

# TWIN SISTER OF FT, GIGANTEA, and CONSTANS Have a Positive But Indirect Effect on Blue Light-Induced Stomatal Opening in Arabidopsis<sup>1</sup>[W][OA]

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*FLOWERING LOCUS T (FT)* is the major regulatory component controlling photoperiodic floral transition. It is expressed in guard cells and affects blue light-induced stomatal opening induced by the blue-light receptor phototropins *phot1* and *phot2*. Roles for other flowering regulators in stomatal opening have yet to be determined. We show in *Arabidopsis thaliana* that *TWIN SISTER OF FT (TSF)*, *CONSTANS (CO)*, and *GIGANTEA (GI)* provide a positive effect on stomatal opening. *TSF*, which is the closest homolog of *FT*, was transcribed in guard cells, and light-induced stomatal opening was repressed in *tsf-1*, a T-DNA insertion mutant of *TSF*. Overexpression of *TSF* in a *phot1 phot2* mutant background gave a constitutive open-stomata phenotype. Then, we examined whether *CO* and *GI*, which are upstream regulators of *FT* and *TSF* in photoperiodic flowering, are involved in stomatal opening. Similar to *TSF*, light-induced stomatal opening was suppressed in the *GI* and *CO* mutants *gi-1* and *co-1*. A constitutive open-stomata phenotype was observed in *GI* and *CO* overexpressors with accompanying changes in the transcription of both *FT* and *TSF*. In photoperiodic flowering, photoperiod is sensed by photoreceptors such as the cryptochromes *cry1* and *cry2*. We examined stomatal phenotypes in a *cry1 cry2* mutant and in *CRY2* overexpressors. Light-induced stomatal opening was suppressed in *cry1 cry2*, and the transcription of *FT* and *TSF* was down-regulated. In contrast, the stomata in *CRY2* overexpressors opened even in the dark, and *FT* and *TSF* transcription was up-regulated. We conclude that the photoperiodic flowering components *TSF*, *GI*, and *CO* positively affect stomatal opening.

Stomatal pores are surrounded by a pair of guard cells in the plant epidermis and control gas exchange in response to environmental and endogenous signals (Schroeder et al., 2001; Shimazaki et al., 2007). Previous studies have revealed that blue light acts as a signal that induces stomatal opening. Blue light is perceived by the receptor kinase phototropins *phot1* and *phot2* and activates the plasma membrane H<sup>+</sup>-ATPase (Kinoshita and Shimazaki, 1999; Kinoshita et al., 2001). Blue light-activated H<sup>+</sup>-ATPase creates inside-negative electrical potential across the plasma membrane and drives K<sup>+</sup> uptake through voltage-gated inward-rectifying K<sup>+</sup>

channels. As a consequence, changes in guard cell turgor and volume lead to stomatal opening (Kinoshita and Hayashi, 2011). Meanwhile, the plasma membrane anion channel has been shown to play a major role in abscisic acid (ABA)-induced stomatal closure. The anion channel causes depolarization of the plasma membrane itself and drives K<sup>+</sup> efflux through voltage-gated outward-rectifying K<sup>+</sup> channels (Schroeder et al., 1987, 2001). In addition, the plasma membrane anion channel is reported to be inhibited by blue light in a phototropin-dependent manner (Marten et al., 2007). Despite these recent studies, however, the mechanism controlling stomatal aperture has yet to be completely determined.

Recently, the *suppressor of closed-stomata phenotype in phot1 phot2 (scs1-1)* mutant was isolated from among ethyl methanesulfonate-treated *phot1 phot2* double mutants. The *scs1-1* mutant exhibits a constitutive open-stomata phenotype and carries a novel null allele of *early flowering3 (elf3)* (Kinoshita et al., 2011). *ELF3* is reported to act as a negative regulator of the florigen *FLOWERING LOCUS T (FT)* during floral induction (Kim et al., 2005), and the guard cells of *scs1-1 (elf3 phot1 phot2)* triple mutant have high levels of *FT* transcription and H<sup>+</sup>-ATPase activity. Overexpression of *FT* in guard cells promotes stomatal opening. In the loss-of-function *FT* mutant *ft-1*, both blue light-induced stomatal opening and activation of H<sup>+</sup>-ATPase were

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repressed, with no effect on the amounts of phototropins or H<sup>+</sup>-ATPase. These results suggest that *FT* provides a positive effect on stomatal opening via the activity of H<sup>+</sup>-ATPase in guard cells (Kinoshita et al., 2011). Overexpression of *APELATA1* (*API1*), which is a MADS box transcription factor known to be a downstream target of *FT* in floral induction (Abe et al., 2005; Wigge et al., 2005), showed an open-stomata phenotype, suggesting that *FT*-mediated stomatal opening involves transcriptional regulation (Kinoshita et al., 2011).

*FT* was identified as the floral inducer florigen (Corbesier et al., 2007). *FT* encodes a small protein of approximately 20 kD that belongs to a subfamily in the phosphatidylethanolamine-binding protein family (Kardailsky et al., 1999; Kobayashi et al., 1999; Ahn et al., 2006). *FT* is synthesized in the phloem companion cells of leaves in response to an appropriate photoperiod for flowering and is then transported to the shoot apical meristem, where it interacts with FLOWERING LOCUS D (*FD*), a bZIP-type transcriptional factor, to induce the expression of floral identity genes such as *API1* (Abe et al., 2005; Wigge et al., 2005; Turck et al., 2008). *TWIN SISTER OF FT* (*TSF*) is the closest known homolog of *FT*; it is regulated by photoperiod and induces floral transition redundantly with *FT* (Yamaguchi et al., 2005).

