

Phytochrome B increases drought tolerance by enhancing ABA sensitivity in *Arabidopsis thaliana*

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ABSTRACT

Phytochrome B (phyB) can adjust morphological and physiological responses according to changes in the red : far-red (R:FR) ratio. phyB-driven acclimation of plants to open environments (high R:FR ratio) increases carbon gain at the expense of increased water loss. This behaviour alleviates stressful conditions generated by an excess of light, but increases the chances of desiccation. Here we evaluated how phyB modulates this drought-tolerance response by comparing wild-type *Arabidopsis thaliana* adult plants to the null *phyB* in response to water shortage. *phyB* wilted before the wild type, and this was due to *phyB* maintaining open stomata under a reduction in soil water availability. Although *phyB* presented enhanced ABA levels under well-watered conditions, this mutant was less sensitive than the wild type in diminishing stomatal conductance in response to exogenous ABA application. Reduced sensitivity to ABA in *phyB* correlated with a lower expression of *ABCG22*, which encodes a putative ABA influx transporter, and *PYL5*, which encodes a soluble ABA receptor. Furthermore, the expression of *RABI8* and *RD29A*, both typical ABA-induced genes, was lower in *phyB* than the wild type after ABA treatment. We propose that phyB contributes to the acclimation of plants to open environments by enhancing ABA sensitivity when soil water becomes limiting.

Key-words: ABA signaling elements; R:FR ratio; water shortage.

Abbreviations: ABA, abscisic acid; D, drought = water shortage treatment; d, day s⁻¹; FW, fresh weight; G, genotype; phyB, phytochrome B; PPF, photosynthetic photon flux density; R:FR, red to far-red light ratio.

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INTRODUCTION

As incident solar radiation penetrates the canopy, there is a reduction of photosynthetic photon flux density (PPFD) of light and of red : far-red ratio (R:FR) caused by the selective absorption of visible light by photosynthetic pigments and FR light reflection and transmission (Holmes & Smith 1977). Actual shading or the presence of neighbour plants also reduce the vertical or lateral R:FR ratio (Ballaré *et al.* 1987), a signal that is mainly perceived by phytochrome B (phyB) (Yanovsky, Casal & Whitelam 1995). Low R:FR ratios sensed by phyB evoke the 'shade avoidance syndrome' (SAS) involving responses such as petiole and stem elongation and relocation of leaves to an erect position (Smith 2000). The SAS, in response to detection of plant crowding, is a potentially adaptive trait for shade-intolerant plants, as it increases their chances of foraging for photosynthetic light, and so increase CO₂ fixation (Schmitt *et al.* 2003; Franklin & Whitelam 2005).

In addition to the central role of phytochromes in the regulation of plant development in shaded environments, they play a role in regulating plant water relations and carbon economy in open environments. For example, recent evidence showed that phyB can adjust various morphological and physiological responses that affect acquisition, transport, loss of water and carbon gain according to changes in the R:FR ratio. We previously demonstrated that phyB increases stomatal density (number of stomata per unit area), stomatal index (ratio between stomatal and epidermal cell number) and the level of amphistomy (presence of stomata on both leaf blade surfaces) in *Arabidopsis thaliana* plants exposed to high R:FR ratio (Boccalandro *et al.* 2009). Moreover, phyB rice (*Oriza sativa* L.) mutants showed lower stomatal density than the wild type (Liu *et al.* 2012). phyB also promotes red light-induced stomatal opening in *A. thaliana* (Wang *et al.* 2010); *Phaseolus vulgaris* (Holmes & Klein 1985); *Commelina communis* (Roth-Bejerano, Nejdat & Itai 1982); and orchids of the genus *Paphiopedilum* (Talbot *et al.* 2002). Furthermore, the *lh* mutant of *Cucumis sativus*, which possesses a chromosome

deletion that includes phyB, shows reduced diameter and number of xylem vessels (Casal *et al.* 1994), suggesting that phyB regulates both traits facilitating water transport. At root level, phyB increases the number of lateral roots (Salisbury *et al.* 2007) and root hair formation (De Simone, Oka & Inoue 2000), although it reduces root hair length in *Arabidopsis* (Reed *et al.* 1993). This evidence strongly suggests that phyB plays an important role in plant water relations. In accordance to this idea, it was demonstrated that several of these morphological changes induced by phyB under high R:FR ratios produced functional consequences, increasing stomatal conductance, transpiration and photosynthetic rates at the expense of water-use efficiency at high photosynthetically active radiation (PAR) (Thiele *et al.* 1999; Boccalandro *et al.* 2003, 2009). In well-watered *Arabidopsis* plants, these effects operated at the level of unit leaf area, but were partially compensated at whole plant level due to the absence of phyB-reduced total leaf area (Boccalandro *et al.* 2009). Several studies showed that phytochromes can affect ABA levels or signalling (Kraepiel *et al.* 1994; Seo *et al.* 2006; Sawada *et al.* 2008; Piskurewicz *et al.* 2009; Dubois *et al.* 2010; Lau & Deng 2010). In plants exposed to drought, abscisic acid (ABA) increases in the tissues inducing stomatal closure and promoting the expression of many stress-related genes, such as *RAB18* and *RD29A* that enhance water stress tolerance or avoidance (Lång & Palva 1992; Yamaguchi-Shinozaki & Shinozaki 1994; Acharya & Assmann 2009). More recently, it has been demonstrated that early ABA signalling components contribute to drought tolerance in plants. For example, the mutation of *HABI*, a negative regulator of ABA signalling, and the overexpression of *PYL5* and *PYL8* ABA-binding receptors, members of the *PYR/PYL/RCAR* protein family (Ma *et al.* 2009; Park *et al.* 2009), increase drought tolerance in *Arabidopsis* (Saez *et al.* 2004, 2006; Santiago *et al.* 2009; Saavedra *et al.* 2010). Besides, the alteration of the levels of ABA transporters, such as *ABCG22*, *ABCG25* or *ABCG40*, can also affect tolerance to water shortage (Kang *et al.* 2010; Kuromori *et al.* 2010; Kuromori, Sugimoto & Shinozaki 2011).

