Running title: Phytochrome regulation of photosynthesis and transpiration

Corresponding author: Jorge J. Casal
IFEVA, Facultad de Agronomía, Universidad de Buenos Aires, and Consejo Nacional de
Investigaciones Científicas y Técnicas (CONICET), Av. San Martin 4453, 1417-Buenos Aires,
Argentina
Phone nº ++54 1145248070 ext 1223
Email casal@ifeva.edu.ar

Research Area
Whole Plant and Ecophysiology
Phytochrome B enhances photosynthesis at the expense of water use efficiency in Arabidopsis

Hernán E. Boccalandro, Matías L. Rugnone, Javier E. Moreno, Edmundo L. Ploschuk, Laura Serna, Marcelo J. Yanovsky and Jorge J. Casal

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*Corresponding author: Jorge J. Casal; e-mail casal@ifeva.edu.ar

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: Jorge J. Casal (casal@ifeva.edu.ar)

Present address: Cátedra de Química Orgánica y Biológica, Facultad de Ciencias Agrarias and Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina

These authors contributed equally to the article

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In open places, plants are exposed to higher fluence rates of photosynthetically active radiation and to higher red to far-red ratios than under the shade of neighbor plants. High fluence rates are known to increase stomata density. Here we show that high, compared to low red to far-red ratios, also increase stomata density in *Arabidopsis thaliana*. High red to far-red ratios increase the proportion of phytochrome B (phyB) in its active form and the *phyB* mutant exhibited a constitutively low stomata density. phyB increased the stomata index (the ratio between stomata and epidermal cells number) and the level of anphistomy (by increasing stomata density more intensively in the adaxial than in the abaxial face). phyB promoted the expression of *FAMA* and *TOO MANY MOUTHS* genes involved in the regulation of stomata development in young leaves. Increased stomata density resulted in increased transpiration per unit leaf area. However, phyB promoted photosynthesis rates only at high fluence rates of photosynthetically-active radiation. In accordance to these observations, phyB reduced long-term water use efficiency estimated by the analysis of isotopic discrimination against $^{13}$CO$_2$. We propose a model where active phyB promotes stomata differentiation in open places allowing plants to take advantage of the higher irradiances at the expense of a reduction of water use efficiency, which is compensated by a reduced leaf area.
Photosynthesis, transpiration and transpiration efficiency, the ratio of carbon fixation to water loss, are key physiological traits considered by plant breeders when selecting productive and water-use efficient plants (Rebetzke et al., 2002; Richards, 2006; Passioura, 2007). Opening of the stomata allows the uptake of CO₂ necessary for photosynthesis but it simultaneously increases the loss of water and the potential deterioration of the water status. Plants are finely tuned to efficiently face this dilemma. Under low levels of photosynthetically-active radiation (PAR), stomata open just enough to prevent the limitation of photosynthesis by CO₂ influx and the photochemical phase of photosynthesis is the limiting step. If PAR increases allowing higher rates of photochemical reactions, which leads to more ATP and NADPH, stomatal conductance also increases to allow sufficient CO₂ to use these products in the Calvin Cycle (Donahue et al., 1997; Yu et al., 2004). If instead of following this response coordinated to photosynthetic rates, stomata opened maximally in response to low PAR, more CO₂ than needed would be allowed to reach the chloroplast at the expense of unnecessary water loss.

Canopy shade-light is characterized not only by reduced PAR levels but also by a reduced proportion of red light (R) compared to far-red light (FR) caused by the selective absorption of visible light by photosynthetic pigments and the reflection and transmission of FR (Holmes and Smith, 1977). This low R/FR ratio compared to unfiltered sunlight is perceived by phytochromes (Smith, 1982; Ballaré et al., 1987; Pigliucci and Schmitt, 1999), mainly phytochrome B (phyB) (Yanovsky et al., 1995). In Arabidopsis, the high R/FR signals perceived by phyB decrease the length of the stem and petioles, cause a more prostrate position of the leaves, promote branching and delay flowering, among other responses (Reed et al., 1993; Franklin and Whitelam, 2005).