*CONSTANS* (*CO*), a zinc-finger transcription factor, is one of the key regulators of photoperiodic floral transition. *CO* directly regulates the transcription of *FT* and probably *TSF* (Suárez-López et al., 2001; Yamaguchi et al., 2005). In *Arabidopsis* (*Arabidopsis thaliana*), *CO* is transcribed only in the late afternoon under long-day conditions and induces *FT* and *TSF* transcription (Yamaguchi et al., 2005; Sawa et al., 2007). Expression of *CO* is regulated by the circadian clock component *GIGANTEA* (*GI*; Fowler et al., 1999; Park et al., 1999). The circadian expression of *GI* reaches a peak before dusk under long-day conditions (Turck et al., 2008). The *GI* protein interacts with the FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (*FKF1*) protein and binds the *CO* promoter in late afternoon. The *GI*-*FKF1* complex degrades CYCLING DOF FACTOR1, which is a clock-controlled transcriptional repressor of *CO*. Thus, formation of the *GI*-*FKF1* complex in late afternoon is crucial for the expression of *CO* with appropriate timing (Sawa et al., 2007). The *GI*-*FKF1* complex also binds the *FT* promoter and promotes *FT* transcription in a *CO*-independent manner (Sawa and Kay, 2011). Moreover, *FKF1* stabilizes the *CO* protein to promote *FT* transcription (Song et al., 2012). Daylength is sensed by photoreceptors such as phytochromes (*phyA* and *phyB*) and cryptochromes (*cry1* and *cry2*; Mockler et al., 2003; Boss et al., 2004). Cryptochromes are known to be involved in the entrainment of the circadian clock (Somers et al., 1998). The regulation of *GI* protein stability by *cry2* and *ELF3* confers an accurate circadian rhythm on *GI* expression (Yu et al., 2008). *CO* protein is stabilized in late afternoon by *cry2* and *phyA* but is destabilized by *phyB* in the early morning (Valverde et al., 2004; Liu

et al., 2008). Thus, many components are involved in the control of photoperiodic floral transition. The floral transition genes *PHYA*, *PHYB*, *CRY1*, *CRY2*, *GI*, and *CO* are all known to be transcribed in guard cells (Kinoshita et al., 2011), but a potential role for these genes in the regulation of stomatal opening in terms of photoperiodic flowering components had not been studied.

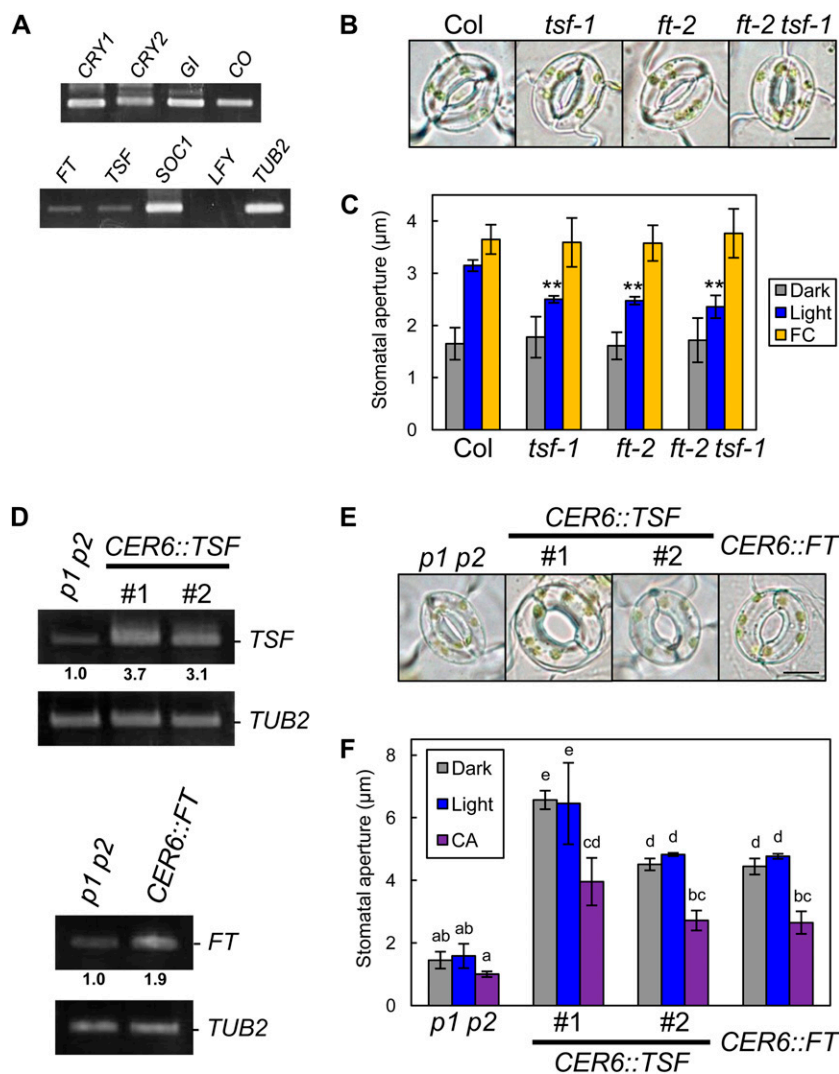
In this study, we show that the *FT* homolog *TSF* is also transcribed in guard cells and has a positive effect on stomatal opening. Our results indicate that the flowering regulators *GI* and *CO* are involved in stomatal opening, with accompanying changes in the transcription of both *FT* and *TSF*. We also show that blue light-induced stomatal opening is suppressed in a *cry1 cry2* double mutant with down-regulation of *FT* and *TSF* transcription and that *CRY2*-overexpressing plants show up-regulation of *FT* and *TSF* transcription and an open-stomata phenotype even in the dark. From these results, we conclude that stomatal opening is affected by a mechanism similar to that of the photoperiodic floral transition involving photoreceptor cryptochromes, the floral inducers *FT* and *TSF*, and the upstream flowering components *GI* and *CO*.

## RESULTS

### *TSF* Has a Positive Effect on Stomatal Opening

*FT* is expressed in guard cells and provides a positive effect on stomatal opening (Kinoshita et al., 2011). In *Arabidopsis*, *TSF* is the closest known homolog of *FT*, and it regulates photoperiodic flowering redundantly with *FT* (Yamaguchi et al., 2005). Transcription of *TSF*, like *FT*, was detected in highly purified guard cell protoplasts (GCPs), as shown in Figure 1A. Stomata in wild-type plants opened in response to light, but light-induced stomatal opening was suppressed in *ft-2*, a loss-of-function mutant of *FT* (Kinoshita et al., 2011). Moreover, stomatal responses in *tsf-1*, a T-DNA insertion mutant of *TSF* (Yamaguchi et al., 2005), were also suppressed (Fig. 1, B and C). These results suggest that *TSF* also has a positive effect on stomatal opening similar to *FT*. To test the functional redundancy between *TSF* and *FT*, we analyzed the stomatal responses of an *ft-2 tsf-1* double mutant. Unexpectedly, we could not detect significant differences between the *ft-2 tsf-1* double mutant and the individual single mutants, although the average stomatal aperture in *ft-2 tsf-1* was slightly lower than those in the single mutants. Note that the stomata in these mutants opened in response to the fungal toxin fusaric acid (FA), which induces stomatal opening via irreversible activation of H<sup>+</sup>-ATPase (Kinoshita and Shimazaki, 2001), suggesting that these mutations may not affect signal transduction components downstream of the H<sup>+</sup>-ATPase.

