OPEN ALL NIGHT LONG: The Dark Side of Stomatal Control[1]

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Stomata are mouth-like cellular complexes at the epidermis that regulate gas transfer between plants and atmosphere. In leaves, they typically open during the day to favor CO_2 diffusion when light is available for photosynthesis, and close at night to limit transpiration and save water. Despite the importance of stomatal closure at night for plant fitness and ecosystem water fluxes (Caird et al., 2007), it remains unclear whether this dark response is simply a passive consequence of the absence of light stimulus, or an active process recruiting other mechanisms of stomatal closure or involving independent signaling events (Tallman, 2004; Kollist et al., 2014). Here, we report the isolation and characterization of five Arabidopsis (*Arabidopsis thaliana*) mutants that maintain stomata open the whole night and were named *open all night long1* (*opal1*) to *opal5*. Importantly, stomata of the *opal* mutants closed normally in response to abscisic acid (ABA) and atmospheric CO_2. We propose that dedicated regulators enforce nighttime stomatal closure.

Transpiration drives evaporative cooling. Based on this property, thermal imaging has permitted screening for mutants impaired in leaf transpiration, and thus discovering new signaling players implicated in stomatal response to drought, atmospheric CO_2, or light quality (Merlot et al., 2002; for review, see Negi et al., 2014). So far, no genetic screen has attempted to isolate mutants insensitive to darkness, a situation that plants encounter every night. Here, we screened a mutagenized population of Arabidopsis seedlings by imaging shoot temperature during the night period. Candidates with lower temperature than the wild type were selected, and 37 of them showed a heritable, cool phenotype in darkness (Supplemental Table S1). To avoid mutations with pleiotropic effects, we focused on the group of six cool mutants with similar growth as in the wild type (Fig. 1; Supplemental Fig. S1, A and B).

The six mutants maintained a stable cooler temperature throughout the nighttime and even when the night period was extended for several hours (tested up to 18 h of darkness; Fig. 1B). One of them exhibited an extremely cool phenotype, with a rosette temperature up to 2°C less than in the wild type. Segregation analysis revealed that the mutation was dominant. This prompted us to hypothesize that this mutant was allelic to *open stomata2* (*ost2*), which was confirmed upon sequencing of the gene (Merlot et al., 2007). *OST2* encodes the plasma membrane H^+-ATPase AHA1, which drives the polarization of the plasma membrane that activates inward ion channels, thereby triggering water influx and thus stomatal opening. A dominant mutation in *ost2-2D* triggers constitutive activity of AHA1 and extreme stomatal opening, regardless of external stimuli (Merlot et al., 2007). The five other mutants showed a milder phenotype, with shoots being cooler by 0.5°C to 0.7°C compared with the wild type (Supplemental Fig. S1B). Backcrosses between the wild type and each mutant resulted in F1 plants showing wild-type temperature, whereas each F2 progeny segregated in a 3:1 hot:cool ratio (Supplemental Table S1). These data indicated that the causal mutations were single and recessive. Furthermore, all pairwise crosses between mutants generated plants with a wild-type temperature (data not shown), indicating that the mutations occurred in five distinct loci. These five mutants were therefore named *opal1* to *opal5*.

We confirmed the stomatal origin of the cooler temperature in the *opal* mutants. Because the mutants displayed an unaffected or lower stomatal density (Supplemental Fig. S1C), their cool phenotype was likely caused by a misregulation in guard cell functioning. Gas

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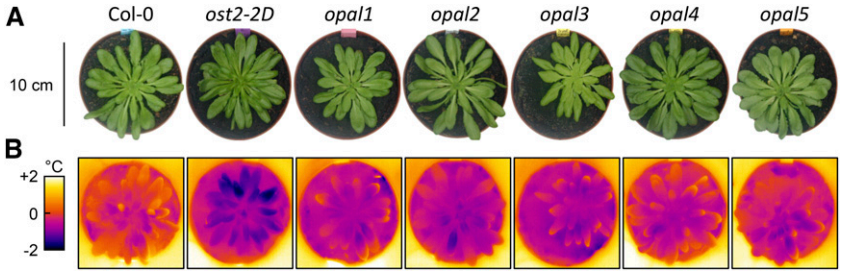

Figure 1. Isolation and characterization of mutants with stomata open all night long. Six mutants were isolated from a thermography screen in dark conditions. A, Top-view pictures of wild-type and mutant (*ost2-2D* and *opal1* to *opal5*) mature plants. B, False-color infrared images of the same plants after 18 h in darkness. The color scale is adjusted so that zero corresponds to the average rosette temperature of Col-0.

