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Short Communication

Isolation and characterization of suppressors of the *early flowering 3* in *Arabidopsis thaliana*

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Abstract EARLY FLOWERING 3 (ELF3) is a circadian clock protein with a major role in maintaining circadian rhythms in plants. In this work, *elf3-101* was mutagenized by EMS in plants of the Landsberg *erecta* (L*er*) background to isolate suppressors of *elf3* and to understand the molecular mechanisms of flowering time controlled by ELF3. Two suppressors, *sel106* (L*er*) obtained from this screen and *sel5* (Col) from a previous study, were chosen for further analysis. Genetic mapping, gene expression analysis, and sequencing identified *sel106* and *sel5* as new alleles of *gi* and *fca*, respectively. Genetic interactions between *elf3* and *gi* and between *elf3* and *fca* in the control of the floral activator *FLOWERING LOCUS T* (*FT*) were also investigated. Six suppressors of *elf3* were classified at least into four subgroups based on the expression of such floral regulators as GI, CO, FT, SVP, and FLC, and on their flowering times under LL, LD, and SD. This classification scheme is useful for the characterization of unidentified suppressor mutations.

Key words: Circadian clock, ELF3, EMS-mutagenesis, FCA, GI, suppressors.

The circadian clock generates endogenous rhythms with an approximately 24-h period and controls many processes in plants and animals. In *Arabidopsis thaliana*, LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), and PSEUDO RESPONSE REGULATOR (PRR) proteins are believed to regulate the circadian clock (Fujiwara et al. 2008; Mizoguchi et al. 2002; Mizoguchi et al. 2005; Niinuma et al. 2007; Niinuma et al. 2008; Yoshida et al. 2009). *EARLY FLOWERING3* (*ELF3*), a component of the circadian clock in *Arabidopsis*, encodes a novel protein that may function as a transcriptional regulator (Hicks et al. 2001; Tajima et al. 2007). *elf3* mutants exhibit a wide range of phenotypes, including early flowering, hypocotyl and leaf elongation, and a loss of circadian rhythm under continuous light (LL). On the basis of these results, *ELF3* was proposed to act in the gating of light input to the circadian clock during the early part of the subjective night (Covington et al. 2001; Hall et al. 2003; McWatters et al. 2000). According to Reed et al. (2000), *ELF3* may affect red- and blue-light responses by altering phytochrome or cryptochrome signaling, or by altering circadian rhythms under LL, since, when transferred to continuous dark (DD), *elf3* mutants retain rhythmicity. Nevertheless, Thines and Harmon (2010) found that under DD and following temperature entrainment, the circadian rhythm was disrupted in *elf3* mutant plants, suggesting that *ELF3* is also involved in temperature signaling. Recently, we have identified *elf3* mutations as suppressors of late-flowering phenotype of plants with mutations in *LHY* and *CCA1* genes (*lhy;cca1*) under continuous light (LL; Fujiwara et al. 2008; Yoshida et al. 2009). Mutations in two MADS box genes, *SHORT VEGETATIVE PHASE* (*SVP*) and *FLOWERING LOCUS C* (*FLC*), encoding floral repressors also suppressed the late flowering phenotype of the *lhy;cca1* under LL (Fekih et al. 2009a, 2009b; Fujiwara et al. 2008). However, the mechanisms by which *ELF3* might accomplish these tasks are poorly understood.

To identify suppressors of *elf3-101* (L*er*; Yoshida et al. 2009), approximately 5,000 *elf3-101* (L*er*) seeds were mutagenized by imbibition in 0.3% EMS (Sigma Aldrich, St. Louis, MO) for 9 h followed by washing

Abbreviations: CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CO, CONSTANS; ELF3, EARLY FLOWERING 3; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUST; GI, GIGANTEA; LHY, LATE ELONGATED HYPOCOTYL; LD, long-days, LL, continuous light, SD, short-days; SVP, SHORT VEGETATIVE PHASE.