We generated transgenic plants with the *CER6* promoter (Hooker et al., 2002) to obtain constitutive *TSF* expression in the epidermis, including the guard cells in a *phot1 phot2* background. As expected, *TSF*-overexpressing



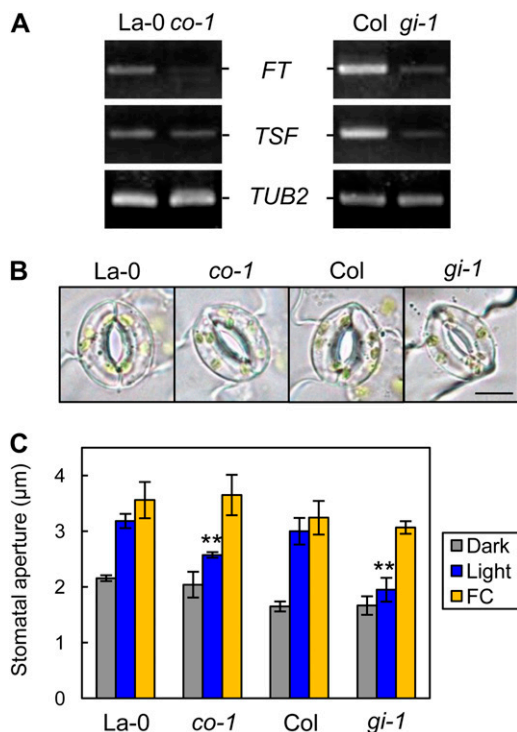
**Figure 1.** *TSF* is transcribed in guard cells and provides positive regulation of stomatal opening. A, Reverse transcription (RT)-PCR analyses of flowering components (*CRY1*, *CRY2*, *CO1*, *GI*, *CO*, *FT*, *TSF*, *SOC1*, and *LFY*) in GCPs. *TUBELIN BETA CHAIN2* (*TUB2*) was used as a control. B, Typical stomata in Col, *tsf-1*, *ft-2*, and the *ft-2 tsf-1* double mutant. The epidermis was illuminated as described below. Bar = 10 μm. C, Stomatal apertures under different treatments. Epidermal fragments were kept in the dark (Dark), illuminated with 10 μmol m<sup>-2</sup> s<sup>-1</sup> blue light under a background 50 μmol m<sup>-2</sup> s<sup>-1</sup> red light (Light), or treated with 10 μM FC in the dark (FC) for 3 h. Data represent means of three independent experiments with SD. Asterisks indicate significant differences between each mutant and Col under the same condition (\*\**P* < 0.01; Student's *t* test). D, RT-PCR analyses of *FT*, *TSF*, and *TUB2* in leaf epidermal fragments of *phot1 phot2* (*p1 p2*), *pCER6::TSF/phot1 phot2* (*CER6::TSF*), and *pCER6::FT/phot1 phot2* (*CER6::FT*). #, Line number of transgenic plants. Epidermal fragments were isolated at zeitgeber time 12. Numbers below each signal represent the relative expression levels for the same genes. Signal values of each gene were estimated by ImageJ software (National Institutes of Health) and normalized to those of *TUB2* and secondarily normalized to corresponding *phot1 phot2* values set to 1.0. Similar results were obtained in independent experiments. E, Typical stomata of *phot1 phot2* and the transgenic plants described in D. The epidermis was kept in the dark as in C. Bar = 10 μm. F, Stomatal apertures of the transgenic plants under different treatments. Dark or light conditions were the same as in C. CA was at 0.5 μM. The epidermis was treated with CA under light-exposure conditions for 3 h. Data represent means of three independent experiments with SD. Different letters indicate significant differences among means (*P* < 0.05; Tukey's test).

plants (*pCER6::TSF/phot1 phot2*) showed a constitutive open-stomata phenotype, as seen in *FT*-overexpressing plants (Kinoshita et al., 2011; Fig. 1, D–F). The stomata in *TSF*-overexpressing plants closed in response to the type 1/type 2A protein phosphatase inhibitor calyculin A (CA; Ishihara et al., 1989),

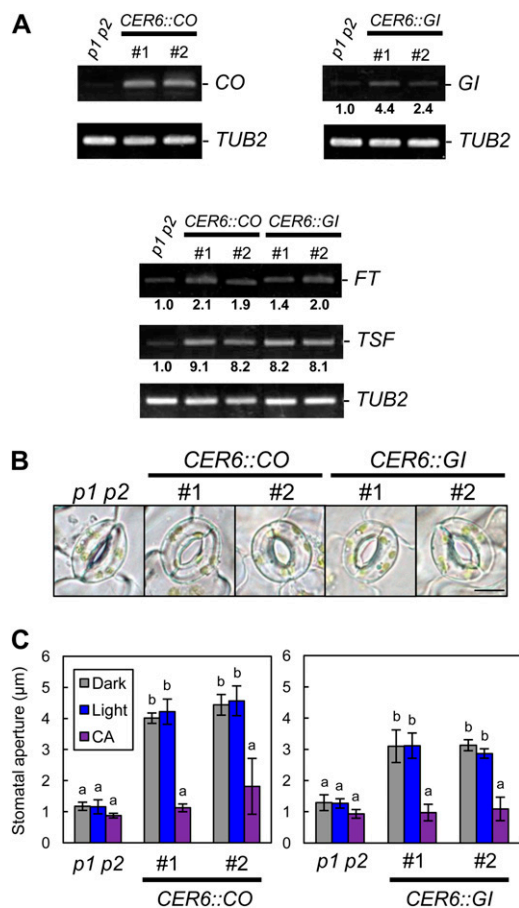
which inhibits the blue light-dependent activation of H<sup>+</sup>-ATPase in guard cells (Kinoshita and Shimazaki, 1997; Hayashi et al., 2011; Fig. 1F) and ABA (Supplemental Fig. S1). Note that an early-flowering phenotype was observed in *TSF*-overexpressing plants (Supplemental Fig. S2).

