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Corresponding author:
Dr Hernán Boccalandro
Instituto de Biología Agrícola Mendoza (IBAM) CONICET-UNCU, Facultad de Ciencias Agrarias and Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina
Phone nº ++54 261 4135010 ext 1307
Email hboccalandro@gmail.com

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Phototropins but not cryptochromes mediate the blue light-specific promotion of stomatal conductance, while both enhance photosynthesis and transpiration under full sunlight\(^1\)[OA]

Hernán E. Boccalandro, Carla V. Giordano, Edmundo L. Ploschuk, Patricia N. Piccoli, Rubén Bottini, Jorge J. Casal

IBAM, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Alte. Brown 500, 5507 – Chacras de Coria, Argentina (H.E.B, P.N.P., R.B.);
Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina (H.E.B.)
IADIZA – CCT Mendoza (CONICET), Av. Ruiz Leal s/n, Mendoza, Argentina (C.V.G.);
Cátedra Cultivos Industriales, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417-Buenos Aires, Argentina (E.L.P.)
IFEVA, Facultad de Agronomía, Universidad de Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. San Martín 4453, 1417-Buenos Aires, Argentina (J.J.C.);
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*Corresponding author: hboccalandro@gmail.com
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: hboccalandro@gmail.com

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ABSTRACT

Leaf epidermal peels of Arabidopsis mutants lacking either phototropins 1 and 2 (phot1, phot2) or cryptochromes 1 and 2 (cry1, 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 and it is therefore 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 non-stomatic. 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 (ABA) 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 ABA levels.
INTRODUCTION

The stomata provide a key point of control the exchange of water and CO₂ 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., 2010; 2011). In isolated epidermal peels of Arabidopsis leaves, the blue-light induced promotion of the aperture of the stomatal pore is severely impaired in the 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 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. Lasceve et al. (1999) measured stomatal conductance in *Arabidopsis thaliana* 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 (Lasceve 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 support 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 CO₂ 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, non-shaded plants, only at both extremes of the photoperiod. However, this prediction requires experimental evaluation.

The aim of this paper was to characterize the light response of stomatal conductance in intact leaves of the *phot1 phot2* and *cry1 cry2* double mutants to elucidate: (a) whether cry is a photoreceptor involved in the blue-light system that controls stomatal conductance of intact leaves, and (b), 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 WT and the double mutants *cry1 cry2* and *phot1 phot2* plants were grown under natural radiation in a glasshouse (winter experiment, photoperiod ~10h, Fig. 1A, B, C) or outdoors (summer experiment, photoperiod ~14.5h, Fig. 1D, E, F) for 6 or 5 weeks, respectively. The diurnal pattern of stomatal conductance (Fig. 1A, D), transpiration per unit leaf area (Fig. 1B, E), irradiance and air temperature (Fig. 1C, 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 WT, 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. 1C, F). Leaf water potential at midday was not significantly affected by the different genotypes (MPa, mean ± SE, winter experiment, WT: -0.12±0.02; *cry1 cry2*: -0.14±0.03 and *phot1 phot2*: -0.16±0.03; *P*=0.28 n=8; summer experiment, WT: -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 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 were observed in WT 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 ± SEM in mmol m⁻² s⁻¹, n ≥ 4; WT: 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 WT and the cry1 cry2 or phot1 phot2 mutants increased towards midday (Fig. 1). This could be due to time- and/or environmental (irradiance, temperature, humidity)-dependent 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 WT (Fig. 2A). These differences were established at the beginning of the photoperiod and remained stable afterwards, suggesting that the increased impact of the mutations observed at midday compared to the extremes of the photoperiod under natural radiation (Fig. 1A, D) are caused by the diurnal fluctuations of the environment (e.g. higher irradiances at midday). Lowering PPFD to 17 µmol m⁻² s⁻¹ reduced stomatal conductance in the WT and the cry1 cry2 mutant, without seriously affecting the difference between these genotypes compared to 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 WT (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, Fig. 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. 1B, 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 CO₂. To investigate the long-term transpiration efficiency (CO₂ fixed
/ H$_2$O loss ratio) it was analysed the isotopic discrimination against $^{13}$CO$_2$ respect to $^{12}$CO$_2$ ($\Delta$) (Farquhar and Richards, 1984; Masle et al., 2005) in plants grown under natural radiation. The WT, the cry1 cry2 and the phot1 phot2 mutants presented similar $\Delta$ values (mean ± SEM in $\Delta$ per mil; n=3; WT: 22.1±0.1; cry1 cry2: 21.6±0.5; phot1 phot2: 21.8±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, B, C) were analysed. Net CO$_2$ uptake was lower in the cry1 cry2 and phot1 phot2 double mutants than in the WT (Fig. 3). The single mutants cry1, cry2, phot1 and phot2 showed no differences with the WT (mean ± SEM in $\mu$mol m$^{-2}$ s$^{-1}$; n≥ 4; WT: 18.6±1.3; phot1: 21.3±2.4; phot2: 18.2±1.7; cry1: 17.5±1.4; cry2: 19.7±3.2; phot1 phot2: 15.9±0.7; cry1 cry2: 8.2±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 chamber under photoperiods of 170 $\mu$mol m$^{-2}$ s$^{-1}$. In the WT, stomatal conductance increased with PPFD, reaching the maximum values at ~500 $\mu$mol m$^{-2}$ s$^{-1}$ (Fig. 4A). The cry1 cry2 and phot1 phot2 mutants presented lower stomatal conductance than WT 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 (Fig. 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 (see Fig. 2) but it failed to respond to changes in irradiance in the order of minutes, which are involved in the measurements done with the IR 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 WT reached maximum photosynthetic rates at ~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 as compared to the WT.

