Physiologia Plantarum
An International Journal for Plant Biology

Phytochrome A increases tolerance to high evaporative demand

Gabriela Alejandra Auge[a], Matías Leandro Rugnone[a,†], Leandro Emanuel Cortés[b,c,†], Carina Verónica González[b,†], Gabriela Zarlavsky[d], Hernán Esteban Boccalandro[b,c] and Rodolfo Augusto Sánchez[a,*]

[a]IFEVA-CONICET, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina
[b]IBAM-CONICET, Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina
[c]Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[d]Laboratorio de Anatomía Vegetal, Cátedra de Botánica Agrícola, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

Correspondence
*Corresponding author,
e-mail: sanchez@ifeva.edu.ar

Received 14 October 2011;
revised 1 February 2012

doi:10.1111/j.1399-3054.2012.01625.x

Stresses resulting from high transpiration demand induce adjustments in plants that lead to reductions of water loss. These adjustments, including changes in water absorption, transport and/or loss by transpiration, are crucial to normal plant development. Tomato wild type (WT) and phytochrome A (phyA)-mutant plants, *fri1-1*, were exposed to conditions of either low or high transpiration demand and several morphological and physiological changes were measured during stress conditions. Mutant plants rapidly wilted compared to WT plants after exposure to high evaporative demand. Root size and hydraulic conductivity did not show significant differences between genotypes, suggesting that water absorption and transport through this organ could not explain the observed phenotype. Moreover, stomatal density was similar between genotypes, whereas transpiration and stomatal conductance were both lower in mutant than in WT plants. This was accompanied by a lower stem-specific hydraulic conductivity in mutant plants, which was associated to lower xylem vessel number and transversal area in *fri1-1* plants, producing a reduction in water supply to the leaves, which rapidly wilted under high evaporative demand. PhyA signaling might facilitate the adjustment to environments differing widely in water evaporative demand in part through the modulation of xylem dimensions.

Introduction

The capacity to adjust their functioning to large variations in environmental conditions is essential for the normal development of plant populations. Phenotypic plasticity offers the opportunity of matching phenotype to environment and so increasing fitness in the diverse ecological scenarios that a plant may encounter (Schmitt et al. 2003). Plants are equipped with a wide array of mechanisms to perceive environmental cues and to translate them into morphological and physiological changes allowing adjustment to actual or future conditions. Stand density is one of the environmental attributes that can show extreme variations between densely crowded and open sites, and plant phenotype is strongly affected by these conditions (Harper 1977, Ballaré et al. 1988). Plants of many species display a number of morphological and developmental responses when exposed

Abbreviations – FR, far-red light; PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux density; phyA, phytochrome A; phyB, phytochrome B; R, red light; SAS, shade avoidance syndrome; VPD, vapor pressure deficit; WT, wild type.

[†]These authors contributed equally to this work.

to high density, collectively termed shade avoidance syndrome (SAS), such as stem and petiole elongation, reduced branching and shortened time to flowering (Smith 1982). One central element in the perception of density is the change in light spectral composition after interaction with green tissues. The selective absorption of visible light and the increased transmission and reflection of far-red light (FR; 700–800 nm) causes a reduction in the proportion of red light (R, 600–700 nm) vs FR (Smith 1982). This reduced R/FR ratio is perceived by the phytochromes, mainly phytochrome B (phyB), which triggers SAS. It has been shown that phytochrome-mediated SAS has adaptive value (Schmitt et al. 1995). Increased plant height, in particular, is recognized as a positive trait in adjusting to high density (Schmitt et al. 1999).