The aim of this study was to evaluate how phyB affects drought tolerance and to elucidate the physiological and molecular bases of its action. We demonstrated that phyB enhances drought tolerance in adult plants of *A. thaliana* by increasing stomatal sensitivity to ABA when water becomes a scarce resource.

MATERIALS AND METHODS

Plant material

Landsberg *erecta* (*Ler*), Columbia (Col) and Nossen accessions of *A. thaliana* were used as the wild type. *phyB-1* (Reed *et al.* 1993) and *phyB-5* (Koornneef, Rolf & Spruit 1980; Reed *et al.* 1993) are in the *Ler* background; *phyB-9* (Reed *et al.* 1993) and the transgenic line overexpressing the *35S:PHYB:GFP* (*PHYB:GFP*) transgene (Mas *et al.* 2000) are in the Col background; and the line overexpressing

the *35S:PHYB* (*PHYB:OX*) transgene is in the Nossen background (Wagner, Tepperman & Quail 1991).

Growth experimental conditions

Seeds were sown in clear plastic boxes on 0.8% agar water, stratified at 4 °C for 3 d and then exposed to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of white light at 22 ± 2 °C. Four-day-old seedlings were transplanted to 180 cm³ perforated plastic pots containing two parts perlite no. 4 (San Carlos, Mendoza, Argentina), two parts blonde peat moss (Tierra del Fuego, Argentina) and one part sand (San Carlos, Mendoza, Argentina), and transferred to a growth room, 12 h white light/12 h darkness, PAR: 170–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$, temperature: 22 ± 2 °C and HR ~50%. Light was provided by white fluorescent lamps (36W/765 Osram, Osasco, SP, Brazil) giving an R:FR = 3.81. Light conditions were measured with a LI-250 light meter with a LI-190SA quantum sensor (Li-Cor Inc., Lincoln, NE, USA) and the R:FR with an USB 4000 spectroradiometer (Ocean Optics Inc., Dunedin, FL, USA). Average air temperature was assessed with a Hobo sensor (Hobo Pro series, Onset Computer Corporation, Bourne, MA, USA). Plants were watered daily, twice a week with a solution containing 1 g L⁻¹ of Hakaphos Red (COMPO, Barcelona, Spain).

Thirty-day-old well-watered plants of wild type and phyB mutants were used for water shortage experiments. For the unwrapped pot experiments, watering was ceased for 7 d. In a second set of experiments, soil evaporation was strongly reduced by covering pots with a plastic wrapping film before imposing water-shortage treatment during 16 d.

The ABA experiments consisted in spraying leaves of 30-day-old well-watered plants with different solutions of ABA in the middle of the photoperiod. ABA (90%, Kelinon Agrochemical Co., Beijing, China) was dissolved in a small volume of 96% ethanol to prepare solutions of 0, 1, 20 and 100 μM of ABA, containing 0.01% Triton X-100.

Stomatal conductance

Stomatal conductance ($\text{mol of air m}^{-2} \text{s}^{-1}$) was measured with a steady-state diffusion porometer (SC-1, Decagon Devices, Pullman, WA, USA) on both leaf surfaces of fully expanded leaves. Stomatal conductance was calculated as the sum of the adaxial and abaxial leaf conductance values for each leaf (Mott & O'leary 1984; Mott 1988).

Relative soil water content

During the water-shortage experiments, pots plus plants were weighed daily. Changes in pot weight were taken to reflect soil water loss, assuming that plant weight was negligible. Once the experiments had finished, the soil of each pot was placed in a paper bag, kept at 60 °C during 4 d and the resultant dried soil was weighed. Relative soil water content was calculated daily as: [(potted plant weight – dry soil and empty pot weight) / potted plant weight].

Table 1. qRT-PCR primer sequences

Gene	RP	LP
<i>UBC21</i>	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC
<i>PYL5</i>	ACCACAGGCTCAAGAACTACCG	ACCACAGGCTCAAGAACTACCG
<i>PYL8</i>	ACGCTCCTGTTTCATATGTGTGG	GTGCTTCTAGTTGCTGGTAGTCC
<i>HAB1</i>	AGAGGAATACAGGAGAGGGTAGGC	TGAGCAAACCAAGGCAACAACAG
<i>RAB18</i>	ACCCGATCCAGCAGCAGTATG	ACCACCACAGTTCGGTATCC
<i>ABCG22</i>	AAATAAGGAGAGAGCAGCGGATATG	GACGACAAGAAGGAAGAGAGAAGG
<i>RD29</i>	GTGACGACGAAGTTACCTATCTCC	TCTCCGCCACATAATCTCTACCC

Morphological and anatomical determinations

Total leaf area, petiole length, leaf angle and soil cover of 30-day-old well-watered plants were assessed. To determine total leaf area, leaves from the whole plant were removed and scanned. Individual leaf area (cm²) was calculated using Adobe Photoshop (v. 7.0) by comparison with a reference area. Petiole length (mm) of the third fully expanded leaf was measured with a ruler. Leaf angle (degrees relative to the horizontal plane) of the third or fourth leaf was measured using a protractor. Photographs of the pots were taken from above with a reference area placed at leaf level. Soil cover (%) was calculated using Adobe Photoshop (v. 7.0).

Epidermal imprints of fully expanded leaves treated with ABA were obtained using transparent nail varnish, before (ABA⁻) and 3.5 h after ABA sprays (ABA⁺). Leaves were not detached from the plant until the varnish dried. Stomata were observed under a Nikon Eclipse E200 optical microscope (Tokyo, Japan) and photographed with a Micrometrics 318 CU digital camera (Shanghai, China) at 1000 \times .

