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Report

FLOWERING LOCUS T Regulates Stomatal Opening

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Summary

Stomatal pores surrounded by a pair of guard cells in the plant epidermis control gas exchange for photosynthesis in response to light, CO_2, and phytohormone abscisic acid [1, 2]. Phototropins (phot1 and phot2) are plant blue-light receptor kinases and mediate stomatal opening via activation of the plasma membrane H^+-ATPase [3, 4]. However, the signaling mechanism from phototropins to the H^+-ATPase has yet to be determined. Here, we show that *FLOWERING LOCUS T* (*FT*) is expressed in guard cells and regulates stomatal opening. We isolated an *scs* (*suppressor of closed-stomata phenotype in phot1 phot2*) *1-1* mutant of *Arabidopsis thaliana* and showed that *scs1-1* carries a novel null *early flowering 3* (*elf3*) allele in a *phot1 phot2* background. *scs1-1* (*elf3 phot1 phot2* triple mutant) had an open-stomata phenotype with high H^+-ATPase activity and showed increased levels of *FT* mRNA in guard cells. Transgenic plants overexpressing *FT* in guard cells showed open stomata, whereas a loss-of-function *FT* allele, *ft-1*, exhibited closed stomata and failed to activate the H^+-ATPase in response to blue light. Our results define a new cell-autonomous role for *FT* and demonstrate that the flowering time genes *ELF3* and *FT* are involved in the regulation of H^+-ATPase by blue light in guard cells.

Results and Discussion

Stomatal opening is mediated by an inside-negative electrical potential-dependent K^+ accumulation through the K^+ channel in guard cells [1, 2]. Phototropins (phot1 and phot2) activate the plasma membrane H^+-ATPase that creates electrical potential through phosphorylation of its C terminus, which allows subsequent binding of a 14-3-3 protein [3, 4]. Since the *phot1 phot2* double mutant of *Arabidopsis thaliana* exhibits both downward curling leaves and closed stomata under light [3, 5] (Figures 1A and 1B), we expected that the disruption of common negative regulators of leaf flattening and stomatal opening would restore both phenotypes in *phot1 phot2*. To obtain such mutants, we selected revertants showing leaf flattening from 160,000 M_2 *phot1 phot2* plants treated with ethyl methanesulphonate (Figure 1A). We next examined their stomatal apertures in the epidermis and successfully isolated eight open-stoma mutants, which we designated as *scs* (*suppressor of closed stomata phenotype in phot1 phot2*) (Figure 1B). Of these, *scs1-1* was recessive and showed early flowering, suggesting that *SCS1* is a common suppressor of leaf flattening, stomatal opening, and early flowering (see Figure S1 available online).

We then analyzed the stomatal responses of *scs1-1* in more detail (Figure 1C). The wild-type stomata opened when exposed to light, whereas those of *phot1 phot2* did not. The stomata of *scs1-1* opened widely in darkness, and exhibited a closing response with the application of either the phytohormone abscisic acid (ABA) or vanadate and erythrosine B (EB), which inhibit plasma membrane H^+-ATPase [4, 6]. Both ATP hydrolysis and H^+-ATPase phosphorylation increased in response to blue light in wild-type guard-cell protoplasts (GCPs), but this was not the case in the GCPs of *phot1 phot2* (Figures 1D and 1E). Note that both processes (i.e., ATP hydrolysis and H^+-ATPase phosphorylation) were enhanced in *scs1-1* not exposed to blue light, but the amount of H^+-ATPase remained unchanged. The results indicate that stomatal opening in *scs1-1* is due to the sustained enhancement of H^+-ATPase activity in guard cells and not to an inhibition of ABA-induced stomatal closure.

To identify the *SCS1* locus in *phot1 phot2*, we performed map-based cloning, which revealed that *scs1-1* had a single nucleotide substitution in *EARLY FLOWERING 3* (*ELF3*) (Figure S2D) [7]. This mutation was localized in the splicing recognition site of the second intron and produced a missplicing of *ELF3* mRNA [8] (Figure 2A). The ELF3 protein was found by immunological assay in the guard cells of wild-type and *phot1 phot2*, but was not seen in *scs1-1* (Figure 2B; see also Figure S2G). The transformation of the wild-type genomic *ELF3* gene with its own promoter into *scs1-1* (*gELF3*/*scs1-1*) complemented all phenotypes that had been lost in *scs1-1* (Figure 2D; see also Figure S1), indicating that *ELF3* is a common negative regulator of phototropin-mediated leaf flattening and stomatal opening. Therefore, we renamed the *scs1-1* mutation as *elf3-201*. The stomata of the *elf3-201* single mutant, which had been obtained by crossing *scs1-1* with *gl1*, opened as widely as those of *scs1-1* (*elf3-201 phot1 phot2* triple mutant) (Figure S2E). An *ELF3* nonsense mutant, *elf3-1* [7], also exhibited the open-stomata phenotype (Figure S2E). These results demonstrate that *ELF3* is expressed in guard cells and that an ELF3 deficiency is responsible for the open-stomata phenotype. We obtained four allelic mutants of *ELF3* in this screening (Figures S2A–S2D).

