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BRIEF COMMUNICATION

Phytochromes influence stomatal conductance plasticity in *Arabidopsis thaliana*

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Abstract The ability to sense and respond effectively to drought stress can be important for plant fitness. Here, phytochrome loss-of-function mutants were grown in dry and moist conditions to examine the role of three photoreceptors (phyA, phyB, and phyE) in stomatal conductance (g_{ST}) and abscisic acid (ABA) concentration. Overall, drought treatment plants had lower g_{ST} than moist treatment plants. However, the wild-type Landsberg erecta line had a less pronounced conductance response to drought treatment than the phytochrome mutants, suggesting a role for phytochrome in suppressing drought tolerance. Phytochrome gene effects were potentially additive for g_{ST}; however, *PHYB* and *PHYE* effects were nonadditive for ABA concentration.

Keywords Phytochrome · Drought stress · Stomatal conductance · Abscisic acid

Introduction

For weedy annuals, the ability to sense and respond to changes in water availability can be deeply tied to plant fitness (Heschel et al. 2002; Heschel and Riginos 2005; Caruso et al. 2006). *Arabidopsis thaliana* is an annual plant that persists across a wide range of moisture climates in North America and Eurasia (Mitchell-Olds and Schmitt 2006). Soil moisture gradients within *A. thaliana* populations themselves may be quite coarse, thus persistence of populations may depend upon plastic responses to a range of stressors, most notably light and moisture availability (Mitchell-Olds and Schmitt 2006). Whether such responses are adaptive can depend upon accurate sensing of environmental conditions (Schmitt et al. 2003).

Plants possess environmental sensors that affect physiological characteristics. For example, the photosensor phytochrome "senses" seasons by responding to changes in light quality (red and far-red), light quantity (e.g. low fluence responses), and temperature (Franklin et al. 2003; Heschel et al. 2007). In *A. thaliana* the genes *PHYA*, *PHYB*, *PHYC*, *PHYD*, and *PHYE* encode five distinct phytochromes (Mathews and Sharrock 1997) that affect traits associated with seasonal environments such as flowering time and germination (Smith 2000; Franklin et al. 2003; Donohue et al. 2007). These traits are also associated with drought response, indicating that phytochrome may have a role in "sensing" moisture conditions; *PHYE* in particular has been associated with local adaptation to dry environments like alpine habitats (Ikeda et al. 2009). Moreover, phyB seems to be involved in the regulation of abscisic acid (ABA) metabolism, such that the expression of the ABA biosynthesis gene *AtNCED6* is *PHYB*-mediated (Seo et al. 2006). This phyB effect on ABA indirectly links phytochrome to transpiration (Kriedemann et al. 1972). phyB seems to have a direct role in gas exchange rates as well (Boccalandro et al. 2003; Sokolskaya et al. 2003), and this regulatory role may be linked to circadian rhythm phase control (Salome et al. 2002). Therefore, phyB and phyE are reasonable candidates for environmental sensors that might influence drought response.

In this study, we used phytochrome loss-of-function mutants to examine the effect of specific phytochome genes on drought response traits. We hypothesized that phytochrome loss-of-function mutants would show increased stomatal conductance and decreased ABA concentration in drought conditions over wild-type (WT).

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Materials and methods

Study system

The Landsberg erecta (Ler WT) ecotype of *A. thaliana* was used in this experiment. phyA, phyB, and phyE loss-of-function mutants (see Figs. 1, 2 for lists of lines) were chosen for this study based upon primary literature and preliminary data indicating that these three phytochromes might influence drought response physiology (M.S. Heschel, unpublished data). All the mutants in this study were isolated in the Ler background. TAIR provided Ler WT and the *hy2-1, phyA$_1$, phyB$_1$, phyB$_5$*, and *phyA$_1$/phyB$_5$* mutants. The G.C. Whitelam laboratory supplied the *phyE$_1$, phyA$_2$/phyE$_1$,* and *phyB$_1$/phyE$_1$* mutants. The R.A. Sharrock laboratory provided the *phyA$_1$/phyB$_1$* mutant. The *phyA$_1$* and *phyA$_2$* loss-of-function mutants contained the *PHYA-201* and *PHYA-202* alleles, respectively.