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with 0.1 M Na_2SO_3 (twice) and distilled water for 30 min (five times). The M_2 seeds were collected into pools, each of which contained approximately 25 M_1 plants. Approximately 12,000 M_2 seeds representing ~500 M_1 plants after mutagenesis of the *elf3-101* seeds were sown on soil and screened for plants that produced more leaves than *elf3-101* under LL. Two candidate suppressor lines were isolated (S#106 and 107) from two independent M_2 pools. Plants of each type were allowed to self-fertilize and the phenotypes of the mutants were confirmed in the M_3 generation under LL. The M_3 progeny were confirmed by sequencing to carry the *elf3-101* mutation (data not shown); therefore, these suppressor lines, which were obtained from M_2 plants that produced significantly more leaves than the *elf3-101* mutant, were derived from *elf3-101*. The M_3 progeny of the two suppressor lines exhibited a late-flowering phenotype similar to that of the M_2 plants, indicating that the suppressor phenotype was heritable. S#106 produced more leaves than S#107, and was therefore selected for further analysis. The characterization of S#107 will be reported elsewhere.

To test whether the suppressor mutation in S#106 was recessive or dominant, S#106 was crossed with the *elf3-101* progenitor line. F_1 plants derived from these crosses flowered at almost the same time as *elf3-101* plants and earlier than wild-type (L*er*) plants under LL (data not shown). F_2 progeny from the cross between S#106 and *elf3-101* were grown under LL, and their flowering times were scored and compared to those of S#106 (M_3) and *elf3-101* plants. The ratio of late-flowering plants with a flowering time similar to that of M_3 and other plants with a flowering time similar to that of *elf3-101* was close to 1 : 3. Thus, the suppressors behaved as monogenic recessive mutations in *elf3-101* to suppress the early-flowering phenotype under LL. The suppressor mutation was named *suppressor of elf3 106* (*sel106*).

Next, the flowering times of S#106 (*sel106;elf3-101*, L*er*) and wild-type L*er* under long-days (LD) and LL were compared (Figure 1A, B). The recently isolated S#5 (*sel5;elf3-1*, Col), obtained by the mutagenesis of *elf3-1* (Col) with heavy ion beams (Nefissi et al. submitted), was also characterized in this work. Under LL, the controls, elf3-101, wild-type (L*er*), elf3-1 and Wild type (Col), flowered when the plants had 3, 7, 5, and 21 rosette leaves, respectively. Plants carrying either of the suppressors flowered later than did those of the progenitor lines *elf3-101* or *elf3-1* under LL (Figure 1B). These differences in flowering time were statistically significant (Student's *t*-test; $p<0.05$). The number of rosette leaves produced by the suppressor candidate lines was 19 (S#106) and 31 (S#5).

The two mutant lines produced a greater number of total leaves not only under LL but also under LD (Figure 1; Nefissi et al. submitted). The flowering time for S#106 was almost the same under LL and LD (Figure 1). The

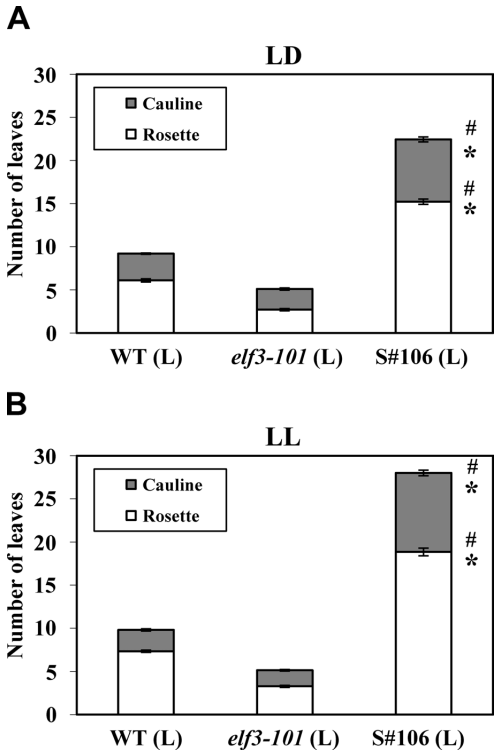

Figure 1. Flowering time of S#106 under LD and LL. (A, B) Flowering times of wild-type L*er*, *elf3-101* (L*er*), and S#106 (*elf3-101;sel106*, L*er*) plants grown under LD (A) and LL (B). Plants were grown on soil in growth chambers at 22°C. The light conditions were LD (16 h of light/8 h of dark) or LL as described previously (Fujiwara et al. 2005, 2008; Mizoguchi et al. 2002). Flowering time was scored by counting the total number of rosette (open boxes) and cauline (dark boxes) leaves on the main stem after bolting. The means±SE are shown. Each experiment was performed at least twice with similar results. Asterisks and squares denote statistical significance in comparison to value of wild-type and *elf3-101*, respectively (Student's *t*-test, $p<0.05$).

