Phototropins But Not Cryptochromes Mediate the Blue Light-Specific Promotion of Stomatal Conductance, While Both Enhance Photosynthesis and Transpiration under Full Sunlight[1,2][C][W][OA]

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Leaf epidermal peels of Arabidopsis (*Arabidopsis thaliana*) mutants lacking either phototropins 1 and 2 (phot1 and phot2) or cryptochromes 1 and 2 (cry1 and cry2) exposed to a background of red light show severely impaired stomatal opening responses to blue light. Since phot and cry are UV-A/blue light photoreceptors, they may be involved in the perception of the blue light-specific signal that induces the aperture of the stomatal pores. In leaf epidermal peels, the blue light-specific effect saturates at low irradiances; therefore, it is considered to operate mainly under the low irradiance of dawn, dusk, or deep canopies. Conversely, we show that both *phot1 phot2* and *cry1 cry2* have reduced stomatal conductance, transpiration, and photosynthesis, particularly under the high irradiance of full sunlight at midday. These mutants show compromised responses of stomatal conductance to irradiance. However, the effects of phot and cry on photosynthesis were largely nonstomatic. While the stomatal conductance phenotype of *phot1 phot2* was blue light specific, *cry1 cry2* showed reduced stomatal conductance not only in response to blue light, but also in response to red light. The levels of abscisic acid were elevated in *cry1 cry2*. We conclude that considering their effects at high irradiances cry and phot are critical for the control of transpiration and photosynthesis rates in the field. The effects of cry on stomatal conductance are largely indirect and involve the control of abscisic acid levels.

The stomata provide a key point of control the exchange of water and CO_2 between the plant and the atmosphere. Stomatal conductance depends on the number of stomata per unit area and the aperture of the stomatal pore, and both are affected by the light environment. High irradiances, typical of open places and seasons with clear skies, increase stomatal density (Willmer and Fricker, 1996; Lake et al., 2001; Thomas et al., 2004; Casson and Gray, 2008). The high red to far-red ratios of open places also increase stomatal density compared to the low ratios of dense vegetation canopies (Boccalandro et al., 2009). These effects of irradiance (Casson et al., 2009) and red/far-red ratio (Boccalandro et al., 2009) are at least partially mediated by phytochrome B (phyB) perception of the light signals. Cryptochrome 1 (cry1) and 2 (cry2) increase stomatal index (Kang et al., 2009), i.e. the ratio between stomata number and total epidermal cells, but whether they also affect stomata density remains to be elucidated.

In addition to its long-term effects on stomatal density, light has rapid effects on stomatal conductance by enhancing the aperture of the stomatal pore. This light effect has two components, one sensitive to photosynthetically active radiation and the other responsive specifically to blue light (Zeiger and Field, 1982). The first component is often called the red light effect (Shimazaki et al., 2007). The nature of the receptor involved in the red light effect has not been definitely established (Baroli et al., 2008; Wang et al.,

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2010, 2011). In isolated epidermal peels of Arabidopsis (*Arabidopsis thaliana*) leaves, the blue light-induced promotion of the aperture of the stomatal pore is severely impaired in the *phototropin1 phototropin2* (*phot1 phot2*) double mutant (Kinoshita et al., 2001) and the *cry1 cry2* mutant (Mao et al., 2005). Furthermore, stomatal conductance fails to respond to blue light in intact leaves of the *phot1 phot2* mutant (Doi et al., 2004). Since cry (Cashmore et al., 1999) and phot (Huala et al., 1997; Kagawa et al., 2001) are blue light photoreceptors, they could be directly involved in the perception of the blue light signal inducing stomatal aperture. Cry would primarily function under relatively high fluence rates of blue light, whereas phot would function under both low and high fluence rates of blue light (Mao et al., 2005).

The proposed function of cry1 and cry2 in blue light-induced stomata opening has been questioned. Ohgishi et al. (2004) compared the response to blue light of epidermal peels of the *phot1 phot2 cry1 cry2* quadruple mutant with that of triple mutants containing either cry1 or cry2 and concluded that none of the latter photoreceptors was able to mediate the blue light-induced stomatal aperture. Shimazaki et al. (2007) argued that the residual effect of blue light observed in the *phot1 phot2* double mutant could be mediated by chlorophyll in the guard cells because the background red light intensity employed by Mao et al. (2005) would not have sufficed to saturate guard cell photosynthesis. Shimazaki et al. (2007) also suggested that cry might function in a blue light-independent manner to inhibit stomatal closure and thereby promote stomatal opening. Lascève et al. (1999) measured stomatal conductance in Arabidopsis intact leaves and observed apparently normal responses to blue light in the *cry1 cry2* double mutant. The *cry1 cry2* double mutant did show a light-independent reduction of stomatal conductance that was assigned by the authors to variations in leaf temperature among plants, inaccuracy in estimation of exposed leaf surface, or some consequence of the mutations (Lascève et al., 1999). Differences between the stomatal responses in isolated epidermal peels compared to intact leaves are well documented and could result from the presence of the mesophyll in intact leaves (Mott, 2009). Therefore, whether cry actually affects stomatal responses in intact leaves remains to be elucidated.

The blue light-dependent system controlling stomatal aperture saturates well below 1% of full sunlight, which supports the idea that predawn increases in stomatal conductance are a response to blue light (Zeiger and Field, 1982). The effect of blue light on stomatal conductance is rapid and would reduce the limitation of photosynthesis by CO_2 at dawn, when light reactions reach maximum rates faster than stomatal opening (Zeiger and Field, 1982; Shimazaki et al., 2007). The blue light-dependent system would also be important for stomata opening under low irradiances, such as the understory of dense canopies, where light levels might not exceed the threshold for the red-light response (Zeiger and Field, 1982). This idea is consistent with the promotion of plant growth capacity and photosynthetic rates by phot in low light environments (Takemiya et al., 2005). Based on these arguments, one would expect blue light photoreceptors to affect stomatal conductance of fully exposed, nonshaded plants, only at both extremes of the photoperiod. However, this prediction requires experimental evaluation.