Transgenic plants of potato expressing the PHYB gene of Arabidopsis thaliana show higher stomatal conductance, transpiration rates and photosynthesis rates per unit leaf area than the wild type (WT) (Thiele et al., 1999; Boccalandro et al., 2003; Schittenhelm et al., 2004). Stomata density is unaffected, indicating that phyB enhances the aperture of the stomatal pore in these transgenic plants. Stomatal conductance is higher in Fuchsia magellanica plants exposed to R than to FR pulses at the end of the photoperiod (Aphalo et al., 1991). However, there are no general effects of R/FR treatments on the aperture of the stomatal pore. The stomata of Commelina communis (Roth-Bejerano, 1981) and of the orchid of the genus Paphiopedilum (Talbott et al., 2002) open in response to R and this effect is reversed by FR, indicating a control by phytochrome. Nevertheless, this FR reversal of the effect of R is absent in WT Arabidopsis thaliana (Talbott et al., 2003). In Phaseolus vulgaris, FR
accelerates stomatal movements during dark to light (opening) and light to dark (closing) transitions and this effect is R reversible, but phytochrome status has no effects under constant conditions of light or darkness (Holmes and Klein, 1985). In the latter species, prolonged FR added to a white-light background promotes stomatal conductance but this effect cannot be ascribed to phytochrome (Holmes et al., 1986).

In addition to this rapid adjustment of the CO₂ and water vapor fluxes to daily fluctuations in light levels via the regulation of the stomatal pore aperture, plants acclimate to the prevailing PAR conditions by changing stomatal density (number of stomata per unit area) and stomatal index (the ratio between the number of stomata in a given area and the total number of stomata and other epidermal cells in that same area). Stomatal density and stomatal index are higher in plants grown in full sunlight at high levels of PAR than in plants grown in shade (Willmer and Fricker, 1996; Lake et al., 2001; Thomas et al., 2004; Casson and Gray, 2008). Mature leaves sense the environment (light intensity and CO₂) and produce a systemic signal that regulates stomatal density and index in young leaves (Coupe et al., 2006). A change in CO₂ concentrations or PAR levels affects photosynthesis and therefore it was suggested that a metabolic compound associated to this process (i.e. a sugar) may regulate stomatal development (Coupe et al., 2006). However, there is no correlation between photosynthetic rate and stomatal index in poplar (Miyazawa et al., 2006) and transgenic anti-SSU tobacco plants, show reduced photosynthesis and normal responses of stomatal density and stomatal index to PAR, suggesting that other photoreceptors could be involved in this regulation (Baroli et al., 2008).

Here we demonstrate that high, compared to low R/FR ratios, perceived by phyB increase stomata density, stomata index and amphistomy in the leaves of Arabidopsis thaliana. This behavior results in an enhanced photosynthetic rate at high PAR at the expense of reduced water use efficiency.

RESULTS

Light signals perceived by phyB increase transpiration rate

Plants of Arabidopsis thaliana of the WT Ler and of the phyB-4 and phyB-5 mutants were grown under white light photoperiods (12 h) terminated with or without a pulse of FR (+FR). The classical end-of-day pulse of FR (Downs et al., 1957) provides a simulation of canopy shade-light that is more rudimentary than FR given simultaneously with white light throughout the photoperiod but it avoids potential effects linked to differential excitation of photosystems I and II by FR, which are known to be
important in the acclimation of photosynthesis-related traits (Dietzel and Pfannschmidt, 2008; Wagner et al., 2008).

Transpiration per plant depends on leaf area per plant and transpiration per unit leaf area. The +FR treatment and the phyB mutation increased the leaf area per plant (Fig. 1A) and reduced the rate of transpiration per unit leaf area (Fig. 1B), compared to WT plants grown under the high R/FR ratio control conditions. A highly significant interaction occurred between light conditions and genotype because under the +FR conditions, the phyB mutation had no effects compared to the WT, and in the phyB mutant, the +FR treatment had no effect compared to the high R/FR ratio control. These observations indicate a control of leaf area and transpiration per unit leaf area by the +FR treatment perceived by phyB, without any obvious participation of other phytochromes in the response to R/FR ratio.

**phyB increases stomata density**

To investigate the basis for the differences in the rate of transpiration per unit leaf area we recorded the number of stomata per unit leaf area. The +FR treatment (Fig. 2A) and the phyB mutation (Fig. 2B) reduced stomata density compared to WT plants grown under the high R/FR ratio control conditions. Light and genotype effects on stomata density correlate positively with the effects on the rate of transpiration per unit leaf area (cf Fig. 2A, B, and Fig 1B). The phyA-201 mutant presented a WT-like phenotype and the phyB-5 phyA-201 double mutant behaved as the phyB single mutants (Fig. 2B), indicating no significant role of phyA.