## Photoperiodic Flowering Regulators *CO* and *GI* Are Involved in *FT*- and *TSF*-Mediated Stomatal Opening

We examined whether *CO* and *GI*, which are upstream regulators of *FT* and *TSF* during the photoperiodic floral transition (Yamaguchi et al., 2005; Turck et al., 2008), are involved in stomatal opening. As shown in Figure 1A, expression of *CO* and *GI* was observed in GCPs (Kinoshita et al., 2011). A loss-of-function mutant of *CO*, *co-1* (Robson et al., 2001), showed decreased *FT*, and *TSF* transcript levels in the epidermis and light-induced stomatal opening were repressed (Fig. 2). In contrast, the stomata in *CO*-overexpressing plants (*pCER6::CO/phot1 phot2*) opened as in *TSF*-overexpressing plants, with high levels of both *FT* and *TSF* transcripts detected in the epidermis (Fig. 3). Similarly, a loss-of-function mutant of *GI*, *gi-1* (Fowler et al., 1999), had low levels of *FT* and *TSF* transcripts in the epidermis; light-induced stomatal opening was



**Figure 2.** Light-induced stomatal opening was suppressed in *co-1* and *gi-1*, and the transcription of *FT* and *TSF* was down-regulated. A, RT-PCR analyses of *FT*, *TSF*, and *TUB2* in leaf epidermal fragments of *La-0*, *co-1*, *Col*, and *gi-1*. Epidermal fragments were isolated at zeitgeber time 12. B, Typical stomata of the mutant and corresponding background plants described in A. The epidermis was illuminated as described below. Bar = 10  $\mu\text{m}$ . C, Stomatal apertures under different treatments. Epidermal fragments were kept in the dark (Dark), illuminated with 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light under a background 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  red light (Light), or treated with 10  $\mu\text{M}$  FC in the dark (FC) for 3 h. Data represent means of three independent experiments with SD. Asterisks indicate significant differences between each mutant and the corresponding wild type under the same conditions (\*\* $P < 0.01$ ; Student's *t* test).



**Figure 3.** Overexpression of *CO* or *GI* promoted stomatal opening and the up-regulation of *FT* and *TSF* transcription. A, RT-PCR analyses of *GI*, *CO*, *FT*, *TSF*, and *TUB2* in leaf epidermal fragments of *phot1 phot2* (*p1 p2*), *pCER6::CO/phot1 phot2* (*CER6::CO*), and *pCER6::GI/phot1 phot2* (*CER6::GI*). #, Line number of transgenic plants. Epidermal fragments were isolated at zeitgeber time 4 for the detection of *CO* and *GI* and at zeitgeber time 12 for *FT* and *TSF*. Signal values are estimated as in Figure 1D. B, Typical stomata of *phot1 phot2* and the transgenic plants described in A. The epidermis was kept in the dark for 3 h. Bar = 10  $\mu\text{m}$ . C, Stomatal apertures of the transgenic plants under different treatments. Epidermal fragments were kept in the dark (Dark) or illuminated with 10  $\mu\text{mol m}^{-2} \text{s}^{-1}$  blue light under a background 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  red light (Light) for 3 h. CA was at 0.5  $\mu\text{M}$ . The epidermis was treated with CA under light-exposure conditions for 3 h. Data represent means of three independent experiments with SD. Different letters indicate significant differences between the means of *phot1 phot2* and each transgenic plant ( $P < 0.05$ ; Tukey's test).

suppressed, and a constitutive open-stomata phenotype was observed in *GI*-overexpressing plants (*pCER6::GI/phot1 phot2*), with high levels of *FT* and *TSF* transcripts detected in the epidermis (Figs. 2 and 3). Note that *CO*- and *GI*-overexpressing plants showed early-flowering phenotypes and that the transcription of *CO* was up-regulated in the epidermis of *GI*-overexpressing plants (Supplemental Fig. S3). In addition, FC induced stomatal opening in *gi-1* and *co-1* (Fig. 2C), and both CA and ABA induced stomatal closure in the *GI*- and *CO*-overexpressing plants (Fig. 3C; Supplemental



Fig. S1). These results suggest that *GI* and *CO* are also involved in stomatal opening and that the sequence of regulatory action is *GI*, *CO*, and then *FT/TSF*, as it is for the regulation of photoperiodic floral transition. Note that *TSF*-, *GI*-, and *CO*-overexpressing plants and *tsf-1*, *ft-2 tsf-1*, *co-1*, and *gi-1* mutants showed similar stomatal index compared with background plants (Supplemental Table S4).

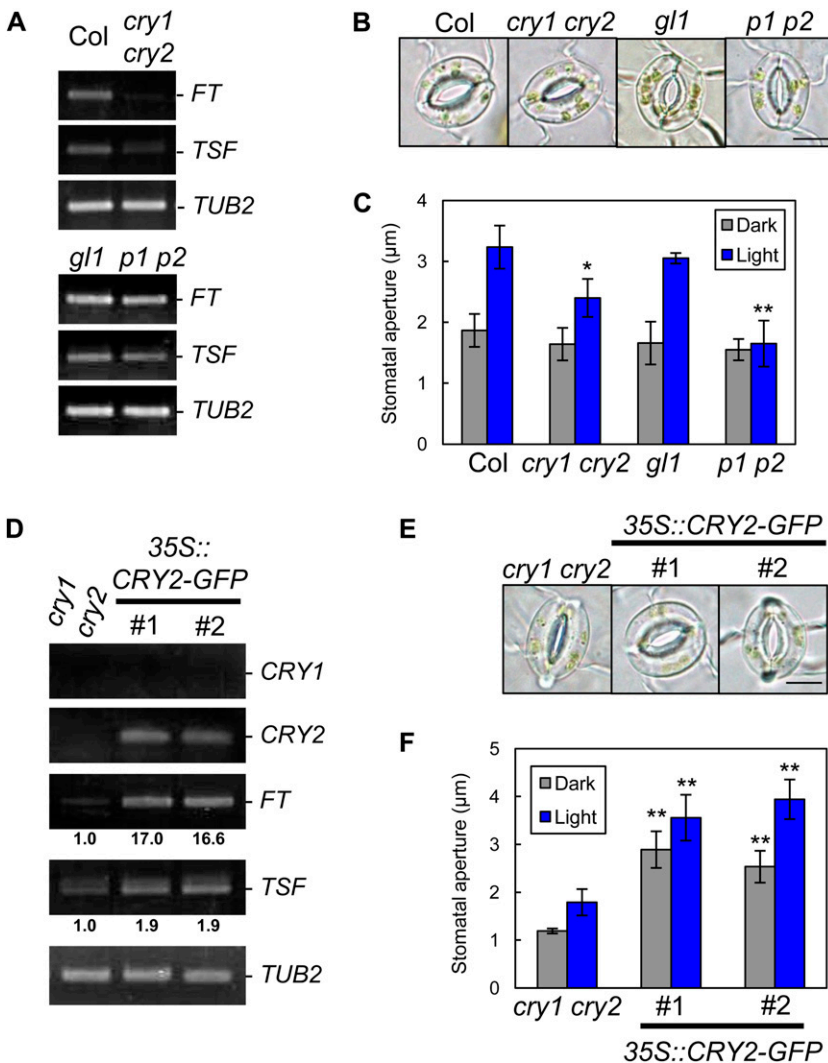
**Cryptochromes Affect Stomatal Aperture via the Regulation of *FT/TSF* Transcription**

