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A Flowering Integrator, *SOC1*, Affects Stomatal Opening in *Arabidopsis thaliana*

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Stomatal movements are regulated by multiple environmental signals. Recent investigations indicate that photoperiodic flowering components, such as *CRY*, *GI*, *CO*, *FT* and *TSF*, are expressed in guard cells and positively affect stomatal opening in *Arabidopsis thaliana*. Here we show that *SOC1*, which encodes a MADS box transcription factor and integrates multiple flowering signals, also exerts a positive effect on stomatal opening. *FLC* encodes a potent repressor of *FT* and *SOC1*, and *FRI* acts as an activator of *FLC*. Thus, we examined stomatal phenotypes in *FRI*-Col, which contains an active *FRI* allele of accession Sf-2 by introgression. We found higher expression of *FLC* and lower expression of *FT*, *SOC1* and *TSF* in guard cells from *FRI*-Col than in those from Col. Light-induced stomatal opening was significantly suppressed in *FRI*-Col. Interestingly, vernalization of *FRI*-Col partially restored light-induced stomatal opening, concomitant with a decrease of *FLC* and increase of *FT*, *SOC1* and *TSF*. Furthermore, we observed the constitutive open-stomata phenotype in transgenic plants overexpressing *SOC1-GFP* (green fluorescent protein) in guard cells (*SOC1-GFP* overexpressor), and found that light-induced stomatal opening was significantly suppressed in a *soc1* knockout mutant. RNA sequencing using epidermis from the *SOC1-GFP* overexpressor revealed that the expression levels of several genes involved in stomatal opening, such as *BLUS1* and the plasma membrane H^+-ATPases, were higher than those in background plants. From these results, we conclude that *SOC1* is involved in the regulation of stomatal opening via transcriptional regulation in guard cells.

Keywords: *Arabidopsis thaliana* • *FRI* • *FT* • Plasma membrane H^+-ATPase • *SOC1* • Stomata.

Abbreviations: *AHA*, *ARABIODOPSIS H^+-ATPASE*; *AP1*, *APETALA1*; *BLUS1*, *BLUS LIGHT SIGNALING1*; CA, calyculin A; *CO*, *CONSTANS*; Col, Columbia; *CRY*, *CRYPTOCHROME*; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; FC, fusicoccin; *FLC*, *FLOWERING LOCUS C*; *FT*, *FLOWERING LOCUS T*; *FUL*, *FRUITFULL*; *FRI*, *FRIGIDA*; GCP, guard cell protoplast; GFP, green fluorescent protein; *GI*, *GIGANTEA*; L*er*, Landsberg *erecta*; *LFY*, *LEAFY*; PP1, protein phosphatase 1; *PRSL1*, *PP1 REGULATORY SUBUNIT2-LIKE PROTEIN1*; RT–PCR, reverse transcription–PCR; RNA-seq, RNA sequencing; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CO1*; *TOPP*, *TYPE ONE PROTEIN PHOSPHATASE*; *TSF*, *TWIN SISTER OF FT*; *TUB2*, *TUBLIN2*; ZT, Zeitgeber time.

RNA-seq data reported herein have been submitted to the Gene Expression Omnibus under accession number GSE60183.

Introduction

Stomata on the plant surface regulate gas exchange between plants and the atmosphere, such as uptake of CO_2 for photosynthesis, efflux of O_2 and evaporation. Stomatal movements are regulated by various environmental signals, including light, drought conditions, CO_2, pathogens and temperature (Schroeder et al. 2001, Shimazaki et al. 2007). Thus, stomatal guard cells integrate environmental signals and show clear responses, such as opening and closing. Light is an important environmental signal in promoting stomatal opening, and blue light acts as a direct signal through blue light receptor phototropin-mediated activation of the plasma membrane H^+-ATPase in guard cells (Kinoshita and Shimazaki 1999, Kinoshita et al. 2001). There are 11 H^+-ATPase isoforms in Arabidopsis (Palmgren 2001), all of which are expressed in Arabidopsis guard cell protoplasts (GCPs; Ueno et al. 2005). Blue light activates the H^+-ATPase through phosphorylation of the penultimate threonine in the H^+-ATPase, causing subsequent binding of the 14-3-3 protein (Kinoshita and Shimazaki 1999, Kinoshita and Shimazaki 2002). Blue light-activated H^+-ATPase creates inside-negative electrical potential across the plasma membrane and drives K^+ uptake through voltage-gated inward-rectifying K^+ channels. As a consequence, changes in guard cell turgor and volume lead to stomatal opening (Kinoshita and Hayashi 2011).

BLUE LIGHT SIGNALING1 (*BLUS1*) and protein phosphatase 1 (PP1) are involved in the blue light signaling pathway between phototropins and the plasma membrane H^+-ATPase. *BLUS1* is a

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substrate of phototropin kinases in guard cells and interacts with phototropins. *BLUS1* encodes a protein kinase, and phosphorylation of BLUS1 by phototropins and BLUS1 kinase activity are both essential for activation of the plasma membrane H^+-ATPase (Takemiya et al. 2013a). PP1 functions as a holoenzyme of both a catalytic and a regulatory subunit. Both PP1 subunits may regulate signal transduction from phototropins to the plasma membrane H^+-ATPase (Takemiya et al. 2006, Takemiya et al. 2013b).

In addition to the direct effect of the blue light signaling pathway, recent investigations indicate that photoperiodic flowering components, such as *GIGANTEA* (*GI*), *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*) and *TWIN SISTER OF FT* (*TSF*), are expressed in guard cells and positively affect stomatal opening in *Arabidopsis thaliana* (Kinoshita et al. 2011, Ando et al. 2013). Loss-of-function or knockout mutants of these components generally showed reduced light-induced stomatal phenotypes. In contrast, overexpressors of these components showed constitutively open-stomata phenotypes. Furthermore, *CRYPTOCHROME* (*CRY*), a well-understood blue light photoreceptor that regulates 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), is also involved in the regulation of stomatal aperture via *FT* and *TSF* regulation in response to photoperiod (Ando et al. 2013). Moreover, overexpression of *APELATA1* (*AP1*), 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). These results suggest that this component-mediated stomatal opening involves transcriptional regulation. However, there are no data on the target genes of these components in guard cells.