**phyB increases stomata index**

Differences in stomata density can result either from a general effect on cell density per unit leaf area (larger cells would reduce density and account for the enhanced area in response to +FR or the phyB mutation) or from a specific reduction in stomata differentiation. We calculated the stomatal index, i.e. the ratio between the number of stomata and the number of epidermal cells in a given area. The +FR treatment (Fig. 2C) and the phyB mutation (Fig. 2D) reduced stomatal index revealing the existence of a specific control by phyB of the proportion of epidermal cells that differentiate into stomata. The phyA-201 mutant presented a WT-like phenotype and the phyB-5 phyA-201 double mutant was similar to the phyB single mutants (Fig. 2), indicating no significant role of phyA. The phyA and phyB mutant backgrounds caused small but statistically significant increments or reductions, respectively, of non-guard
epidermal cell density (cells per mm², mean ±SE, WT Ler: 764 ±19; phyB-5: 710 ±26; phyB-4: 772 ±25; phyA-201: 849 ±29; phyB-5 phyA-201: 772 ±21, P <0.05 for phyA vs PHYA and phyB vs PHYB). The phyB mutation caused no obvious stomata aberrant distribution phenotype (e.g. contiguous stomata).

**phyB enhances the amphistomy level**

Arabidopsis has amphistomatous leaves, with higher stomata density in the abaxial respect to the adaxial leaf surface (Hetherington and Woodward, 2003). Depending on the species, PAR levels increases not only stomata density but also the level of amphistomy, a trait that affects acclimation to sunny environments (Mott et al., 1982; Mott and Michaelson, 1991). The +FR treatment and the phyB mutation reduced stomata density more in the adaxial than in the abaxial face of the leaf and therefore increased the abaxial / adaxial ratio (Fig. 3).

**phyB reduces water use efficiency**

Transpiration efficiency (the ratio between fixed CO₂ and water vapor lost by transpiration) was estimated through the analysis of isotopic discrimination against ^13CO₂ respect to ^12CO₂ (Δ). This parameter is a reliable and sensitive marker negatively correlated with water use efficiency or photosynthesis per unit transpiration (Farquhar and Richards, 1984; Masle et al., 2005). The phyB mutation decreased isotope discrimination (Δ) (Fig. 4), indicating that phyB decreases water use efficiency.

**Overexpression of PHYB increases stomatal density and stomatal index and reduces water use efficiency**

Transgenic plants overexpressing PHYB (PHYB OX) had a phenotype opposite in many respects to that of the phyB mutants (Supplementary Figure 1). Compared to the WT, the transgenic overexpressors showed reduced leaf area per plant, increased transpiration rate per unit leaf area, increased stomata density, increased stomatal index, increased amphistomatous character and increased isotope discrimination (Δ). The proportion between abaxial and adaxial stomata decreased because stomata density increased more in the adaxial (147 %) than in the abaxial face (76 %). In addition, PHYB overexpression increased total non-guard epidermal cell density (WT Nossen, 468±30; PHYB OX, 645±46, P <0.05). PHYB overexpression caused no obvious aberrant stomata distribution phenotype (e.g. contiguous stomata).
phyB increases the expression of FAMA and TMM

FAMA (FAMA) causes the guard mother cell to divide into the guard cells that define the stomatal pore (MacAlister et al., 2007; Casson and Gray, 2008). TOO MANY MOUTHS (TMM) negatively regulates the development of supernumerary stomata and enhances spacing among stomata (Geisler et al., 2000; Nadeau and Sack, 2002). We investigated the level of expression of these genes in developing leaves of WT and phyB mutant plants. Other genes involved in stomatal development were also investigated but the expression levels did not allow a clear resolution. The phyB mutation reduced both FAMA and TMM expression compared to the WT (Fig. 5A, B). At first glance it was surprising to see that both genes with contrasting consequences on stomata density were affected in the same direction in the phyB mutant, which shows reduced stomata density. To explore this issue in further detail we analyzed the correlation of expression between FAMA and TMM across 633 conditions representing different tissues, developmental stages and differentially treated plants (www.arabidopsis.org). Publicly available data for each of the two genes were normalized to the median of each experiment and ln (x+1) transformed as described (Buchovsky et al., 2008). This analysis demonstrates that a highly significant positive correlation between the expression of FAMA and TMM beyond the action of phyB (Figure 5C).