The *elf3* mutants have been identified previously as early-flowering mutants under short days [9]. *ELF3* negatively regulates the transcription of a florigen *FLOWERING LOCUS T* (*FT*), and therefore the *elf3* mutation greatly elevates the transcription of *FT* in leaves, leading to accelerated flowering [10]. Moreover, it has been demonstrated that *FT*-overexpressing plants show up-curled rosette leaves in addition to early flowering phenotype [11]. These results suggest that leaf flattening and open stomata phenotypes in *scs1-1* is probably due to

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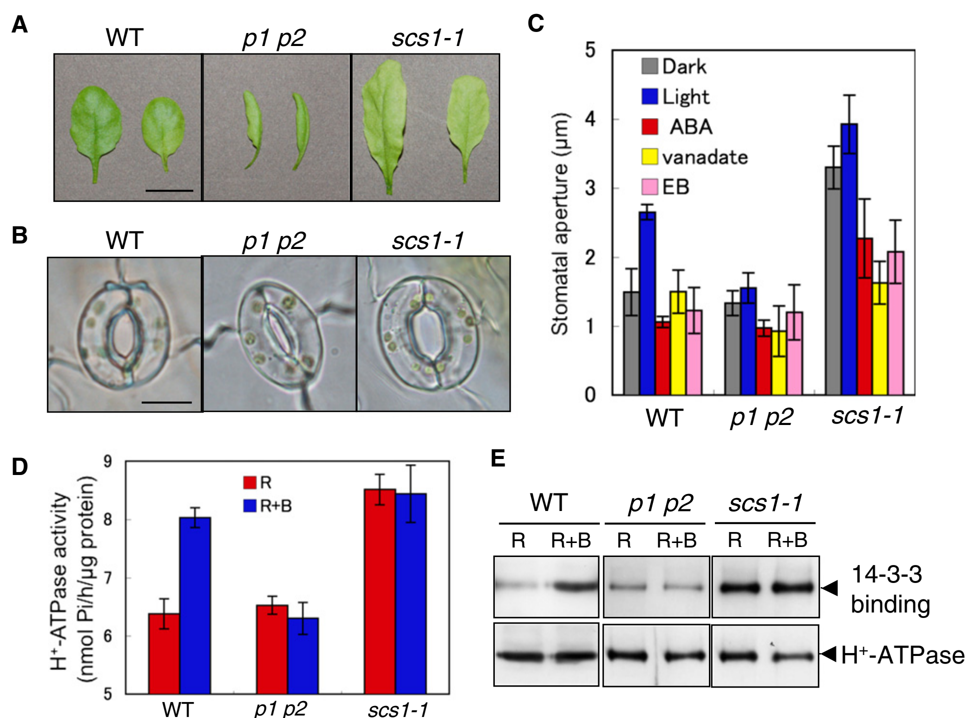

Figure 1. Stomatal Aperture and Plasma Membrane H^+-ATPase Activity of *scs1-1*

(A) Mature rosette leaves of wild-type (WT), *phot1 phot2* (*p1 p2*), and *scs1-1* plants. Scale bar represents 1 cm.

(B) Typical stomata in the epidermis, which was illuminated with blue light under background red light for 3 hr. Scale bar represents 5 μm.

(C) Stomatal apertures under various conditions. Light conditions were the same as in (B). ABA, 20 μM ABA; vanadate, 1 mM vanadate; EB, 30 μM EB. Data represent the means of 45 measurements with standard deviations (SDs). Experiments repeated on three occasions yielded similar results.