In Arabidopsis, the vernalization requirement/winter-annual habit is typically established by active alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) (Napp-Zinn 1979, Koornneef et al. 1994, Lee et al. 1994). *FLC* encodes a potent flowering repressor, and *FRI* is required for *FLC* to be transcribed to a level that effectively suppresses flowering (Michaels and Amasino 1999, Sheldon et al. 1999, Johanson et al. 2000). The Columbia (Col) wild type, the most commonly used 'lab strain' of Arabidopsis, possesses a null allele of *fri*, and therefore exhibits low *FLC* expression and rapid flowering behavior in inductive long days (Johanson et al. 2000). When an active *FRI* is introgressed into Col, it increases *FLC* transcription and delays flowering in non-vernalized plants (Lee and Amasino 1995). FRI activates *FLC* transcription by recruiting chromatin modification factors (Choi et al. 2011), although the molecular mechanism of the *FLC* activation by FRI is not fully understood. *FLC* regulates the expression of downstream genes, the so-called flowering pathway integrators, including *FT*, *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *LEAFY* (*LFY*). These three genes and *TSF* integrate signals from multiple flowering pathways, and their expression levels eventually determine the exact flowering time (Araki 2001, Simpson and Dean 2002, Parcy 2005, Yamaguchi et al. 2005, Hiraoka et al. 2013).

SOC1 encodes a multifunctional MADS box protein (Borner et al. 2000, Lee et al. 2000, Onouchi et al. 2000, Samach et al. 2000) that regulates not only flowering time but also floral patterning and floral meristem determinacy (Melzer et al. 2008, Liu et al. 2009, Lee and Lee 2010). The *SOC1* transcript has been observed along with *FT* and *TSF* in Arabidopsis guard cells, while the *LFY* transcript has not (Ando et al. 2013). However, it remains unclear if *SOC1* is involved in stomatal regulation.

In this study, we performed phenotypic analysis of *FRI*-Col, and found that it shows significantly reduced stomatal apertures at lower expression levels of *FT*, *TSF* and *SOC1*. Next, we examined stomatal aperture in *SOC1* overexpressors and *soc1-10* mutants, and found that *SOC1* expressed in guard cells affects stomatal aperture. Furthermore, we examined *SOC1*-dependent gene expression in guard cells and found that the expression level of several key genes involved in stomatal opening is increased by *SOC1*. From these results, we conclude that stomatal opening is positively regulated by transcriptional regulation of *SOC1* expression in guard cells.

Results

FRI-Col shows a closed stomatal phenotype

FRI elevates expression level of *FLC*, which represses flowering. Plants with an active *FRI* allele showed reduced expression of the major flowering components, such as *FT* and *SOC1*, without vernalization or late flowering phenotype (Johanson et al. 2000). In addition, it has been reported that *FT* affects stomatal opening positively in *A. thaliana* (Kinoshita et al. 2011). These results suggest that if *FRI* also elevates the expression level of *FLC* and reduces the expression level of *FT* via *FLC* in stomatal guard cells, the plants with an active *FRI* allele would show a closed-stomata phenotype. Therefore, we examined expression levels of *FLC*, *FT*, *TSF* and *SOC1* in Col and *FRI*-Col GCPs by reverse transcription–PCR (RT–PCR) (**Fig. 1A**). We observed higher expression of *FLC* in *FRI*-Col. In contrast, expression levels of *FT*, *TSF* and *SOC1* in GCPs of *FRI*-Col were lower than those of Col. These results indicate that *FRI* affects the expression of these components in guard cells as well as leaves, and suggest that light-induced stomatal opening would be reduced in *FRI*-Col since positive regulators of stomatal opening, *FT* and *TSF* (Kinoshita et al. 2011, Ando et al. 2013), were reduced in GCPs of *FRI*-Col. Next, we investigated light-induced stomatal opening in Col and *FRI*-Col (**Fig. 1B**). As expected, light-induced stomatal opening was significantly suppressed in *FRI*-Col. However, the stomata in these mutants opened in response to the fungal toxin fusicoccin (FC) (**Fig. 1C**), which induces stomatal opening via irreversible activation of H^+-ATPase (Kinoshita and Shimazaki 2001). Moreover, *FRI*-Col showed similar stomatal density and index compared with those in Col (**Fig. 1D**). These results suggest that *FRI*-Col exhibits reduced light-induced stomatal opening without affecting stomatal morphology and development, and that the