late-flowering phenotype of S#5 was much more pronounced under SD than under LD or LL (Nefissi et al. submitted). These results suggest that the s*el106* and *sel5* defects are in genes involved in the photoperiodic and autonomous/vernalization pathways, respectively.

elf3 mutant plants of the L*er* background were crossed with those of the Col background to obtain a segregating population for mapping. For each F_2 population, the aberrant-flowering phenotype under LL was scored. F_2 plants with a late-flowering phenotype were used to map recessive suppressor mutations in S#106. The SSLP markers shown in Supplemental Table 1 were used to analyze pooled DNA for an initial linkage test. The *sel106* mutation was placed on the genetic map, and additional recombination analyses were performed with newly created SSLP markers to fine-map the mutated region to a small physical interval. This approach resulted in the mapping of *sel106* to the upper side of Chromosome 1, between the markers Ch1-8037559 (8.0 Mb) and Ch1-10860088 (10.8 Mb). This region includes

the gene encoding GIGANTEA (GI), the predominant floral activator in the photoperiodic flowering pathway (Fowler et al. 1999; Mizoguchi et al. 2005). *gi* mutants flower later than wild-type plants under LL and LD but not under SD (Fowler et al. 1999; Fujiwara et al. 2008), suggesting *gi* as a candidate gene responsible for the *sel106* mutation. *GI* was sequenced in the S#106 mutant and a point mutation in the 8th exon was identified (Figure 2A) that generates a premature stop codon (Figure 2A). The *gi-1* mutation together with the *ft-1*, *fwa-1*, *ld-1*, and *fca-9* mutations was shown to suppress the early-flowering phenotype of *elf3-1* (Chou and Yang 1999). These results suggest that the late-flowering mutation contained in the *sel106* line is the result of a *gi* mutant allele.

The *sel5* mutation was initially mapped to the middle of Chromosome 4, between the markers Ch4-7549144 (7.54 Mb) and Ch4-11022419 (11.02 Mb) (Nefissi et al. submitted). This region includes the gene encoding the RNA-binding protein FCA (Marknight et al. 1997). FCA was shown to be the predominant floral regulator in the autonomous pathway and to regulate the expression of *FLC*, a major floral repressor (Marknight et al. 1997; Michaels and Amasino 2001). In fact, *fca* mutants flower later than wild-type plants under LD and LL, and the late-flowering phenotype of the mutants is enhanced under the non-inductive condition SD (Koornneef et al. 1991; Fujiwara et al. 2008). Late-flowering phenotype of the S#5 was much pronounced under SD (Nefissi et al. submitted). Thus, *fca* was considered a candidate gene for the *sel5* mutation. Sequencing of *FCA* in the S#5 mutant revealed a 14-bp deletion in the intron 1 (Figure 2B).

To confirm that the *fca* mutation in S#5 was responsible for the delayed flowering of the *elf3-1* plants, allelism between *fca-1* (L*er*) and S#5 (*elf3-1;sel5*, Col) was analyzed. All three mutations, *elf3*, *fca*, and *sel5*, were found to be recessive (Marknight et al. 1997; Nefissi et al. submitted; Zagotta et al. 1996). F_1 plants obtained from crosses between *fca-1* (L*er*) and S#5 (*elf3-1;sel5*, Col) flowered later than F_1 control plants obtained from crosses between wild-type L*er* and S#5 (*elf3-1;sel5*, Col) under LL (Figure 2C). As controls, wild-type Col, wild-type L*er*, and *elf3-1* (Col) plants were grown under LL. The flowering time of the F_1 control plants (S#5×wild-type L*er*) was similar to that of wild-type L*er*, indicating that the late-flowering mutation of the *sel5* line was indeed due to an *fca* mutant allele.