exchange was monitored on intact leaves of plants exposed to extended darkness. Compared with the wild type, stomatal conductance in darkness was 2 times higher in the *opal* mutants and 5 times higher in the extreme *ost2-2D* (Fig. 2A). Bioassays on epidermal strips confirmed that stomata of the *opal* mutants remain open in darkness (Fig. 2B). In light conditions, the *opal* mutants showed higher stomatal conductance than the wild type (Fig. 2A), whereas their stomatal aperture on epidermal strips was similar or only slightly increased (Fig. 2B), a discrepancy commonly observed for stomatal response to light, CO_2, or ABA (Mott et al., 2008; Fujita et al., 2013; Pantin et al., 2013a). These results suggest that the mechanisms ruling nighttime stomatal closure also constrain daytime stomatal movements when in contact with the mesophyll.

The sustained stomatal opening of *opal* mutants indicates that their phenotype prevails over transient or circadian effects. Opening stomata in darkness is a typical trait of several mutants affected in the regulation of photomorphogenesis. Photomorphogenesis in darkness is repressed by CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1), an E3 ubiquitin ligase that interacts with a large spectrum of photoreceptors. Although most photoreceptor mutants or overexpressors show the same basal stomatal aperture in the dark as in wild-type plants (Kinoshita et al., 2001; Ohgishi et al., 2004; Mao et al., 2005; Wang et al., 2010, 2014), down-regulation of COP1 activity induces constitutively open stomata in darkness (Mao et al., 2005; Wang et al., 2010). However, *cop1* mutants show severe growth reduction (Mao et al., 2005) or altered stomatal patterning (Kang et al., 2009), ruling out *COP1* as a possible candidate for the *OPAL* genes. Photomorphogenesis in darkness as well as stomatal closure are also controlled by the vacuolar H^+-ATPase, a subunit of which is encoded