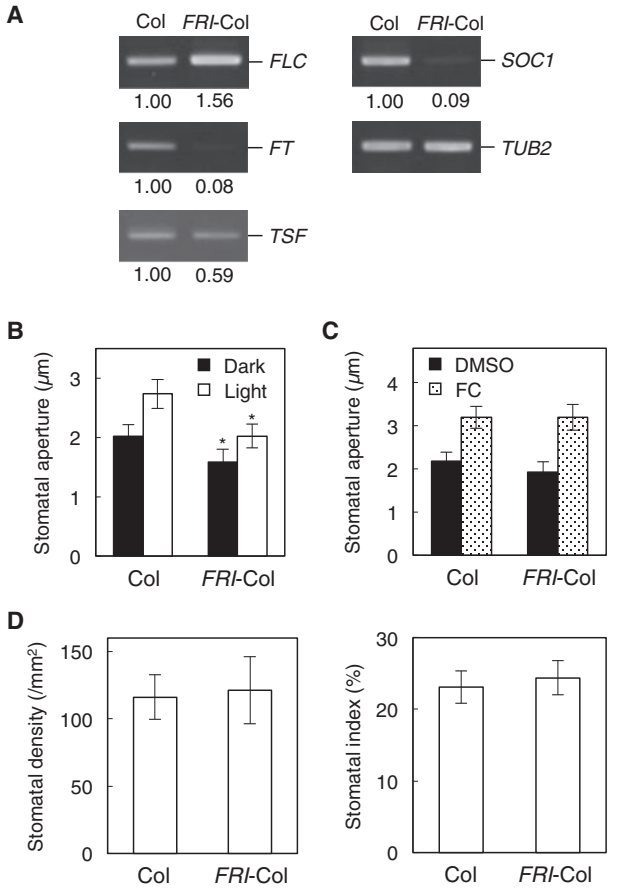

Fig. 1 Gene expression and stomatal phenotypes in *FRI*-Col. (A) RT–PCR analysis of *FLC*, *FT*, *TSF* and *SOC1* in GCPs of Col and *FRI*-Col. *TUBLIN BETA CHAIN2* (*TUB2*) was used as a control. The expression level was quantified as the ratio of the signal intensity from *FRI*-Col to that from Col. Numbers below each signal represent the relative expression levels for the same genes. Signal values of each gene were estimated using the ImageJ software (National Institutes of Health), normalized to those of *TUB2*, and secondarily normalized to corresponding Col values set to 1.0. PCR was performed three times on biologically independent occasions, yielding similar results each time (**Supplementary Fig. S1A**). (B) Light-induced stomatal opening in Col and *FRI*-Col. Epidermal fragments were kept in the dark (Dark) or illuminated with 10 μmol m^{-2} s^{-1} blue light under a background 50 μmol m^{-2} s^{-1} red light (Light). Data represent means of three independent experiments with the SD. Asterisks indicate significant differences between Col and *FRI*-Col under the same conditions (*$P < 0.05$; Student's *t*-test). (C) FC-induced stomatal opening in Col and *FRI*-Col. Epidermal fragments were treated with 0.25% (v/v) DMSO as a vehicle (DMSO) or 10 μM FC (FC) in the dark for 3 h. (D) Stomatal density and index of Col and *FRI*-Col. Stomatal density and index were calculated according to a previous method (Ando et al. 2013).

H^+-ATPase and its immediate regulatory mechanisms are not affected in *FRI*-Col.

Vernalization of *FRI*-Col restores the closed stomatal phenotype

Enhancement of *FLC* expression by *FRI* is canceled by vernalization in leaves (Sung and Amasino 2005). Therefore, we carried out vernalization of Col and *FRI*-Col, and then examined the

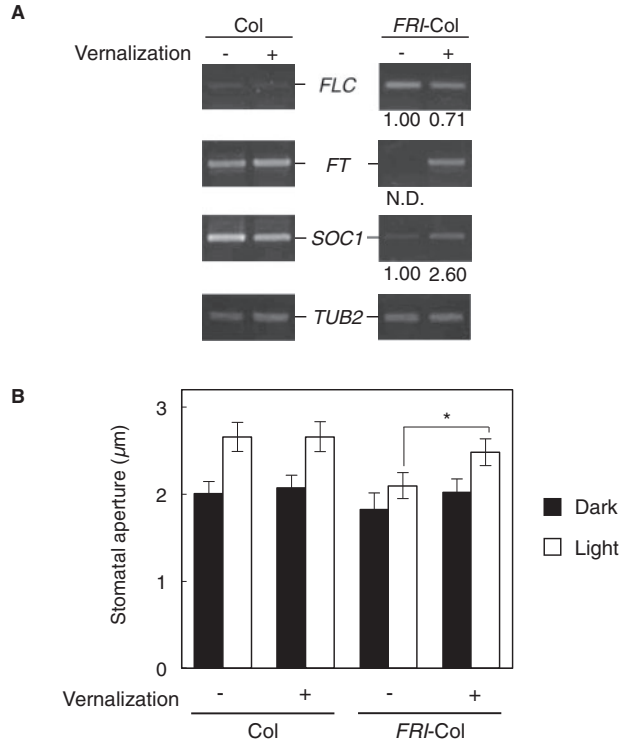

Fig. 2 Effect of vernalization of *FRI*-Col on gene expression and stomatal opening. For vernalization, 2-week-old plants under normal conditions were transferred to a cold room at 4°C for 2 weeks. Then, the plants were returned to normal conditions for 14–20 d (+Ver). −Ver represents plants grown only under normal conditions. (A) RT–PCR analysis of *FLC*, *FT* and *SOC1* in leaf epidermal fragments of Col and *FRI*-Col. Signal values are estimated as in **Fig. 1A**. N.D., not detected. PCR was performed three times on biologically independent occasions, yielding similar results each time (**Supplementary Fig. S1B**). (B) Light-induced stomatal opening in Col and *FRI*-Col. Asterisks indicate significant differences between −Ver and + Ver (*$P < 0.05$; Student's *t*-test). Other conditions are the same as in **Fig. 1B**.

expression level of *FLC*, *FT* and *SOC1* in the epidermis, including guard cells (**Fig. 2A**). Vernalization (4°C for 2 weeks) reduced *FLC* expression and increased *FT* and *SOC1* expression. Next, we examined light-induced stomatal opening (**Fig. 2B**). In Col plants, vernalization had no effect on stomatal phenotype. Interestingly, light-induced stomatal opening was significantly enhanced in vernalized *FRI*-Col. These results suggest that both *FT* and *SOC1* in guard cells are correlated with stomatal aperture.

Note that expression levels of *FT* and *SOC1* in vernalized *FRI*-Col were lower than those in vernalized Col (**Fig. 2A**). Consistent with this, the stomatal aperture under light condition in vernalized *FRI*-Col was smaller than that in vernalized Col (**Fig. 2B**). These results indicate that vernalization (4°C for 2 weeks) is not sufficient for reduction of *FLC* expression in *FRI*-Col. In fact, the expression level of *FLC* in *FRI*-Col was reduced only 29% by vernalization (**Fig. 2A**). Further investigation will be needed to examine the effects of vernalization period on the expression levels of *FLC*, *FT* and *SOC1* and light-induced stomatal aperture in *FRI*-Col.

Overexpression of *SOC1* in guard cells induces stomatal opening

Figs. 1 and 2 indicate a correlation between expression levels and stomatal opening in both *FT* and *SOC1*. However, there is no experimental evidence to show regulation of stomatal movement by *SOC1*. Therefore, we generated *SOC1*–green fluorescent protein (GFP) transgenic plants with the *GC1* promoter, a strong guard cell promoter (Yang et al. 2008, Wang et al. 2014), to express *SOC1* specifically in guard cells. We used a *phot1 phot2* double mutant as the background plant. Since the light-induced stomatal opening in *phot1 phot2* is very small (Kinoshita et al. 2001), it is easy to monitor the effect of a transgene on stomatal aperture. As shown in Fig. 3A, the expression level of epidermal *SOC1* including the endogenous *SOC1* and *SOC1-GFP* in *pGC1::SOC1-GFP* plants was 2.5- to 2.8-fold higher than the endogenous *SOC1* of the background plants. *SOC1-GFP* signals were observed mainly from nuclei, stained by the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI), and secondarily from the cytosol in guard cells (Fig. 3B). We then analyzed the stomatal responses of *SOC1-GFP*-overexpressing plants. The stomata of *phot1 phot2* plants did not show light-induced stomatal opening, as this mutant lacked functional phototropins (Kinoshita et al. 2001). Interestingly, the stomata of *SOC1-GFP*-overexpressing plants opened widely in both dark and light conditions (Fig. 3C), as seen in *FT*- and *TSF*-overexpressing plants (Kinoshita et al. 2011, Ando et al. 2013). To examine how *SOC1* overexpression induces stomatal opening, we tested the effects of ABA and the type 1/type 2A protein phosphatase inhibitor calyculin A (CA; Ishihara et al. 1989), which inhibit blue light-dependent activation of H^+-ATPase in guard cells (Kinoshita and Shimazaki 1997, Zhang et al. 2004, Hayashi et al. 2011) on stomatal aperture, and found that ABA and CA induced stomatal closure in *SOC1-GFP*-overexpressing plants (Fig. 3D). These results suggest that *SOC1* may affect stomatal aperture, at least in part, by affecting the H^+-ATPase activity in guard cells.

