips, A. L., Hedden, P., Sun, T.-p. et al. (2006). Genetic characterization and functional analysis of the GID1 gibberellin receptors in *Arabidopsis*. *Plant Cell* **18**, 3399-3414.
- He, Y., Michaels, S. D. and Amasino, R. M. (2003). Regulation of flowering time by histone acetylation in *Arabidopsis*. *Science* **302**, 1751-1754.
- Imazumi, T., Schulz, T. F., Harmon, F. G., Ho, L. A. and Kay, S. A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in *Arabidopsis*. *Science* **309**, 293-297.
- Ishikawa, M., Kiba, T. and Chua, N.-H. (2006). The *Arabidopsis* SPA1 gene is required for circadian clock function and photoperiodic flowering. *Plant J.* **46**, 736-746.
- Jang, S., Marchal, V., Panigrahi, K. C. S., Wenkel, S., Soppe, W., Deng, X.-W., Valverde, F. and Coupland, G. (2008). *Arabidopsis* COP1 shapes the temporal pattern of CO accumulation conferring a photoperiodic flowering response. *EMBO J.* **27**, 1277-1288.
- Jiang, D., Yang, W., He, Y. and Amasino, R. M. (2007). *Arabidopsis* relatives of the human lysine-specific Demethylase1 repress the expression of FWA and FLOWERING LOCUS C and thus promote the floral transition. *Plant Cell* **19**, 2975-2987.
- Jung, J.-H., Ju, Y., Seo, P. J., Lee, J.-H. and Park, C.-M. (2012). The SOC1-SPL module integrates photoperiod and gibberellic acid signals to control flowering time in *Arabidopsis*. *Plant J.* **69**, 577-588.
- Karimi, M., De Meyer, B. and Hilson, P. (2005). Modular cloning in plant cells. *Trends Plant Sci.* **10**, 103-105.
- Kim, D.-H. and 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. USA* **107**, 17029-17034.
- Kim, D.-H., Doyle, M. R., Sung, S. and Amasino, R. M. (2009). Vernalization: winter and the timing of flowering in plants. *Annu. Rev. Cell Dev. Biol.* **25**, 277-299.
- Lange, T., Hedden, P. and Graebe, J. E. (1993). Gibberellin biosynthesis in cell-free extracts from developing *Cucurbita maxima* embryos and the identification of new endogenous gibberellins. *Planta* **189**, 350-358.
- Lange, T., Kappler, J., Fischer, A., Frisse, A., Padelfke, T., Schmidtke, S. and Pimenta Lange, M. J. (2005). Gibberellin biosynthesis in developing pumpkin seedlings. *Plant Physiol.* **139**, 213-223.
- Laubinger, S., Marchal, V., Gentilhomme, J., Wenkel, S., Adrian, J., Jang, S., Kulajta, C., Braun, H., Coupland, G. and Hoecker, U. (2006). *Arabidopsis* SPA proteins regulate photoperiodic flowering and interact with the floral inducer CONSTANS to regulate its stability. *Development* **133**, 3213-3222.

- Lazaro, A., Valverde, F., Pineiro, M. and Jarillo, J. A.** (2012). The Arabidopsis E3 ubiquitin ligase HOS1 negatively regulates CONSTANS abundance in the photoperiodic control of flowering. *Plant Cell* **24**, 982-999.
- Li, M., An, F., Li, W., Ma, M., Feng, Y., Zhang, X. and Guo, H.** (2015). DELLA proteins interact with FLC to repress the flowering transition. *J. Integr. Plant Biol.* doi:10.1111/jipb.12451
- Lim, M.-H., Kim, J., Kim, Y.-S., Chung, K.-S., Seo, Y. H., Lee, I., Kim, J., Bong Hong, C., Kim, H.-J. and Park, C.-M.** (2004). A new Arabidopsis gene, FLK, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. *Plant Cell* **16**, 731-740.
- Liu, B., Zuo, Z., Liu, H., Liu, X. and Lin, C.** (2011). Arabidopsis cryptochrome 1 interacts with SPA1 to suppress COP1 activity in response to blue light. *Genes Dev.* **25**, 1029-1034.
- Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C. et al.** (1997). FCA, a gene controlling flowering time in Arabidopsis, encodes a protein containing RNA-binding domains. *Cell* **89**, 737-745.