There are substantial differences in photosynthetically active radiation (PAR), leaf temperature and wind speed between open and crowded habitats; consequently, carbon and water economies are strongly influenced by plant density. Adjustments to those conditions in both processes take place very frequently, and it is not surprising that phytochromes may mediate some of them. In fact, higher R/FR ratios detected by phyB have been shown to increase stomatal density, stomatal index and amphistomy in leaves of *Arabidopsis thaliana* plants, resulting in a greater photosynthetic rate at high PAR at the expense of a reduction in water use efficiency (Boccalandro et al. 2009). On the other hand, potato plants over-expressing phyB showed no changes in stomatal density but higher stomatal conductance and transpiration and photosynthesis rates; in this case, the relationship between phyB and carbon and water economies may be an indirect result of phytochrome influence on other processes such as assimilate demand or leaf senescence (Boccalandro et al. 2003). There are more examples that phyB and/or the stable phytochromes can influence plant attributes related to carbon and/or water balance (Roth-Bejerano et al. 1982, Casal et al. 1994, Rousseaux et al. 2000, Sokolskaya et al. 2003). The situation of phytochrome A (phyA) is markedly different. Unlike other family members, phyA is unstable in the Pfr form and therefore it has been suspected that its main functions were control of seed germination and seedling de-etiolation (Smith and Whitelam 1990). But it has been established that phyA controls a number of functions throughout development, including day-length perception (Johnson et al. 1994, Lin 2000) and inhibition of internode elongation (Yanovsky et al. 1995). However, these functions were detected in double or triple mutants since monogenic phyA-mutant *Arabidopsis* plants grown under natural light are very similar to the wild type (WT). More recently, Franklin et al. (2007) have shown that under relatively high photon irradiances phyA protein degradation is retarded, opening the possibility of a significant phyA contribution to behavior regulation of full-daylight-grown plants. Nevertheless, there is almost no information indicating a dominant role of phyA on the adjustment to situations affecting water balance in full-daylight-grown plants. *Arabidopsis* phyA mutants have been shown to have reduced transpiration compared with WT plants (Eckert and Kaldenhoff 2000), although to the best of our knowledge the impact on the water budget has not been investigated further.

Here, we show that phyA plays a prominent part in acclimation to full sunlight, enhancing the hydraulic conductivity necessary to cope with conditions of high atmospheric demand, therefore revealing that phyA has a key role in the water economy of tomato plants.

Materials and methods

Plant material and growth conditions

Solanum lycopersicum 'Moneymaker' seeds were incubated at 25°C in plastic boxes on a 0.5 cm cotton layer saturated with distilled water. After 12 days, the seedlings were planted in 3 l pots with a mixture of organic matter-enriched soil and perlite (3:1). Plants were grown in a temperature controlled greenhouse at 25 ± 2°C until they had eight to nine fully expanded leaves for hydraulic conductivity measurements or four to five fully expanded leaves for water loss, leaf water potential and leaf temperature measurements. For experiments under high evaporative demand, treated plants were transferred to a hotter greenhouse, i.e. without temperature control.

Experiments were carried out at IFEVA-CONICET, Facultad de Agronomía, Universidad de Buenos Aires, Argentina (34°25′S and 58°25′W at 25 m asl), and Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo, Mendoza, Argentina (33°0′S and 68°52′W at 940 m asl). All experiments were conducted at least once in each location with similar results. Figures represent the results of one representative experiment.

Air temperature and relative humidity were recorded every 10 min with three Hobo sensors (Hobo Pro series; Onset Computer Corporation, Bourne, MA). Photosynthetic photon flux density (PPFD) was measured with a LI-COR LI-190SA quantum sensor and a LI-250 light meter (LI-COR, Lincoln, NE).