(D) ATP hydrolysis by H^+-ATPase in GCPs. The GCPs were illuminated by red light (600 $\mu mol\ m^{-2}\ s^{-1}$), with a 30 s blue-light pulse (100 $\mu mol\ m^{-2}\ s^{-1}$) superimposed on the red light. R, red light; R+B, 2 min after the onset of blue light. Data represent the means of three independent measurements, with SDs.

(E) Phosphorylation-dependent binding of 14-3-3 protein to the H^+-ATPase and the amount of the H^+-ATPase in the GCPs were assayed with protein blots and immunoblots, respectively. Since the amount of 14-3-3 protein bound to H^+-ATPase is proportional to the phosphorylation levels of H^+-ATPase [4], phosphorylation levels were estimated by protein blot analysis with GST-14-3-3 as a probe. The GCPs were illuminated as in (D).
Experiments repeated on three occasions yielded similar results. See also Figure S1.

overexpression of *FT*. Then, we examined expression of *FT* in guard cells. RT-PCR experiments showed that the *FT* transcript was present in GCPs isolated from either the wild-type or *phot1 phot2* (Figure 2A). There was no contamination of these GCPs by mesophyll cells (Figure S2F) [12–14]. To show the expression of *FT* in guard cells, we performed a promoter analysis with the *FT* promoter (*FT::GUS*/wild-type) [15]. A GUS stain was found in the guard cells of young rosette leaves but was not seen in mature leaves (Figure 2E). This result was most probably due to the low levels of expression of GUS in mature rosette leaves; the *GUS* transcript was detected by RT-PCR in GCPs from mature leaves (Figure 2F). In addition, FT protein having a molecular mass of 19 kDa was detected in GCPs by immunological assay (Figure 2G). These results indicate that *FT* is expressed in guard cells. Interestingly, the levels of the *FT* transcript were greatly enhanced in the *scs1-1* GCPs (Figure 2A). Quantitative-PCR analysis revealed that expression levels of *FT* were 50 times higher in GCPs from *scs1-1* than in those from wild-type and the background *phot1 phot2* (Figure 2C). Hence, we suspected that *FT* acts as a positive regulator of stomatal opening.

To test our hypothesis, we generated transgenic lines with the *CaMV35S* promoter for constitutive *FT* expression in the whole plant [16] (*35S::FT/phot1 phot2*), the *CER6* promoter for epidermal tissue containing guard cells [17] (*CER6::FT/phot1 phot2*), and the *KAT2* promoter for the guard cells and phloem of a minor vein [18] (*KAT2::FT/phot1 phot2*) (Figures S3A and S3B). As expected, the stomata of all of the *FT*-transgenic plants opened widely, whereas ABA and H^+-ATPase inhibitors decreased the aperture (Figures 3A and 3B). To detect the expression of the *FT* transgene in guard cells, we transformed the plants with *FT-GFP* under *CER6* promoter or a strong guard-cell promoter, *GC1* [14] (Figures 3C–3F). Both *CER6::FT-GFP/phot1 phot2* and *GC1::FT-GFP/phot1 phot2* plants showed an open-stomata phenotype, and an FT-GFP signal was detected in the nucleus and cytosol of guard cells. By contrast, similar expression of *ft-1-GFP* (*CER6::ft-1-GFP/phot1 phot2*), which exhibited a loss of FT function by a missense mutation (Gly171 to Glu) [19], did not induce stomatal opening. The results indicate that an overexpression of *FT* in guard cells induces stomatal opening and that *FT* serves as an H^+-ATPase activator. Note that both early flowering and open stomata phenotypes were observed in *FT*-transgenic plants (Figures 3A and 3B; see also Figure S3A). It would initially seem possible that flowering influences stomatal aperture. However, this is not the case. We compared light-induced stomatal opening between 4-week-old nonflowering plants and 6-week-old flowering plants. Both plants exhibited similar stomatal responses and expressed *FT* at the same levels (Figures S3C and S3D).