- Mai, Y.-X., Wang, L. and Yang, H.-Q.** (2011). A gain-of-function mutation in IAA7/AXR2 confers late flowering under short-day light in Arabidopsis. *J. Intergr. Plant Biol.* **53**, 480-492.
- McNellis, T. W., von Arnim, A. G., Araki, T., Komeda, Y., Miséra, S. and Deng, X. W.** (1994). Genetic and molecular analysis of an allelic series of cop1 mutants suggests functional roles for the multiple protein domains. *Plant Cell* **6**, 487-500.
- Michael, T. P., Mockler, T. C., Breton, G., McEntee, C., Byer, A., Trout, J. D., Hazen, S. P., Shen, R., Priest, H. D., Sullivan, C. M. et al.** (2008). Network discovery pipeline elucidates conserved time-of-day-specific cis-regulatory modules. *PLoS Genet.* **4**, e14.
- Michaels, S. D. and Amasino, R. M.** (1999a). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.
- Michaels, S. D. and Amasino, R. M.** (1999b). The gibberellin acid biosynthesis mutant ga1-3 of Arabidopsis thaliana is responsive to vernalization. *Dev. Genet.* **25**, 194-198.
- Michaels, S. D., Himmelblau, E., Kim, S. Y., Schomburg, F. M. and Amasino, R. M.** (2005). Integration of flowering signals in winter-annual Arabidopsis. *Plant Physiol.* **137**, 149-156.
- Moon, J., Suh, S.-S., Lee, H., Choi, K.-R., Hong, C. B., Paek, N.-C., Kim, S.-G. and Lee, I.** (2003). The SOC1 MADS-box gene integrates vernalization and gibberellin signals for flowering in Arabidopsis. *Plant J.* **35**, 613-623.
- Moon, J., Zhu, L., Shen, H. and Huq, E.** (2008). PIF1 directly and indirectly regulates chlorophyll biosynthesis to optimize the greening process in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **105**, 9433-9438.
- Morris, K., Thornber, S., Codrai, L., Richardson, C., Craig, A., Sadanandom, A., Thomas, B. and Jackson, S.** (2010). DAY NEUTRAL FLOWERING represses CONSTANS to prevent Arabidopsis flowering early in short days. *Plant Cell* **22**, 1118-1128.
- Mutasa-Göttgens, E. and Hedden, P.** (2009). Gibberellin as a factor in floral regulatory networks. *J. Exp. Bot.* **60**, 1979-1989.
- Nelson, D. C., Lasswell, J., Rogg, L., Cohen, M. and Bartel, B.** (2000). FKF1, a clock-controlled gene that regulates the transition to flowering in Arabidopsis. *Cell* **101**, 331-340.
- Noh, B., Lee, S.-H., Kim, H.-J., Yi, G., Shin, E. A., Lee, M., Jung, K.-J., Doyle, M. R., Amasino, R. M. and Noh, Y.-S.** (2004). Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. *Plant Cell* **16**, 2601-2613.
- Osnato, M., Castillejo, C., Matías-Hernández, L. and Pelaz, S.** (2012). TEMPRANILLO genes link photoperiod and gibberellin pathways to control flowering in Arabidopsis. *Nat. Commun.* **3**, 808.
- Park, J., Nguyen, K. T., Park, E., Jeon, J.-S. and Choi, G.** (2013). DELLA proteins and their interacting RING Finger proteins repress gibberellin responses by binding to the promoters of a subset of gibberellin-responsive genes in Arabidopsis. *Plant Cell* **25**, 927-943.
- Plackett, A. R. G., Ferguson, A. C., Powers, S. J., Wanchoo-Kohli, A., Phillips, A. L., Wilson, Z. A., Hedden, P. and Thomas, S. G.** (2014). DELLA activity is required for successful pollen development in the Columbia ecotype of Arabidopsis. *New Phytol.* **201**, 825-836.
- Porri, A., Torti, S., Romera-Branchat, M. and Coupland, G.** (2012). Spatially distinct regulatory roles for gibberellins in the promotion of flowering of Arabidopsis under long photoperiods. *Development* **139**, 2198-2209.
- Ranjan, A., Fiene, G., Fackendahl, P. and Hoecker, U.** (2011). The Arabidopsis repressor of light signaling SPA1 acts in the phloem to regulate seedling de-etiolation, leaf expansion and flowering time. *Development* **138**, 1851-1862.