Hydraulic conductivity measurements

Hydraulic conductivity of whole root systems and stem segments (between third and fourth nodes) was measured as described in Fernández and Gyenge (2010),

using a low pressure (water column) conductivity meter ($n = 11$). The root system (whole roots plus the first stem internode segment) or stem segments from plants with eight to nine fully expanded leaves were cut under water to avoid embolisms due to dissection. Roots were completely submerged in a closed water-filled container, and the cut-end of the root pushed through an opening in the lid of the container, which was connected to a low pressure water column. After conductivity measurements, roots were dried at 70°C for 2 days and their dry weight determined. Stem segments were connected to a rubber hose, the other end of which was attached to a low pressure water column. After measurements, stem length and diameter were recorded. Hydraulic conductivity was assessed by measuring the volume of water that passed through the tissues under a known pressure during a given period (1–5 min) using the following equations for roots and stems, respectively:

$$k_r = J/(P \times M_r)\ mg_{water}\ s^{-1}kPa^{-1}g_r^{-1} \quad (1)$$

$$k_s = J/[(P/L_s) \times A_s]\ mg_{water}\ s^{-1}kPa^{-1}\ cm_s^{-1} \quad (2)$$

where k_r and k_s are the root and stem hydraulic conductivity, respectively, J is the measured water flux, P is the known pressure applied to the system, M_r is the root dry weight and L_s and A_s are the length and transversal area of the stem segment, respectively.

Plant water loss measurements

Loss of water per pot (in grams) was calculated by weighing every pot at the beginning (6:30 h) and at the end of the experimental period (13:30 h). Adaxial and abaxial leaf conductance to water vapor (g_l) was measured with a steady-state diffusion porometer (SC-1; Decagon Devices, Pullman, WA) in the terminal leaflet of the third fully expanded sun-exposed leaf ($n = 8$). Stomatal conductance, g_l, was calculated as the sum of the adaxial and abaxial leaf conductance values for each leaf. Stomatal aperture was assessed by taking imprints using transparent nail varnish applied to the fourth fully expanded leave from 50-day-old plants at 9:00 h (low atmospheric demand) and at 14:00 h (high atmospheric demand). Leaves were not detached until the varnish dried. Stomatal aperture imprints were measured under a microscope (40×/100×) in the middle portion of the abaxial leaf surface. Representative photographs were taken using a Micrometrics 318 CU camera (China) attached to a Nikon Eclipse E200 optical microscope (Tokyo, Japan). After plant water relation measurements, leaves were harvested and immediately scanned. Individual leaf area was calculated using Adobe Photoshop (v. 7.0) by comparison with a reference area.

Water status measurements

Leaf water potential (Ψ_w) measurements were recorded for each genotype at pre-dawn (6:00 h), mid-morning (10:30 h) and mid-day (around solar noon for each location) using the third fully expanded leaf of plants with four to five leaves, with a pressure chamber (PMS Instruments Co., Corvallis, OR). Cut leaves were measured within 1–2 min.

Thermographic pictures

Digital thermal images were obtained using a Fluke TiR Thermal Imager (Fluke Co., Everett, WA). Plants were photographed from 7:00 to 13:00 h. Digital thermograms were analyzed with SMARTVIEW software (Fluke Co.).

Preparation of stem cross sections

Stem sections between the stem/root junction and the cotyledon node were cut from plants with eight to nine fully expanded leaves and conserved in 20% v/v ethanol solution until sectioned. Cross sections were cut by hand or microtome. Hand-cut sections (around 15–20 µm thick) were treated with 50% v/v commercial sodium hypochlorite to clarify the cells and then washed twice with distilled water and incubated with safranin solution until conducting tissues with secondary growth were stained. Cross sections were mounted on slides with mounting medium (gelatin:pure glycerol:distilled water, 1:7:6). Sections were observed using a stereoscopic microscope (CETI, Medline Scientific Ltd., Oxfordshire, UK) and photographed with a PowerShot A520 digital camera (Canon Inc., Ontario, Canada) at 40× to assess xylem vessel number and area with Adobe Photoshop (v 7.0) by comparison with a reference area. For microtome-cut stem sections, stem segments were fixed in FAA buffer (ethanol 96% v/v:distilled water:formaldehyde:acetic acid, 50:35:10:5) for at least 48 h before processing. After the material had been washed and rinsed with distilled water, it was submitted to a gradual dehydration process with a series of 50, 70, 80, 96 and 100% v/v ethanol solutions. Subsequently, the material was gradually embedded in paraffin by transferring it sequentially to xylene 100% v/v, xylene:paraffin (3:1), xylene:paraffin (1:1) and finally to pure paraffin. The time necessary for each stage was variable and depended on the tissue size and traits. Paraffin and xylene:paraffin solutions were kept at 60°C during the process. After paraffin blocks had been cooled, they were sectioned using a microtome (SM 2000 R; Leica Microsystems, Wetzlar, Germany) and the cross sections (14 µm thick) were mounted on slides. The sections were dewaxed with xylene 100% v/v