The aim of this article was to characterize the light response of stomatal conductance in intact leaves of the *phot1 phot2* and *cry1 cry2* double mutants to elucidate (1) whether cry is a photoreceptor involved in the blue light system that controls stomatal conductance of intact leaves, and (2), the contribution of phot and cry to the diurnal dynamics of stomatal conductance, transpiration, and photosynthesis in plants grown under full natural radiation.

RESULTS

Diurnal Pattern of Stomatal Conductance and Transpiration in *cry1 cry2* and *phot1 phot2* under Natural Radiation

Arabidopsis wild-type and the double mutants *cry1 cry2* and *phot1 phot2* plants were grown under natural radiation in a glasshouse (winter experiment, photoperiod of approximately 10 h; Fig. 1, A–C) or outdoors (summer experiment, photoperiod of approximately 14.5 h; Fig. 1, D–F) for 6 or 5 weeks, respectively. The diurnal pattern of stomatal conductance (Fig. 1, A and D), transpiration per unit leaf area (Fig. 1, B and E), and irradiance and air temperature (Fig. 1, C and F) were recorded. Blue light (400–500 nm) at maximum irradiance was 302 and 798 μmol m^{-2} s^{-1} in the winter and summer experiments, respectively. In the wild type, maximum stomatal conductance and transpiration rate occurred at midday and were higher in summer than in winter. Since the proportion of blue light is higher at the extremes of the photoperiod and previous experiments with epidermal peels had shown saturation of the blue light effect at relatively low irradiances (Mao et al., 2005; Kinoshita et al., 2001), it was expected a higher impact of the *cry1 cry2* and *phot1 phot2* mutations at dawn and/or at dusk. Contrary to this, both the *cry1 cry2* and *phot1 phot2* reduced stomatal conductance mainly at midday, when irradiance was high (Fig. 1, C and F). Leaf water potential at midday was not significantly affected by the different genotypes (MPa, mean $\pm$ SE; winter experiment: the wild type, -0.12 ± 0.02; *cry1 cry2*, -0.14 ± 0.03; and *phot1 phot2*, -0.16 ± 0.03; $P = 0.28$ $n = 8$; summer experiment: the wild type, -2.0 ± 0.29; *cry1 cry2*, -1.6 ± 0.08; and *phot1 phot2*, -1.4 ± 0.14; $P = 0.5$; $n = 5$).

Additional glasshouse experiments were conducted to test the effect of the above mutations under different

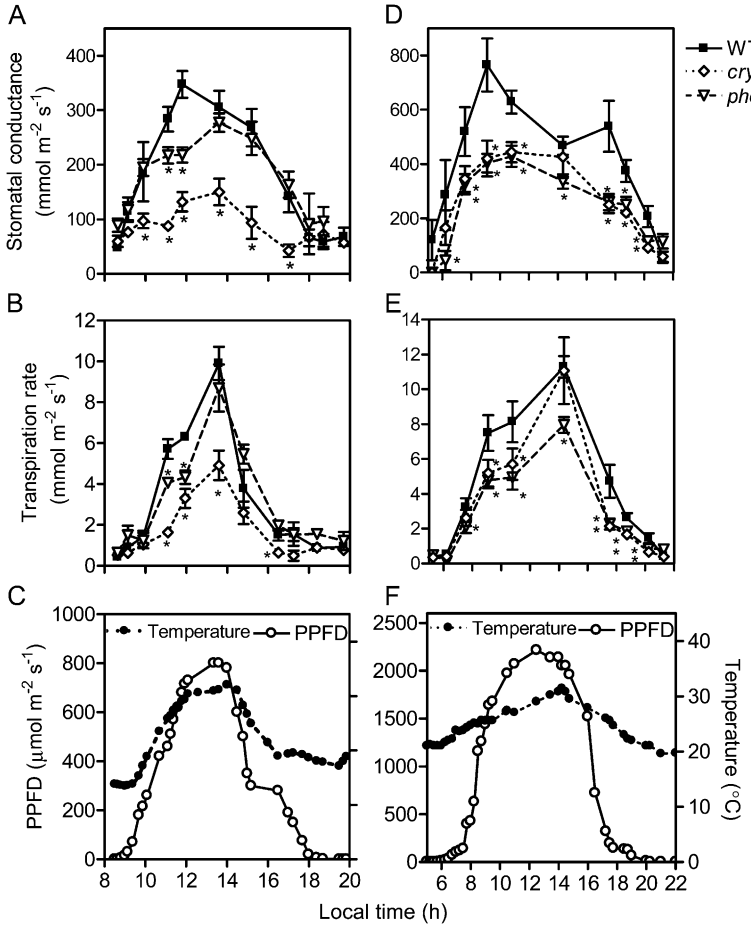

Figure 1. Cryptochromes and phototropins control stomatal conductance and transpiration rates under natural radiation. Diurnal course of stomatal conductance (A and D) and transpiration per unit leaf area (B and E) in adult plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants recorded under the indicated PPFD and temperatures (C and F) in a glasshouse in winter (A–C) or outdoors in summer (D–F). Data are means and SE of at least three plant replicates. An asterisk close to a mutant genotype symbol denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests.

combinations of cloudiness, temperature, and humidity. The *cry1 cry2* mutant showed reduced midday stomatal conductance in all cases, but the *phot1 phot2* mutant was significantly affected only in the sunny and temperate days when conductance was higher (Supplemental Fig. S1). This indicates that the impact of cry on stomatal conductance is more robust than that of phot. Variation in abaxial rather than in adaxial stomatal conductance was observed in wild-type plants subjected to different environmental conditions, indicating that stomata located in the abaxial leaf surface are responsible for most of the dynamic adjustment to a changing environment (Supplemental Fig. S1). cry promoted stomatal conductance in both leaf surfaces under different environmental conditions, while phot mainly stimulate stomatal conductance under maximum irradiance in the lower epidermis (Supplemental Fig. S1).