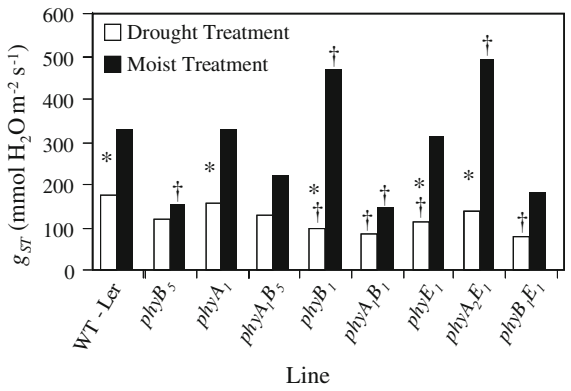

Fig. 1 Stomatal conductance (g_{ST}) in drought versus moist conditions for phytochrome mutant and wild-type (*WT*) lines. * indicates $P < 0.05$ for contrasts between drought and moist conditions for each line. † indicates $P < 0.05$ for contrasts between mutant lines and WT for drought or moist conditions. Data are taken from the 2006 conductance experiment (see "Materials and methods")

Stomatal conductance experiment

Individuals were grown in four moist and four drought treatment trays, divided between two Percival growth chambers in 2006 (Percival Scientific, Perry, IA, USA). The Percival chambers used incandescent and fluorescent lamps that generated a photon flux density of about 200 µmol photons m^{-2} s^{-1}. Light intensity was measured with a PAR quantum sensor (LI-COR Biosciences, Lincoln, NE, USA). There were 6 replicates for each line per treatment and the replicates were evenly distributed between chambers. Individual trays were rotated within chambers weekly to reduce microenvironmental effects. Each tray contained randomly distributed replicates for each line plus three randomly distributed "dummy" cells; dummy cells were used to measure soil moisture differences between drought and moist treatment trays with a TDR moisture meter (Campbell Scientific, model CS620, Logan, UT, USA). Tray cells contained Canadian Growing Mix 2 (Fafard Inc., Agawan, MA, USA) and were 8 cm × 5.7 cm × 5.5 cm (L × W × H) in size.

In June 2006, four seeds were planted per cell using a micropipette with 5 µl distilled water for each seed; seeds were dark and wet-stratified at 4°C for 5 days to break dormancy and then transferred to chambers maintained at 14 h light, 19°C, and 36–40% relative humidity (RH). After 5 days in these light conditions, 10 ml of 100 ppm 24:8:16 Nitrogen: Phosphorous: Potassium (N:P:K; Scotts-Sierra Hort. Products, Marysville, OH, USA) were given to each cell; after 10 days in these light conditions, 10 ml of 200 ppm N:P:K were given to each cell. The fertilizer concentration was increased after 10 days to ensure that rapidly growing plants had sufficient nutrients. After thinning and transplanting, each cell contained one seedling. Moist treatment trays were bottom-watered every 2 days to maintain constant soil moisture availability. Drought treatment cells were individually top-watered with 25 ml