A *soc1* knockout mutant shows reduced light-induced stomatal opening

Next, we examined stomatal phenotypes in a T-DNA insertional mutant of *SOC1* (GABI_466F09) (Fig. 4A). As shown in Fig. 4B, there was no detectable *SOC1* transcript, indicating that this mutant is a knockout mutant of *SOC1*. Therefore, we designated it as *soc1-10* (Fig. 4A, B). Light-induced stomatal opening was significantly suppressed in the *soc1-10* mutant, and in the loss-of-function mutant of *FT*, *ft-2* (Imura et al. 2012) (Fig. 4C). However, the stomata of these mutants opened in response to an activator of H^+-ATPase, FC (Fig. 4D). Moreover, the *soc1-10* mutant showed similar stomatal density and index compared with Col (Fig. 4E). These results suggest that the *soc1-10* mutant shows reduced light-induced stomatal opening without affecting morphology and stomatal development, and that the H^+-ATPase and its immediate regulatory mechanisms are not affected in the *soc1-10* mutant.

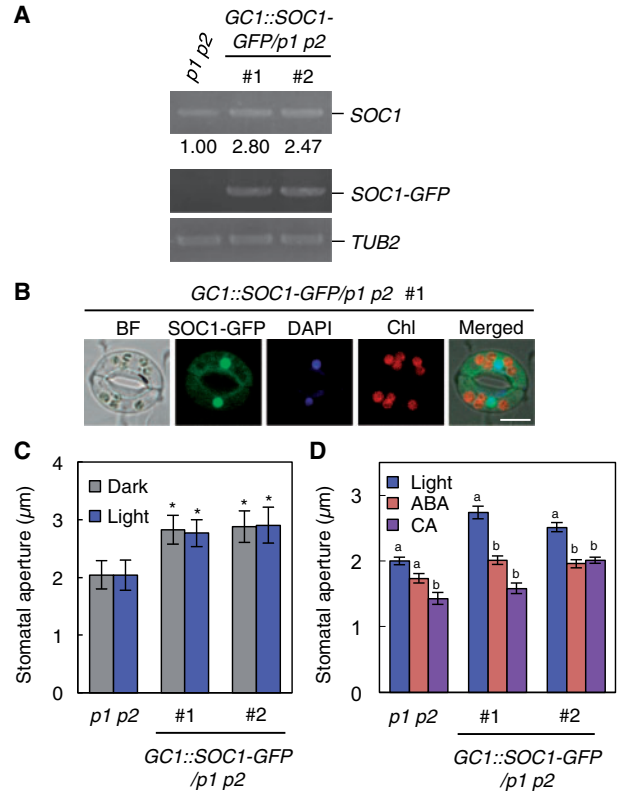

Fig. 3 Stomatal phenotypes in *SOC1-GFP*-overexpressing plants. (A) RT–PCR analysis of *SOC1* and *SOC1-GFP* in leaf epidermal fragments of *phot1 phot2* (*p1 p2*) and *pGC1::SOC1-GFP/phot1 phot2* (*GC1::SOC1-GFP/p1 p2*). #, line number of transgenic plants. Epidermal fragments were isolated at Zeitgeber time (ZT) 12. (B) Subcellular localization of SOC1–GFP in guard cells; bright field (BF), GFP fluorescence (SOC1–GFP), DAPI fluorescence (DAPI) and Chl fluorescence (Chl) images of a typical stoma from *GC1::SOC1-GFP/p1 p2* #1. Scale bar = 10 μm. (C) Stomatal apertures of *SOC1-GFP*-overexpressing plants (*pGC1::SOC1-GFP/phot1 phot2*). *p1 p2*, *phot1 phot2*. #, Line number of transgenic plants. Data represent means of three independent experiments with the SD. Asterisks indicate significant differences between *phot1 phot2* and *SOC1-GFP*-overexpressing plants under the same conditions (*$P < 0.05$; Student's *t*-test). Other conditions are the same as in Fig. 1B. (D) Effects of ABA and CA on stomatal aperture in *SOC1-GFP*-overexpressing plants. Epidermal fragments were treated with 0.25% (v/v) DMSO as a vehicle (Light) or 20 μM ABA (ABA) or 0.5 μM CA (CA) under 10 μmol m^{-2} s^{-1} blue light under a background 50 μmol m^{-2} s^{-1} red light for 3 h. Data represent means of 45 stomata with the SD. Two independent experiments were performed, yielding similar results. Different letters indicate significant differences among means for each genotype ($P < 0.01$; Tukey's test).

Overexpression of *SOC1* in guard cells induces gene expression

SOC1 encodes the MADS domain transcription factor (Samach et al. 2000, Hepworth et al. 2002), and microarray and chromatin immunoprecipitation (ChIP)-seq analyses show that *SOC1* regulates gene expression in seedlings (Seo et al. 2009, Immink et al. 2012). Additionally, SOC1–GFP was expressed primarily in the nuclei of guard cells (Fig. 3B). Together, these results suggest that *SOC1* in guard cells also affects gene expression.