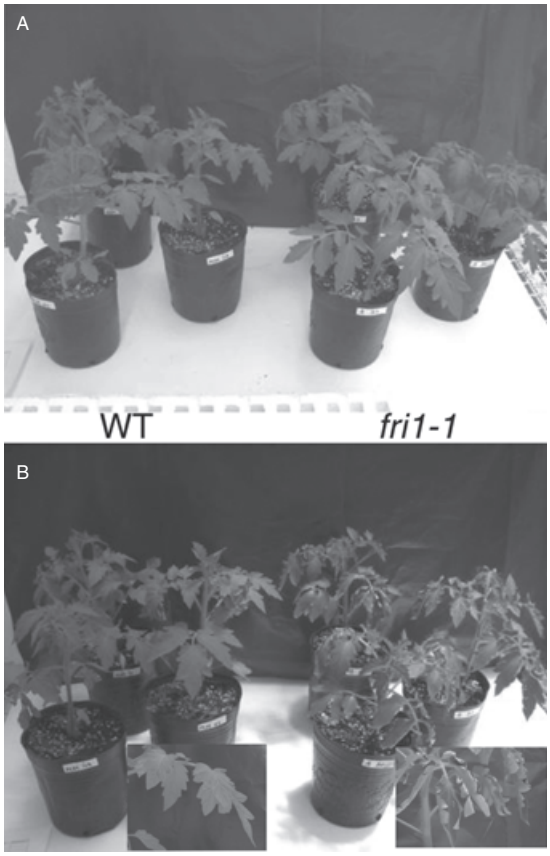

Fig. 1. PhyA increases tolerance to environments of high evaporative demand. Phenotypic differences between WT and phyA-mutant *fri1-1* plants. Pictures of one representative experiment before (A) and after (B) 30 min of exposure to stressful conditions (higher temperature, see Fig. S1). Insets in (B) show a detail of the phenotype observed after exposure to stressful conditions in leaves of both genotypes.

and incubated in safranin solution until the conducting tissues with secondary growth were stained, then rinsed twice with distilled water. Finally, stem cross sections were mounted on slides with Natural Canada Balsam and observed under a Nikon Eclipse E200 optical microscope (Tokyo, Japan) and photographed with a Micrometrics 318 CU digital camera (Shanghai, China) at 40×.

Stomatal density measurements

The first pair of fully expanded leaves was used to determine stomatal density in WT and phyA-mutant plants. The number of stomatal cells was counted in clarified leaves under a clear-field Leica DMIRB inverted microscope at 600× and photographed with a Leica DC 300F camera (Leica Camera AG, Solms, Germany). Clarification was carried out immersing leaves in an NaOH 1% p/v solution at 70°C for 30 s, and then, keeping them in ethanol 70% v/v until measurements were made. If necessary, safranin staining was performed to help stomatal cell visual identification.

Statistical analysis of the data

Student's *t*-test or ANOVA followed by LSD Fisher post-test was performed when appropriate, in order to asses minimum differences between means with a $P < 0.05$ (*) significance level using INFOSTAT software (www.infostat.com.ar).