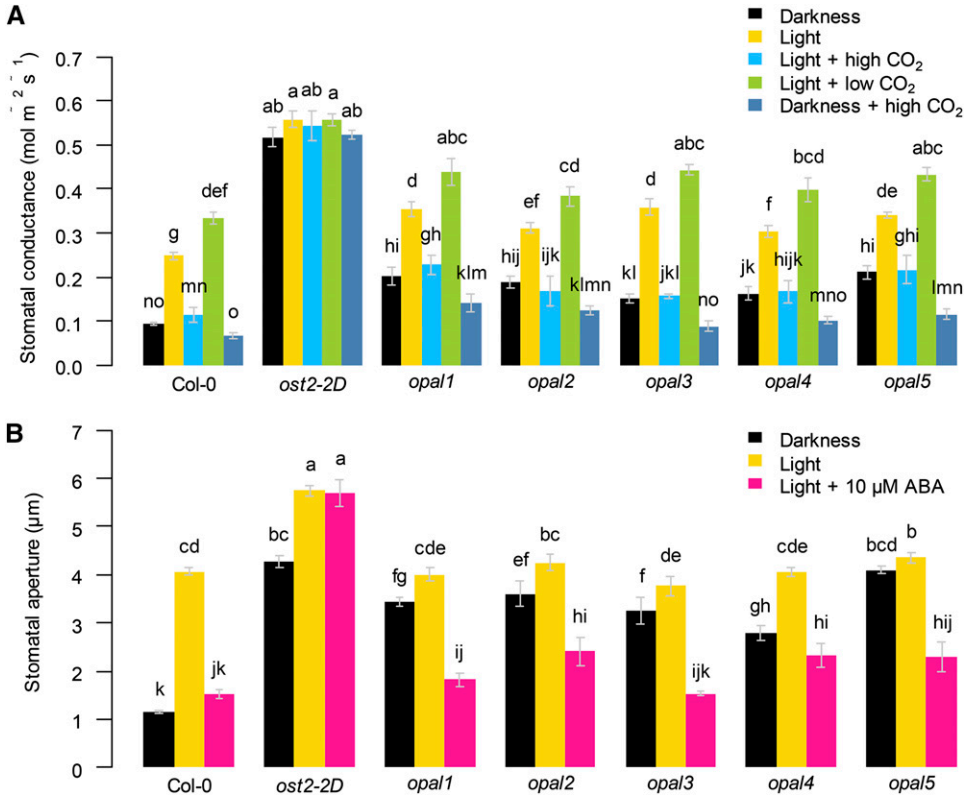

Figure 2. Stomatal response to dark, light, CO_2, and ABA in the *opal* mutants. A, Gas exchange analysis on individual leaves attached to mature plants. Stomatal conductance to water vapor was measured in dark or light (500 μmol m^{-2} s^{-1}) conditions at control (360 μL L^{-1}), low (75 μL L^{-1}), or high (2,000 μL L^{-1}) CO_2 concentration ($41 \leq n \leq 4$). B, Stomatal aperture was measured on epidermal peels in darkness or light (250 μmol m^{-2} s^{-1}) with or without 10 μM ABA ($36 \leq n \leq 6$). Error bars are means ± SE. Letters denote significant differences after a Kruskal-Wallis test ($\alpha = 0.05$), with *P* values adjusted using the Benjamini and Hochberg method for multiple comparisons.

by *DE-ETIOLATED3* (*DET3*; Schumacher et al., 1999; Allen et al., 2000). The *det3* mutant can be rescued by down-regulation of *MYB61* (Newman et al., 2004), a gene coding for an R2R3-MYB transcription factor also involved in stomatal closure (Liang et al., 2005). The *myb61* mutant shows enhanced stomatal conductance in the dark (Liang et al., 2005), but also pleiotropic developmental alterations (Romano et al., 2012), thereby decreasing *MYB61*'s chance as a candidate for the *OPAL* genes.

Stomatal response to darkness might recruit other mechanisms leading to stomatal closure, such as the pathways controlling ABA and CO_2 responses (Tallman, 2004). In line with this, stomata of mutants severely impaired in ABA synthesis (aba type) or sensitivity (abi type) remain largely open in the dark (Leymarie et al., 1998; Pantin et al., 2013b), and mutants with defective ABA receptors (*pyr/pyl/rcar*) show deficient stomatal response to CO_2 and darkness (Merilo et al., 2013). Moreover, disruption of the guard cell slow-type anion channel SLAC1 strongly decreases stomatal response to ABA, CO_2, and darkness (Negi et al., 2008; Vahisalu et al., 2008; Merilo et al., 2013). Similarly, alteration of actin dynamics in guard cells of the *high sugar response3* mutant reduces stomatal response to several closure stimuli, including ABA and darkness (Jiang et al., 2012). Thus, stimuli, such as darkness, ABA, and CO_2, may promote stomatal closure through shared terminal molecular events, triggering solute movements and cytoskeleton rearrangements that result in guard cell deflation.

We, therefore, tested the possibility of *opal* mutants being impaired in stomatal sensitivity to ABA or CO_2. ABA content in these lines did not significantly differ from the wild type (Supplemental Fig. S1E). Epidermal bioassays showed that stomata of the *opal* mutants close in response to 10 μM ABA, contrasting with *ost2-2D* (Fig. 2B). Moreover, the *opal* mutants had similar or even reduced levels of seed germination in the presence of ABA (Supplemental Fig. S1D). Thus, the *opal* mutants are neither strongly ABA deficient nor strongly ABA insensitive. We then probed *opal* responsiveness to contrasting CO_2 concentrations. In the presence of light, the *opal* mutants showed intact responsiveness to both low and high CO_2 (Fig. 2A). Likewise, in darkness, high CO_2 triggered similar stomatal closure in the *opal* mutants as in the wild type, suggesting that these mutants are not impaired in CO_2 signaling. Thus, the *opal* mutants clearly deviate from the classical behavior of mutants impaired in ABA or CO_2 signaling pathways, although it still could be that the *OPAL* genes encode alternative components involved in guard cell ABA metabolism or remote signals related to mesophyll metabolism (Tallman, 2004; Lawson et al., 2014).