We analyzed the stomatal responses of *ft-1*, an *FT* loss-of-function mutant [19]. As expected, the stomata in *ft-1* and

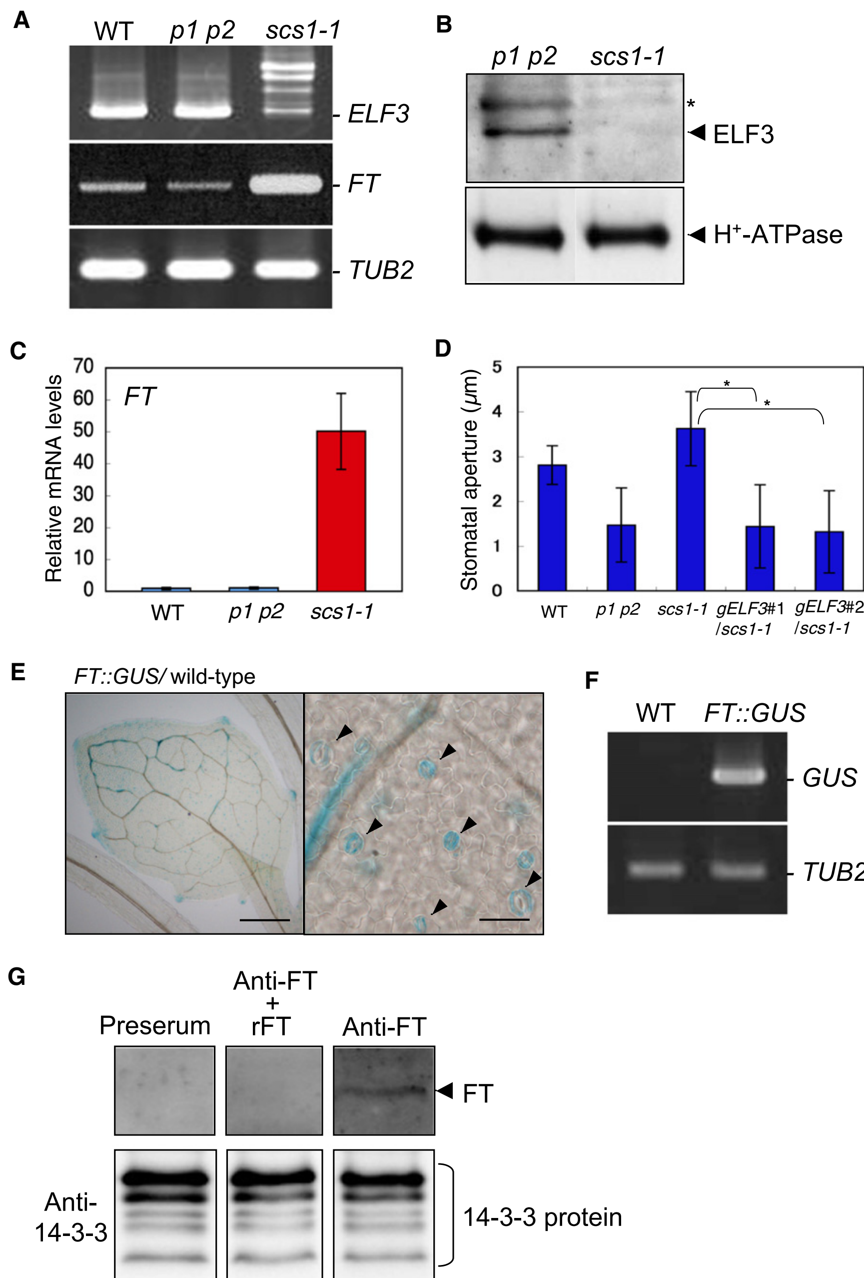

Figure 2. High *FT* Expression and Absence of ELF3 Protein in Guard Cells of *scs1-1*

(A) RT-PCR analyses of *ELF3*, *FT*, and *TUB2* in GCPs from the wild-type (WT), *phot1 phot2* (*p1 p2*), and *scs1-1*. *TUB2*, *TUBELIN BETA CHAIN2* as a control.

(B) Immunoblots of ELF3 and the H^+-ATPase proteins in GCPs. Asterisk indicates an unknown protein recognized by ELF3 antibodies. The solubilized protein (50 μg protein per lane for ELF3 and 10 μg protein per lane for the H^+-ATPase) was subjected to SDS-PAGE.

(C) Quantitative-PCR analysis of *FT* in GCPs from WT, *p1 p2*, and *scs1-1*. Data represent the means of three independent measurements, with SDs.

(D) Stomatal apertures of WT, *p1 p2*, *scs1-1*, and genomic *ELF3/scs1-1* (*gELF3/scs1-1*) under light-exposure conditions. *gELF3/scs1-1*, wild-type genomic *ELF3* gene with the native promoter, was transformed into *scs1-1*. Data represent the means of 45 measurements with SDs (Student's t test, *p < 0.01, pairs for Student's t test are indicated with brackets). #, line number of transgenic plants.