**Stomatal and non-stomatal 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 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, WT 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 WT and cry1 cry2 narrowed down with increasing CO\(_2\), and the difference between the WT and phot1 phot2 disappeared for CO\(_2\) levels above 1000 μmol mol\(^{-1}\) (Fig. 5A). Net CO\(_2\) uptake increased with CO\(_2\) levels in WT, 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 non-stomatal 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 WT (Fig. 5C), while stomatal limitations would yield a low ratio. As well, the ratio between intercellular and ambient CO\(_2\) concentrations were unaffected by the cry1 cry2 mutations, indicating that the limitations to photosynthesis are largely non-stomatal (Fig. 5C).

**Reduced stomatal conductance of cry1 cry2 under red light**

The reduced stomatal conductance of the cry1 cry2 double mutant compared to the WT in darkness (Fig. 2) encouraged the analysis of red light effects, i.e. in the absence of blue-light to activate cry and phot. WT, cry1 cry2 and phot1 phot2 plants were grown under white light for 32 days. 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 WT, the phot1 phot2 mutant showed reduced conductance under white light, and conductance values
similar to WT 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 WT) (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 WT (sum of stomata of both leaf surfaces mm$^{-2}$; WT: 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 WT, leaves of the cry1 cry2 mutant had significantly higher levels of ABA measured either at midday (Fig. 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 WT 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 phot 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 cry are not directly 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 determinate 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 have 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; León-Kloosterziel et al., 1997) and stomatal responses to light (Shimazaki et al., 2007). Addition of ABA eliminated the differences in conductance between the WT 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 cryptochrome mutants had been reported for gene expression (Yang et al., 2008) and seedling morphology (Botto et al., 2003) during de-etiolation 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 (Kobayashi and Weigel, 2007; Endo et al., 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 had shown 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 shadelight. 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 WT 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 vapour partial pressure deficit accompany different sunlight levels but a defect in the response to irradiance was also observed under controlled conditions (Fig. 2, Fig. 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 CO₂ diffusion is more likely to limit photosynthesis and the higher radiation load augments leaf temperature and water vapour partial pressure deficit, thus increasing transpiration particularly if stomata are open. Close to midday of a winter clear day the phot1 phot2 and cry1 cry2 mutations respectively reduced instant transpiration rates 29 and 72% (Fig. 1) and instant photosynthesis rates 29 and 39% compared to the WT (Fig. 3). The long-term transpiration efficiency was unaffected in cry1 cry2 or phot1 phot2 indicating that transpiration and CO₂ fixation suffered quantitatively similar reductions. phot had been shown to affect plant growth at low irradiance (Takemiya et al., 2005) and present
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 non-stomatal (Fig. 5), i.e. although stomatal conductance was reduced by the mutations to levels that in the WT 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 non-stomatal limitation of these mutants (Boonman, et al., 2009). In rice, the phot1 mutant shows reduced photosynthetic rates associated to elevated H2O2 accumulation (Goh et al., 2009). At low irradiance levels phot1 is required to optimize chloroplast exposure to light (Königer et al., 2008; Christie et al., 2007).