Although genetic interactions between *elf3* and *gi* and between *elf3* and *fca* have been investigated (Chou and Yang 1999; Kim et al. 2005; Yu et al. 2008), analysis on gene expression levels of floral activators and repressors in double mutants *elf3;gi* and *elf3;fca* have not been reported. Therefore, these were analyzed in S#106 (*sel106;elf3-101*; L*er*), S#5 (*sel5;elf3-1*; Col), *elf3-101* (L*er*), *elf3-1* (Col), and wild-type L*er* and Col plants under LL. As reported previously, the mRNA level of *FT* in the *elf3* mutants was higher than that in wild type (Figure 3; Kim et al. 2005; Yoshida et al. 2009). Both suppressor mutations (*sel106* and *sel5*) decreased *FT* expression in *elf3* (Figure 3). In S#5, the increased level of expression of the floral repressor *FLC* suggested that

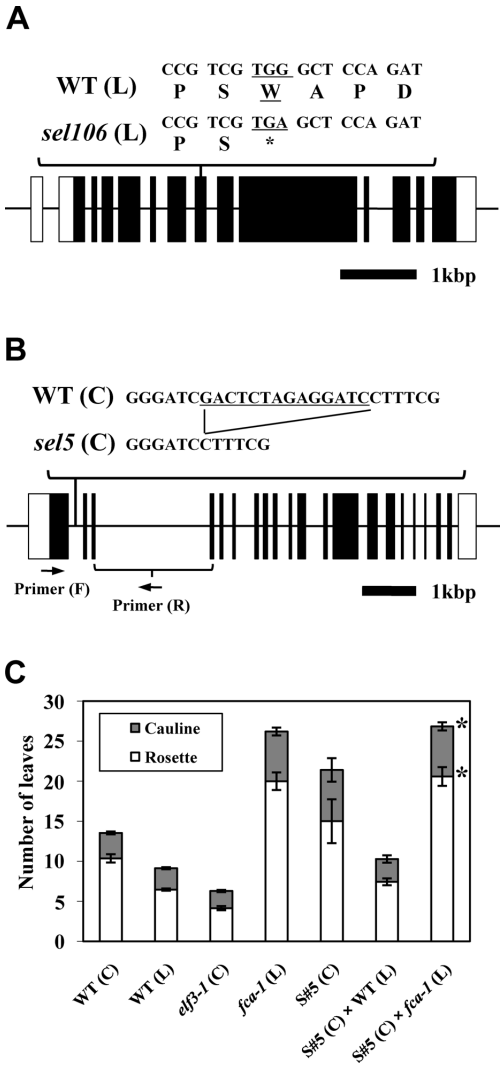

Figure 2. Identification of *sel106* and *sel5* as candidate new alleles of *gi* and *fca*. (A) Identification of *sel106* as a point mutation in *GI*. (B) Identification of *sel5* as a deletion mutation in *FCA*. Black and white boxes represent coding and non-coding regions in exons, respectively, and black lines between these boxes indicate introns in (A) and (B). Amino acid and nucleotide sequences are shown. Primers (F: GAGGTTTCCGGCAGATGAAT and R: AAACAACAGCCTTGCTGCTG) were used for RT-PCR analysis of *FCA* transcripts in Figure 3B. (C) Analysis of allelism between *fca-1* (L*er*) and S#5 (*elf3-1;sel5*, Col). Flowering times of wild-type Col (WT [C]), wild-type L*er* (WT [L]), *elf3-1* (Col), *fca-1* (L*er*), S#5 (*elf3-1;sel5*, Col), S#5×L*er* wild-type (F_1), and S#5×*fca-1* (F_1) plants grown under LL. Plants were grown and flowering time was scored as indicated in the legend of Figure 1. The means±SE are shown. Each experiment was performed at least twice with similar results. Asterisks denote statistical significance in comparison to value of F_1 plants obtained by crossing between S#5 and L*er* wild-type (Student's *t*-test, $p<0.05$).