Osmotic adjustment

Three whole leaves of each plant replicate were homogenate. Samples were centrifuged at 8000 g during 5 min before measuring total osmolytes in the cell sap (10 μ L) using a vapour pressure osmometer (Wescor 5500, Wescor Inc., Logan, UT, USA). For osmolytes measurement, leaves from 30-day-old well-watered plants cultivated in wrapped pots were taken, before suspending watering and after 7 d of water shortage.

ABA determination

Approximately 100 mg fresh weight (FW) of freeze-dried plant material from the aerial parts was processed per sample as stated in Berli *et al.* (2010). ABA concentration was measured using capillary gas chromatography-electron impact mass spectrometry (GC-EIMS) with ([2H₆]-ABA) as internal standard. Sample measurements were performed using three biological replicates. Replicate consisted of a sample from four to six plants. For ABA determination, leaves were taken from 30-day-old well-watered plants cultivated in wrapped pots, before suspending watering and after 7 d of water shortage.

Gene expression analysis

Leaves of 30-day-old well-watered plants were collected, frozen in liquid nitrogen and stored at -80°C until processing. Each of the three biological replicates consisted in a leaf sample from three plants. Total RNA was extracted with RNEasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and subjected to a DNase treatment with RQ1 RNase-Free Dnase (Promega, Madison, WI, USA). cDNA derived from this RNA was synthesized using M-MLV RT (Promega) and an oligo-dT primer. The synthesized cDNAs were amplified with Fast Start Universal SYBR Green Master (Roche, Indianapolis, IN, USA) using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) cycler. UBIQUITIN-CONJUGATING ENZYME 21 (*UBC21*) gene was used as the normalization control, as reported by Czechowski *et al.* (2005). Gene expression analysis was performed by means of $\Delta\Delta\text{CT}$ relative quantification method (Pfaffl 2001). The primer sequences are listed in Table 1.

Statistical Analysis

Student's *t*-test and one or two-way ANOVA followed by Tukey honestly significant difference (HSD) post-test were performed when appropriate in order to assess differences between means. Analyses were carried out with InfoStat/p 2008 version (<http://www.infostat.com.ar>).

RESULTS

phyB increases drought tolerance in *Arabidopsis*

Stomatal conductance of well-watered plants was reduced by the phyB mutation and promoted by *PHYB* overexpression (Fig. 1). *phyB-5*, *phyB-1* and *phyB-9* plants presented lower stomatal conductance than their respective wild types (Fig. 1a). In contrast, stomatal conductance was promoted in two *PHYB* overexpressor lines (Fig. 1b). These responses provided evidence that phyB induces higher stomatal conductance in well-watered plants and is independent of the accession assessed.

To evaluate whether phyB affects drought tolerance, we exposed 30-day-old plants to water shortage. phyB mutants, in spite of having lower stomatal conductance when well

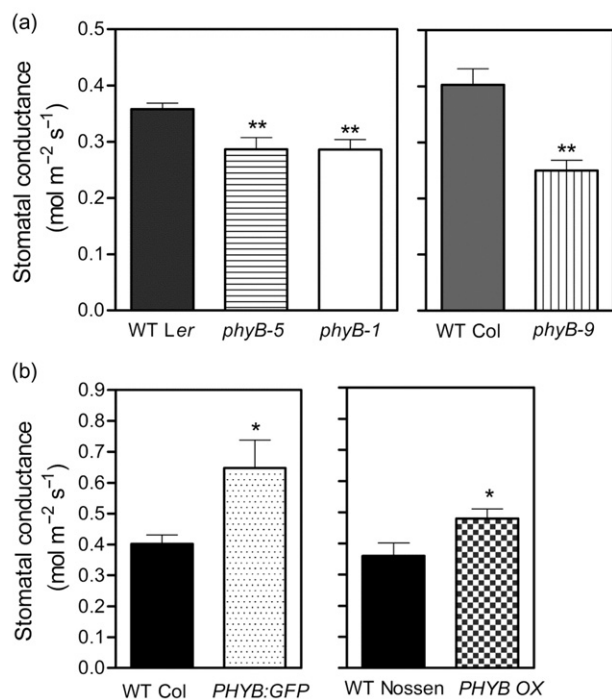


Figure 1. *phyB* increases stomatal conductance. Stomatal conductance of (a) *phyB* mutants in the *L.erecta* (left) and Columbia (right) background and (b) *PHYB* overexpressors in the Columbia (left) and Nossen background (right), measured in 30-day-old well-watered plants in the middle of the photoperiod (12:12 h light : dark cycle). Data are means \pm SE, $n = 8$. The experiment was repeated three times showing similar results. Asterisks denote significant differences (* $P < 0.05$; ** $P < 0.001$).

watered, showed less tolerance to water stress than the wild type, wilting after 5 to 7 d without watering while wild-type plants continued to display a normal phenotype (Fig. 2a). Total leaf area was similar between genotypes (mean \pm SE in cm²; $n = 20$, *Ler*: 30 ± 4 , *phyB-1*: 24 ± 3 , *phyB-5*: 20 ± 2 , $P > 0.05$). As *PHYB* overexpressors presented a strong reduction in leaf area, they were not included in water shortage assays.

We measured stomatal conductance during the period of water shortage. Under well-watered conditions, *phyB* mutants had lower stomatal conductance than the wild type (Fig. 1a), so it was expressed relative to its initial value. In consequence, the relative soil water content at which each genotype started to reduce stomatal conductance was detected. This occurred when relative soil water content fell below 46% in wild-type plants (Fig. 2b) and 37% in *phyB* mutants (i.e. *phyB1* and *phyB5*), which suggested that *phyB* is involved in stomatal closure when plants are subjected to water shortage.

It is known that the *phyB* mutation results in the display of constitutive shoot morphological changes, such as petiole elongation and hyponastic leaves (Reed *et al.* 1993), which reduce soil cover when plants are cultivated under white light (Supporting Information Fig. S1). The differences observed might be related to the higher soil evaporation of *phyB* plants than the wild type. To avoid soil water loss

through evaporation, we wrapped the pots with plastic wrapping film before the water shortage treatment (in this and in the following experiments).