In independent experiments, the single *cry1*, *cry2*, *phot1*, and *phot2* mutants were included. Reduced stomatal conductance was only observed for the *cry1 cry2* and *phot1 phot2* double mutants (mean ± SE in mmol $m^{-2}\,s^{-1}$, $n \geq 4$; the wild type, 310 ± 33; *phot1*, 398 ± 58; *phot2*, 385 ± 20; *cry1*, 222 ± 18; *cry2*, 279 ± 62; *phot1 phot2*, 193 ± 35; *cry1 cry2*, 117 ± 13). This indicates that while cry and phot are not redundant, the members of these families are.

Diurnal Pattern of Stomatal Conductance in *cry1 cry2* and *phot1 phot2* under Constant Irradiance

Under natural radiation, the differences between the wild type and the *cry1 cry2* or *phot1 phot2* mutants increased toward midday (Fig. 1). This could be due to time-dependent and/or environmental-dependent (irradiance, temperature, and humidity) effects. To discriminate between these possibilities, plants were cultivated in a growth chamber under a photosynthetic photon flux density (PPFD) of 170 μmol $m^{-2}\,s^{-1}$, and immediately prior to the onset of the photoperiod in which the measurements were taken, half of the plants were transferred to a lower PPFD (17 μmol $m^{-2}\,s^{-1}$). At 170 μmol $m^{-2}\,s^{-1}$, stomatal conductance was lower in the *cry1 cry2* and *phot1 phot2* mutants than in the wild type (Fig. 2A). These differences were established at the beginning of the photoperiod and remained stable afterward, suggesting that the increased impact of the mutations observed at midday compared to the extremes of the photoperiod under natural radiation (Fig. 1, A and D) are caused by the diurnal fluctuations of the environment (e.g. higher irradiances at midday). Lowering PPFD to17 μmol $m^{-2}\,s^{-1}$ reduced stomatal conductance in the wild type and the *cry1 cry2* mutant, without seriously affecting the difference between these genotypes compared to

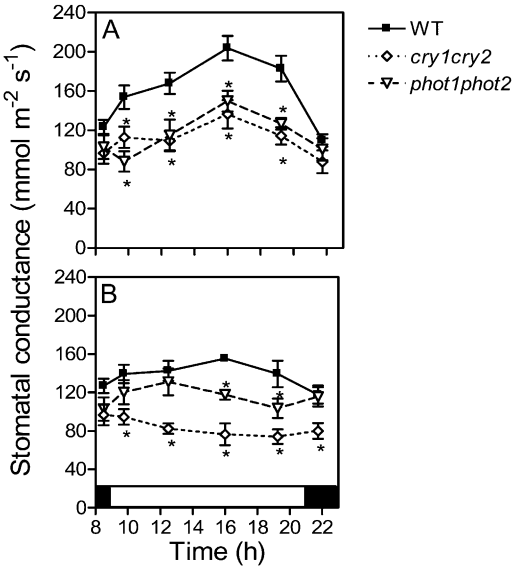

Figure 2. Diurnal course of stomatal conductance in plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants exposed to different levels of constant irradiance. Plants were grown in a growth chamber under a PPFD = 170 μmol m^{-2} s^{-1} (12 h light/12 h dark, relative humidity [HR] approximately 50%, 22°C) for 32 d and either remained at that irradiance (A) or were transferred to 17 μmol m^{-2} s^{-1} (B) before the onset of the photoperiod in which stomatal conductance was recorded. Data are means and SE of six plant replicates. An asterisk close to a mutant genotype symbol denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests.

that observed at 170 μmol m^{-2} s^{-1} (Fig. 2B). Conversely, lowering PPFD only slightly reduced stomatal conductance of the *phot1 phot2* mutant, which therefore showed values closer to those of the wild type (Fig. 2B). Despite constant environmental conditions throughout the photoperiod, in plants exposed to 170 μmol m^{-2} s^{-1}, stomatal conductance reached a maximum at midday (all genotypes; Figure 2A). This fluctuation was weak or undetectable in plants exposed to 17 μmol m^{-2} s^{-1} during the day when measurements were recorded (Fig. 2B), indicating that rhythmic fluctuations in stomatal conductance depend on irradiance.

Photosynthesis in *cry1 cry2* and *phot1 phot2*

Both *cry1 cry2* and *phot1 phot2* showed reduced rates of transpiration under natural radiation (Fig. 1, B and E). Since these effects were observed close to midday, when irradiance levels were high, *cry1 cry2* and *phot1 phot2* could limit photosynthesis by lowering the flux of CO_2. To investigate the long-term transpiration efficiency (CO_2 fixed/water loss ratio), the isotopic discrimination against $^{13}CO_2$ with respect to $^{12}CO_2$ (Δ; Farquhar and Richards, 1984; Masle et al., 2005) in plants grown under natural radiation was analyzed. The wild type and the *cry1 cry2* and *phot1 phot2* mutants presented similar Δ values (mean $\pm$ SE in Δ per million, $n = 3$; the wild type, 22.1 $\pm$ 0.1; *cry1 cry2*, 21.6 $\pm$ 0.5; *phot1 phot2*, 21.8 $\pm$ 0.4; $P = 0.69$), indicating that lower photosynthetic rates should accompany the lower transpiration rates presented by *cry1 cry2* and *phot1 phot2*.

Based on these long-term observations, the daily fluctuations in net CO_2 uptake in plants grown under natural radiation (Fig. 3, same day displayed in Fig. 1, A–C) were analyzed. Net CO_2 uptake was lower in the *cry1 cry2* and *phot1 phot2* double mutants than in the wild type (Fig. 3). The single mutants *cry1*, *cry2*, *phot1*, and *phot2* showed no differences from the wild type (mean $\pm$ SE in μmol m^{-2} s^{-1}, $n \geq 4$; the wild type, 18.6 $\pm$ 1.3; *phot1*, 21.3 $\pm$ 2.4; *phot2*, 18.2 $\pm$ 1.7; *cry1*, 17.5 $\pm$ 1.4; *cry2*, 19.7 $\pm$ 3.2; *phot1 phot2*, 15.9 $\pm$ 0.7; *cry1 cry2*, 8.2 $\pm$ 0.7), indicating a redundant role for both members of the cry and phot families in the control of photosynthetic rate. Differences in the rate of net CO_2 uptake were confirmed in an independent experiment involving a different set of plants in a sunny day of winter (Supplemental Fig. S2).