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 report). 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 neighbours (shade) or season (cloudiness), complementing the rapid adjustment mediated by phot.
MATERIALS AND METHODS

Plant material

The WT accession Columbia (Col) of Arabidopsis 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); 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 days-old seedlings were transplanted to 230 cm³ pots containing equal amounts of perlite (Perlome, Perfiltra, Rosario, Argentina) peat moss (Ciudad Floral, Escobar, Argentina) and vermiculite (Intersum, Aislater, Córdoba, Argentina) and watered as needed with a solution containing 1 g L⁻¹ of Hakaphos Red (COMPO, Spain).

Experimental conditions and light treatment

Field experiments were conducted in the experimental field of the IFEVA - 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 950m).

Temperature and relative humidity were recorded using iButton data loggers (iButton, Maxim Integrated Products, CA) and Hobo (Onset Computer Corporation, Bourne, MA, USA) respectively.

Growth chamber experiments were performed with plants cultivated under fluorescent lamps (36W Osram, Brazil) that provide 170 μmol m⁻² s⁻¹ of PPFD. PPFD was measured with a LI-COR Li-188B sensor, Lincoln, Nebraska. Photoperiod was 12 h and temperature was 23 ± 1°C.

Red light treatment (110 μmol m⁻² s⁻¹ in Fig. 7A and indicated irradiances in Fig. 7B) was obtained by filtering fluorescent lamps with a combination of orange and yellow filters (Lee filters, USA, number 105 and 101 respectively). Light spectrum was determined with an Ocean Optics spectrometer (model USB4000, Fl, USA) to corroborate blue light was completely cut-off in the red light treatment. Blue light was
obtained filtering fluorescent lamps with a blue filter (Lee filters, USA, number 119). To obtain different irradiances of red, blue or white light, neutral filters (Lee filters, USA, numbers 298, 210 and 299) were added to the different light treatments.

**Gas exchange measurements: conductance, transpiration and photosynthesis**

Stomatal conductance, transpiration rate and CO₂ exchange presented in figures 1, 3, 4, 5 and Supplemental 2, were measured by using an open infrared gas analysis system (LI-COR 6400, LI-COR Inc., Lincoln, NE.). CO₂ exchange at 0, 50, 100, 200, 400, 800, 1000 and 1200 μmol m⁻² s⁻¹ PPFD were measured in fully expanded leaves of cry1 cry2, phot1 phot2 and WT Ler 35-d-old plants, using a 0.25-L chamber attached to a regulated portable light power (QB1205LI-670, Quantum Devices Inc., Barneveld, WI). CO₂ during measurements were set at 400 ppm in the reference cell. Dose-response to CO₂ levels was determined with the following sequence of CO₂ concentrations at the reference cell: 400, 300, 200, 100, 50, 400, 800, 1200, 1600 and 2000 μmol mol⁻¹ s⁻¹, setting PPFD at 700 μmol m⁻² s⁻¹. Stomatal conductance to water vapour presented in figures 2, 6, 8 and Supplemental 1 was measured with a steady state diffusion porometer (SC-1, Decagon Devices, Pullman, WA, USA) in 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 SIRFER (Stable Isotope Ratio Facility for Environmental Research, University of Utah) following the standard protocol to determine stable isotopes (http://ecophys.biology.utah.edu/sirfer.html). The δ values were then converted to carbon isotopic discrimination values (Δ). Δ was calculated according to Masle et al., (2005), using the equation Δ = (δₐ - δₚ) / 1+ δₚ where δₐ and δₚ are the δ of the source air and the plant, respectively. δ of the source air (δₐ) was assumed to be -8 per mil.

**Stomata density determinations**

Fully expanded leaves of the first pair were collected from 40-d-old plants. The number of stomata and epidermal cells were counted in imprints performed with transparent nail varnish, under an optical microscope Axiostar Plus at 40X, (Carl Zeiss, Göttingen)
in 6 portions of the adaxial surface of the leaf blade, at both sides of the midrib (two
determinations in the distal, medium and proximal zone). 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 FW of freeze dried aerial parts for each sample (that combine leaves from 4 plants) was processed as stated in Berli et al. (2010) to assess ABA levels by capillary gas chromatography-electron impact mass spectrometry (GC-EIMS) with ([2H6]-ABA) as internal standard; measurements on samples from each treatment were performed using three biological repetitions.