In the experiment with wrapped pots, plants reached their wilting point later than those in unwrapped pots (i.e. 10 d versus 6 d in *phyB* mutants, respectively), indicating that soil evaporation was effectively reduced. Again, *phyB* plants displayed drought stress symptoms and wilted before the wild type (Fig. 3a), showing an uncoupled regulation of stomatal closure by lower soil water content (Fig. 3b,c). Wild-type plants showed reduction of stomatal conductance at higher values of relative soil water content than

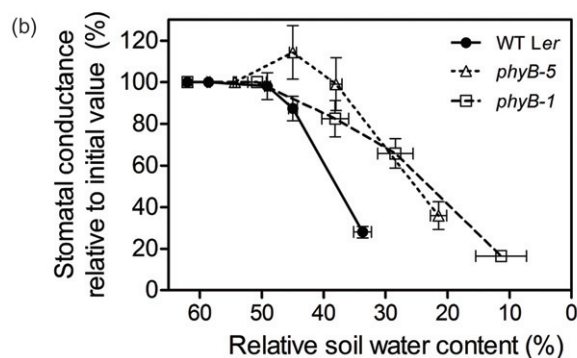
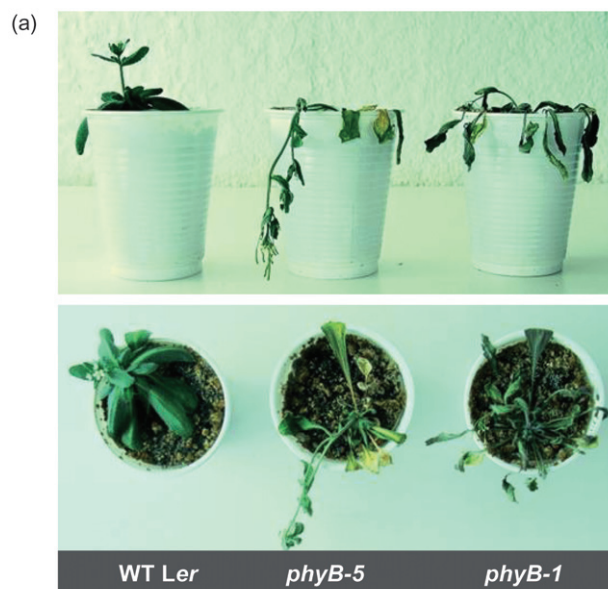


Figure 2. *phyB* enhances drought tolerance. (a) Photographs of wild-type, *phyB-5* and *phyB-1* plants cultivated in unwrapped pots were taken after 7 d of water shortage treatment.

(b) Stomatal conductance relative to stomatal conductance under well-watered conditions as a function of relative soil water content. Initial absolute values in mol m⁻² s⁻¹: wild type = 0.338 ± 0.01 , *phyB-5* = 0.270 ± 0.01 and *phyB-1* = 0.270 ± 0.02 . Stomatal conductance was measured in the middle of the photoperiod (12:12 h light : dark cycle). Each point corresponds to 0, 1, 3, 4 and 6 d since the start of the water shortage treatment. Data are means \pm SE, $n = 8$. The experiment was repeated twice showing similar results.

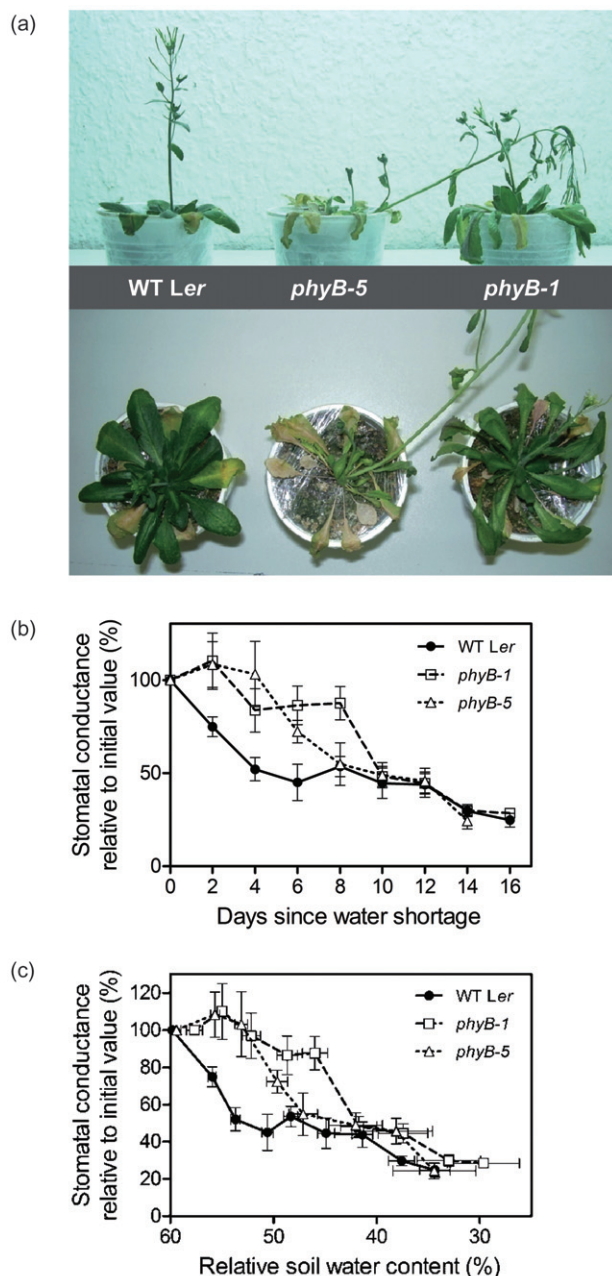


Figure 3. *phyB* enhances sensitivity to detect water content depletion in soil. (a) Photographs of wild-type, *phyB-5* and *phyB-1* plants taken after 14 d of water shortage. Pots were covered with wrapping film to avoid soil water evaporation before the beginning of the drought treatment. Stomatal conductance relative to stomatal conductance under well-watered conditions as a function of (b) days since water shortage or (c) relative soil water content. Initial absolute values in $\text{mol m}^{-2} \text{s}^{-1}$: wild type = 0.411 ± 0.03 , *phyB-5* = 0.317 ± 0.03 and *phyB-1* = 0.244 ± 0.01 . Stomatal conductance was measured in the middle of the photoperiod (12:12 h light : dark cycle). Each point corresponds to 0, 2, 4, 6, 8, 10, 12, 14 and 16 d since water shortage was imposed. Data are means \pm SE, $n = 8$. The experiment was repeated twice showing similar results.