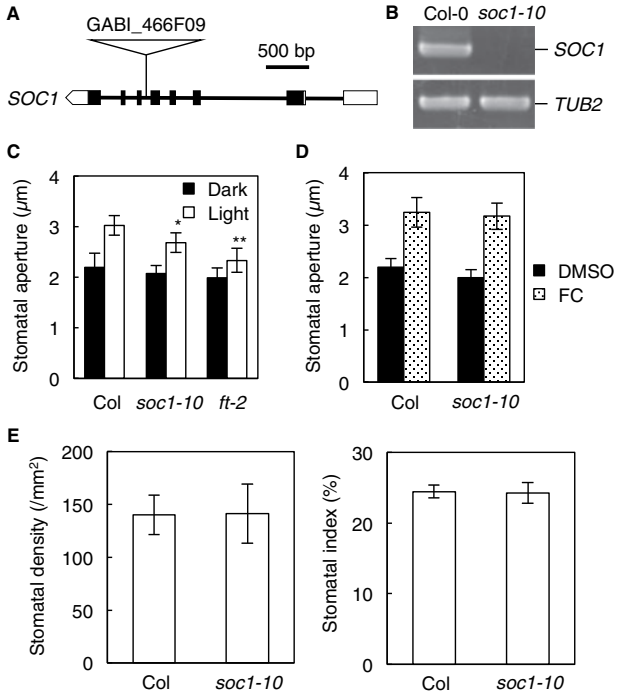

Fig. 4 Stomatal phenotype in the *soc1* mutant. (A) Schematic structure of the *SOC1* gene. Exons and introns are indicated by boxes and bars, respectively. White and black boxes represent untranslated regions and coding regions, respectively. The T-DNA insertion site of the *soc1* mutant (*soc1-10*; GABI_466F09) is indicated. (B) RT–PCR analysis of *SOC1* in leaf epidermal fragments of Col and *soc1-10*. (C) Stomatal apertures of *soc1-10* and *ft-2*. Data represent means of three independent experiments with the SD. Asterisks indicate significant differences between Col and each mutant under the same conditions (*$P < 0.05$; **$P < 0.01$; Student's *t*-test). Other conditions are the same as in **Fig. 1B**. (D) FC-induced stomatal opening in Col and *soc1-10*. Data represent means of three independent experiments with the SD. Other conditions are the same as in **Fig. 1C**. (E) Stomatal density and index of Col and *soc1-10*.

To identify the genes controlled by *SOC1* in guard cells, we performed RNA sequencing (RNA-seq) experiments using RNAs from the epidermis, including guard cells of *SOC1-GFP*-overexpressing (*pGC1::SOC1-GFP/phot1 phot2*) and *phot1 phot2* plants. We found that read numbers of numerous genes are increased in *SOC1-GFP*-overexpressing plants. **Supplementary Table S1** lists the top 20 genes, in which the read numbers are increased in *SOC1-GFP*-overexpressing plants based on fold change. However, to our knowledge, no gene has been conclusively implicated in stomatal opening and closing. Therefore, we focused on the genes involved in blue-light-induced stomatal opening, such as *BLUS1*, a primary component mediating phototropin signaling (Takemiya et al. 2013a); catalytic subunits of PP1 (*TOPP1–TOPP8*), a positive signaling component between phototropins and plasma membrane H^+-ATPase (Takemiya et al. 2013b); regulatory subunits of PP1 (*PRSL1*), a positive signaling component with TOPPs (Takemiya et al. 2013b); plasma membrane H^+-ATPases (*AHA1–AHA11*), which induce hyperpolarization of the plasma membrane for K^+ uptake (Palmgren 2001, Wang et al. 2014); and inward-rectifying K^+ channels (*KAT1*, *KAT2* and *AKT1*), which take up K^+ in response to hyperpolarization (Kwak et al. 2001, Szyroki et al. 2001) (**Table 1**). Interestingly, the read numbers of *BLUS1*, some H^+-ATPase isoforms (*AHA1*, *AHA2*, *AHA3*, *AHA5*, *AHA6*, *AHA10* and *AHA11*) and *KAT2* were higher in *SOC1-GFP*-overexpressing plants. Furthermore, we confirmed an increase in the expression levels of *SOC1* including the endogenous *SOC1* and *SOC1-GFP*, *BLUS1* as a direct downstream component of phototropins (Takemiya et al. 2013), and *AHA2* as a typical guard-cell H^+-ATPase isoform in Arabidopsis (Wang et al. 2014) in *SOC1-GFP*-overexpressing plants by RT–PCR (**Fig. 5**; **Supplementary Fig. S1C**). In contrast, the read numbers of *KAT1* and *AKT1* were lower in *SOC1-GFP*-overexpressing plants. The read numbers of *TOPP* genes and *PRSL1* were similar between *SOC1-GFP*-overexpressing and *phot1 phot2* plants. These results indicate that actually *SOC1* regulates gene expression in stomatal guard cells including the genes involved in blue-light-induced stomatal opening.

Discussion

FRI and *FLC* affect stomatal opening

FRI–FLC suppression of flowering is required for winter-annual plants to take maximum advantage of favorable growing conditions in the spring (Sung and Amasino 2005). In this study, we examined the stomatal phenotype in *FRI*-Col plants and found that *FRI*-Col plants show a closed-stomata phenotype concomitant with increased *FLC* expression and decreased expression of *FT*, *TSF* and *SOC1* in guard cells (**Fig. 1**), and that vernalization of *FRI*-Col plants significantly induces light-induced stomatal opening concomitant with decreased *FLC* expression and increased expression of *FT* and *SOC1* (**Fig. 2**). These results indicate that *FRI* and *FLC* affect stomatal opening, and that vernalization affects *FRI* and *FLC* in guard cells the same as in the leaves. It may be interesting to analyze whether increased stomatal opening in spring is also beneficial for plants.

SOC1 functions as a positive regulator of stomatal opening

The results in **Figs. 1** and **2** suggest that *FLC* regulates flowering components, such as *FT* and *SOC1*, in stomatal guard cells and there is a correlation between expression levels and stomatal opening in both *FT* and *SOC1*. Previously, we showed that *FT* and *TSF* are involved in stomatal opening (Kinoshita et al. 2011, Ando et al. 2013). Therefore, we investigated the role of *SOC1* in stomatal guard cells. As expected, *SOC1-GFP*-overexpressing plants that express *SOC1-GFP* under the guard cell strong promoter *GC1* showed a constitutive open-stomata phenotype (**Fig. 3**). In contrast, the *SOC1* knockout mutant, *soc1-10*, showed reduced light-induced stomatal opening, much like the *ft-2* mutant (**Fig. 4**). These results indicate that *SOC1* functions as a positive regulator for stomatal opening. To our knowledge, this is the first evidence of a novel function of *SOC1* in stomatal opening, although *SOC1* is a multifunctional protein, which regulates both flowering time and floral patterning, and

Table 1 Comparison of read numbers of genes related to blue-light-induced stomatal opening