**ABA sensitivity experiments**

ABA (Sigma Chem Co, St. Louis, MO) was either sprayed (100 µM solution) to 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 measure at the indicated times with a steady state diffusion porometer (SC-1, Decagon Devices, Pullman, WA, USA) in 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 ~50%, 22°C).

**Water Potential determination**

Midday leaf water potential ($\Psi_w$) was determined in expanded leaves that were cut and immediately measured with a pressure chamber (PMS Instruments Co., Corvallis, OR, USA) based on Scholander et al. (1965).

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FIGURE LEGENDS

Figure 1. Cryptochromes and phototropins control stomatal conductance and transpiration rates under natural radiation. Diurnal course of stomatal conductance (A, D) and transpiration per unit leaf area (B, E) in adult plants of the WT and of the cry1 cry2 and phot1 phot2 double mutants recorded under the indicated PPFD and temperatures (C, F) in a glasshouse in winter (A, B, C) or outdoors in summer (D, E, F). Data are means and SE of at least 3 plant replicates. * close to a mutant genotype symbol denotes significant differences (P <0.05) with the WT according to ANOVA and Bonferroni post tests.

Figure 2. Diurnal course of stomatal conductance in plants of the WT 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, HR ~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 6 plant replicates. * close to a mutant genotype symbol denotes significant differences (P <0.05) with the WT according to ANOVA and Bonferroni post tests.

Figure 3. Diurnal course of net CO₂ uptake in plants of the WT and of the cry1 cry2 and phot1 phot2 double mutants grown under natural radiation in a glasshouse (same experiment involved in Fig. 1A, B, C). Data are means and SE of at least 3 plant replicates for each time and genotype. * close to a mutant genotype denotes significant differences (P <0.05) with the WT according to ANOVA and Bonferroni post tests.

Figure 4. Fluence-rate response curves of net CO₂ uptake in plants of the WT, and of the cry1 cry2 and phot1 phot2 double mutants. Stomatal conductance (A), and net CO₂ 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 ~50%, 22°C). Data are means and SE of at least 4 plant replicates. * close to a mutant genotype denotes significant differences (P <0.05) with the WT according to ANOVA and Bonferroni post tests.

Figure 5. CO₂-response curves of net CO₂ uptake in plants of the WT, and of the cry1 cry2 and phot1 phot2 double mutants. Stomatal conductance (A), net CO₂ uptake (B) and ratios between intercellular (Cᵢ) and ambient (Cₐ) CO₂ concentrations as a function of ambient CO₂ concentration (C).
Plants were grown in a growth chamber (12 h light / 12 h dark, PPFD= 170 µmol m\(^{-2}\) s\(^{-1}\), HR ~50%, 22°C). Data are means and SE of at least 5 plant replicates. * close to a mutant genotype denotes significant differences (\(P<0.05\)) with the WT according to ANOVA and Bonferroni post tests.

**Figure 6.** The cry1 cry2 double mutant has reduced stomatal conductance even in the absence of blue light. Plants of the WT 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 ~50%, 22°C). During the photoperiod when stomatal conductance was recorded, the plants were exposed for 6 h: A), to red light or white light (both at 110 µmol m\(^{-2}\) s\(^{-1}\)), or B) to different irradiances of blue or red light. Data are means and SE of at least 5 plant replicates. In A, * close to a mutant genotype denotes significant differences (\(P<0.05\)) with the WT according to ANOVA and Bonferroni post tests. In B, the slope and SE is indicated.

**Figure 7:** Increased ABA levels in the leaves of the cry1 cry2 mutant. Plants of the WT 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 ~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 3 replicates. * denotes significant differences (\(P<0.05\)) with the WT according to \(t\) tests.

**Figure 8:** Exogenously applied ABA eliminates the differences in stomatal conductance between the cry1 cry2 and the WT. Plants of the WT 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 ~50%, 22°C) and ABA was either sprayed (100 µM) to the leaves (A) or added with watering (35 µM; B) at the indicated times (arrows). Data are means and SE of 5 Replicates. * denotes significant differences (\(P<0.05\)) between the WT and cry1 cry2 according to \(t\)-tests.
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