(E) GUS stain of *FT::GUS*/wild-type plants. Plants were subjected to GUS staining at ZT12 on day 16 [15]. Left, young rosette leaf. Right, magnification of leaf showing GUS stain in guard cells.

(F) RT-PCR analyses for transcripts of *GUS* and *TUB2* in GCPs from WT and promoter *FT::GUS/* wild-type (*FT::GUS*) plants. 35 cycles of PCR were performed.

(G) Immunoblots of FT in GCPs from WT. The solubilized protein (100 μg protein) was subjected to SDS-PAGE. Preserum: preserum at 1:3000 was used for first antibodies, anti-FT + rFT; FT antibodies at 1:3000 incubated with 0.5 μM recombinant FT as an antigen was used for first antibodies, anti-FT; FT antibodies at 1:3000 was used for first antibodies. Lower panels represent immunoblots of 14-3-3 protein as a loading control using 14-3-3 antibodies [4]. All experiments repeated on three occasions yielded similar results. See also Figure S2.

scs1-1 ft-1 (*elf3-101 phot1 phot2 ft-1* quadruple mutant) exhibited reduced light-induced stomatal opening (Figures 4A and 4B; see also Figure S4A). The activation and phosphorylation of the H^+-ATPase by blue light were severely inhibited in *ft-1* GCPs (Figures 4C and 4D). However, the H^+-ATPase activator, a fungal toxin fusicoccin (FC) [3], induced activation of the H^+-ATPase and stomatal opening in *ft-1* (Figures 4B-4D). The amounts of H^+-ATPase and phototropins in *ft-1* did not differ from those of the wild-type GCPs (Figure 4D; see also Figure S4B). These results suggest that the *ft-1* mutation does not affect phototropins, H^+-ATPase, or any of the other downstream components responsible for regulating stomatal opening. An *FT* nonsense mutant, *ft-2* [19], and an *FT* knockout mutant, *ft-101* [15], also exhibited the closed-stomata phenotype (Figure S4A). Thus, we conclude that a component of blue-light signaling is deficient or inactive in guard cells of *ft* mutants and that *FT* acts either as a regulator of this component or as the component itself. Open-stomata phenotype of *scs1-1* and closed-stomata phenotype of *ft* mutants are probably not due to alternations in endogenous concentrations of ABA, because the ABA levels in whole leaves did not differ from those in background plants (Figure S2H). Note that *MOTHER OF FT AND TFL1* (*MFT*), which is a homolog of *FT* and *TERMINAL FLOWER 1* (*TFL1*), has been found to regulate seed germination via ABA signaling pathway [20]. Further investigations are needed to examine detailed ABA sensitivity of stomata in *scs1-1* and *ft* mutants.

ELF3 encodes a nuclear protein of unknown biochemical activity and regulates photoperiodic floral induction [7, 21–23]. In photoperiodic floral induction, leaf sensing of day length via photoperiod-sensing photoreceptors, i.e., phytochrome A (phyA), phyB, cryptochrome1 (cry1), and cryptochrome2 (cry2), generate a signal that regulates *FT* expression [24, 25]. Downstream of photoreceptors, *ELF3* negatively regulates the expression of both *GIGANTEA* (*GI*) and *CO* [10]. It has been