phyB promotes photosynthesis at high PAR

phyB increased stomata density and the level of amphistomy. Both traits potentially favor CO₂ diffusion from the atmosphere to the chloroplasts. We investigated whether phyB affects CO₂ uptake in WT and phyB mutant leaves exposed to a range of PAR (Fig. 6). Net CO₂ uptake was unaffected by the phyB mutation at or below the PAR that the plants had experienced during the growth period (250 μmol m⁻² s⁻¹). However, the phyB mutants presented lower photosynthetic rates than the WT under higher PAR (380 μmol m⁻² s⁻¹ or more) (Fig. 6).

To investigate whether the differences in CO₂ uptake were caused by stomatal limitations to CO₂ diffusion, consistent with the lower stomata density, we obtained curves of net CO₂ uptake against the intercellular CO₂ concentration (Fig. 7A). The WT showed higher CO₂ than the phyB-5 mutant (mean ±SE in mol m⁻² s⁻¹ for 400 μmol mol⁻¹ of CO₂ in the reference infra-red gas analyzer; n= 5; WT= 0.36 ±0.02; phyB-5= 0.21 ±0.02; P <0.0005). The lower ratios between intercellular and ambient CO₂
concentrations in the phyB mutant compared to the WT indicate stomatal limitations to CO₂ diffusion (Fig. 7B). The residual differences in the curves of net CO₂ uptake against the intercellular CO₂ concentration (Fig. 7A) suggest that additional non-stomatal effects of phyB on photosynthesis could occur.

DISCUSSION

In Arabidopsis thaliana, the density of stomata and the stomata index increase in response to PAR and in response to low CO₂ (Willmer and Fricker, 1996; Lake et al., 2001). Low R/FR ratios are typical of canopy shade-light and establish low proportions of active phytochrome (Holmes and Smith, 1977). Here we show that light treatments (+FR) that reduce the proportion of phytochrome in its active form also reduce stomata density and stomata index of Arabidopsis thaliana leaves (Fig. 2A, C). The phyB mutants having reduced or null levels of phyB, the main photoreceptor of R/FR ratios, showed low stomata density and stomata index under high R/FR ratios (Fig. 2B, D, E). The level of amphistomy (i.e. the presence of stomata on both leaf blade surfaces) can increase with PAR (Mott et al., 1982). The +FR treatment and the phyB mutation reduced stomata density more in the adaxial than in the abaxial face and therefore reduced the level of amphistomy (Fig. 3). This picture is completed by the observation that lines overexpressing the PHYB gene increased stomata density, stomata index, and the level of amphistomy compared to the WT (Supplementary Fig. 1). Therefore, active phyB increases stomata density, stomata index and amphistomy in Arabidopsis.

Low irradiance or low R/FR ratio do not change stomata density in the leaves of Commelina communis (Assmann, 1992), indicating that not all the species follow the pattern of response to shade-light signals exhibited by Arabidopsis. However, given the contribution of Arabidopsis as a model experimental system to study the regulation of transpiration and drought tolerance (Nilson and Assmann, 2007) it is important to uncover the environmental signals and the receptors controlling stomatal density and the physiological consequences of this regulation in Arabidopsis.