Irradiance Dependency of the Effects of cry and phot on Stomatal Conductance and Photosynthetic Rates

The responses of stomatal conductance and photosynthesis to irradiance were investigated in plants cultivated in a growth camber under photoperiods of 170 μmol m^{-2} s^{-1}. In the wild type, stomatal conductance increased with PPFD, reaching the maximum values at approximately 500 μmol m^{-2} s^{-1} (Fig. 4A). The *cry1 cry2* and *phot1 phot2* mutants presented lower stomatal conductance than wild-type plants throughout the whole range of irradiances tested here and did not respond to increasing PPFD levels (slope not significantly different from zero; Fig. 4A). Stomatal conductance consistently failed to respond to irradiance in the *phot1 phot2* mutant (Figs. 2 and 4A). The *cry1 cry2* mutant showed normal responses to differences in irradiance established at least 1 h before the measurements of stomatal conductance (Fig. 2), but it

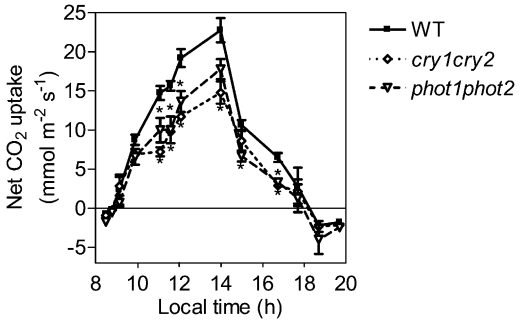

Figure 3. Diurnal course of net CO_2 uptake in plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants grown under natural radiation in a glasshouse (same as the experiment in Fig. 1, A–C). Data are means and SE of at least three plant replicates for each time and genotype. An asterisk close to a mutant genotype denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests.

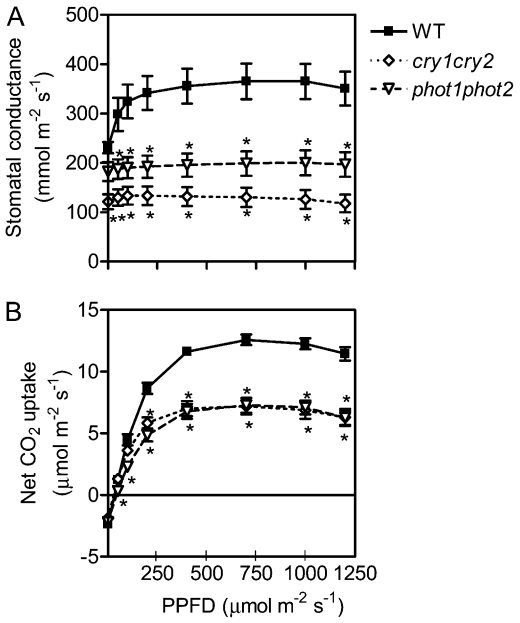

Figure 4. Fluence rate response curves of net CO_2 uptake in plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants. Stomatal conductance (A) and net CO_2 uptake (B) as a function of PPFD. Plants were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C). Data are means and SE of at least four plant replicates. An asterisk close to a mutant genotype denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests.

failed to respond to changes in irradiance in the order of minutes, which are involved in the measurements done with the infrared gas analyzer in combination with its portable light source of variable irradiance (Fig. 4A).

Net CO_2 exchange in darkness was unaffected by the *cry1 cry2* or *phot1 phot2* mutations, which therefore had no significant effects on mitochondrial respiration rates (Fig. 4B). The rates of net CO_2 exchange increased with PPFD in the three genotypes, (Fig. 4B). The *phot1 phot2* double mutant presented significantly lower photosynthetic rates at all the irradiances tested here, while *cry1 cry2* showed significantly lower photosynthetic rates only at high irradiances (200 μmol m^{-2} s^{-1} or more; Fig. 4B). Both mutants and the wild type reached maximum photosynthetic rates at approximately 400 μmol m^{-2} s^{-1}, but these rates were significantly lower in *cry1 cry2* and *phot1 phot2* (Fig. 4B), indicating that they have lower carboxylation capacity compared to the wild type.

Stomatic and Nonstomatic Effects of *phot1 phot2* and *cry1 cry2* on Photosynthetic Rate

To investigate whether at high irradiance (700 μmol m^{-2} s^{-1}) the effects of *phot1 phot2* and *cry1 cry2* mutants are the result of their reduced stomatal conductance that limits the flux of CO_2 to the mesophyll, the responses of stomatal conductance and net CO_2 uptake to CO_2 levels were measured. When exposed to increasing CO_2 levels, wild-type plants gradually reduced stomatal conductance, the *cry1 cry2* mutant responded poorly, and the *phot1 phot2* mutant completely failed to respond (Fig. 5A). As a result of this, the differences in stomatal conductance between the wild type and *cry1 cry2* narrowed down with increasing CO_2, and the difference between the wild type and *phot1 phot2* disappeared for CO_2 levels above 1,000 μmol mol^{-1} (Fig. 5A). Net CO_2 uptake increased with CO_2 levels in the wild type, *cry1 cry2*, and *phot1 phot2*, but the mutants showed lower rates of CO_2 uptake even in the range of high CO_2 levels, though differences in stomatal conductance were small or null (Fig. 5B). This indicates that the *phot1 phot2* mutant has