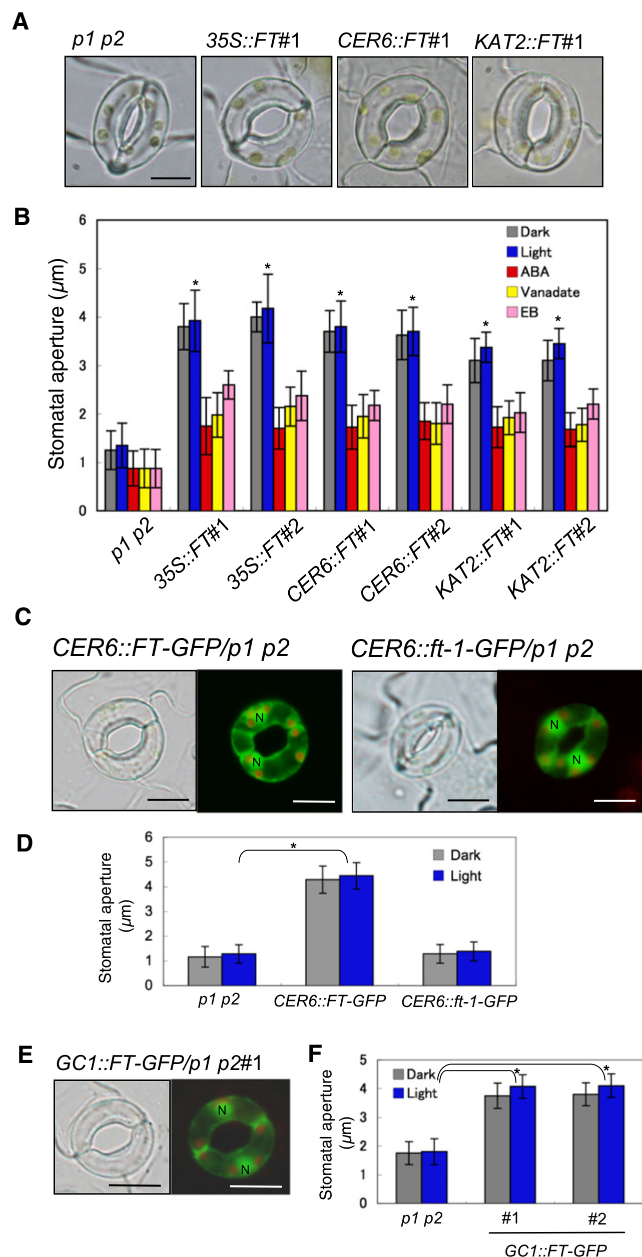

Figure 3. Wide Opening of Stomata of *FT*-Transgenic Plants

(A) Typical stomata of *phot1 phot2* (*p1 p2*), *CaMV35S::FT/p1 p2* (*35S::FT*), *CER6::FT/p1 p2* (*CER6::FT*), and *KAT2::FT/p1 p2* (*KAT2::FT*). Epidermis was illuminated as described in Figure 1B. Scale bar represents 5 μm. #, line number of transgenic plants.

(B) Stomatal apertures of various transgenic plants under different treatment conditions. The conditions were the same as in Figure 1C. Data represent the means of 45 measurements with SDs (Student's t test, *$p < 0.01$, Student's t test was performed between *p1 p2* and transgenic plants under light condition).

(C) Bright-field and fluorescent images of typical stomata from *CER6::FT-GFP/p1 p2* and *CER6::ft-1-GFP/p1 p2*. The epidermis was illuminated as described in Figure 1B. Scale bars represent 5 μm. N, nucleus.

(D) Stomatal apertures of *phot1 phot2* (*p1 p2*), *CER6::FT-GFP/p1 p2* (*CER6::FT-GFP*), and *CER6::ft-1-GFP/p1 p2* (*CER6::ft-1-GFP*). Conditions were the same as in Figure 1C. Data represent the means of 45 measurements with SDs (Student's t test, *$p < 0.01$, a pair for Student's t test is indicated with bracket).

(E) Bright-field and fluorescent images of typical stomata from *GC1::FT-GFP/p1 p2*. The epidermis was illuminated as described in Figure 1B. Scale bars represent 5 μm. N, nucleus.

(F) Stomatal apertures of *phot1 phot2* (*p1 p2*) and *GC1::FT-GFP/p1 p2* (*GC1::FT-GFP*). Conditions were the same as in Figure 1C. Data represent the means of 45 measurements with SDs (Student's t test, *$p < 0.01$, pairs for Student's t test are indicated with brackets). #, line number of transgenic plants.

All experiments repeated on three occasions yielded similar results. See also Figure S3.

demonstrated that CO directly upregulates expression of *FT* [26, 27]. The GI-CO-FT proteins act in this order at the core of the photoperiodic pathway [24]. When FT protein expresses in the phloem companion cells of leaves, FT protein moves through the phloem to the shoot apex, and, upon arriving at the shoot apical meristem (SAM), becomes bound to the bZIP transcription factor, *FD* [28, 29]. The resulting *FT/FD* complex induces the transcription of floral meristem identity genes such as a MADS-box transcription factor *APETALA1* (*AP1*), which promotes the transition from the vegetative to the reproductive stage in the SAM [30]. The present results indicate that *FT* is expressed in guard cells and acts as a positive regulator of stomatal opening. We had expected to detect in guard cells the expression of other components involved in photoperiodic floral induction. We thus identified by RT-PCR the transcripts of major components (*PHYA, PHYB, CRY1, CRY2, GI, CO, FD, TFL1, AP1*, and *FUL*) in the floral induction pathway [24, 25]. As shown in Figure 4E, all of these components were transcribed in both the wild-type and the *phot1 phot2* guard cells, suggesting that *FT* induces stomatal opening via a similar pathway to that of floral induction (Figure 4F). It is worthy of note that expression levels of *FD* and *TFL1*, a key antagonist of *FT*, are greatly reduced in *scs1-1* guard cells.