Stomata index is defined relatively early during the development of the leaves (Larkin et al., 1997; Serna et al., 2002) and therefore, the adjustment to the future conditions that the leaf is more likely to face would be beneficial. In closing canopies, the reduction in R/FR ratio anticipates actual shading among neighbors due to selective FR reflection on green leaves (Ballaré et al., 1987). The perception of low R/FR signals by phyB would provide a mechanism to adjust stomata density of developing leaves to the likely occurrence of shade, before mutual shading (i.e. reduced irradiance) actually takes place.
We are largely ignorant of the genes that relate environmental signals to the control of stomata density (Wang et al., 2007). One exception is the *HIGH CARBON DIOXIDE* (*HIC*) gene, which encodes a putative 3-keto acyl coenzyme A synthase involved in the synthesis of very-long-chain fatty acids, involved in the response to carbon dioxide (Gray et al., 2000). Here we place phyB between light quality signals and genes involved in stomata differentiation. The expression of *FAMA*, a BHLH transcription factor that promotes stomata differentiation (MacAlister et al., 2007; Casson and Gray, 2008), is reduced in developing leaves of the phyB mutant compared to the WT (Fig. 5A). *SPEECHLESS* (*SPCH*) is a BHLH transcription factor that positively regulates the asymmetric divisions that form stomata and the expression of *FAMA* (MacAlister et al., 2007; Casson and Gray, 2008). The expression of *SPCH* in 8-days-old seedlings with very young primordia is reduced by exposure to 1 h or 4 d to low R/FR ratio (Carabelli et al., 2007). Based on these observations we propose a model where phyB-mediated promotion of stomata differentiation involves positive regulation of BHLH transcription factors that play a key role in stomata development. Interestingly, the expression of *TMM*, a putative cell-surface leucine-rich repeat (LRR)-containing receptor-like protein that negatively regulates stomata differentiation (Geisler et al., 2000; Nadeau and Sack, 2002) was also lower in the phyB mutant than in the WT (Fig. 5B). The analysis of the expression levels of *FAMA* and *TMM* across different developmental, abiotic and biotic conditions revealed that, despite their opposite effects on stomata density, the positive expression correlation between these genes is not limited to the comparison between the WT and the phyB mutant (Fig. 5C). *TMM* is not only a repressor of stomatal development, it also seems to provide competence to enter into the stomatal pathway (Nadeau and Sack, 2002). It is likely that phyB positively regulates stomata formation by increasing the number of cells from which the stomatal lineage may develop. The fact that *TMM* also represses stomatal development, playing an opposite role to phyB, suggests that its expression might be positively controlled by a feedback mechanism during stomata differentiation. These findings suggest that the expression of *TMM* might be positively controlled by a feedback mechanism during stomata differentiation. There are several examples where an environmental cue initiates downstream signaling and sets into motion mechanisms that negatively regulate that signaling (Casal et al., 2004). The complex interaction among *TMM* and three *ERECTA* family of leucine-rich repeat containing receptor kinases revealed by the mutant phenotypes is not fully understood (Nadeau, 2008) and this sort of feedback regulations could be part of the complexity. The *ERECTA* gene product is a putative partner of TMM in the regulation of stomata development (Casson and Gray, 2008). We observed changes in stomata density and index in response to
+FR or to the phyB mutation in the Landsberg erecta background indicating that phyB-mediated responses of these traits can occur in the absence of a functional ERECTA gene.

Changes in stomata density do not necessarily translate into changes in water vapor and carbon dioxide fluxes. In transgenic Arabidopsis plants over-expressing the STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1) gene and in the sdd1 mutant stomata density is significantly decreased or increased, respectively, compared to the WT (Bussis et al., 2006). However, carbon dioxide assimilation rate and stomatal conductance of over-expressers and sdd1 mutant were unchanged compared with WT because changes in stomatal density were compensated for by opposite changes in stomatal aperture (Bussis et al., 2006). Conversely, the control of stomatal density by phyB correlated with changes in the rate of transpiration when the WT is compared to either the phyB mutant (Fig. 1B) or the PHYB OX (Supplementary Fig. 1). This resulted in a negative regulation of water use efficiency by phyB (Fig. 4, Supplementary Fig. 1) consistent with the positive regulation by ERECTA, which reduces stomata density (Masle et al., 2005).

The activity of phyB is higher in open places, where the radiation load is stronger and atmospheric water demand is more intense. Therefore, the increased stomatal density caused by phyB, which results in reduced water use efficiency, cannot easily be associated with a strategy aimed to conserve water. Along the same line of arguments, increased amphistomy is a feature often linked to adequate water availability (Mott et al., 1982; Lynn and Waldren, 2002). Low R/FR ratios and the phyB mutation increased leaf area per plant (Figure 1A). Increased leaf area in response to +FR has been observed in other species such us Cucumis sativus (López Juez et al., 1990), Taraxacum officinale (Cogliatti and Sánchez, 1987), Terminalia ivorensis (Kwesiga and Grace, 1986) and Petunia axilaris (Casal et al., 1987), and for some leaf positions in Arabidopsis thaliana (Robson et al., 1993). However, the opposite pattern has also been found (Kasperbauer, 1971; Holmes and Smith, 1977; Frankland and Letendre, 1978; Robson et al., 1993; Devlin et al., 1999), suggesting that the actual effect may depend on the species and growing conditions. The negative regulation of leaf area by phyB observed here would help to reduce water loss per plant in open places. However, the opposite regulation of leaf area and transpiration per unit leaf area by phyB largely compensate each other, indicating that the main function of these changes is not the adjustment of water use. Rather, phyB perception of the high R/FR ratios of open places allows Arabidopsis plants to increase photosynthesis in response to the higher PAR levels that the plant may experience in those conditions (Fig. 6). The increased stomata density and amphistomy mediated by phyB would be an adaptation
to the high PAR of open places by favoring the diffusion of carbon dioxide (Fig. 7B) and by facilitating leaf cooling due to the concomitant increase in transpiration rate reducing the stress imposed by high radiation loads (Mott et al., 1982; Parkhurst and Mott, 1990; Mott and Michaelson, 1991). Excitation energy that is not used for photochemistry and not dissipated as fluorescence or heat can be transferred to molecular oxygen, creating highly damaging reactive oxygen species ((Golan et al., 2006) and references therein). Therefore, the higher rate of photosynthesis at high PAR caused by phyB could also reduce the diversion of excitation energy to the generation of reactive species of oxygen in open places.