Cryptochromes are well-understood blue light photoreceptors that regulate photoperiodic floral transition through entrainment of the circadian clock (Somers et al., 1998; Mockler et al., 2003) and by the regulation of *GI* and *CO* protein stability (Valverde et al., 2004; Liu et al., 2008; Yu et al., 2008). Cryptochromes are also transcribed in GCPs (Fig. 1A; Kinoshita et al., 2011). We hypothesized that cryptochromes affect stomatal

opening through their activity as photoperiod-sensing light receptors. To test this hypothesis, we examined stomatal responses in a *cry1 cry2* double mutant. In *cry1 cry2*, *FT* and *TSF* transcript levels decreased in the epidermis and light-induced stomatal opening was suppressed compared with the wild type (Fig. 4, A–C). Overexpression of *CRY2* in the *cry1 cry2* background (*35S::CRY2-GFP/cry1 cry2*) gave increased *FT* and *TSF* transcript levels in the epidermis, and the stomata of the *CRY2*-overexpressing plants opened even in the dark (Fig. 4, D–F). These results suggest that cryptochromes most likely affect stomatal aperture via the regulation of *FT* and *TSF* transcription in response to photoperiod.

**Phototropins Are the Blue Light Receptors That Mediate a Fast Stomatal Response to Blue Light**

Cryptochromes are suggested to function additively with phototropins in blue light-induced stomatal opening



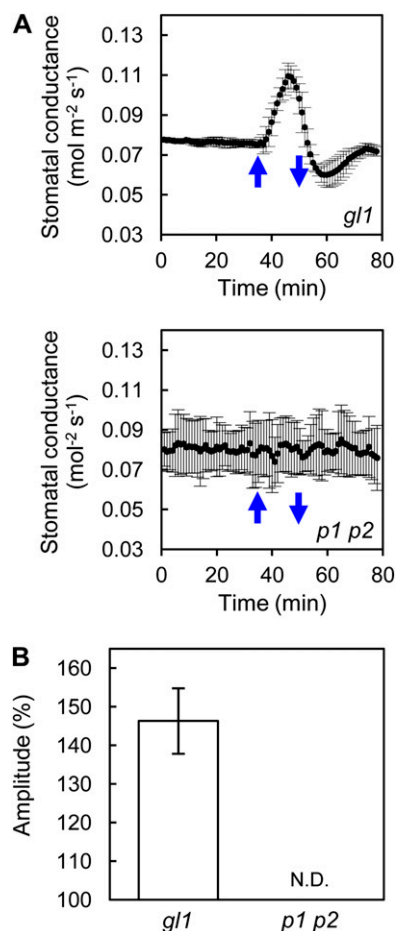
**Figure 4.** Regulation of stomatal aperture by cryptochromes is likely to be mediated by the regulation of *FT* and *TSF*. A, RT-PCR analyses of *FT*, *TSF*, and *TUB2* in leaf epidermal tissue of Col, *cry1 cry2*, *gl1*, and *phot1 phot2* (*p1 p2*). Epidermal fragments were isolated at zeitgeber time 12. B, Typical stomata of the same plants described in A. The epidermis was illuminated as described below. Bar = 10 µm. C, Stomatal apertures under different treatments. Epidermal fragments were kept in the dark (Dark), illuminated with 10 µmol m<sup>-2</sup> s<sup>-1</sup> blue light under a background 50 µmol m<sup>-2</sup> s<sup>-1</sup> red light (Light), or treated with 10 µM FC in the dark (FC) for 3 h. Data represent means of three independent experiments with sd. Asterisks indicate significant differences between each mutant and the corresponding background plants under the same conditions (\**P* < 0.05, \*\**P* < 0.01; Student's *t* test). D, RT-PCR analyses of *CRY1*, *CRY2*, *FT*, *TSF*, and *TUB2* in leaf epidermal tissue of *cry1 cry2* and *CaMV35S::CRY2-GFP/cry1 cry2* (*35S::CRY2-GFP*). #, Line number of transgenic plants. Epidermal fragments were isolated at zeitgeber time 12. Signal values are estimated as in Figure 1D. E, Typical stomata of the same plants described in D. The epidermis was kept in the dark for 3 h. Bar = 10 µm. F, Stomatal apertures of the transgenic plants under different treatments. Epidermal fragments were kept in the dark (Dark) or illuminated with 10 µmol m<sup>-2</sup> s<sup>-1</sup> blue light under a background 50 µmol m<sup>-2</sup> s<sup>-1</sup> red light (Light) for 3 h. Data represent means of three independent experiments with sd. Asterisks indicate significant differences between transgenic plants and *cry1 cry2* under the same conditions (\*\**P* < 0.01; Student's *t* test).

(Mao et al., 2005). However, as shown in Figure 4C, stomata in *phot1 phot2* completely failed to open in response to light, even though they expressed cryptochromes at wild-type levels (Kinoshita et al., 2011). The transcription of *FT* and *TSF* was not affected in *phot1 phot2* (Fig. 4A), indicating that the loss of light-induced stomatal opening in *phot1 phot2* was not due to a down-regulation of *FT* and *TSF* transcription. To examine the stomatal response to blue light in more detail in *phot1 phot2* and a corresponding background plant, *glabra1* (*gl1*), we measured stomatal conductance in intact leaves using a gas-exchange system. As shown in Figure 5, stomatal conductance in the background plant *gl1* showed a clear increase in response to blue light illumination for 15 min at  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$  superimposed on background red light. In contrast, blue light-induced fluctuation of stomatal conductance was completely abolished in *phot1 phot2* leaves even under a high fluence rate of blue light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 15 min. These results are consistent with those found for stomatal aperture (Fig. 4C) and demonstrate that phototropins, but not cryptochromes, are the only blue light receptors that can directly induce a fast stomatal response to blue light.