phyB mutants (Fig. 3c). These results demonstrate that *phyB* contributes to adjust stomatal closure when water becomes scarce, thus increasing drought tolerance.

phyB does not evoke osmotic adjustment under water stress

The mechanism underlying *phyB*-enhanced drought tolerance could be the result of a higher capacity of wild-type plants to extract soil water. Osmotic adjustment is a typical response to drought, which can improve chances of acquiring water from a drying soil (Zhang, Nguyen & Blum 1999). Bulk osmolyte content was measured in 30-day-old wild-type and *phyB* leaves under well-watered conditions and after 7 d of water shortage before plants showed any signs of wilting. Although the wild-type and *phyB* mutants displayed identical quantities of osmolytes in well-watered conditions, after 7 d of suspending watering, *phyB* mutants showed higher osmolyte concentration in green tissues than the wild type (Fig. 4). We thus rejected the hypothesis that osmotic adjustment is involved in the drought tolerance of wild-type plants. Root biomass and shoot-to-root biomass ratio (calculated as FW or dry weight) of plants grown in a sand substrate – to facilitate root washing – were similar in both wild-type and *phyB* mutants (Gonzalez *et al.*, unpublished data). Taken together, these data indicate that the root system of *phyB* mutants was able to explore the whole soil volume of the pot, suggesting that the cause/s of their worse performance under water stress might be due to their shoot morphology and/or physiology.

phyB reduces leaf ABA concentration under well-watered conditions

Low sensitivity to drought observed in *phyB* mutants could be due to a lower capacity to synthesize or to respond to ABA, a key molecule which induces stomatal closure in

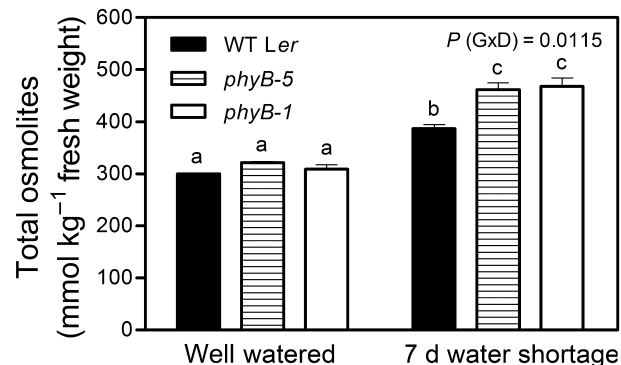


Figure 4. *phyB* mutants display higher osmotic adjustment. Total osmolytes (mmol kg^{-1} fresh weight) of 30-day-old-plants exposed to well-watered condition and after 7 d water shortage. Different letters denote significant differences at $P < 0.001$. P value of the interaction between genotype and drought treatment is shown in the figure. Data are means \pm SE, $n = 4$.

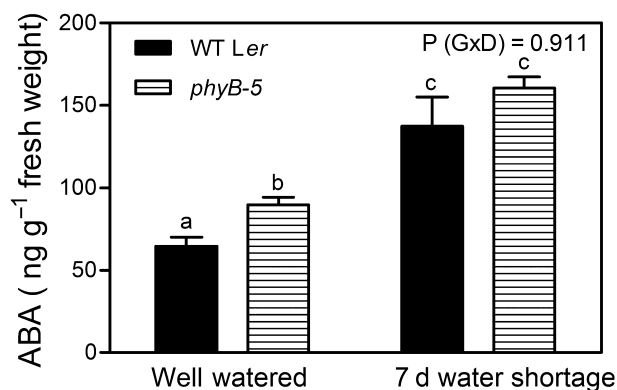


Figure 5. *phyB* mutants present enhanced endogenous ABA. ABA concentration was measured in leaves of 30-day-old plants exposed to well-watered condition and after 7 d of water shortage treatment. Different letters denote significant differences at $P < 0.001$. P value of the interaction between genotype and drought treatment is presented in the graph. Data are means \pm SE, $n = 3$.

response to drought (Zeevaart 1980). Unexpectedly, we detected that *phyB-5* possessed ~39% higher leaf ABA concentration than the wild type ($P < 0.001$) under well-watered conditions (Fig. 5). After 7 d without watering, both wild type and *phyB-5* increased their leaf ABA concentration reaching similar endogenous contents (Fig. 5).

***phyB* promotes the expression of early ABA signalling components**

As *phyB-5* plants showed higher ABA levels than the wild type under well-watered conditions (Fig. 6), and also displayed a lower capacity to diminish stomatal conductance in response to depletion of soil water content (Figs 2b and 3c), we evaluated whether *phyB* controls the expression of ABA signalling components. We studied the expression of certain ABA influx and efflux transporters and ABA signalling elements that alter drought tolerance when they are mutated or overexpressed. *ABCG22* expression, an ATP-binding cassette (ABC) transporter gene that putatively acts as an ABA influx transporter in stomata contributing to increase drought tolerance in *A. thaliana* (Kuromori *et al.* 2011), was higher in the wild type than in *phyB-5* (Fig. 5). Expression of another ABA efflux transporter (*ABCG25*) and the ABA influx transporter *ABCG40* (Kang *et al.* 2010; Kuromori *et al.* 2010) were not detectable under our experimental conditions. We also examined the expression of different members of the *PYR/PYL/RCAR* family, which are expressed in stomata (*Arabidopsis* eFP browser at <http://www.bar.utoronto.ca>), and they are key pieces in ABA perception and promotion of drought tolerance (Santiago *et al.* 2009; Saavedra *et al.* 2010). *PYL5* expression was three times lower in *phyB-5* than in the wild type (Fig. 5). This suggested a central role in the *phyB*-induced mechanism of drought tolerance. Expression of *PYL8* was similar between wild-type and *phyB-5* plants (Fig. 5); while the