MATERIALS AND METHODS

Plant material

The accessions Landsberg erecta (Ler), Columbia (Col) and Nossen (No) of Arabidopsis were used as WT in this study. The phyA-201 (formerly fre-1 (Nagatani et al., 1993), phyB-5 (formerly hy3), phyB-4 (Koornneef et al., 1980; Reed et al., 1993) and the double mutant phyA-201 phyB-5 are in the Ler background; the transgenic line expressing 35S:PHYB:GFP (PHYB:GFP) (Más et al., 2000) is in the Columbia background and the overexpressor line 35S:PHYB (PHYB OX) is in the Nossen background (Wagner et al., 1991).

Seeds were sown on 0.8% agar-water and four-day-old seedlings were transplanted to 230 cm³ pots containing equal amounts of perlite (Perlome, Perfiltra, Rosario, Argentina) peat moss (Cuidad floral, Escobar, Argentina) and vermiculite (Intersum, Aislater, Córdoba, Argentina) and watered as needed with a solution containing 1 g per L of Hakaphos R (COMPO, Spain).

Light conditions

Plants were grown under white light photoperiods of 12 h at 250 µmol m⁻² s⁻¹ of photosynthetically active radiation (LI-COR Li-188B sensor, Lincoln, Nebraska) provided by a combination of mercury and sodium lamps and temperature was 23±1°C. The R/FR ratio (Skye meter SKR 100, remote probe SKR110; Skye Instruments, England), was 4.1. During one hour after the end of white light photoperiod, FR (10 µmol m⁻² s⁻¹, R/FR ratio = 0.04) was provided from one side of the plants by incandescent lamps in combination with a water filter (10 cm width), a red filter (Lee
filters, number 026), and two blue acrylics filters (1mm thick, Paolini 2031, Buenos Aires Argentina).

Stomata density, stomata index and amphistomy

Fully expanded leaves of the first pair were collected from 25-d-old plants. The number of stomata and epidermal cells were counted in clarified leaves or in imprints performed with transparent nail varnish, under the microscope (40 X) 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). In some experiments, epidermal cell counting was performed in the adaxial and abaxial sides of the second pair of leaves to investigate the amphistomy level (Fig. 3) or the effect of +FR on stomata density and index (Fig. 2). Representative photographs were taken using Nomarsky optics with a Leica DC 300F camera attached to a Leica DMIRB inverted microscope. To improve their visualization, cell walls were draw on the image, using the brush tool of the Photoshop v.7.0.

Carbon isotope discrimination

Analysis of carbon isotope composition was performed on 35-d-old rosette leaves (vegetative stage). 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 determinate 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.

Gene expression

Total RNA was isolated from 200 mg of developing leaves (less than 5 mm in length), the upper part of the shoot and shoot apex of 25-days-old plants by using RNeasy kit (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using 0.1 μg oligo (dT) primer and reverse transcriptase (Super script 3, Invitrogen). RT-PCR was run for 40 cycles. Real Time RT-PCR analysis was carried out with a 7500 Real-Time PCR System with Fast Universal PCR Master Mix (Applied Biosystems). The
expression of TMM1 (At1g80080) and FAMA (At3g24140) was normalized to the expression of ACT8. The primers sequences were: TMM1Fw, 5´AACAGTCTTCGGGTCTTCAC 3´; TMM1Rv, 5´GCTTTCTCTCATCCTCCACA 3´; FAMA1Fw, 5´GACCATAACCAACCCCAACA 3´; FAMA1Rv, 5´GCTCTCTCTTGTGCTCTTCA 3´; ACT8Fw, 5´AGTGGTCGTACAACCGTATTGT 3´; ACT8Rv, 5´GAGGATAGCATGTGGAACGTGAA 3´.