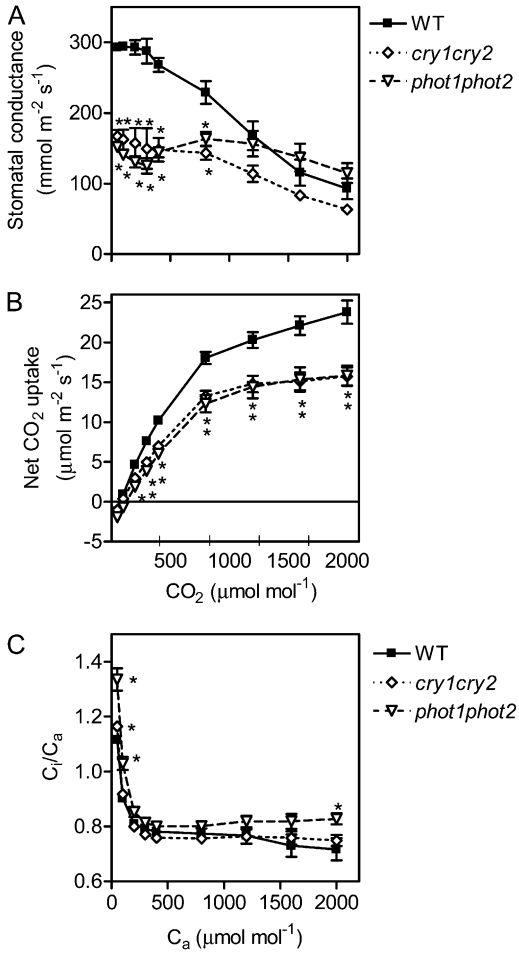

Figure 5. CO_2 response curves of net CO_2 uptake in plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants. Stomatal conductance (A), net CO_2 uptake (B), and ratios between intercellular (C_i) and ambient (C_a) CO_2 concentrations as a function of ambient CO_2 concentration (C). Plants were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C). Data are means and SE of at least five plant replicates. An asterisk close to a mutant genotype denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests.

nonstomatic effects on photosynthesis. In accordance with this conclusion, the ratio between intercellular and ambient CO_2 concentrations was higher in the *phot1 phot2* mutant than in the wild type (Fig. 5C), while stomatal limitations would yield a low ratio. Also, the ratio between intercellular and ambient CO_2 concentrations was unaffected by the *cry1 cry2* mutations, indicating that the limitations to photosynthesis are largely nonstomatic (Fig. 5C).

Reduced Stomatal Conductance of *cry1 cry2* under Red Light

The reduced stomatal conductance of the *cry1 cry2* double mutant compared to the wild type in darkness (Fig. 2) encouraged the analysis of red light effects, i.e. in the absence of blue light to activate cry and phot. Wild-type, *cry1 cry2*, and *phot1 phot2* plants were grown under white light for 32 d. At day 33, half of the plants were exposed since the onset of the photoperiod to 6 h of white light, while the other half was exposed for 6 h to red light before measurements of stomatal conductance. Compared to the wild type, the *phot1 phot2* mutant showed reduced conductance under white light and conductance values similar to the wild type under red light (Fig. 6A). This is consistent with a direct role of phot1 and phot2 on the perception of current blue light levels. Conversely, the *cry1 cry2* mutant presented low stomatal conductance even under red light (Fig. 6A). In a similar experimental setting, the response of stomatal conductance to different irradiance levels of red or blue light was measured. The *phot1 phot2* mutant responded normally to red light but it failed to exhibit the blue light-specific effect (evidenced by the higher slope of the response to blue compared to red light in the wild type; Fig. 6B). The *cry1 cry2* mutant not only lacked the blue light-specific component, but it also severely failed in the response to red light (Fig. 6B).

Reduced Stomata Density in *cry1 cry2*

The *cry1 cry2* mutant has reduced stomatal index, i.e. the ratio between stomata density and epidermal cell density (Kang et al., 2009). Stomatal conductance is not necessarily affected by stomatal index (Morrison, 1998; Poole et al., 2000). We therefore recorded stomata density and observed a statistically significant reduction in *cry1 cry2* compared to *phot1 phot2* or the wild type (sum of stomata of both leaf surfaces mm^{-2}; the wild type, 292 ± 12; *cry1 cry2*, 251 ± 5; *phot1 phot2*, 316 ± 14; $n = 16$). However, the magnitude of this effect (14% reduction) accounts only partially for the effects on stomatal conductance under red light (17% to 38% reduction; Fig. 6).

Increased ABA Levels in *cry1 cry2*

We investigated the levels of abscisic acid (ABA) in *cry1 cry2* because ABA alters stomatal responses to light (Shimazaki et al., 2007). Compared to the wild type, leaves of the *cry1 cry2* mutant had significantly higher levels of ABA measured either at midday (Fig.