Results

It has been reported that in *Arabidopsis* and potato, phytochromes, specially phyB, affect several morphological (e.g. root:shoot biomass ratio and stomatal density) and physiological characteristics (e.g. stomatal conductance and transpiration) (Salisbury et al. 2007, Boccalandro et al. 2009) that could be involved in plant water use efficiency. In order to elucidate if phyA is playing any role in plant water relations, we grew WT and phyA-mutant (*fri1-1*) tomato plants under stressful conditions. We observed that under conditions of high evaporative demand, leaves of *fri1-1* plants wilted, while the leaves of WT plants remained unaffected (Fig. 1).

Loss of turgor in leaves could be the consequence of differences in water uptake, transport or transpiration between *fri1-1* and WT plants. We did not observe significant differences either in root biomass or root hydraulic conductivity, suggesting that there was no difference in water acquisition capacity between genotypes (Fig. 2). In contrast, loss of water per pot during the experiment (7:00 h to 13:00 h) was different between genotypes but, surprisingly, WT plants lost more water than *fri1-1* plants (Fig. 3A). The larger water loss was not due to a larger leaf area or stomatal density

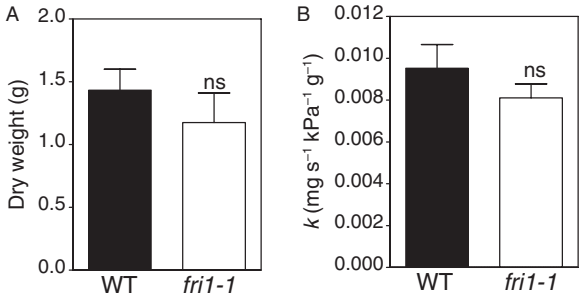

Fig. 2. Influence of genotype on attributes related to water uptake. (A) Root mass (dry weight, grams) and (B) root system hydraulic conductivity (k, mg s^{-1} kPa^{-1} g^{-1}) of WT and *fri1-1* plants. Bars are means ($n \geq 10$), narrow vertical extensions represent one standard error. Values are from one representative experiment; ns, differences not significant ($P > 0.05$).

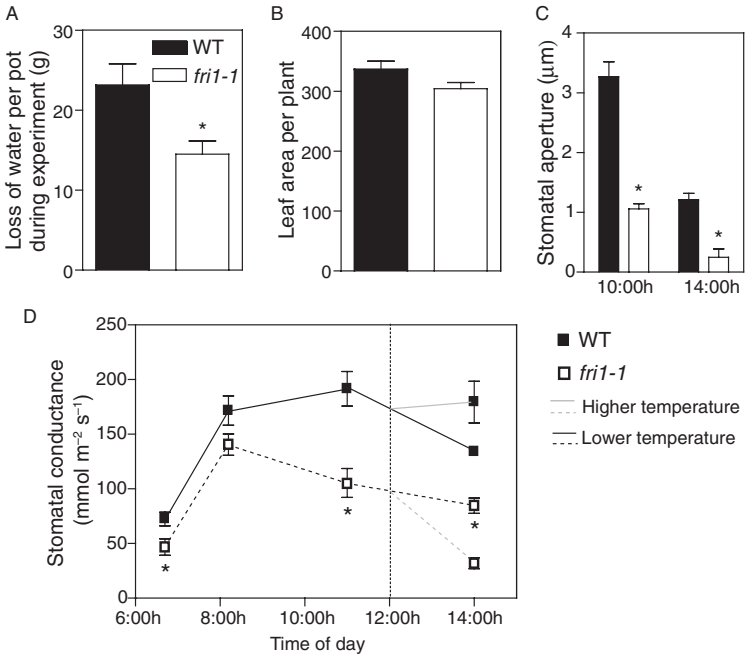

Fig. 3. Influence of genotype on water loss. (A) Water loss per pot during the experiment (7:00 h to 13:00 h) in normal (lower temperature) conditions, (B) leaf area per plant and (C) stomatal aperture (μm) before (10:00 h) and after (14:00 h) the beginning of exposure to stressful conditions (i.e. lower and higher temperatures, respectively, see Fig. S1). (D) Time course of stomatal conductance during the experiment. Data points are means, vertical bars represent ± one standard error. Values are from one representative experiment. Asterisks indicate significant ($P < 0.05$) differences between means.