expression of *PYL6* was not detectable in our experimental conditions. *HABI*, a negative regulator of ABA signalling (Saez *et al.* 2004, 2006), showed a significantly enhanced expression in the wild type compared with *phyB-5* (Fig. 5). Previous reports demonstrated that overexpression of both *PYL5* and *HABI* enhances the response to ABA (Santiago *et al.* 2009), and the expression of *PYL5* and *HABI* in our experimental conditions also suggested that the net result of an enhanced expression of both genes favour the response to ABA.

***phyB* increases ABA sensitivity responses**

To evaluate the specific effects of *phyB* in ABA signalling, we analysed whether *phyB* affects responses to exogenous ABA applications. We first assessed ABA-induced stomatal closure spraying the leaves of 30-day-old plants with a solution of 0, 1, 20 or 100 μM of ABA. Wild-type plants were more effective in diminishing stomatal conductance than *phyB-5* after 3.5 h of ABA application (Fig. 7a). This was due to reductions in stomatal aperture (Fig. 7c). ABA concentrations of less than 1 μM were effective in reducing

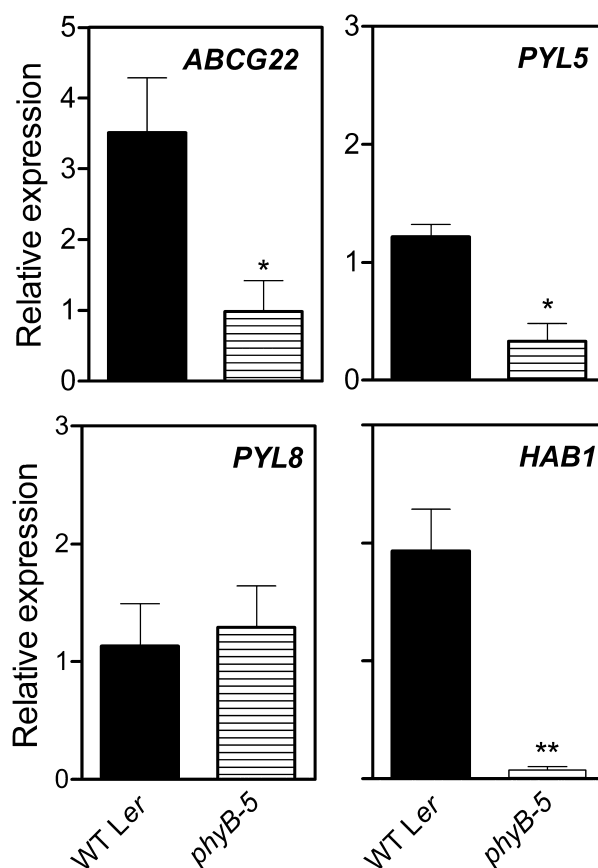


Figure 6. *phyB* enhances the expression of early ABA signalling components. *ABCG22*, *PYL5*, *PYL8* and *HABI* expression relative to *UBC21* measured in 30-day-old well-watered plants. Asterisks denote significant differences (* $P < 0.05$; ** $P < 0.01$). Data are means \pm SE, $n = 3$.

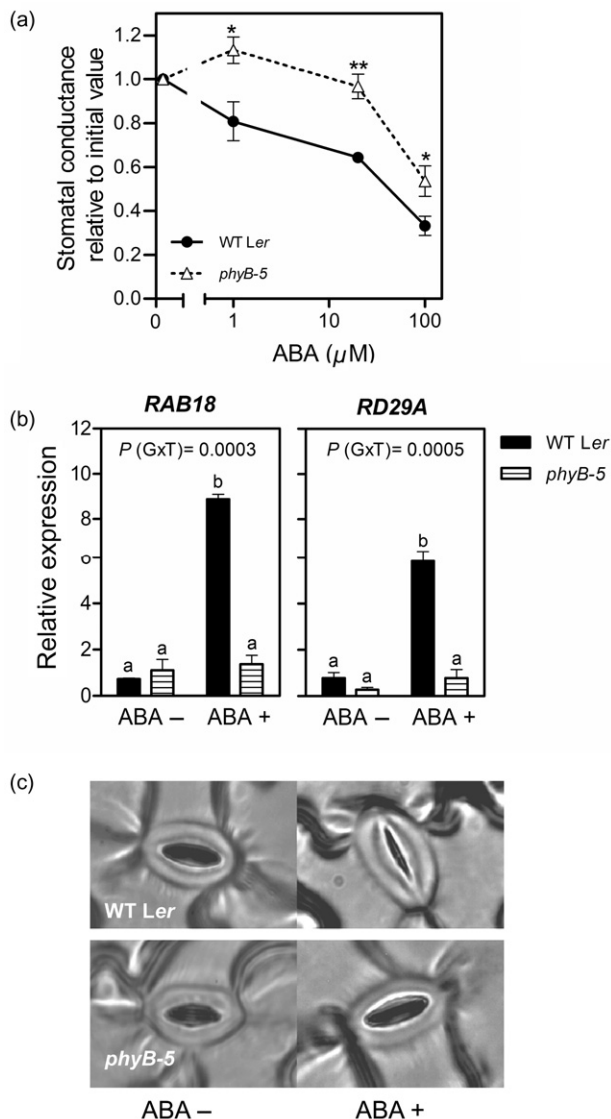


Figure 7. *phyB* enhances sensitivity to ABA. (a) Stomatal conductance relative to initial value was calculated measuring stomatal conductance before and 3.5 h after spraying leaves of 30-day-old plants with ABA solutions 0, 1, 20 and 100 μM (log10 scale) in the middle of the photoperiod. Asterisks denote significant differences ($*P < 0.05$; $**P < 0.01$). Data are means \pm SE, $n = 5$. (b) *RAB18* and *RD29A* expression relative to *UBC21*, before (ABA $-$) and 1.5 h after spraying the leaves with 20 μM ABA (ABA $+$). P value of the interaction between genotype and ABA treatment is shown in the figure. Different letters denote significant differences at $P < 0.001$. Data are means \pm SE, $n = 3$. The experiments were repeated twice showing similar results. (c) Photographs of stomata taken from imprints performed on the abaxial leaf surface of wild-type and *phyB-5* leaves before (ABA $-$) and after 3.5 h (ABA $+$) of spraying leaves with a solution of 20 μM ABA (1000 \times).