We found that the transcript of *AP1*, which is known to be a downstream component of *FT* in floral induction [28, 29], was enhanced in the guard cells of *scs1-1* (Figure 4E) and was decreased in those of *ft-1* (Figure S4C). The stomata in *AP1*-transgenic plants (*CER6::AP1/phot1 phot2*) opened widely (Figures S4D and S4E). These findings suggest that *FT* may induce stomatal opening via *AP1* in guard cells. However, it should be noted that a loss-of-function mutant, *ap1-10*, showed normal stomatal responses (Figure S4F), which was probably due to redundant role(s) of other MADS-box transcription factor(s) such as *FUL* [31] with respect to the role played by *API* in guard cells. Indeed, we found expression of *FUL* in guard cells (Figure 4E). Further investigations are needed to clarify this.

ELF3 is also shown to be involved in the gating of photic input to the circadian clock via physical interaction with phytochrome B (phyB) [7, 21–23]. The stomatal aperture shows circadian rhythms under constant-light conditions [32] and the amount of *FT* transcript also reflects circadian rhythm under the same conditions [33]. Therefore, the circadian rhythm of the stomatal aperture may be brought about by diurnal changes in the amount of *FT* via *ELF3*. In support of this hypothesis, *elf3-201* exhibited a continuously open-stomata phenotype under conditions of constant exposure to light. In contrast, *ft-1*, an *FT* loss-of-function mutant, lacked rhythmicity, and instead exhibited a continuously closed-stomata phenotype, and wild-type plants exhibited a clear circadian rhythmicity of the stomatal aperture under the same conditions (Figure S4G). In addition, our results imply that guard cells can sense photoperiods through a similar pathway to that of floral induction and that photoperiodic