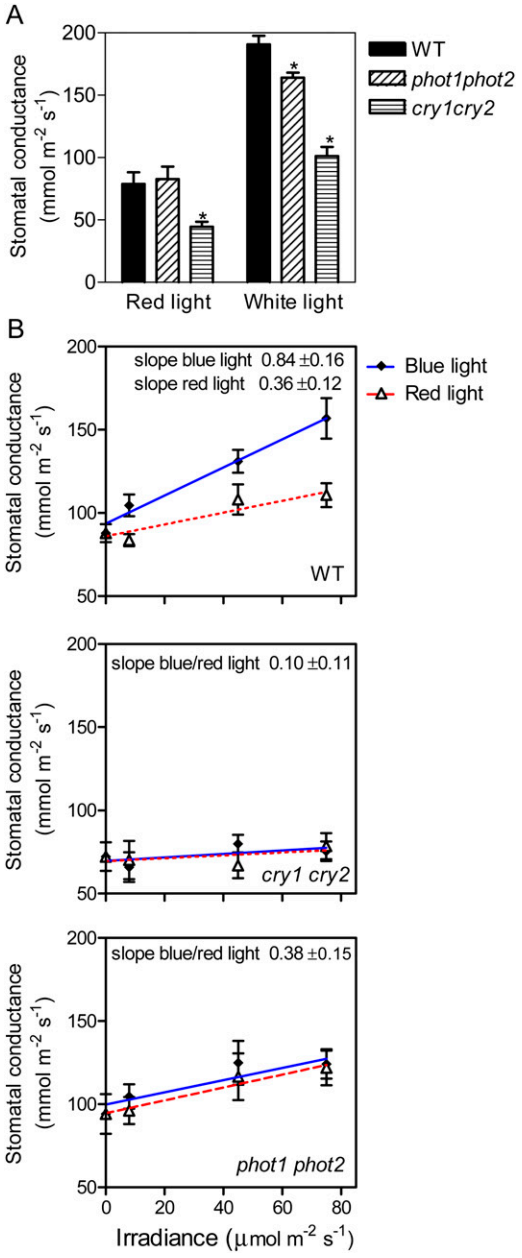

Figure 6. The *cry1 cry2* double mutant has reduced stomatal conductance even in the absence of blue light. Plants of the wild type and of the *cry1 cry2* and *phot1 phot2* double mutants were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C). During the photoperiod when stomatal conductance was recorded, the plants were exposed for 6 h to red light or white light (A; both at 110 μmol m^{-2} s^{-1}) or to different irradiances of blue or red light (B). Data are means and SE of at least five plant replicates. In A, an asterisk close to a mutant genotype denotes significant differences ($P < 0.05$) with the wild type according to ANOVA and Bonferroni post tests. In B, the slope and SE are indicated. [See online article for color version of this figure.]

7A) or during the night (Fig. 7B). This suggests that differences in ABA generated under white light persist even in the absence of light absorbed by cry and affect stomata responses. Consistently with differences in stomatal conductance generated by different ABA levels, ABA applications to foliage (spray of 100 μM solution; Fig. 8A) or to roots (watering with 35 μM solution; Fig. 8B) eliminated the differences between the wild type and *cry1 cry2*.

DISCUSSION

It has been shown that both the stomatal pore of leaf epidermal peels of the *phot1 phot2* (Kinoshita et al., 2001) and *cry1 cry2* (Mao et al., 2005) double mutants of Arabidopsis and the stomatal conductance of intact *phot1 phot2* leaves (Doi et al., 2004) have severely impaired responses to blue light added to a background of red light. Based on these observations, it would be reasonable to predict that phot and cry are the receptors of the blue light-specific system involved in stomatal opening (although the role of cry had already been challenged; Shimazaki et al., 2007). Here, we confirm that the effects of phot on stomatal conductance are blue light specific (Fig. 6), indicating that phots are the direct receptors of the blue light stimulus. However, the *cry1 cry2* mutant showed severely reduced responses to blue as well as to red light (Fig. 6). In some experiments, the *cry1 cry2* mutant showed reduced stomatal conductance even in darkness (Fig. 2). These results indicate that crys are not directly

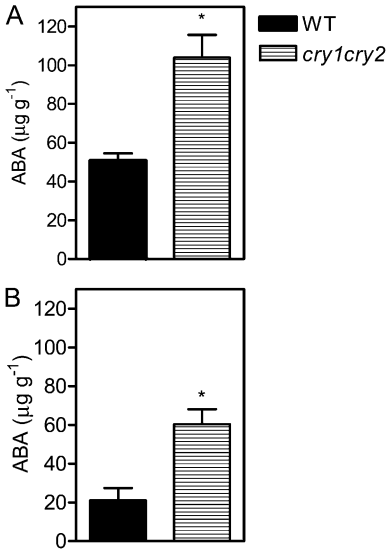

Figure 7. Increased ABA levels in the leaves of the *cry1 cry2* mutant. Plants of the wild type and of the *cry1 cry2* double mutant were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C) and harvested either at midday of day 33 (A) or the following night, 9 h after the end of the photoperiod (B). Data are means and SE of three replicates. An asterisk denotes significant differences ($P < 0.05$) with the wild type according to *t* tests.

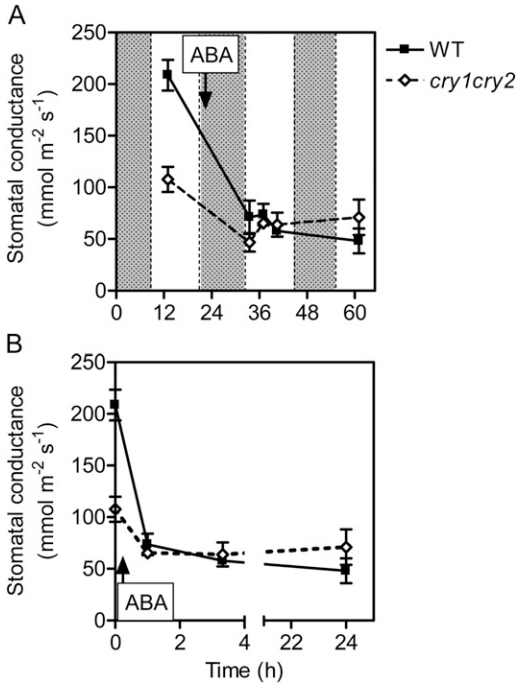

Figure 8. Exogenously applied ABA eliminates the differences in stomatal conductance between the *cry1 cry2* and the wild type. Plants of the wild type and of the *cry1 cry2* double mutant were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C), and ABA was either sprayed (100 μM) on the leaves (A) or added with watering (35 μM; B) at the indicated times (arrows). Data are means and SE of five replicates. An asterisk denotes significant differences ($P < 0.05$) between the wild type and *cry1 cry2* according to *t* tests.

involved in the perception of the blue light signal that stimulates stomatal aperture. We propose that the perception of previous blue light by cry causes persistent changes that determine subsequent stomatal conductance and its response to both red and blue light. One of the persistent changes in *cry1 cry2* is the reduced stomatal density. This result complements previous observations of a reduced stomatal index in *cry1 cry2* (Kang et al., 2009). However, differences in stomatal density were too small to account for the larger effects on stomatal conductance. Therefore, the effects of cry were largely on stomatal aperture. We observed increased levels of ABA in the *cry1 cry2* double mutant (Fig. 7). In turn, increased ABA has been shown to reduce stomatal density (Bradford et al., 1983; Léon-Kloosterziel et al., 1996) and stomatal responses to light (Shimazaki et al., 2007). Addition of ABA eliminated the differences in conductance between the wild type and *cry1 cry2* (Fig. 8). Therefore, we propose that light perception by cry reduces ABA levels, which in turn cause increased stomatal density and stomatal responsiveness to light signals, inducing the opening of the pore. Blue light-independent phenotypes of cry mutants had been reported for gene expression (Yang et al., 2008) and seedling morphol-

ogy (Botto et al., 2003) during deetiolation, and it would be interesting to elucidate whether the latter effects are also related to differences in ABA levels.