## DISCUSSION

### Roles for Photoperiodic Flowering Regulators in the Regulation of Stomatal Opening

A previous study indicated that the Arabidopsis floral pathway integrator *FT* is expressed in stomatal guard cells and provides a positive effect on stomatal opening by affecting plasma membrane  $\text{H}^+$ -ATPase activity in the guard cells, and a transcriptional factor, such as *AP1*, is suggested to be involved in *FT*-mediated stomatal opening (Kinoshita et al., 2011). In this study, we showed that the *FT* homolog *TSF* is also transcribed in guard cells and has a positive effect on stomatal opening (Fig. 1). We also showed that the photoperiodic flowering components *GI* and *CO* are involved in stomatal opening, with accompanying changes in the transcription of both *FT* and *TSF*, and that cryptochromes, which function in sensing photoperiod, affect *FT/TSF* transcription and stomatal aperture (Figs. 2–4). Stomatal opening is induced by blue light under background red light; however, it is also stimulated by red light only in both intact leaves and the isolated epidermis (Kinoshita et al., 2001; Shimazaki et al., 2007). In our conditions, stomatal responses to red light in the isolated epidermis were not affected in the mutants used in this study (Supplemental Fig. S4). These results indicate that a regulatory mechanism similar to that employed for photoperiodic floral transition has a positive but indirect effect on blue light-induced stomatal opening. We propose a possible model for stomatal opening directly induced by blue light signaling and indirectly affected by the photoperiodic pathway (Fig. 6). In this model, *FT* or *TSF* affects the blue light signaling component(s) between phototropins and  $\text{H}^+$ -ATPase. Note that overexpression



**Figure 5.** Stomatal conductance in *phot1 phot2* in response to short-term blue light irradiation. A, Fluctuation of stomatal conductance in *phot1 phot2* (*p1 p2*) and corresponding background plant (*gl1*) leaves in response to blue light. Leaves were illuminated with a strong red light ( $550 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) as a background to stabilize the conductance before illumination with blue light. Upward and downward blue arrows indicate the start and termination, respectively, of illumination for 15 min with blue light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  for *phot1 phot2* and  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$  for *gl1*). Data represent means of three independent experiments with sd. B, Amplitude of the stomatal conductance in response to blue light. The amplitude is shown as a percentage against a steady state set to 100%. Data represent means of three independent experiments with sd. N.D., Not detected.

of *FT* or *TSF* promoted stomatal opening even in the absence of functional phototropins (Figs. 1F and 3C). *FT* or *TSF* overexpression might lead to the up-regulation or activation of an unidentified component(s) in blue light signaling independent of the perception of blue light.

In this study, light-induced stomatal opening in *tsf-1* was suppressed to the same extent as in *ft-2*, indicating that the contributions of *TSF* and *FT* to stomatal opening are comparable (see white arrows from *FT* and *TSF* in Fig. 6). However, we could not detect significant differences between the *ft-2 tsf-1* double mutant and the individual single mutants, although the



A recent study indicated that cryptochromes affect stomatal conductance by controlling the amount of endogenous ABA in leaves (Boccalandro et al., 2012). Endogenous ABA has been suggested to affect the activity of the plasma membrane  $H^+$ -ATPase in guard cells even in the absence of drought stress (Hayashi et al., 2011). These findings suggest that endogenous ABA may have an important role in the regulation of stomatal aperture even under nonstress conditions. The relationship between photoperiod and ABA content in guard cells is an interesting problem for further investigation.

### Physiological Significance of *FT*/*TSF*-Mediated Stomatal Opening

Several studies have shown that *FT* and its orthologs have multiple physiological functions apart from the regulation of floral transition (Pin and Nilsson, 2012). Such functions include the regulation of growth (Gyllenstrand et al., 2007; Shalit et al., 2009), seed germination (Chiang et al., 2009), fruit set (Lifschitz et al., 2006), stomatal opening (Kinoshita et al., 2011), and tuberization (Navarro et al., 2011; González-Schain et al., 2012). Our results suggest that in addition to *FT*, *TSF* and the floral regulators *GI* and *CO* promote stomatal opening. The promotion of stomatal opening by these components may lead to the acceleration of photosynthesis prior to both flowering and seed development. Plant growth and reproduction are thought to entail the regulation of metabolism, and *FT*/*TSF* may play a part via the promotion of photosynthesis. Further study on multiple physiological functions of *FT*/*TSF* will provide a better understanding of the mechanisms involved in plant growth and reproduction.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

*Arabidopsis* (*Arabidopsis thaliana*) *gl1*, Columbia (Col), and Landsberg (La-0) plants were used as the background ecotypes of the mutants and overexpression plants. Col is the background ecotype of *gi-1*, *ft-2* (introgressed; Kobayashi et al., 1999), *tsf-1* (SALK\_087522; Yamaguchi et al., 2005), and *cry1-304 cry2-1* (*cry1 cry2*; Mockler et al., 2003). *phot1-5 phot2-1* (*phot1 phot2*; Kinoshita et al., 2001) is in the *gl1* background. La-0 is the background ecotype of *co-1*. Seeds for *gi-1* and *co-1* were obtained from the Arabidopsis Biological Resource Center. All plants were grown in soil under a 16-h-white light ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ )/8-h-dark cycle at 22°C to 24°C in 50% to 70% relative humidity in a growth room. The *ft-2 tsf-1* double mutant was obtained by crossing *tsf-1* with *ft-2*.

### Measurement of Stomatal Apertures

Epidermal fragments were isolated from mature rosette leaves of 4- to 6-week-old plants as described previously (Inoue et al., 2008). For the analysis of stomatal apertures, epidermal fragments isolated from dark-adapted plants were dipped into a basal buffer [5 mM MES-1,3-bis(tris[hydroxymethyl]methylamino)propane [pH 6.5], 50 mM KCl, and 0.1 mM  $\text{CaCl}_2$ ] and illuminated with blue light at  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  superimposed on a background red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  for 3 h at room temperature. CA (0.5  $\mu\text{M}$ ) was added to the basal buffer under light-exposure conditions, and 10  $\mu\text{M}$  FC was added in the dark. CA and FC were dissolved in dimethyl sulfoxide. Twenty-five stomatal apertures (five stomata per epidermal fragment) in the abaxial epidermis were measured microscopically for each independent experiment,

treatment, and genotype. All data represent means of three independent experiments with SD.

### Gas-Exchange Measurements

Gas-exchange measurements were performed using the Li-6400 system (Li-Cor), and parameters were calculated with the software supplied by the manufacturer. Mature leaves from *Arabidopsis* plants were clamped in a standard Li-6400 chamber and illuminated from the adaxial side with background red light at  $550 \mu\text{mol m}^{-2} \text{s}^{-1}$ . When the photosynthetic rate and stomatal conductance reached their steady states, a weak blue light ( $20 \mu\text{mol m}^{-2} \text{s}^{-1}$  for *phot1 phot2* and  $5 \mu\text{mol m}^{-2} \text{s}^{-1}$  for *gl1*) was superimposed on the background red light for 15 min. Flow rate, leaf temperature, relative humidity, and ambient  $\text{CO}_2$  concentration were kept constant at  $500 \mu\text{mol s}^{-1}$ , 24°C, 30% to 40%, and  $400 \mu\text{L L}^{-1}$ , respectively. Data represent means of three independent experiments with SD.