Gene name	ID	RPM in *SOC1-GFP*	RPM in *phot1 phot2*	Fold change
BLUS1	AT4G14480	28.31	11.93	2.37
TOPP1	AT2G29400	103.89	85.97	1.21
TOPP2	AT5G59160	13.09	13.41	0.98
TOPP3	AT1G64040	48.04	42.31	1.14
TOPP4	AT2G39840	28.64	29.09	0.98
TOPP5	AT3G46820	19.14	19.91	0.96
TOPP6	AT5G43380	5.03	4.55	1.11
TOPP7	AT4G11240	30.78	31.76	0.97
TOPP8	AT5G27840	20.52	18.28	1.12
PRSL1	AT4G40100	0.07	0	0
AHA1	AT2G18960	689.17	494.57	1.39
AHA2	AT4G30190	282.05	160.95	1.75
AHA3	AT5G57350	562.31	387.02	1.45
AHA4	AT3G47950	5.80	8.23	0.70
AHA5	AT2G24520	45.93	24.70	1.86
AHA6	AT2G07560	1.34	0.25	5.36
AHA7	AT3G60330	3.21	3.74	0.83
AHA8	AT3G42640	18.73	23.46	0.80
AHA9	AT1G80660	2.90	3.24	0.89
AHA10	AT1G17260	2.45	1.50	1.64
AHA11	AT5G62670	66.67	35.68	1.87
KAT1	AT5G46240	39.09	51.40	0.78
KAT2	AT4G18290	11.82	4.99	2.37
AKT1	AT2G26650	18.73	45.63	0.41

PHOT1 and *PHOT2* are not included in this list, because we used *phot1-5 phot2-1* double mutants (Kinoshita et al. 2001) as background plants. The *SOC1-GFP*-overexpressing plant #1 and *phot1 phot2* were used for analysis.
The actual read counts were normalized into reads per million (RPM).
The data represent means from three independent experiments.
BLUS1, *BLUS LIGHT SIGNALING1*; *TOPP*, *TYPE ONE PROTEIN PHOSPHATASE*; *PRSL1*, *PP1 REGULATORY SUBUNIT2-LIKE PROTEIN1*; *AHA*, *ARABIODOPSIS* H^+-*ATPASE*.

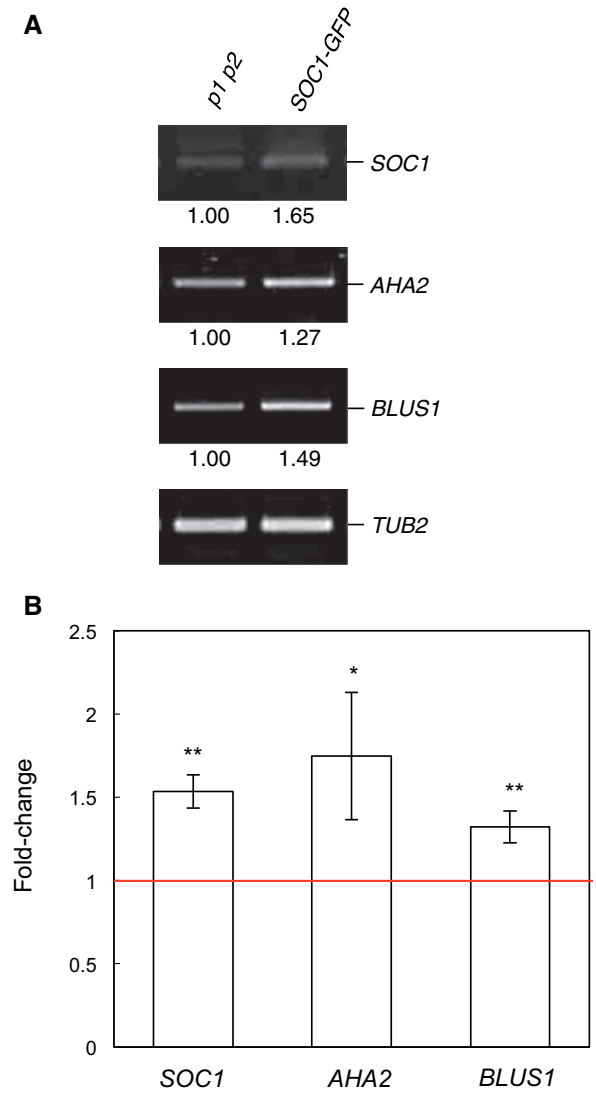

Fig. 5 *AHA2* and *BLUS1* are likely to be up-regulated in *SOC1-GFP*-overexpressing plants. (A) Up-regulation of *BLUS1* and *AHA2* was confirmed by RT–PCR analysis in leaf epidermal fragments of *phot1 phot2* (*p1 p2*) and *GC1::SOC1-GFP/phot1 phot2* (*SOC1-GFP*) #1 used in RNA-seq experiments. Signal values are estimated as in **Fig. 1A**. PCR was performed three times on biologically independent occasions, yielding similar results each time (**Supplementary Fig. S1C**). (B) Fold changes in gene expression described in (A). Signal values for *GC1::SOC1-GFP/phot1 phot2* estimated in (A) were used as fold change. Data represent means with the SEM from three independent PCRs. Asterisks indicate that the fold change is statistically higher than 1.0 (red line) assessed by one-tailed one-sample *t*-test ($^*P < 0.1$, $^{**}P < 0.05$).

floral meristem determinacy (Melzer et al. 2008, Liu et al. 2009, Lee and Lee 2010). In floral induction, *FT*, *TSF* and *SOC1* are known to act redundantly as flowering pathway integrators (Yamaguchi et al. 2005, Truck et al. 2008, Hiraoka et al. 2013). Therefore, further study is needed to investigate the stomatal phenotype in *ft soc1* double mutants or *ft tsf soc1* triple mutants. We note that *LFY* is also known as a flowering pathway integrator (Araki 2001, Simpson and Dean 2002, Parcy 2005), but we did not detect an *LFY* transcript in wild-type GCPs (Ando et al. 2013).

SOC1 is likely to mediate stomatal opening through transcriptional regulation

To clarify how *SOC1* affects stomatal opening, we performed comprehensive expression analysis in *SOC1-GFP*-overexpressing plants by RNA-seq, since *SOC1* is the MADS box transcription factor. However, no gene has been conclusively implicated in stomatal opening and closing in the top genes, in which the read numbers are increased in *SOC1-GFP*-overexpressing plants based on fold change (**Supplementary Table S1**). Further investigations are needed to examine whether these genes are involved in stomatal opening. Interestingly, we found that the read numbers of the genes which are involved in blue-light-induced stomatal opening, such as *BLUS1*, some H^+-ATPase isoforms (*AHA1*, *AHA2*, *AHA3*, *AHA5*, *AHA6*, *AHA10* and *AHA11*) and *KAT2*, were increased in epidermis containing guard cells of *SOC1-GFP*-overexpressing plants, compared

with that of the background plant (Table 1). Since *SOC1-GFP* was expressed using a strong guard cell promoter, these genes increased in guard cells.