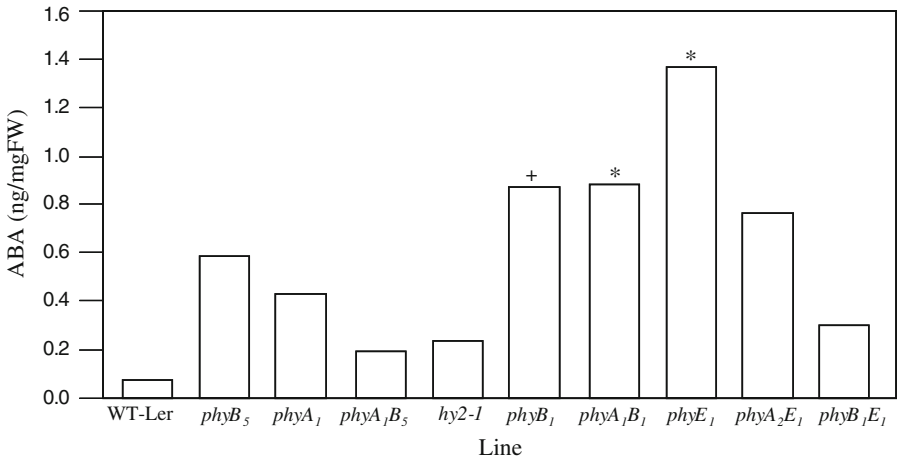

Fig. 2 Mean abscisic acid (*ABA*) foliar content (ng per mg fresh weight (*FW*)) for drought stressed mutant and wild-type (*WT*) lines. * indicates $P < 0.05$ and + indicates $P < 0.10$ for *t*-tests between mutant lines and WT in drought conditions. Data are taken from the 2007 ABA experiment (see "Materials and methods")

water when dryness was detected in the dummy cells. Moist cell soil conditions were maintained at 38–40% volumetric water content (VWC) and drought cells were dried to 4–6% VWC. After 4 weeks of growth, stomatal conductance was measured inside each chamber on the most-recent-fully-expanded rosette leaf of each replicate; it was at 4 weeks that plants had a significant rosette across both treatments. Stomatal conductance measurements (g_{ST} = mmol H_2O m^{-2} s^{-1}) were taken with a steady-state leaf porometer (Decagon Devices, model SC-1, Pullman, WA, USA) on abaxial leaf surfaces. Transpiration rates were assessed between 1000 and 1500 h. The degree of plasticity was defined as the difference in stomatal conductance between drought and moist conditions.

ABA experiment

The 2007 experimental design was similar to the design of the 2006 conductance experiment, with three exceptions. First, dummy cells were replaced with *hy2-1* lines, which contain a mutation in a chromophore biosynthetic gene, impairing phytochrome activity. Second, line replicates for ABA quantification were grown under drought conditions in one chamber. Third, bulbs were changed between experiments, resulting in slightly higher chamber temperatures in 2007 (21°C in 2007 vs. 19°C in 2006). Planting, cold imbibition, thinning, and maintenance procedures were identical to those performed in the 2006 conductance experiment. In fact, porometer readings on the 2007 plants were nearly identical to those taken in 2006, indicating that plants were behaving similarly across experiments (note: all reported porometer data are from the 2006 conductance experiment). After 4 weeks of growth in 2007, the lines were harvested for ABA extraction and quantification.

ABA extraction and quantification

From the 2007 ABA experiment, four samples per line grown in drought conditions within a single chamber (about 17 mg leaf tissue per sample) were lyophilized for 23.5 h. Dried tissue was ground with a Polytron tissue grinder in an ABA extraction buffer containing methanol, butylated hydroxytoluene, and citric acid monohydrate (Walker-Simmons 1987). The lyophilized material was incubated in the extraction buffer for 12 h at 4°C and then spun at 2,000 g for 10 min; dried supernatant was resuspended in TBS (with $MgCl_2$) (Walker-Simmons 1987). ELISA was used to quantify ABA in leaf extracts. Serial dilutions of ABA (± mixed isomers, Sigma–Aldrich, St. Louis, MO, USA) were used to generate standard curves with a Fluostar Optima Plate Reader set to absorbance at 405 nm (BMG Labtech, Offenburg Germany). ELISA reactions were performed in low light (to prevent ABA degradation) in microtiter trays. ABA tracer and substrate antigen/antibody reactions (Agdia Inc., Elkhart, IN, USA) were quantified with the Optima plate reader. ABA foliar content was determined as ng ABA per mg fresh weight or ng/mg FW.