in WT plants (Figs 3B and S2) but was related with a greater leaf conductance and stomatal aperture in WT plants during the day (Fig. 3C, D). In Fig. 3D it is shown that early in the morning (8:00 h), stomatal conductance of *fri1-1*-mutant plants was similar to the WT (Fig. 3D), but when at noon air temperature increased (Fig. S1), a significant decrease in *fri1-1* plant leaf conductance was observed, something that did not occur in WT (Fig. 3D).

Although genotype might affect stomatal behavior in several ways, two of the more likely possibilities are that somehow leaf water potential may decrease during the day to lower values in *fri1-1* or that in the mutants the stomata are more sensitive to a decrease in water potential. At sunrise, leaf water potential was the same in both genotypes but later in the day it became lower in *fri1-1* leaves (Fig. 4). As expected, leaf temperature was significantly higher in *fri1-1* leaves (Fig. 5). Taken together, the observations that *fri1-1* plants had lower rate of water loss and leaf water potential than the WT suggested that water transport to the leaves was affected. Shoot hydraulic conductivity was significantly lower in the *fri1-1* plants (Fig. 6B) and this was not related with stem diameter (Fig. 6A) but with the area of xylem elements in the stem (Fig. 6D). Therefore the marked tendency to leaf wilting under high transpiration demand of *fri1-1* plants seems to be related to phyA control

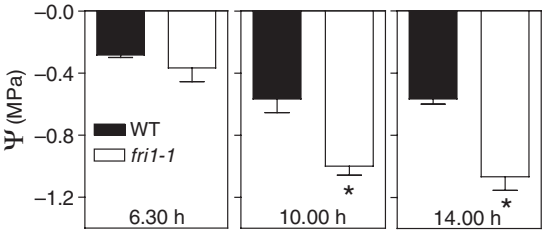

Fig. 4. Plants lacking phyA have less control of leaf water potential. Time course of leaf water potential during one representative experiment. Bars are means ($n \geq 10$), narrow vertical extensions represent one standard error. Asterisks indicate significant ($P < 0.05$) differences between means.

of xylem development that causes a higher resistance to water transport through the xylem vascular tissue, precluding an adequate water provision to the leaves when the transpiration rate is high.

Discussion

Adjustment to stand density provided by the SAS of the phytochromes has been shown to be adaptive. The fitness advantage in sites with high density is mainly related with plant height due to its influence in sunlight capture. Improving the ability for light competition is certainly