stomatal conductance in wild-type plants, while the *phyB* mutant required more than 100 μM of ABA to induce stomatal closure (Fig. 7a). The expression of *RAB18* and *RD29A*, two typical genes induced by ABA, was strongly induced in the wild type compared with *phyB-5*, after 1.5 h

of spraying the leaves of 30-day-old plants with 20 μM of ABA (ABA $+$ treatment; Fig. 7b). Taken together, these results show that *phyB* enhances ABA sensitivity, increasing ABA-induced responses that promote drought tolerance in *A. thaliana*.

DISCUSSION

In plant-crowded environments, SAS evocation by *phyB* is crucial to enhance light interception to improve carbon gain when light is the photosynthesis-limiting factor. On the other hand, during acclimation to open environments in well-watered conditions, *phyB* adjusts plant growth to increase carbon gain at the expense of a higher water loss (Boccalandro *et al.* 2003, 2009). Until now, the role of *phyB* when water became scarce had not been clarified. Plants growing under those conditions are subjected to a trade-off between: (1) avoiding light excess by fixing more CO_2 at the expense of enhanced water loss; and (2) preserving soil water, reducing the risk of water stress at the expense of decreasing the capacity of CO_2 fixation to deal with light excess. Excitation energy not used in the photochemical phase of photosynthesis or not dissipated as fluorescence or heat can be transferred to molecular oxygen, generating a highly damaging reactive oxygen species with detrimental functional consequences for plants (Golan, Müller-Moulé & Niyogi 2006 and references therein). Our results showed that *A. thaliana* plants can resolve this trade-off in part due to the contribution of *phyB*, under either high or low water availability. Wild-type *A. thaliana* plants acclimated to high R:FR ratios, despite using more water per unit CO_2 fixed, are able to rapidly sense a decline in soil water content, exhibiting early stomatal closure mediated by an increase in ABA sensitivity.

phyB mutants were found to constitute a paradoxical phenotype, because in spite of displaying lower stomatal conductance under well-watered conditions (Fig. 1a), they presented a reduced capacity for stomatal closure under water stress conditions. During soil desiccation, they were less sensitive than the wild type, wilting at higher soil water content than wild-type plants (Figs 2a and 3a). The lower capacity of *phyB* mutants to respond to drought was not due to a lower ABA biosynthetic capacity (Fig. 5); we detected that *phyB-5* possesses higher endogenous ABA concentrations than the wild type at field capacity, similar to what occurs with the *ABA INSENSITIVE-1* mutant, *abi-1* (Verslues & Bray 2006).

We present physiological and molecular evidence supporting the idea that the lower drought tolerance observed in *phyB* mutants was mainly due to a lower sensitivity to ABA. At a physiological level, *phyB-5* plants were less effective in diminishing stomatal conductance than the wild type in response to spraying with increasing ABA doses (Fig. 7a,c). At a molecular level, we found that the mechanism underlying the rapid stomatal closure response is the early expression of signalling genes related to ABA transport and perception.

In well-watered plants, phyB highly promoted the expression of *ABCG22*, a putative ABA influx transporter. It was reported that *abgc22* mutants were more susceptible to drought stress, suggesting that enhanced levels of *ABCG22* transcripts found in wild-type plants could contribute to its increased drought tolerance (Kuromori *et al.* 2011; Fig. 6). The expression of *PYL5*, a member of the ABA-binding receptor-like proteins known as PYR/PYL/RCAR (Ma *et al.* 2009; Park *et al.* 2009), was also higher in the wild type than in *phyB-5* (Fig. 6). *PYL5* activates ABA signalling through direct inhibition of proteins of the clade A PP2Cs which are negative ABA signalling element, such as *HAB1* (Santiago *et al.* 2009; Umezawa *et al.* 2010; Qin, Shinozaki & Yamaguchi-Shinozaki 2011). It was demonstrated that *PYL5* enhances resistance to drought in *Arabidopsis* plants (Santiago *et al.* 2009). The expression of *HAB1* is higher in the wild type than in *phyB-5* (Fig. 6). When *PYL5* and *HAB1* are overexpressed, the net result of increments in the expression of both genes was an enhancement of ABA responses (Santiago *et al.* 2009).

Some other ABA genes are induced by the phyB action that increases ABA sensitivity in the leaves. In fact, the expression of *RAB18* and *RD29A*, both typical genes induced by ABA, was much higher in the wild-type than in *phyB-5* plants sprayed with 20 μM ABA solution (Fig. 7b), indicating that phyB clearly promotes ABA sensitivity.

Under our experimental conditions, we could not detect consistent differences in leaf area between *phyB* mutants and the wild type, as previously observed by Boccalandro *et al.* (2009). This might be due to the different light sources and irradiances used between these studies. R:FR ratio and phyB mutation are known to affect leaf area; nevertheless, this depends on the species and the growing condition. For example, leaf area increased in response to +FR in many species (Kwesiga & Grace 1986; Casal, Aphalo & Sánchez 1987; Cogliatti & Sánchez 1987; López Juez *et al.*, 1995). However, the opposite pattern (i.e. reduction of leaf area in response to +FR) has also been found even in *Arabidopsis* (Kasperbauer 1971; Holmes & Smith 1977; Frankland & Letendre 1978; Devlin *et al.* 1999).