Phosphorylation of BLUS1 by phototropins is essential for signaling (Takemiya et al. 2013a). Therefore, *BLUS1* may be the integrator of the phototropin- and the *SOC1*-dependent pathways for the stomatal opening, where *SOC1* contributes to the transcription, and phototropins function in the phosphorylation. We used a *phot1 phot2* double mutant as a background for the *SOC1-GFP*-overexpressing plants. It is possible that the increase of *BLUS1* in the *SOC1-GFP*-overexpressing plants is unrelated to the open-stomata phenotype. Furthermore, it is likely that elevated *KAT2* in the *SOC1-GFP*-overexpressing plant is unrelated to stomatal phenotype, because *KAT2* is not a major inward-rectifying K^+ channel in guard cells, based on the number of sequence reads, and the number of sequence reads of the inward-rectifying K^+ channels including *KAT1*, *KAT2* and *AKT1* was lower in *SOC1-GFP*-overexpressing plants (Table 1).

The plasma membrane H^+-ATPase is a key enzyme in the regulation of stomatal opening in guard cells (Kinoshita and Hayashi 2011). In *SOC1-GFP*-overexpressing plants, the number of sequence reads of the major H^+-ATPase isoforms, *AHA1*, *AHA2* and *AHA3*, increased by 1.39-, 1.75- and 1.45-fold, respectively. The H^+-ATPase is activated by phototropin-dependent phosphorylation of the penultimate threonine in the C-terminus (Kinoshita and Shimazaki 1999, Ueno et al. 2005). Therefore, H^+-ATPase may be the other integrator of the phototropin- and the *SOC1*-dependent stomatal opening pathways. The H^+-ATPase possesses the basal activity without a blue light signal in guard cells or in guard cells from *phot1 phot2* (Kinoshita and Shimazaki 1999, Ueno et al. 2005). It is possible that such increases in the major H^+-ATPase isoforms enhanced stomatal aperture even in *SOC1-GFP*-overexpressing plants with a *phot1 phot2* background. We note that the overexpression of a single isoform, *AHA2*, in wild-type plants using the guard cell strong promoter enhances light-induced stomatal opening, but the stomata close in the dark (Wang et al. 2014). Further investigation is needed to clarify the relationship between total H^+-ATPase amounts in guard cells and stomatal phenotype in more detail. Additionally, previous studies suggested that *FT* affects stomatal aperture, at least in part, by affecting the phosphorylation status of the penultimate threonine of H^+-ATPase in guard cells (Kinoshita et al. 2011), and that *SOC1* directly binds to the target promoter region (Immink et al. 2012). Further investigations are needed to clarify whether *SOC1* also affects the phosphorylation status of H^+-ATPase in guard cells and whether *AHA* expression is directly regulated by *SOC1*.

In this study, we performed comprehensive expression analysis only in *SOC1-GFP*-overexpressing plants. However, *FT*, *TSF* and *SOC1* are known to act redundantly as flowering pathway integrators in floral induction (Yamaguchi et al. 2005, Hiraoka et al. 2013). Moreover, *FT*- or *TSF*-overexpressing plants showed a stronger open-stomata phenotype than did *SOC1-GFP*-overexpressing plants (Kinoshita et al. 2011, Ando et al. 2013). Therefore, comprehensive expression analyses in the guard cells of *FT*- or *TSF*-overexpressing plants pose an interesting challenge in elucidating the relationships among the molecular mechanisms of *FT*/*TSF*/*SOC1*-mediated stomatal opening.

Physiological significance of flowering component-mediated stomatal opening

Our study suggests that *SOC1* in guard cells, regulated, as with flowering, by photoperiod and the *FLC*-mediated pathway, promotes stomatal opening through indirect transcriptional regulation. We previously showed that photoperiodic flowering components such as *CRY* genes, *GI*, *CO*, *FT* and *TSF* promote stomatal opening (Kinoshita et al. 2011, Ando et al. 2013). Moreover, enhancement of light-induced stomatal opening by manipulating the expression of plasma membrane H^+-ATPase in guard cells increases photosynthesis and plant growth under relatively high light conditions ($>200\ \mu mol\ m^{-2}\ s^{-1}$) (Wang et al. 2014). This indicates that the enhancement of stomatal aperture by these flowering components increases photosynthesis in the reproduction phase. Why do plants require enhanced stomatal opening in the reproduction phase? It is possible that increased photosynthesis is useful for flower and seed development in plants. Further study of the multiple physiological functions of *SOC1* and photoperiodic components will provide a better understanding of the mechanisms involved in plant growth and reproduction.

In conclusion, we demonstrated a novel function for *SOC1*, a flowering integrator, in stomatal opening. Stomatal guard cells integrate environmental signals and show clear stomatal responses. Our study provides new insight into stomatal responses and function during reproduction. Identifying the target of *SOC1* in guard cells will be the next important step in elucidating the molecular mechanism of *SOC1*-mediated stomatal opening.

Materials and Methods

Plant materials and growth conditions

FRI-Col was the Col genetic background into which an active *FRI* allele of accession Sf-2 was introgressed (Lee and Amasino, 1995). *phot1-5 phot2-1* (*phot1 phot2*; Kinoshita et al. 2001) was the *gl1* background. We obtained a *SOC1* knockout mutant, *soc1-10* (GABI_466F09), from the Arabidopsis Biological Resource Center. Homozygous *soc1-10* mutant plants were identified by genomic PCR using the T-DNA left border primer, 5′-ATATTGACCATCATACTCATTGC-3′, and the *SOC1* gene-specific primers 5′-TTTGAGAACACCATTGGGATC-3′ and 5′-CCAAAAAGAACCCCAACTTTC-3′. Col is the background ecotype of *soc1-10* and *ft-2* (introgressed; Imura et al. 2012). Seeds were incubated in water at 4°C for 3 d and sown directly on the soil surface. Plants were grown in soil under a 16 h white light ($50\ \mu mol\ m^{-2}\ s^{-1}$)/8 h dark cycle at 22–24°C in 50–70% relative humidity in a growth room.