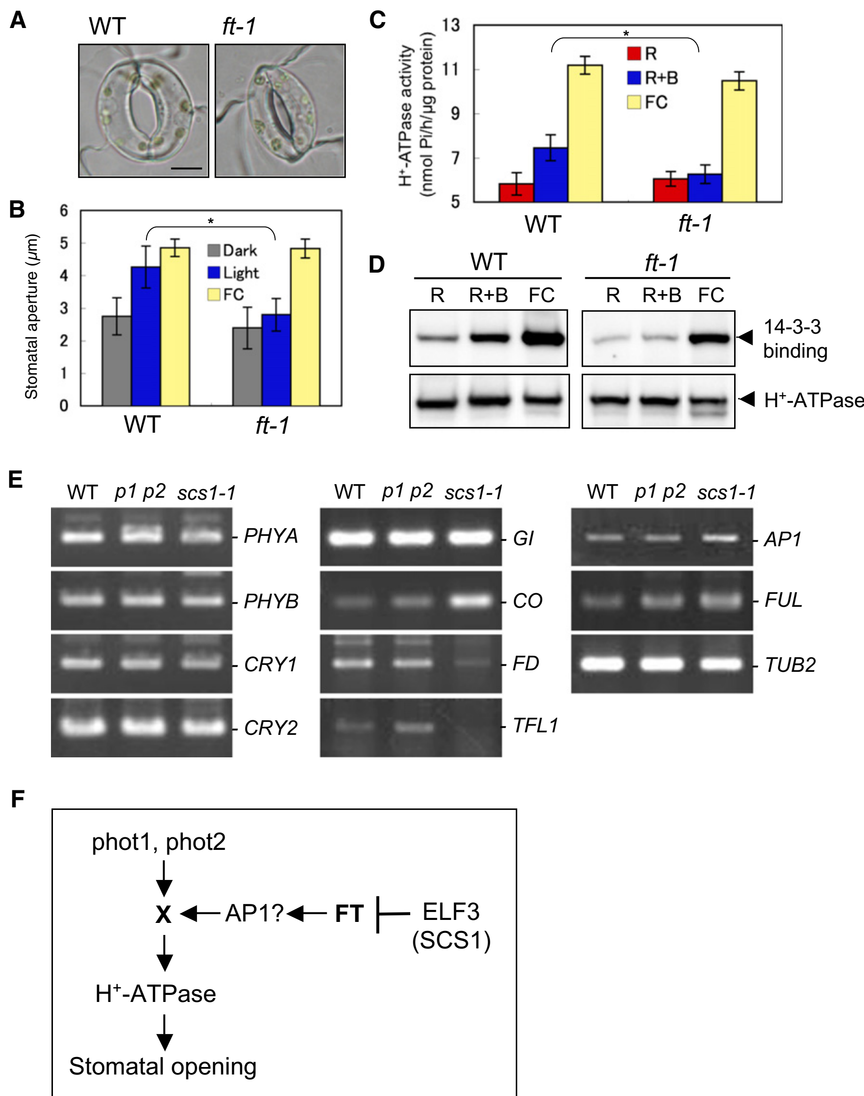

Figure 4. *FT* Is Required for Blue Light-Induced Stomatal Opening and H^+-ATPase Activation

(A) Typical stomata in wild-type (WT) and *ft-1* plants. The epidermis was illuminated as in Figure 1B. Scale bar represents 5 μm.

(B) Stomatal apertures under different treatments. Light, red and blue light as in (A). FC, the epidermis was incubated with 10 μM FC for 3 hr. Data represent the means of 45 measurements with SDs (Student's t test, *p < 0.01, a pair for Student's t test is indicated with bracket). Experiments repeated on three occasions yielded similar results.

(C) ATP hydrolysis by the H^+-ATPase in GCPs. The GCPs were illuminated as in Figure 1D. FC, 5 min after addition of 10 μM FC. Data represent the means of three independent measurements with SDs (Student's t test, *p < 0.05, a pair for Student's t test is indicated with bracket).

(D) Phosphorylation-dependent binding of 14-3-3 protein to the H^+-ATPase and the amount of H^+-ATPase in the GCPs, as in (C). Experiments repeated on three occasions yielded similar results.

(E) RT-PCR analyses of transcripts of *PHYA, PHYB, CRY1, CRY2, GI, CO, FD, TFL1, AP1, FUL*, and *TUB2* in GCPs from WT, *phot1 phot2* (*p1 p2*), and *scs1-1* plants. Experiments repeated on three occasions yielded similar results.

(F) A possible convergence of phototropin-mediated signaling and the photoperiodic pathway including *ELF3* and *FT* in guard cells. X represents an unidentified *FT*- and/or *AP1*-regulated component in blue-light signaling.

See also Figure S4.

information significantly affects stomatal movement via *FT*. In accordance with this, we found that light-induced stomatal openings were larger in plants grown under long-day compared to short-day conditions and the magnitude of response was related to the amount of *FT* transcript (Figures S4H and S4I). These results suggest that larger stomatal opening, which results in enhancement of photosynthesis and transpiration, may be beneficial to the flowering plants. Further investigations are needed to clarify the physiological significance of regulation of stomatal movements by *FT*.

Notably, FT protein and its orthologs, which are expressed in leaves, act as a mobile long-distance signaling component of flowering [34–38]. Our study suggests that *FT* is expressed autonomously in guard cells and may induce the expression of downstream components such as *AP1.* Since guard cells are not connected to neighboring cells via plasmodesmata [39], FT protein is probably synthesized and functional in guard cells in a manner independent of mobile FT. To the best of our knowledge, this is the first evidence of a cell-autonomous role for *FT*.

Recently, *FT* and its orthologs have been suggested to function as general growth regulators in addition to their florigenic activity [40–42]. Here, we provide evidence that *FT* plays a role in regulating the stomatal aperture via activation of the plasma membrane H^+-ATPase (Figure 4F). The H^+-ATPases, which are ubiquitous in all plant cell types investigated, provide the driving force for the uptake of numerous nutrients, including K^+, NO_3^-, SO_4^{2-}, PO_4^{3-}, amino acids, peptide, and sucrose, by coupling with organ-specific transporters [43]. Such activities are essential for cell growth and development [44]. Thus, *FT* may act as a general growth regulator by modulating H^+-ATPase activity. Further investigations will be required whether activation of the H^+-ATPase by *FT* is specific in guard cells or not. In addition, identification of the component(s) regulated by *FT* and/or *AP1* in guard cells will be important for elucidating the molecular mechanisms of blue-light responses in stomata and may provide clues to a better understanding of *FT* involvement in floral induction.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at doi:10.1016/j.cub.2011.06.025.

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