Based on the sensitivity of the *opal* mutants to ABA and CO_2, we propose that stomatal response to darkness is at least partly independent from ABA or CO_2 signaling pathways. Interestingly, lycophyte and fern stomata show low sensitivity to ABA and CO_2 (Doi and Shimazaki, 2008; Brodribb et al., 2009; Brodribb and McAdam, 2011, 2013; Ruszala et al., 2011; McAdam and Brodribb, 2012a; Creese et al., 2014) but do respond to dark-light regime (Doi et al., 2006; Doi and Shimazaki, 2008; McAdam and Brodribb, 2012b; Creese et al., 2014). This may indicate that the dark response of stomata is a primitive regulatory backbone over which seed plants have evolved other signaling pathways to respond to an increasing number of stimuli (McAdam and Brodribb, 2012b; but see also Ruszala et al., 2011; Chater et al., 2013). Several pieces of evidence suggest that stomatal responsiveness has been evolutionary refined through an assembly of signaling modules that preexisted in ancestral clades. For instance, seed plants open their stomata in response to blue light, perception of which by phototropins triggers phosphorylation events that activate plasma membrane H^+-ATPases (Takemiya et al., 2013). By contrast, ferns lack stomatal response to blue light, although they possess functional phototropins and plasma membrane H^+-ATPases (Doi et al., 2006). This suggests that seed plants have evolved components able to bridge these signaling modules. Similarly, only angiosperms show stomatal closure in response to high CO_2, which may result from a recent specialization of Ca^{2+} signaling in the guard cells of angiosperms (Brodribb and McAdam, 2013). According to this evolutionary framework, the dark response of stomata may be controlled by more primitive signaling events.

Plasma membrane depolarization through regulation of proton pumps seems to be a key step for stomatal response to darkness. The strong dark phenotype of *ost2-2D* (Merlot et al., 2007; this work) and a line overexpressing constitutively activated AHA2 in guard cells (Wang et al., 2014) show the necessity of down-regulating the activity of plasma membrane H^+-ATPases to close stomata in darkness. The recent discovery that constitutive stomatal opening in the dark through overexpression of flowering regulators (FLOWERING LOCUS T, TWIN SISTER OF FT, CONSTANS, and GIGANTEA) is mediated by the activation of H^+-ATPases (Kinoshita et al., 2011; Ando et al., 2013) further strengthens this proposition. Altogether, these data are consistent with in silico simulations showing stomatal opening in darkness upon constitutive activity of H^+-ATPases, for instance, by abolishing the sensitivity of H^+-ATPases to Ca^{2+} (Blatt et al., 2014). Regulators of the proton pumps (Fuglsang et al., 2007, 2014; Shimazaki et al., 2007), whose involvement in stomatal response to darkness remains largely unknown, seem therefore to be potential candidates underlying the *opal* mutants.

Downstream regulators of the guard cell solute balance also emerge as relevant candidates for the *opal* behavior. For instance, transport and metabolism of malate have been proven of particular importance for stomatal closure in darkness. Mutants defective in QUAC1, a guard cell malate transporter, show a slower rate of stomatal closure in response to light-dark transitions compared with the wild type, but similar steady-state stomatal conductance after dark adaptation (Meyer et al., 2010) or altered growth (Sasaki et al., 2010).

By contrast, *pck1*, a mutant lacking an isoform of phosphoenolpyruvate carboxykinase involved in malate catabolism in guard cells, shows sustained open stomata in darkness and normal growth (Penfield et al., 2012). Importantly, apoplastic malate produced in the mesophyll has an opposite effect on guard cell movements (Araújo et al., 2011; Lawson et al., 2014). Therefore, effectors poising malate concentration within and around guard cells are key candidates for the opal phenotype, a stomatal trait naturally coselected with singular regulation of malate metabolism in Crassulacean acid metabolism plants.

Nighttime stomatal control is of evolutionary and ecological importance, but Francis Darwin's early conclusion that "the biology of nocturnal closure is obscure" (Darwin, 1898) remains timely. The *opal* mutants reported here credit the existence of specific regulators leading to stomatal closure in darkness. Further characterization of these mutants may well shed some light on the dark side of stomatal behavior.

MATERIALS AND METHODS

Growth Conditions

For the primary screen, plants were grown in a growth chamber with a 14-h photoperiod, an irradiance of 180 μmol m^{-2} s^{-1}, a temperature of 25°C, and a relative humidity (RH) of 65%. The other experiments were conducted on plants grown under an 8-h photoperiod at an irradiance of 270 μmol m^{-2} s^{-1}, a temperature of 22°C:18°C (day:night) and an RH of 65%.