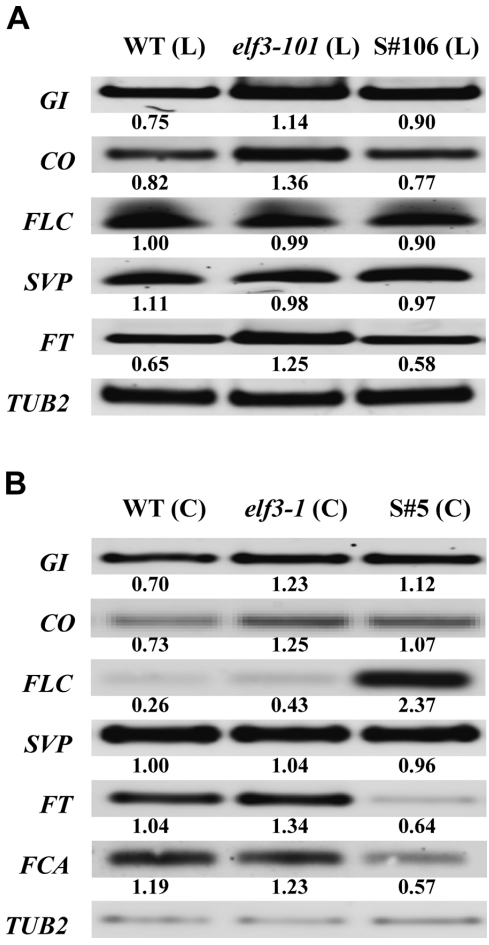

Figure 3. Effects of *sel106* and *sel5* on the expression level of the floral activators *GI*, *CO*, *FT* and *FCA* and the repressors *FLC* and *SVP* in *elf3* under LL. The abundance of *GI*, *CO*, *FLC*, *SVP*, *FT* and *FCA* transcripts relative to that of *TUB2* was measured by semi-quantitative RT-PCR in wild-type L*er* (WT), *elf3-101* (L*er*), and S#106 (*elf3-101;sel106*; L*er*) plants in (A) and wild-type Col (WT), *elf3-1* (Col), and S#5 (*elf3-1;sel5*, Col) plants in (B). RNA extraction, cDNA synthesis, RT-PCR, and gene expression analysis were performed as described (Fujiwara et al. 2008; Nefissi et al. submitted). Numbers of PCR cycles for *GI*, *CO*, *FLC*, *SVP*, *FT* , *FCA* and *TUB2* in were 30, 38, 28, 30, 35, 35 and 25, respectively. Gels were stained with Ethidium bromide and analyzed by Molecular Imager (Bio-Rad, CA, USA). Expression level of each gene relative to that in wild type (WT) is shown. The experiment was performed twice with similar results.

the gene responsible for the suppressor mutation *sel5* was a negative regulator of *FLC* (Figure 3B). FCA is a negative regulator of *FLC*, and *FCA* loss-of-function mutations delay flowering by increasing the expression of *FLC* and decreasing that of the activator gene *FT* (Kardailsky et al. 1999; Kobayashi et al. 1999). *FCA* expression (*FCA*γ and δ) in S#5 was almost half of those in *elf3-1* and wild type plants (Figure 3B). The increased level of *FLC* expression in S#5 is consistent with our finding that *sel5* is a new allele of *fca* (Figure 2B). By contrast, the late-flowering phenotype of S#106 was found to be associated with decreased *FT* expression without any effects on *FLC* (Figure 3A).

Expression of the photoperiod promotion pathway genes *GI* and *CO* in *elf3-101* (L*er*) was higher than that in wild-type L*er* under LL as reported in *elf3-1* (Figure 3A; Kim et al. 2005). In S#106, decreased *CO* expression indicated that the gene responsible for the suppressor mutation, *sel106*, was a positive regulator of *CO* (Figure 3A). GI is a positive regulator of *CO* expression, and *GI* loss-of-function mutations delay flowering by decreasing expression of the floral activators *CO* and *FT* (Mizoguchi et al. 2005). The decrease in *CO* expression in *sel106* is consistent with our finding that the latter is a new allele of *gi* (Figure 2A). By contrast, the *GI* and *CO* mRNA levels detected in S#5 were nearly the same as that in *elf3-1* and higher than that in wild-type Col (Figure 3B).