For vernalization, 2-week-old plants grown under the conditions above were transferred to a cold room under a 16 h white light ($40\ \mu mol\ m^{-2}\ s^{-1}$)/8 h dark cycle at 4°C for 2 weeks. Then, the plants were returned to baseline conditions for 14–20 d. Finally, the plants were used for measuring stomatal aperture and determining gene expression.

Reverse transcription–PCR

GCPs were prepared enzymatically from 4- to 6-week-old plants as described previously (Ueno et al. 2005). 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) (Ando et al. 2013). 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 μm nylon mesh and frozen in a tube with liquid nitrogen, isolated at Zeitgeber time (ZT) 12, because *FT* shows a high expression level around ZT12 (Turck et al. 2008). Total RNAs were extracted from GCPs and epidermal fragments with an RNeasy Plant Mini Kit (Qiagen) and on-column DNase treatment using the RNase-Free DNase Set (Qiagen), and first-strand cDNAs were synthesized using the PrimeScript II First-Strand cDNA Synthesis Kit (TAKARA) following the manufacturer's instructions. In **Fig. 1A**, 30 cycles of PCR were performed for *FLC*, *SOC1* and *TUB2*, and 35 cycles for *FT* and *TSF*. In **Fig. 2A**, 25 cycles of PCR were performed for *FLC*, 30 cycles for *SOC1* and *TUB2*, and 35 cycles for *FT*. In **Fig. 3A**, 25 cycles of PCR were performed for *TUB2*, 30 cycles for *SOC1* and 35 cycles for *SOC1-GFP*. In **Fig. 4B**, 30 cycles of PCR were performed for all genes. Primers are shown in **Supplementary Table S2**.

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). Epidermal tissue isolated from dark-adapted plants was incubated in a basal buffer [5 mM MES-BTP (pH 6.5), 50 mM KCl and 0.1 mM $CaCl_2$] and illuminated with blue light at 10 μmol m^{-2} s^{-1} superimposed on a background red light at 50 μmol m^{-2} s^{-1} for 3 h at room temperature. We started the experiment for stomatal aperture at ZT4. ABA (20 μM) and CA (0.5 μM) were added to the basal buffer under light exposure conditions, and 10 μM FC was added in the dark. ABA, CA and FC were dissolved in dimethylsulfoxide (DMSO). From 25 to 45 stomatal apertures (five stomata per epidermal fragment) in the abaxial epidermis were measured microscopically for each independent experiment. 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. Photon flux densities were determined with a quantum meter (Li-250; Li-Cor).

Construction of transformation vectors and transformation of plants

The plasmid vectors used for plant transformation were constructed according to a previous method (Kinoshita et al. 2011). In brief, a genomic DNA fragment spanning 1,702 to 1 bp upstream of the start codon of *GC1* (At1g22690), flanked by *Hin*dIII and *Xba*I sites, was used to replace the corresponding region of the *Cauliflower mosaic virus* (CaMV) *35S* promoter in *pPZP211* (*pPZP211-GC1*). Before *Hin*dIII digestion, a single-nucleotide substitution (1,453 C to G) was introduced into the *GC1* fragment by site-directed mutagenesis. A cDNA fragment of *SOC1* (At2g45660) was amplified with the following oligonucleotide primers: 5′-GCCTCTAGAGATATGGTGAGGGGCAAAACTC-3′ and 5′-GCCTCTAGATCACTTTCTTGAAGAACAAGGTAACCC-3′. The amplified DNA fragment was inserted into *pPZP211-GC1* using *Xba*I. The stop codon of the *SOC1* coding sequence in *pPZP211-GC1::SOC1* was replaced with a gene encoding synthetic GFP with an S65T mutation (*pPZP211-GC1::SOC1-GFP*). All of the plant transformation plasmid vectors were introduced into *Agrobacterium tumefaciens* (GV3101), which was then transferred into plants using a standard method. F_3 homozygous plants were used for the experiments.

Fluorescence imaging

To localize the SOC1–GFP fusion protein, *pGC1::SOC1-GFP/phot1 phot2* plants were grown on Murashige and Skoog plates with 1% sucrose for 2 weeks. Epidermal fragments of rosette leaves were isolated as described above. Isolated epidermal fragments were incubated in phosphate-buffered saline (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4) containing 1 μM DAPI (Dojindo) for 10 min at 37°C. After incubation, epidermal fragments were collected on 58 μm nylon mesh, washed with Milli-Q water and mounted with 50% (v/v) glycerol.

Specimens were observed under a fluorescence microscope (BX50; Olympus) with the following filter sets: BP460–480HQ BA495–540HQ (U-MGFPHQ; Olympus) for GFP; BP330–385 BA420 (U-MWU2; Olympus) for DAPI; and BP530–550 BA575IF (U-MWIG3; Olympus) for Chl fluorescence, respectively. An Hg arc lamp was used as a source of excitation light. Fluorescent images were collected using a CCD camera system (DP72; Olympus) and processed using DP2-BSW software (Olympus).

RNA-seq experiments

Epidermal fragments that included stomatal guard cells were isolated from mature rosette leaves of 4- to 5-week-old plants using a blender equipped with an MC2 mini container (Waring Commercial). Mature rosette leaves (1.5–2.8 g) were blended for approximately 40 s in 60 ml of ice-cold Milli-Q water. The epidermal fragments were collected on 58 μm nylon mesh and frozen in a tube with liquid nitrogen. To examine the expression profiles when the stomatal aperture is measured, the epidermal fragments were isolated at ZT4. Total RNA was prepared using a NucleoSpin RNA Plant kit (TAKARA). From a 4 μg aliquot of total RNA, poly(A)-containing RNA was enriched by a Dynabeads mRNA DIRECT Micro Purification Kit (Life Technologies). mRNA was then used to generate an RNA sequencing library using an Ion Total RNA-Seq Kit v2 (Life Technologies). The libraries were analyzed by Ion PGM with Ion 318 ChIP (Life Technologies). Basecalling of sequence reads and mapping to the Arabidopsis transcripts (TAIR10) were performed by Torrent Suite (Life Technologies). Mapping files in *bam* format were converted to *bed* format using the R bioconductor package *GenomicAlignments* (Lawrence et al. 2013) (R Development Core Team). The *bed* format files were used to count sequence reads mapped to each transcript (TAIR10) with the R bioconductor package *ShortRead* (Morgan et al. 2009). We obtained from 2.7 to 5.1 million sequence reads per experiment. The actual read counts were normalized into reads per million (RPM). The experiments were repeated three times on three separate occasions.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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