The expression of *FLOWERING LOCUS T* (*FT*) in occlusive cells has recently been shown to be important for the induction of stomatal opening by blue light (Kinoshita et al., 2011). In Arabidopsis plants exposed to long days, cry (mainly cry2) promotes the expression of *FT* in vascular tissues (Endo et al., 2007; Kobayashi and Weigel, 2007), and the FT protein then migrates to the apex to induce the transition to flowering (Corbesier et al., 2007). Therefore, a role of FT in the cry-mediated effects on stomatal opening cannot be ruled out. However, the latter is unlikely to be the main pathway of cry activity because at least in the case of flowering, cry promotion of *FT* expression occurs under long days and here cry effects on stomatal conductance were observed under long or short days (Fig. 1). Furthermore, while cry2 is the main cry in terms of induction of *FT* expression (Endo et al., 2007), cry1 and cry2 redundantly enhance stomatal conductance.

Previous experiments where epidermal peels from Arabidopsis leaves exposed to a background of red light showed saturation of stomatal aperture by low irradiances of blue light (Kinoshita et al., 2001; Mao et al., 2005), suggesting that the relative contribution of blue light photoreceptors would be maximal at the low irradiance levels typical of the extremes of the photoperiod or of deep canopy shade light. However, both *phot1 phot2* and *cry1 cry2* showed impaired promotion of stomatal conductance by irradiance in the field. In these mutants, stomatal conductance was close to the wild type at the extremes of the day and reached the widest difference at midday (Fig. 1), particularly under clear skies (Fig. 1; Supplemental Fig. S2). In the field, changes in temperature and water vapor partial pressure deficit accompany different sunlight levels, but a defect in the response to irradiance was also observed under controlled conditions (Figs. 2 and 4), indicating a light-specific effect. Both *phot1 phot2* and *cry1 cry2* partially responded to irradiance when stomatal conductance was measured on a time scale of hours (Fig. 2), but they completely failed to respond to irradiance on a time scale of minutes (Fig. 4). Therefore, rapid adjustment of stomatal conductance depends entirely on phot and cry. The differences with previous reports where the effects of cry and phot appear to saturate at low irradiance (Kinoshita et al., 2001; Mao et al., 2005) might relate to the use of intact leaves rather than epidermal peels and to acclimation to higher growth irradiances, among other possibilities.

The scenario reported here significantly upgrades the importance of the control of stomatal conductance by phot and cry: At high irradiance, CO_2 diffusion is more likely to limit photosynthesis, and the higher radiation load augments leaf temperature and water vapor partial pressure deficit, thus increasing transpiration particularly if stomata are open. Close to midday of a clear winter day, the *phot1 phot2* and *cry1 cry2* mutations reduced instant transpiration rates 29% and 72% (Fig. 1) and instant photosynthesis rates 29% and 39% compared to the wild type (Fig. 3). The long-term transpiration efficiency was unaffected in *cry1 cry2* or *phot1 phot2*, indicating that transpiration and CO_2 fixation suffered quantitatively similar reductions. phot had been shown to affect plant growth at low irradiance (Takemiya et al., 2005), and our results expand the range where phot and cry influence key physiological processes to high irradiances.

The effects of phot and cry on photosynthesis were largely nonstomatic (Fig. 5), i.e. although stomatal conductance was reduced by the mutations to levels that in the wild type would reduce maximum photosynthesis, other aspects of the photosynthetic process were also affected in the mutants and imposed a limit to photosynthesis. Light-saturated rates of electron transport per unit area were reduced in *cry1 cry2* and *phot1 phot2* and could account for the nonstomatic limitation of these mutants (Boonman, et al., 2009). In rice (*Oryza sativa*), the *phot1* mutant shows reduced photosynthetic rates associated to elevated hydrogen peroxide accumulation (Goh et al., 2009). At low irradiance levels, phot1 is required to optimize chloroplast exposure to light (Christie, 2007; Königer et al., 2008).

In conclusion, phot is directly involved in the perception of the daily fluctuations of irradiance, which triggers rapid stomatal responses. Conversely, both phyB (Boccalandro et al., 2009; Casson et al., 2009) and cry are involved in long-term changes that indirectly condition rapid stomatal responses. In the case of phyB, the effects on stomatal density (Boccalandro et al., 2009; Casson et al., 2009) are larger than the effects on stomatal aperture (Wang et al., 2010). In the case of cry, the effects on stomatal aperture are larger than the effects on stomatal density (this article). Changes in stomatal density involve a slow developmental response, and the cry-mediated changes in ABA do not disappear at night (i.e. in the absence of cry activity; Fig. 7B), also indicating a slower turnover. The longer term kinetics of the changes mediated by phyB and cry would provide a wider temporal window to integrate signals from the environment related to neighbors (shade) or season (cloudiness), complementing the rapid adjustment mediated by phot.