Correlation of expression of FAMA and TMM

The correlation between the expression of FAMA and TMM was analyzing using 633 data points corresponding to different developmental contexts and biotic or abiotic treatments (1 to 3 biological replicates per point) taken from 46 experiments (1388 microarrays, www.arabidopsis.org). Data were normalized to the median of each experiment and transformed as ln (x+1) as it was performed by Buchovsky et al., (2008).

Whole plant transpiration rate

Whole plant transpiration rate was measured as described (Masle et al., 2005). Briefly, the night before transpiration was assayed, the pots containing one 35 d old plant (vegetative stage) was watered at field capacity and the surface of the soil was wrapped with 0.025-mm thick transparent film (Rolopac, Buenos Aires, Argentina) to avoid evaporation. The next day, pots were weighted immediately prior to the beginning of the photoperiod and 12 h later. Relative humidity during the photoperiod was 70 ±4 %. The rosette leaves were harvested immediately and photographed to determinate leaf area of each plant using the Photoshop v.7.0. Transpiration per plant per hour (mg of water h⁻¹) was calculated as the difference between the initial and the final weight of each pot at the end of the photoperiod, divided by the number of hours of the photoperiod (12 h). The transpiration rate (mg of water cm⁻² h⁻¹) was calculated dividing the former parameter by the total leaf area of each plant. Units were converted to mmol of water m⁻² s⁻¹.
Leaf Photosynthesis

Leaf CO₂ exchange responses to PPF were obtained by using a closed infrared gas analysis system (LI-COR 6200, LI-COR Inc., Lincoln, NE.). CO₂ exchange at 0, 200, 380, 710 and 1,150 μmol m⁻² s⁻¹ PAR was measured in fully expanded leaves of phyB-4, phyB-5 and WT Ler 30-d-old plants, using a 0.25-L chamber attached to a regulated portable red light power (QB1205LI-670, Quantum Devices Inc., Barneveld, WI). Actual leaf temperature during measurements was between 27 and 29 °C and CO₂ concentration was 390 μmol mol⁻¹.

A portable gas exchange system (LI-COR 6400; LI-COR, Lincoln, NE) was used to obtain curves of leaf CO₂ exchange against intercellular CO₂ concentration in fully expanded leaves of phyB-5 and WT Ler 30-d-old plants. The area included in the 6 cm² chamber was recorded for each leaf. Measurements of leaf CO₂ exchange started at 400 μmol mol⁻¹ CO₂ in the reference chamber, decreased stepwise to 50 μmol mol⁻¹, returned to 400 μmol mol⁻¹ and increased stepwise to 1,200 μmol mol⁻¹ CO₂. PPFD was 1,200 μmol m⁻² s⁻¹ provided by the red al blue diodes of the gas-exchange system (6400-02B LED Light Source). Actual leaf temperature during measurements was between 27 and 29 °C. The flow rate of air was set at 300 μmol s⁻¹. Ambient and reference CO₂ concentrations are the concentration in the sample and reference infrared gas analyzers, respectively.

Additional Note

While this paper was under review Casson et al. (2009) have demonstrated a role of phyB in the promotion of stomata density by irradiance. The latter is consistent with and complements the function of phyB in the response to light quality reported here.

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LITERATURE CITED


Richards RA (2006) Physiological traits used in the breeding of new cultivars for water-scarce environments. Agric Water Manag 80: 197-211


Figure legends

**Figure 1.** Phytochrome controls leaf transpiration rate. Leaf area per plant (A) and transpiration per unit leaf area (B) in seedlings of the WT and of the *phyB* mutants grown under white light with or without exposure to FR at the end of the photoperiod. Data are means and SE of at least 21 plant replicates. Factorial ANOVA indicates significant interaction (P <0.0001) between the effects of the *phyB* mutations and the +FR treatment because the *phyB* mutation had effects under white light but not under white light +FR (A, B). Different letters denote significant differences (P <0.05) among means according to Bonferroni post-tests.