### Construction of Transformation Vectors and Transformation of Plants

All of the plant transformation plasmid vectors listed in Supplemental Table S1 were generated using standard molecular biology procedures and introduced into *Agrobacterium tumefaciens* (GV3101), which was then transformed into plants using a standard method. Homozygous plants were used for the experiments. Primers are shown in Supplemental Table S2.

### Light Source

Light-emitting photodiodes (ISL-150X150-RB and ISC-201-2 power supply; CCS) served as red and blue light sources for the stomatal opening measurements. Halogen projector lamps (150-W and MHAB-150W power supply; Moritex) with red or blue filters (Daiken) were used as red or blue light sources for the gas-exchange measurements. Photon flux densities were determined with a quantum meter (Li-250; Li-Cor).

### Reverse Transcription-PCR

Epidermal fragments that included stomatal guard cells were isolated from mature rosette leaves of 4- to 6-week-old plants using a blender equipped with an MC1 mini container (Waring Commercial). Mature rosette leaves (0.1–0.2 g) were blended for approximately 20 s in 35 mL of ice-cold Milli-Q water (Millipore). The epidermal fragments were collected on 58- $\mu\text{m}$  nylon mesh and frozen in a tube with liquid nitrogen. The epidermal fragments were isolated at zeitgeber times as indicated. The GCPs were prepared enzymatically from 4- to 6-week-old plants as described previously (Ueno et al., 2005).

Total RNAs were extracted from epidermal fragments and GCPs with an RNeasy Plant Mini Kit (Qiagen) and on-column DNase treatment using the RNase-Free DNase Set (Qiagen), and first-strand complementary DNAs were synthesized using the PrimeScript II First-Strand cDNA Synthesis Kit (Takara) following the manufacturer's instructions. In Figure 1A, 35 cycles of PCR were performed for all genes. In Figures 1D, 2A, 3A, and 4, A and D, 25 cycles of PCR were performed for *GI*, 30 cycles for *CRY1*, *CRY2*, *CO*, and *TUB2*, and 35 cycles for *FT* and *TSF*. Primers are shown in Supplemental Table S3.

### Statistical Significance Test

The significance of differences in means between genotypes was assessed by the two-tailed unpaired Student's *t* test. Asterisks in the figures indicate significant differences for the samples under the same condition. Differences among means were analyzed by Tukey's multiple comparison tests. Different letters in the figures indicate significant differences among means.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *CRY1* (AT4G08920), *CRY2* (AT1G04400), *GI* (AT1G22770), *CO* (AT5G15840), *FT* (AT1G65480), *TSF* (AT4G20370), *SOCI* (AT2G45660), *LFY* (AT5G61850), and *TUB2* (AT5G62690).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** ABA-induced stomatal closure in transgenic plants.