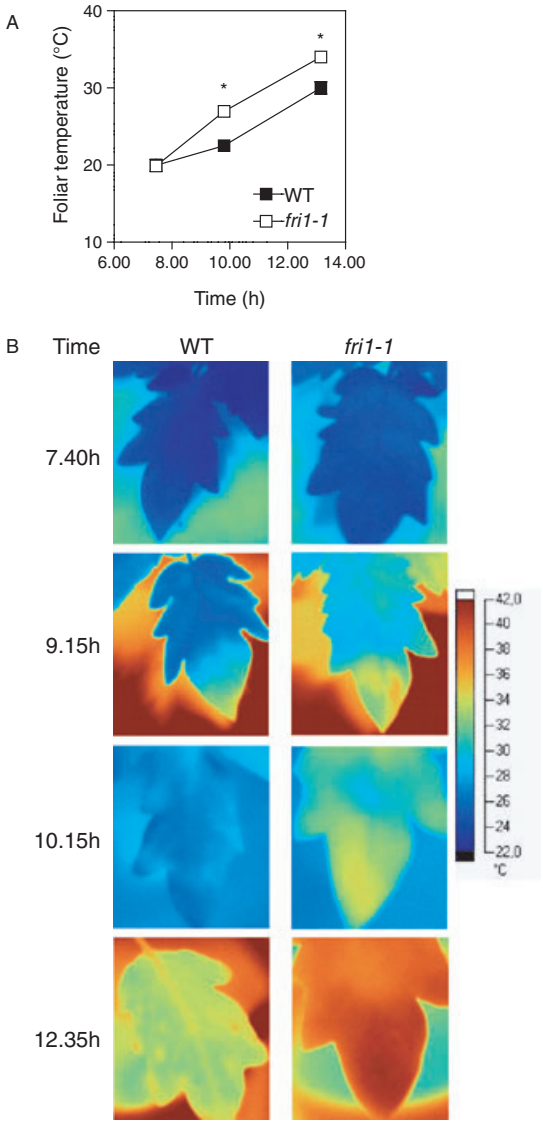

Fig. 5. Leaf temperature is higher in plants lacking phyA. Time course of leaf temperature (A) and thermographic pictures of fully expanded leaves (B) during one representative experiment. Data points in (A) are means ($n \geq 10$). Asterisks indicate significant ($P < 0.001$) differences between means.

a central aspect of the responses to density (Schmitt et al. 1999). However, several other morphological and physiological traits are modified by the environmental differences between open and crowded habitats (Hutchings and de Kroon 1994). Some of them are controlled by phytochromes, although almost all the information available so far is about the involvement of phyB (Ballaré et al. 1997). While evoking SAS, phyB can improve carbon gain in a crowded environment through shoot morphological changes that increase chances to forage for PAR. Nevertheless, phyB effects on carbon and water economies are not limited to promoting a better exposure of leaves to sunlight. Evidence shows that phyB can also adjust structures related to acquisition, transport and loss of water as well as carbon gain. phyB shapes *Arabidopsis* roots, increasing the number of lateral roots (Salisbury et al. 2007) and reducing main root and root hair length (Reed et al. 1993, González and Boccalandro 2008). It can also adjust xylem vessel diameter and number, functionally increasing stem water conductance of adult cucumber plants (Casal et al. 1994). At leaf level, phyB increases stomatal density and index, amphistomy (Boccalandro et al. 2009, Casson et al. 2009) and stomatal aperture (Wang et al. 2010) in *Arabidopsis*. Several of these structural and physiological changes modulated by phyB produce functional consequences on photosynthetic rate and transpiration, modifying water use efficiency (Boccalandro et al. 2009). In addition to these observations, mostly obtained with *Arabidopsis* plants grown in controlled conditions, phyB overexpression in potato increases stomatal conductance, photosynthetic and transpiration rate per unit leaf area under field conditions (Boccalandro et al. 2003). Taken together, this evidence clearly shows that phyB, operating at different levels, acts as a key modulator of water and carbon economies. In contrast, the involvement of phyA on water or carbon economy is largely ignored. It has been reported that *Arabidopsis* phyA-103 mutants display a reduced transpiration rate under red light but not under blue light (Eckert and Kaldenhoff 2000), but its response to sunlight was not reported.

In this study, we observed a significant phenotype of the *fri1-1* mutant when grown in the field. Under conditions of high evaporative demand, leaf wilting was conspicuous (Fig. 1) and at a stage when there were no differences in plant biomass or leaf area, it was observed that transpiration, leaf conductance and leaf water potential were significantly lower in *fri1-1* than in WT plants (Figs 3 and 4). These observations suggest that a reduced water supply to leaves produced an earlier decrease in water potential with consequences for stomatal aperture and hence transpiration (Fig. 3D). The reduced leaf water supply does not appear to be related either to the size or the conductivity of the root system (Fig. 2), while it is consistent with reduced stem water conductivity, associated with a smaller area of xylem elements (Fig. 6).

The structure of the plant hydraulic system can potentially limit water flow through the plant with consequences for the water and carbon economies, so it is not surprising that differences in either stem conductance or leaf-specific conductance have been found to be associated with habitats showing divergent irradiance conditions (like sites under forest canopies or in gaps).