The lower tolerance to drought of *phyB* mutants could be the result of a potential incapacity of these mutants to extract soil water, although at morphological level they present similar root biomass and shoot-to-root biomass ratio as the wild type. *phyB* mutants wilted, extracting equal or more water from the soil than the wild type, showing that the root system of *phyB* was able to explore the soil, suggesting that the different water status dynamics was established at transpiration level. We also observed that *phyB* mutants displayed enhanced osmotic adjustment (Fig. 4). We could not discern if this was the result of an enhanced sensitivity to induce osmolyte accumulation during drought or because *phyB* mutants reached a lower water potential than the wild type. The last is an ABA-independent condition necessary to trigger osmotic adjustment (Verslues & Bray 2006). In conclusion, the wilting phenotype of *phyB* observed in unwrapped and wrapped pots experiments is a consequence of remaining their stomata opened for a

longer time than wild-type plants. This fact conduces to a higher reduction of soil water content in terms of relative (Figs 2 and 3) and absolute stomatal conductance (Supporting Information Fig. S2).

Our results for *Arabidopsis* are not necessarily valid for other species. For example, cotton plants acclimated to an end-of-day FR pulse exhibited reduced transpiration and increased drought resistance. This was proposed to be due to the effect of FR diminishing stomatal aperture (Ouedraogo & Hubac 1982). Nevertheless, R/FR effects on the aperture of the stomatal pore have not been reported. The stomata of *C. communis* (Roth-Bejerano *et al.* 1982) and of *Paphiopedilum* orchids (Talbot *et al.* 2002) open in response to R, and this effect is reversed by subsequent FR light, which indicates phytochrome control. Nevertheless, this reversed effect by FR is absent in wild-type *Arabidopsis* (Talbot *et al.* 2003). It was recently reported by Liu *et al.* (2012) that *phyB* deficiency in rice causes both reduced total leaf area and transpiration per unit leaf area. This can explain the reduced water loss and improved drought tolerance of *phyB* rice mutants.

Using data from previous reports, plus the results of this study, we present a working model (Fig. 8), suggesting that *phyB* is a key player in acclimation to open environments. It induces physiological responses that avoid stressful

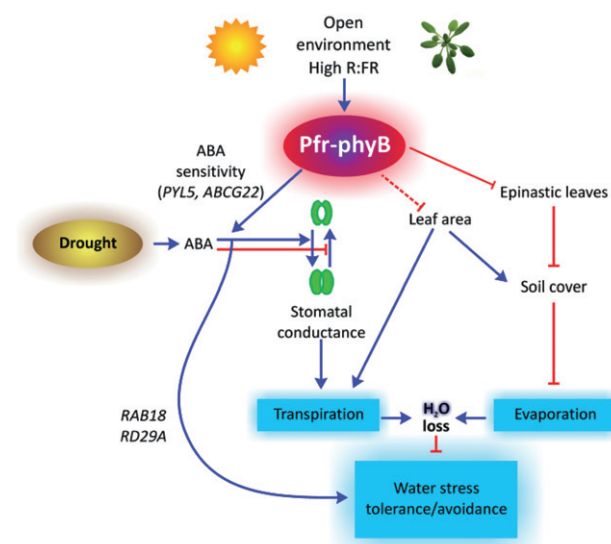


Figure 8. Working model. During acclimation to open environments (high R:FR), phyB induces several morphological and physiological responses which affect carbon gain at the expense of higher water loss. phyB enhances sensitivity, allowing quick detection and response to drought conditions; phyB can eventually modify leaf area and leaf positioning, altering soil cover and consequently reduce soil evaporation. On the other hand, phyB also increases ABA sensitivity, through enhancement of the expression of some early ABA signalling components, such as *PYL5* and *ABCG22*, leading to feed-forward stomatal closure after detection of soil water depletion. As a result, this mechanism controlled by phyB allows plants a fine adjustment of stomata closure to tolerate or avoid water stress in open environments.

conditions provoked by light excess under high water availability and enhances sensitivity to ABA that allow plants to induce a fine-tuning adjustment when soil water becomes a scarce and limited resource.

The function of phyB in plant development was initially and primarily associated to its role in modulating SAS as a function of plant crowding (Smith 2000). Growing evidence shows that phyB can act as an integrator of different environmental signals by modulating responses to different stresses. phyB regulates freezing tolerance, affecting the expression of the *C-REPEAT-BINDING-FACTOR* (CBF) regulon and its downstream targets, the *COLD-REGULATED (COR)* genes (Kim *et al.* 2002; Franklin & Whitelam 2004, 2007). phyB also plays an important role in biotic stress, promoting plant defences to herbivory. By detecting the proximity of neighbours through the action of phyB, plants can induce a selective desensitization to jasmonates attenuating defences under conditions of crowding and competition (Ballaré 2009).

Nowadays, a common strategy pursued by different seed companies to improve crop yield is to increase the number of plants per unit soil. However, beyond certain plant densities, yield increments become null or negative (Tetio-Kagho 1988; Sangoi *et al.* 2002). Part of this yield reduction can be associated to higher SAS evocation that enhances the production of non-productive structures (i.e. stem elongation) and/or the increased susceptibility to stem lodging (Casal *et al.* 1994; Sparkes & King 2008). Considering this evidence, *PHYB* overexpression is a plausible strategy to enable the cultivation of more tolerant plants at a higher crop density (Boccalandro *et al.* 2003). In support of such a strategy, here we demonstrated that phyB enhances responses to abiotic stress, such as water stress. Our results, together with previous reports, suggest that 'blind' plants unable to detect neighbours due to the overexpression of phyB could display a better plant performance in dense crops not only because of SAS repression, but also by enhancing drought tolerance and defences.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Constitutive shade avoidance phenotype of *phyB* mutants reduces soil cover.

Figure S2. *phyB* plants maintained open stomata despite a reduction in soil water content.

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