MATERIALS AND METHODS

Plant Material

The wild-type accession Columbia of Arabidopsis (*Arabidopsis thaliana*) and the single mutants *cry1* (*hy4-B104*; Bruggemann et al., 1996), *cry2-1* (Guo et al., 1998), *phot1-5* (Liscum and Briggs, 1995; Huala et al., 1997), *phot2-1* (Kagawa et al., 2001); and the double mutants *cry1 cry2* (*cry1-hy4-b104 cry2-1*; Buchovsky et al., 2008) and *phot1 phot2* (*phot1-5 phot2-1*; Liscum and Briggs, 1995; Kagawa et al., 2001) were used in this study. The seeds were sown on 0.8% agar, and 4 d-old seedlings were transplanted to 230-cm^3 pots containing equal amounts of perlite (Perlome; Perfiltra), peat moss (Ciudad Floral), and vermiculite (Intersum) and watered as needed with a solution containing 1 g L^{-1} of Hakaphos Red (COMPO).

Experimental Conditions and Light Treatment

Field experiments were conducted in the experimental field of the Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura, Faculty of Agronomy of the University of Buenos Aires (latitude 34° 35′S, 58° 29′W, altitude 10 m). During the experiments, plants were covered with a 6-mm glass combined with a neutral mesh (that reduce 30% of the PPFD), located 60 cm above the plants to cut off rain.

Glasshouse experiments were conducted in the experimental field of the Faculty of Agronomy of the University of Cuyo, Mendoza, Argentina (latitude 33°0′S, longitude 68°52′W, altitude 950 m).

Temperature and HR were recorded using iButton data loggers (Maxim Integrated Products) and Hobo (Onset Computer Corporation), respectively.

Growth chamber experiments were performed with plants cultivated under fluorescent lamps (36 W; Osram) that provide 170 μmol m^{-2} s^{-1} of PPFD.

PPFD was measured with a LI-COR Li-188B sensor. Photoperiod was 12 h, and temperature was 23°C ± 1°C.

Red light treatment (110 μmol m^{-2} s^{-1} in Fig. 7A and indicated irradiances in Fig. 7B) was performed by filtering fluorescent lamps with a combination of orange and yellow filters (Lee Filters; nos. 105 and 101, respectively). The light spectrum determined with an Ocean Optics spectrometer (model USB4000) to corroborate blue light was completely cut off in the red light treatment. Blue light was obtained by filtering fluorescent lamps with a blue filter (Lee Filters; no. 119). To obtain different irradiances of red, blue, or white light, neutral filters (Lee Filters; nos. 298, 210, and 299) were added to the different light treatments.

Gas Exchange Measurements: Conductance, Transpiration, and Photosynthesis

Stomatal conductance, transpiration rate, and CO_2 exchange presented in Figures 1, 3 to 5, and Supplemental Figure S2 were measured by using an open infrared gas analysis system (LI-COR 6400). CO_2 exchange at 0, 50, 100, 200, 400, 800, 1,000, and 1,200 μmol m^{-2} s^{-1} PPFD were measured in fully expanded leaves of *cry1 cry2*, *phot1 phot2*, and wild-type Landsberg *erecta* 35-d-old plants using a 0.25-liter chamber attached to a regulated portable light source (QB1205LI-670; Quantum Devices). CO_2 during measurements were set at 400 ppm in the reference cell. Dose response to CO_2 levels was determined with the following sequence of CO_2 concentrations at the reference cell: 400, 300, 200, 100, 50, 400, 800, 1,200, 1,600, and 2,000 μmol mol^{-1} s^{-1}, setting PPFD at 700 μmol m^{-2} s^{-1}. Stomatal conductance to water vapor presented in Figures 2, 6, 8, and Supplemental Figure S1 was measured with a steady-state diffusion porometer (SC-1; Decagon Devices) on both leaf surfaces.

Carbon Isotope Discrimination

Analysis of carbon isotope composition was performed on 35-d-old rosette leaves grown under natural radiation. Three plants per genotype were pooled for each independent biological replicate. Carbon isotope composition (δ) was measured at the Stable Isotope Ratio Facility for Environmental Research (University of Utah) following the standard protocol to determinate stable isotopes (http://sirfer.utah.edu/). The δ values were then converted to carbon isotopic discrimination values (Δ). The Δ value was calculated according to Masle et al. (2005) using the equation $\Delta = (\delta_a - \delta_p)/1 + \delta_p$, where δ_a and δ_p are the δ of the source air and the plant, respectively; δ of the source air (δ_a) was assumed to be −8 per million.

Stomata Density Determinations

Fully expanded leaves of the first pair were collected from 40-d-old plants. The number of stomata and epidermal cells was determined in imprints performed with transparent nail varnish under an optical microscope (Axiostar Plus at ×40; Carl Zeiss) in six portions of the adaxial surface of the leaf blade, at both sides of the midrib (two determinations in the distal, medium, and proximal zones). Stomata density was calculated as the sum of stomata located in the abaxial plus adaxial per unit leaf area.

ABA Determination

The equivalent of 100 mg fresh weight of freeze-dried aerial parts for each sample (that combine leaves from four plants) was processed as stated by Berli et al. (2010) to assess ABA levels by capillary gas chromatography-electron impact mass spectrometry with ([$^2H_6$]-ABA) as internal standard; measurements on samples from each treatment were performed using three biological repetitions.

ABA Sensitivity Experiments

ABA (Sigma-Aldrich) was either sprayed (100 μM solution) on the leaves or added with watering to the roots (35 μM solution) at the indicated times in the figures. After ABA applications, stomatal conductance was measured at the indicated times with a steady-state diffusion porometer (SC-1; Decagon Devices) on both leaf surfaces of expanded leaves of plants that were grown in a growth chamber (12 h light/12 h dark, PPFD = 170 μmol m^{-2} s^{-1}, HR approximately 50%, 22°C).

Water Potential Determination

Midday leaf water potential (Ψ_w) was determined in expanded leaves that were cut and immediately measured with a pressure chamber (PMS Instruments) based on Scholander et al. (1965).

Supplemental Data

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

Supplemental Figure S1. Cryptochromes and phototropins promote stomatal conductance.

Supplemental Figure S2. Cryptochromes and phototropins promote photosynthesis.

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