**Figure 2.** Phytochrome controls stomata density (A, B) and stomata index (C, D). Plants of the WT were grown under white light and white light +FR (A, C) and plants of the *phyA, phyB, phyA phyB* mutants (B, D) were grown with their respective WT under white light. Data are means and SE of at least 12 plant replicates. * denotes significant differences (P <0.05) between the indicated condition or genotype and its control according to ANOVA followed by Bonferroni post tests. Representative sections of the epidermis are shown for a *phyB* mutant and its WT (E). Stomata density, index and images correspond to the adaxial epidermis of fully expanded leaves.

**Figure 3.** Phytochrome increases the level of amphistomy (A, B). The abaxial and abaxial stomatal densities and the percent decrease caused by +FR or by the *phyB* mutation are also shown (C, D). Plants of the WT were grown under white light and white light +FR (A, C) and plants of the *phyB* mutant were grown with its WT under white light (B, D). Data are means and SE of at least 12 plant replicates. * denotes significant differences (P <0.05) according to ANOVA. *** denotes significant differences (P <0.001) according to Bonferroni post tests.

**Figure 4.** Phytochrome reduces water use efficiency. Plants of the *phyB* mutant and its WT were grown under white light. Data are means and SE of 3 plant replicates (pool of 3 plants each one). * denotes significant differences (P <0.05) according to ANOVA.

**Figure 5.** Phytochrome promotes the expression of *FAMA* and *TMM* in the leaves and the positive correlation in the expression of these two genes extends beyond *phyB* action. (A, B) Plants of the *phyB* mutant and its WT were grown under white light. Data are means and SE of 3 biological replicates. * denotes significant differences (P <0.05)
according to ANOVA. (C) Positive correlation between the expression of FAMA and TMM. The figure includes 633 data points corresponding to different developmental contexts and biotic or abiotic treatments (1 to 3 biological replicates per point) taken from 46 experiments (1388 microarrays, www.arabidopsis.org). Data were normalized to the median of each experiment and transformed as ln (x+1) (Buchovsky et al., 2008). The line shows least square linear fit of the 633 points and the significance is indicated.

**Figure 6.** Phytochrome promotes photosynthesis in Arabidopsis. Plants of the WT and of the phyB mutants were grown under white light and then exposed to the indicated PAR during measurements. Data are means and SE of 6 plant replicates. * denotes significant differences (P <0.05) according to ANOVA and Bonferroni post tests.

**Figure 7.** Analysis of stomatic and non-stomatic limitations to photosynthesis. (A) Curves of leaf net CO2 uptake against intercellular CO2 concentrations were obtained with a Li-COR 6400 system for the WT and the phyB-5 mutant. (B) Ratio between intercellular and ambient CO2 concentrations plotted against ambient CO2 concentration (i.e. the concentration in the sample infrared gas analyzer of the Li-COR 6400 system). Data are means and SE of 5 plant replicates. * denotes significant differences (P <0.05) according to t tests. The tests were done between WT and the phyB having the closest intercellular (A) or ambient (B) CO2 concentrations (in A, this overestimates the significance of the difference for the samples around 280 μmol.mol⁻¹).

**Supplementary Figure 1.** Phytochrome B overexpression increases transpiration per area, stomata density, stomata index, and amphistomy level and reduces leaf area per plant and transpiration efficiency. Leaf area per plant (A) and transpiration per unit leaf area (B) in plants of the WT and in the PHYB OX line grown under white light with or without exposure to FR at the end of the photoperiod. Data are means and SE of at least 21 plant replicates. Factorial ANOVA indicates significant interaction (P <0.0001) between the effects of the PHYB overexpression and the +FR treatment in the control of leaf area because the latter had larger effects in the PHYB OX than in the WT; and significant effects of PHYB overexpression (P <0.0001) and of the +FR treatment (P <0.01) but no interaction in the control of transpiration rate. Stomata density (C), amphistomy level (D) and stomata index (E) in two PHYB OX lines and their respective WT grown under white light. Data are means and SE of at least 12 plant replicates.
Water use efficiency of *PHYB OX* plants and their respective WT (F) grown under white light. Data are means and SE of 3 plant replicates (pool of 3 plants each one). * denotes significant differences (P <0.05) according to ANOVA. Stomata density and index were determined in the adaxial epidermis of fully expanded leaves.
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