We identified nine suppressors (S#1, 3, 5, 7, 14, 15, 20, 106, and 107) of *elf3* in Col and L*er* backgrounds (Nefissi et al. submitted, this work). The mutations responsible for *sel5*, *sel20*, and *sel106* were identified as new alleles of *fca*, *cry2* (Guo et al. 1998), and *gi*, respectively. GI and CRY2 regulate CO in the photoperiodic flowering pathway while FCA plays a key role in the control of *FLC* expression in the autonomous pathway (Calvino et al. 2005; Fekih et al. 2009b; Koornneef et al. 1991; Marknight et al. 1997). CO is a central floral activator in the photoperiodic pathway (Mizoguchi et al. 2006; Putterill et al. 1995), while FLC is a repressor in the autonomous pathway (Michaels and Amasino 2001).

Based on changes in the expression of genes encoding key regulators of flowering and on enhancement of the late-flowering phenotype under SD, the six suppressors (S#5, 7, 14, 15, 20, and 106) were classified at least into four subgroups. S#1, 3 and 107 have not been fully characterized yet and will be classified. In subgroup 1, only *FT* expression was decreased, whereas the expression levels of *GI*, *CO*, and *FLC* were the same as those in *elf3* under LL. The late-flowering phenotype was pronounced under LL, but almost no suppression was observed under LD or SD. The subgroup 1 includes *sel20/cry2* (Guo et al. 1998; Nefissi et al. submitted). The expression of *CO* and *FT* in the photoperiodic pathway was reduced in subgroup 2. Neither *GI* nor *FLC* expression was affected by a suppressor mutation in this subgroup. For these suppressors, similar degrees of late flowering were observed under LD and LL conditions. Thus, this subgroup is comprised of *sel106/gi*. Although the expression of *FLC* increased while that of *FT* decreased, no change was observed in *GI* and *CO* expression in subgroup 3. The mutants in this group produced more leaves under SD than under LD and LL, suggesting that the genes responsible for the mutations belong to the autonomous/vernalization pathway. Thus, this group includes *sel5/fca*, *sel7*, and *sel15* (Nefissi et al. submitted). Mutations in subgroup 4 resulted in the *sel14*

delayed-flowering phenotype, in which *FT* expression was unaffected, suggesting that other floral activators (or repressors) are targets for the control of flowering.

Although *sel106* and *sel5* were due to mutations in well-characterized genes (*GI* and *FCA*, respectively) in an *elf3* background, these mutations are of interest as controls in the characterization of other, as yet unidentified suppressors. In addition, the phenotypes of subgroup 1 are of particular interest because they are quite unusual and resemble those of the double clock mutant *lhy;cca1* (Fujiwara et al. 2008; Mizoguchi et al. 2002; Mizoguchi et al. 2005; Yoshida et al. 2009). To understand the molecular mechanism behind the switch from photoperiodic response type LD to that of SD (Mizoguchi and Yoshida 2009), the identification and characterization of suppressors in subgroup 1 would be useful.

Although a similar level of early flowering was observed in *elf3* and *35S::CO* plants under LL, LD, and SD (Fujiwara et al. 2008; Onouchi et al. 2000; Zagotta et al. 1996), the effect of increased *FLC* expression caused by mutations in the autonomous pathway or *35S::FLC* on the early flowering of *elf3* appeared to be much stronger than that on *35S::CO* (Figure 1; Hepworth et al. 2002; Onouchi et al. 2000). The *elf3* mutation is believed to accelerate flowering time via a mechanism involving multiple pathways (Kim et al. 2005; Mizoguchi and Yoshida 2009; Yoshida et al. 2009). Why the early flowering of *elf3* was apparently more sensitive to the accumulation of *FLC* mRNA is unclear. Further analysis of the uncharacterized suppressors and enhancer of *elf3* and the identification of mutations responsible for the phenotypes are needed to address this issue.

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