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

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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 non-flowering phenotype in SD is rescued either by exogenous application of gibberellin (GA) or by introducing della quadruple mutants in the nfi 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 nfi 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 (L FY)] 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, FT, FPA, FLD, FVE, FLK and REF6 (Noh et al., 2004; Simpson, 2004). FCA, FT, 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. FT 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; Domagal ska 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 (ga1) 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
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 meristems (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 (Andrès 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 FLAWIN-BINDING, KELCH REPEAT AND F-BOX 1 (FKF1), GIIGANTEA (GI) and repressors ELF3, CYCLING DOF FACTOR 1 (CDF1) and RED AND FAR-RED INSENSITIVE 2 (RF2) (Chen and Ni, 2006; Covington et al., 2001; Fowler et al., 1999; Imazumii 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 FKFI and the GI-FKFI 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 crytochromes (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 FKFI stabilizes CO by direct interaction (Song et al., 2014, 2012). TOEI, in turn, prevents FKFI-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 SOCI and downstream floral identity genes, such as AP1 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). vilI and vil2 mutants flower later only under SD conditions, but do eventually flower. However, spaI, 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 homoyogous 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 (Fig. S2A,B). Both alleles (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 to flowering. 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...
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.
Exogenous application of GA_4_ 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 GA_4_ 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 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 hypocotyl 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.
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 AP1 between wt and nfl mutants (Fig. S15). 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 GA12 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 GA24 and GA19, 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 GA15 might be due to reduced activity of GA 20-oxidase, and the slightly higher levels of GA9 might be due to reduced GA 3-oxidase activity (Table 1, Fig. 5A). These data strongly 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
Numbers in parentheses on and ** to plot the data on the same graph. Error bars indicate s.e.m. (axr2-1 mutants display quantitative differences in flowering time 2010; Reed et al., 1993; Sung et al., 2006). However, et al., 2006; Mai et al., 2011; McNellis et al., 1994; Morris et al., 1992) flower late under both LD and SD conditions (Ishikawa et al., 2006; Kim and Sung, 2010; Laubinger et al., 2009). Unlike known late-flowering mutants, under SD. However, they flower very late under LD as well (Kim and Sung, 2010; Mai et al., 2011; Sung et al., 2006). Although the gal1 mutant also failed to flower under SD, gal1 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 genes (Fig. 5). Fifth, measurement of the endogenous 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 GA20ox7) 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 gal1

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

<table>
<thead>
<tr>
<th>GA</th>
<th>wt</th>
<th>nfl-1 mutant</th>
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<tr>
<td>13-H GAs</td>
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<tr>
<td>GA1</td>
<td>0.1±0.0</td>
<td>0*</td>
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<tr>
<td>GA20</td>
<td>0.1±0.0</td>
<td>0*</td>
</tr>
<tr>
<td>GA4</td>
<td>4.2±0.5</td>
<td>3.6±0.2*</td>
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<tr>
<td>GA8</td>
<td>0.3±0.0</td>
<td>0.4±0.3*</td>
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<tr>
<td>GA12</td>
<td>308.0±11.3</td>
<td>167.9±8.4*</td>
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<tr>
<td>GA16</td>
<td>17.6±0.4</td>
<td>25.4±0.3*</td>
</tr>
<tr>
<td>GA19</td>
<td>168.4±9.1</td>
<td>109.6±13.0*</td>
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<tr>
<td>GA20</td>
<td>0.9±0.0</td>
<td>1.1±0.0*</td>
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<tr>
<td>GA29</td>
<td>0.1±0.1</td>
<td>0*</td>
</tr>
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<td>GA34</td>
<td>0.5±0.0</td>
<td>0.3±0.0*</td>
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<tr>
<td>GA51</td>
<td>3.9±0.0</td>
<td>4.0±0.1*</td>
</tr>
<tr>
<td>GA53</td>
<td>28.5±1.4</td>
<td>27.8±0.4*</td>
</tr>
<tr>
<td>GA9</td>
<td>0.9±0.0</td>
<td>1.1±0.0*</td>
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<tr>
<td>GA20</td>
<td>308.0±11.3</td>
<td>167.9±8.4*</td>
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<tr>
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<td>168.4±9.1</td>
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<td>GA29</td>
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<tr>
<td>GA51</td>
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<tr>
<td>GA53</td>
<td>28.5±1.4</td>
<td>27.8±0.4*</td>
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GA analysis was performed three times with individual plant samples, except for *where 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).

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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 vil1, vil2 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, vil1, vil2 and axr2-1 mutants display quantitative differences in flowering time compared with the wt and still flower under SD conditions (Kim and Sung, 2010; Mai et al., 2011; Sung et al., 2006). Although the gal1 mutant also failed to flower under SD, gal1 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.
germplasm 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 µmol m⁻² s⁻¹ and 8 h dark), short day (SD, 8 h light, 200 µmol m⁻² s⁻¹ and 16 h dark), 14 h:10 h, 12 h:12 h or 10 h:14 h lightdark photoperiod at 21±0.5°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-t2 (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.

**Exogenous GA application and vernalization response assays**

GA₃ (100 µM) was applied twice a week directly on the meristem of the wt, nfl-1, nfl-2, gag1 and phyB mutant plants grown under SD starting at day 6 until the plants flowered (increasing amount of GA₃ 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 pB7WG2 (Karmi 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 nft 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^(-ΔCT) where ΔCT=CT(P2A)-CT(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 nft-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 (GA13, GA15, GA20, GA24, GA29, GA34) and seven of the 13-hydroxylated pathway (GA3, GA5, GA10, GA15, GA20, GA24, GA34) were further analyzed.

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