Screen and Mutant Selection

An M_2 population of Arabidopsis (*Arabidopsis thaliana*) seeds (ecotype Columbia-0 [Col-0]) mutagenized with ethyl methanesulfonate was purchased from Lehle Seeds. About 72,000 seeds were sown in pots (9 × 9 × 7 cm, 40 seeds per pot). Seedlings were screened 9 to 18 d after germination by thermal imaging in the growth chamber. The screen was performed in darkness about 2 h after light-to-dark transition. Individuals displaying cooler temperature than the wild type were selected as candidate mutants. The progeny of the fertile candidates was used to produce the M_3 generation. M_3 seedlings were probed again by thermal imaging to validate the heritability of the cool phenotype. All validated candidates were then backcrossed with the wild type, and the resultant F1 plants (M_2 × Col-0) were self-crossed to obtain F2 seeds. Only the mutant lines with cool F2 seedlings and similar growth to the wild type were selected for additional backcrosses (Supplemental Table S1). Segregation in the F2 generation was analyzed with a χ^2 for goodness of fit. The allelism tests between the isolated mutants were performed by genetic crosses covering all possible pairwise combinations. Each resulting F1 progeny was compared for rosette temperature with the wild type and its respective parent mutant lines. The temperature phenotype in darkness was checked 15 to 30 d after germination.

Thermal Imaging

Thermography screening was performed using an Inframetrics 760 infrared camera (Inframetrics) equipped with a Stirling-cooled scanning detector in the 3- to 5-μm spectral band. Image resolution was 256 × 256 pixels, and thermal sensitivity of the camera was such that noise-equivalent differential temperature was below 0.1°C. For subsequent phenotyping of the isolated candidates, thermal imaging was performed using a ThermaCam B20HS camera (FLIR Systems) equipped with an uncooled 320 × 240 microbolometer matrix detector in the 7- to 13-μm band having an improved sensitivity (noise-equivalent differential temperature below 0.05°C). These subsequent experiments were carried out in a dedicated darkroom under 25°C ± 1°C temperature, low RH (45% ± 5%), and low wind speed to ensure temperature contrast between lines.

Leaf Gas Exchange

For gas exchange analysis, single plants were grown in pots, and stomatal conductance to water vapor was measured using a LI-COR 6400 gas analyzer system (LI-COR Inc.) equipped with a clamp-on leaf cuvette (6400-40 Leaf Chamber Fluorometer; LI-COR Inc.). Measurements were performed on individual leaves attached to mature plants (40- to 50-d-old plants). Plants were dark adapted for 18 h, and stomatal conductance to water vapor was determined in the dark. After a 2-h light adaptation, stomatal conductance was measured again. The light-emitting diode source provided 500 μmol m^{-2} s^{-1} of irradiance using 10% blue and 90% red. Leaf temperature was maintained at 23.5°C, and leaf-to-air vapor pressure deficit was at 0.8 kPa. The effect of high and low CO_2 was tested by increasing or decreasing atmospheric CO_2 concentration from 360 to 2,000 or 75 μL L^{-1}, respectively.

Stomatal Aperture Bioassays and Stomatal Density

Stomatal aperture and density were determined on epidermal peels of leaves from 21- to 28-d-old plants. Leaves were harvested at the end of the night period, and strips of the abaxial epidermis were processed as described by Merlot et al. (2007). Stomatal aperture was measured after subjecting epidermal peels to 2 to 3 h of darkness or light (250 μmol m^{-2} s^{-1}). After light treatment, peels were incubated for 2 to 3 h with 10 μM ABA, and stomatal aperture was measured again. Each replicate was the average aperture of at least 60 stomata. Stomatal density was determined on abaxial and adaxial peels by counting stomata on a surface of 0.06 mm^2.

Quantification of ABA

The ABA content in leaves of 5- to 6-week-old plants was determined from fresh material frozen in liquid nitrogen and freeze dried before extraction in 1 mL of sterile distilled water at 4°C overnight. ABA was quantified by an ELISA method using the Phytodetek enzyme immunoassay test kit (Agdia Inc.).

Germination Assays

Sensitivity of seed germination to exogenous ABA was tested on mature seeds of plants grown under identical conditions and harvested at the same time. Seeds were sterilized and plated on one-half-strength Murashige and Skoog medium with contrasting ABA concentrations. They were stratified at 4°C for 4 d and then transferred to a growth chamber under a temperature of 24°C and a 16-h photoperiod. Germination was scored according to radicle emergence after 3 d in the growth chamber.

Statistical Analyses

Data analyses were performed using R 3.0.2 (R Core Team, 2013). Each replicate corresponds to one plant, except for germination, which was scored on 50 plants per replicate.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Additional phenotypic characterization of the *opal* mutants.

Supplemental Table S1. Isolation and characterization of mutants with low temperature in darkness.

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