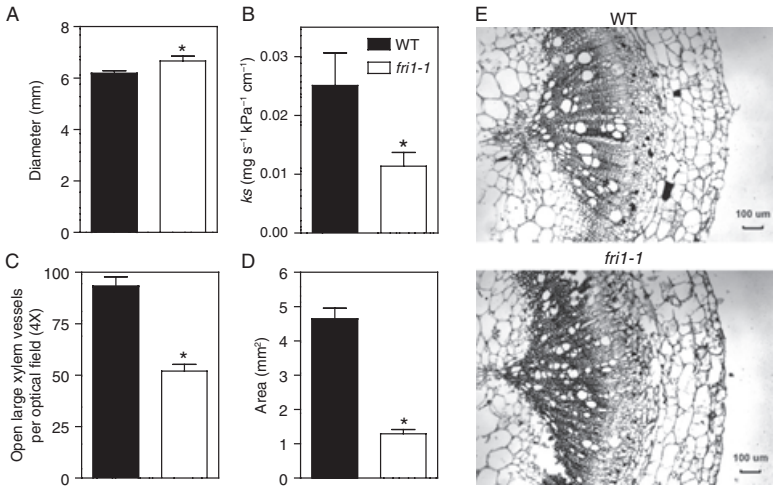

Fig. 6. PhyA affects water transport through the stem. Differences between genotypes of mature plants with eight to nine fully expanded leaves in: stem diameter (A), stem-specific hydraulic conductivity (k_s) (B), number of large xylem vessels observed in hand-cut stem cross sections (C) and estimated area of open xylem vessels (D). (E) Microtome-stem cross section micrographs of WT and mutant mature plants with four to five fully expanded leaves. Bars in (A) and (B) are means ($n \geq 10$), narrow vertical extensions represent one standard error. Values are from one representative experiment. Asterisks indicate significant ($P < 0.05$) differences between means.

That is the case of *Piper trigonum*, a species that shows a greater leaf-specific conductance (K_{la}) when exposed to higher irradiances, and which in addition to being abundant in understory conditions, can invade open areas when water is in adequate supply (Engelbrecht et al. 2000). A similar response is found in *Rhododendron maximum* plants, that when growing in gaps, have a higher proportion of large diameter xylem vessels compared with plants growing under a canopy (Lipp and Nielsen 1997). Also, it has been reported that olive trees have larger xylem cross-sectional area when grown under high light irradiance than in low light conditions (Raimondo et al. 2009). Here, we found that phyA is a key factor promoting xylem vessel diameter under full sunlight, this morphological adjustment being of functional importance to enhanced water conductance to leaves subjected to high evaporative demand.

The positive relationship between phyA and stem conductance that we are describing as well as that reported in cucumber plants with phyB (Casal et al. 1994) suggests that phytochromes might have a role in the adjustment of the plant water economy to a changing environmental scenario. This aspect as well as the mechanisms underlying the modification of the stem water transport capacity clearly deserve more attention and are currently being investigated.

Acknowledgements – We thank Esteban Fernandez for assistance in hydraulic conductivity measurements and Dr Roberto Fernandez-Alduncín for helpful discussions of the results and critical reading of the manuscript. This work was funded by ANPCYT and UBACYT grants to H. E. B. and R. A. S. This paper is dedicated to the memory of our dear friend and colleague Dr Hernán Boccalandro who tragically departed this world on December 10, 2011.

Supporting Information

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

Fig. S1. Greenhouse conditions. PPFD, air temperature, relative humidity (RH) and vapor pressure deficit (VPD) during the course of one representative experiment conducted in Mendoza, Argentina.

Fig. S2. PhyA does not affect stomatal density of tomato leaves. Stomatal density on abaxial (A) and adaxial (B) sides of the second and third fully expanded leaves of mature plants measured at 600×. Bars are means ($n \geq 10$), narrow vertical extensions represent one standard error; ns, not significant.

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Edited by R. Scheibe