**Supplemental Figure S2.** Phenotypes of *TSF*-overexpressing plants.

**Supplemental Figure S3.** Phenotypes of *GI*- and *CO*-overexpressing plants.

**Supplemental Figure S4.** Effect of red light on stomatal opening.

**Supplemental Table S1.** List of transformation vectors.

**Supplemental Table S2.** Primer sets for construction.

**Supplemental Table S3.** Primer sets for RT-PCR.

**Supplemental Table S4.** Stomatal index in the transgenic plants and mutants.

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## LITERATURE CITED

- Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T (2005) FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309**: 1052–1056
- Ahn JH, Miller D, Winter VJ, Banfield MJ, Lee JH, Yoo SY, Henz SR, Brady RL, Weigel D (2006) A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. *EMBO J* **25**: 605–614
- Araki T (2001) Transition from vegetative to reproductive phase. *Curr Opin Plant Biol* **4**: 63–68
- Boccalandro HE, Giordano CV, Ploschuk EL, Piccoli PN, Bottini R, Casal JJ (2012) Phototropins but not cryptochromes mediate the blue light-specific promotion of stomatal conductance, while both enhance photosynthesis and transpiration under full sunlight. *Plant Physiol* **158**: 1475–1484
- Boss PK, Bastow RM, Mylne JS, Dean C (2004) Multiple pathways in the decision to flower: enabling, promoting, and resetting. *Plant Cell (Suppl)* **16**: S18–S31
- Chiang GCK, Barua D, Kramer EM, Amasino RM, Donohue K (2009) Major flowering time gene, *flowering locus C*, regulates seed germination in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **106**: 11661–11666
- 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
- Fowler S, Lee K, Onouchi H, Samach A, Richardson K, Morris B, Coupland G, 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
- González-Schain ND, Díaz-Mendoza M, Żurczak M, Suárez-López P (2012) Potato CONSTANS is involved in photoperiodic tuberization in a graft-transmissible manner. *Plant J* **70**: 678–690
- Gyllenstrand N, Clapham D, Källman T, Lagercrantz U (2007) A Norway spruce *FLOWERING LOCUS T* homolog is implicated in control of growth rhythm in conifers. *Plant Physiol* **144**: 248–257
- Hayashi M, Inoue S, Takahashi K, Kinoshita T (2011) Immunohistochemical detection of blue light-induced phosphorylation of the plasma membrane H<sup>+</sup>-ATPase in stomatal guard cells. *Plant Cell Physiol* **52**: 1238–1248
- Hooker TS, Millar AA, Kunst L (2002) Significance of the expression of the CER6 condensing enzyme for cuticular wax production in *Arabidopsis*. *Plant Physiol* **129**: 1568–1580
- Inoue S, Kinoshita T, Matsumoto M, Nakayama KI, Doi M, Shimazaki K (2008) Blue light-induced autophosphorylation of phototropin is a primary step for signaling. *Proc Natl Acad Sci USA* **105**: 5626–5631
- Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D, et al (1989) Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun* **159**: 871–877
- Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, Chory J, Harrison MJ, Weigel D (1999) Activation tagging of the floral inducer *FT*. *Science* **286**: 1962–1965
- Kim WY, Hicks KA, Somers DE (2005) Independent roles for *EARLY FLOWERING 3* and *ZEITLUPE* in the control of circadian timing, hypocotyl length, and flowering time. *Plant Physiol* **139**: 1557–1569
- Kinoshita T, Doi M, Suetsugu N, Kagawa T, Wada M, Shimazaki K (2001) Phot1 and phot2 mediate blue light regulation of stomatal opening. *Nature* **414**: 656–660
- Kinoshita T, Hayashi Y (2011) New insights into the regulation of stomatal opening by blue light and plasma membrane H<sup>+</sup>-ATPase. *Int Rev Cell Mol Biol* **289**: 89–115
- Kinoshita T, Ono N, Hayashi Y, Morimoto S, Nakamura S, Soda M, Kato Y, Ohnishi M, Nakano T, Inoue S, et al (2011) *FLOWERING LOCUS T* regulates stomatal opening. *Curr Biol* **21**: 1232–1238
- Kinoshita T, Shimazaki K (1997) Involvement of calyculin A- and okadaic acid-sensitive protein phosphatase in the blue light response of stomatal guard cells. *Plant Cell Physiol* **38**: 1281–1285
- Kinoshita T, Shimazaki K (1999) Blue light activates the plasma membrane H<sup>+</sup>-ATPase by phosphorylation of the C-terminus in stomatal guard cells. *EMBO J* **18**: 5548–5558
- Kinoshita T, Shimazaki K (2001) Analysis of the phosphorylation level in guard-cell plasma membrane H<sup>+</sup>-ATPase in response to fusicoccin. *Plant Cell Physiol* **42**: 424–432
- Kinoshita T, Shimazaki K (2002) Biochemical evidence for the requirement of 14-3-3 protein binding in activation of the guard-cell plasma membrane H<sup>+</sup>-ATPase by blue light. *Plant Cell Physiol* **43**: 1359–1365
- Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**: 1960–1962
- Lifschitz E, Eviatar T, Rozman A, Shalit A, Goldshmidt A, Amsellem Z, Alvarez JP, Eshed Y (2006) The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proc Natl Acad Sci USA* **103**: 6398–6403
- Liu LJ, Zhang YC, Li QH, Sang Y, Mao J, Lian HL, Wang L, Yang HQ (2008) COP1-mediated ubiquitination of CONSTANS is implicated in cryptochrome regulation of flowering in *Arabidopsis*. *Plant Cell* **20**: 292–306
- Mao J, Zhang YC, Sang Y, Li QH, Yang HQ (2005) A role for *Arabidopsis* cryptochromes and COP1 in the regulation of stomatal opening. *Proc Natl Acad Sci USA* **102**: 12270–12275
- Marten H, Hedrich R, Roelfsema MRG (2007) Blue light inhibits guard cell plasma membrane anion channels in a phototropin-dependent manner. *Plant J* **50**: 29–39
- Mockler TC, Yang H, Yu XH, Parikh D, Cheng YC, Dolan S, Lin C (2003) Regulation of photoperiodic flowering by *Arabidopsis* photoreceptors. *Proc Natl Acad Sci USA* **100**: 2140–2145
- Navarro C, Abelenda JA, Cruz-Oró E, Cuéllar CA, Tamaki S, Silva J, Shimamoto K, Prat S (2011) Control of flowering and storage organ formation in potato by *FLOWERING LOCUS T*. *Nature* **478**: 119–122
- Park DH, Somers DE, Kim YS, Choy YH, Lim HK, Soh MS, Kim HJ, Kay SA, Nam HG (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis* *GIGANTEA* gene. *Science* **285**: 1579–1582
- Pin PA, Nilsson O (2012) The multifaceted roles of *FLOWERING LOCUS T* in plant development. *Plant Cell Environ* **35**: 1742–1755
- Robson F, Costa MMR, Hepworth SR, Vizir I, Piñeiro M, Reeves PH, Putterill J, Coupland G (2001) Functional importance of conserved domains in the flowering-time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. *Plant J* **28**: 619–631
- Sawa M, Kay SA (2011) *GIGANTEA* directly activates *Flowering Locus T* in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **108**: 11698–11703
- Sawa M, Nusinow DA, Kay SA, Imaizumi T (2007) FKF1 and *GIGANTEA* complex formation is required for day-length measurement in *Arabidopsis*. *Science* **318**: 261–265
- Schroeder JJ, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. *Annu Rev Plant Physiol Plant Mol Biol* **52**: 627–658
- Schroeder JJ, Raschke K, Neher E (1987) Voltage dependence of K channels in guard-cell protoplasts. *Proc Natl Acad Sci USA* **84**: 4108–4112
- Shalit A, Rozman A, Goldshmidt A, Alvarez JP, Bowman JL, Eshed Y, Lifschitz E (2009) The flowering hormone florigen functions as a general

- systemic regulator of growth and termination. *Proc Natl Acad Sci USA* **106**: 8392–8397
- Shimazaki K, Doi M, Assmann SM, Kinoshita T** (2007) Light regulation of stomatal movement. *Annu Rev Plant Biol* **58**: 219–247
- Simpson GG, Dean C** (2002) *Arabidopsis*, the Rosetta stone of flowering time? *Science* **296**: 285–289
- Somers DE, Devlin PF, Kay SA** (1998) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* **282**: 1488–1490
- Song YH, Smith RW, To BJ, Millar AJ, Imaizumi T** (2012) FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science* **336**: 1045–1049
- Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G** (2001) *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* **410**: 1116–1120
- Turck F, Fornara F, Coupland G** (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. *Annu Rev Plant Biol* **59**: 573–594
- Ueno K, Kinoshita T, Inoue S, Emi T, Shimazaki K** (2005) Biochemical characterization of plasma membrane H<sup>+</sup>-ATPase activation in guard cell protoplasts of *Arabidopsis thaliana* in response to blue light. *Plant Cell Physiol* **46**: 955–963
- Valverde F, Mouradov A, Soppe W, Ravenscroft D, Samach A, Coupland G** (2004) Photoreceptor regulation of *CONSTANS* protein in photoperiodic flowering. *Science* **303**: 1003–1006
- Wigge PA, Kim MC, Jaeger KE, Busch W, Schmid M, Lohmann JU, Weigel D** (2005) Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* **309**: 1056–1059
- Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T** (2005) *TWIN SISTER OF FT (TSF)* acts as a floral pathway integrator redundantly with *FT*. *Plant Cell Physiol* **46**: 1175–1189
- Yu JW, Rubio V, Lee NY, Bai S, Lee SY, Kim SS, Liu L, Zhang Y, Irigoyen ML, Sullivan JA, et al** (2008) COP1 and ELF3 control circadian function and photoperiodic flowering by regulating GI stability. *Mol Cell* **32**: 617–630