- Reed, J. W., Nagpal, P., Poole, D. S., Furuya, M. and Chory, J.** (1993). Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout Arabidopsis development. *Plant Cell* **5**, 147-157.
- Sawa, M., Nusinow, D. A., Kay, S. A. and Imaizumi, T.** (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. *Science* **318**, 261-265.
- Schomburg, F. M., Patton, D. A., Meinke, D. W. and Amasino, R. M.** (2001). FPA, a gene involved in floral induction in Arabidopsis, encodes a protein containing RNA-recognition motifs. *Plant Cell* **13**, 1427-1436.
- Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Kröber, S., Amasino, R. A. and Coupland, G.** (2006). The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in Arabidopsis. *Genes Dev.* **20**, 898-912.
- Shen, H., Moon, J. and Huq, E.** (2005). PIF1 is regulated by light-mediated degradation through the ubiquitin-26S proteasome pathway to optimize photomorphogenesis of seedlings in Arabidopsis. *Plant J.* **44**, 1023-1035.
- Shen, H., Luong, P. and Huq, E.** (2007). The F-box protein MAX2 functions as a positive regulator of photomorphogenesis in Arabidopsis. *Plant Physiol.* **145**, 1471-1483.
- Simpson, G. G.** (2004). The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of Arabidopsis flowering time. *Curr. Opin. Plant Biol.* **7**, 570-574.
- Simpson, G. G., Dijkwel, P. P., Quesada, V., Henderson, I. and Dean, C.** (2003). FY is an RNA 3' end-processing factor that interacts with FCA to control the Arabidopsis floral transition. *Cell* **113**, 777-787.
- Song, Y. H., Smith, R. W., To, B. J., Millar, A. J. and Imaizumi, T.** (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science* **336**, 1045-1049.
- Song, Y. H., Estrada, D. A., Johnson, R. S., Kim, S. K., Lee, S. Y., MacCoss, M. J. and Imaizumi, T.** (2014). Distinct roles of FKF1, GIGANTEA, and ZEITLUPE proteins in the regulation of CONSTANS stability in Arabidopsis photoperiodic flowering. *Proc. Natl. Acad. Sci. USA* **111**, 17672-17677.
- Song, Y. H., Shim, J. S., Kinmonth-Schultz, H. A. and Imaizumi, T.** (2015). Photoperiodic flowering: time measurement mechanisms in leaves. *Annu. Rev. Plant Biol.* **66**, 441-464.
- Suárez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F. and Coupland, G.** (2001). CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature* **410**, 1116-1120.
- Sung, S., Schmitz, R. J. and Amasino, R. M.** (2006). A PHD finger protein involved in both the vernalization and photoperiod pathways in Arabidopsis. *Genes Dev.* **20**, 3244-3248.
- Toledo-Ortiz, G., Huq, E. and Quail, P. H.** (2003). The Arabidopsis basic/helix-loop-helix transcription factor family. *Plant Cell* **15**, 1749-1770.
- Tyler, L., Thomas, S. G., Hu, J., Dill, A., Alonso, J. M., Ecker, J. R. and Sun, T.-P.** (2004). DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiol.* **135**, 1008-1019.
- Valverde, F., Mouradov, A., Soppe, W., Ravenscroft, D., Samach, A. and Coupland, G.** (2004). Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. *Science* **303**, 1003-1006.
- Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U. and Weigel, D.** (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. *Science* **309**, 1056-1059.
- Wilson, R. N., Heckman, J. W. and Somerville, C. R.** (1992). Gibberellin is required for flowering in Arabidopsis thaliana under short days. *Plant Physiol.* **100**, 403-408.
- Yoo, S. K., Chung, K. S., Kim, J., Lee, J. H., Hong, S. M., Yoo, S. J., Yoo, S. Y., Lee, J. S. and Ahn, J. H.** (2005). CONSTANS activates SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 through FLOWERING LOCUS T to promote flowering in Arabidopsis. *Plant Physiol.* **139**, 770-778.
- Zhang, B., Wang, L., Zeng, L., Zhang, C. and Ma, H.** (2015). Arabidopsis TOE proteins convey a photoperiodic signal to antagonize CONSTANS and regulate flowering time. *Genes Dev.* **29**, 975-987.
- Zuo, Z., Liu, H., Liu, B., Liu, X. and Lin, C.** (2011). Blue light-dependent interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis. *Curr. Biol.* **21**, 841-847.