Data analyses

All statistical analyses were performed with JMP (version 4.0.4, SAS Institute, Cary, NC, USA). The degree to which drought treatment, line, and chamber identity predicted stomatal conductance was examined with a three-way ANOVA. For conductance data, contrasts (*t*-tests) were conducted within the context of the treatment by line interaction term to determine where specific treatment and line differences lie. Residuals from the ANOVA model were examined for variance heterogeneity and normality. The results of this ANOVA model are not presented but the following results should be noted: (1) chamber was not a significant predictor of conductance ($F = 0.13$, $df = 1$, $P = 0.72$); (2) neither chamber x line ($F = 0.95$, $df = 8$, $P = 0.48$) nor chamber x treatment ($F = 1.10$, $df = 1$, $P = 0.29$) significantly predicted conductance values; (3) both treatment ($F = 47.54$, $df = 1$, $P < 0.0001$) and line x treatment ($F = 2.59$, $df = 8$, $P = 0.02$) were significant predictors of conductance. Student's *t*-tests were conducted on the [ABA] data to determine whether differences between mutant lines and WT were significant. Because ABA was measured only in drought conditions, comparisons between moisture treatments were not possible for [ABA]. To test whether *PHYA*, *PHYB*, and *PHYE* effects on traits were potentially additive, wild-type and single/double mutants were scored for functionality using dummy variables, and ANOVA was used to test for significant interactions between gene effects. Dummy variables were scored as binary predictors in the ANOVA model, such that for a *PHYB* and *PHYE* model a *phyE* mutant would be associated with a "1" for *PHYB* functionality and a "0" for *PHYE* non-functionality. A significant interaction between *PHYB* and *PHYE* predictors, for example, was indicative of nonadditive contributions of phytochromes to a given trait; nonadditive effects might include gene interactions such as pleiotropy and epistasis. Additive effects were defined as independent effects of phytochrome genes on a particular trait.

Results and discussion

The degree of stomatal conductance (g_{ST}) plasticity was greater for mutant lines than WT. Therefore, Ler WT was not the exemplar of a classic drought tolerance strategy of greatly decreased stomatal conductance in drought

conditions (cf. Heschel and Riginos 2005). For example, the *phyB*$_1$ mutant exhibited the greatest degree of g_{ST} plasticity (Fig. 1). In contrast, the WT Ler line had one of the smallest g_{ST} responses to drought of any line, a trait uncharacteristic of drought tolerant taxa such as *A. thaliana* (Fig. 1). In low-moisture settings, the conservation of water through reduced g_{ST} can be adaptive, despite its correlation with reduced carbon assimilation (Maherali et al. 2008). However, smaller degrees of conductance plasticity are not maladaptive per se, but possibly representative of a different drought response strategy—escape via accelerated life history rather than tolerance (McKay et al. 2003; Heschel and Riginos 2005). Meyre et al. (2001) found the Ler WT to employ such a strategy based largely on morphological characteristics, and our physiological data support that interpretation.

PHYA, *PHYB*, and *PHYE* seem to influence drought response plasticity for stomatal conductance (Fig. 1), and their effects might be, at least in some cases, additive (Table 1). Drought-stressed *phyB, phyA, phyE,* and *phyA/phyE* mutants demonstrated significant reductions in g_{ST} relative to moist conditions (Fig. 1). The degree of conductance plasticity was greatest for the *phyB* and *phyA/phyE* mutants, indicating that phyA and phyE together, as well as phyB singly, might influence water relations. Moreover, these data suggest that in WT, functional phyB, phyA, and phyE might collectively have a role in limiting conductance plasticity. It should be noted, however, that phyB behavior might depend on the allele examined; *phyB*$_5$ exhibited lower g_{ST} than *phyB*$_1$ in moist conditions (contrast, $F = 21.69$, $df = 1$, $P < 0.0001$) and a nonsignificant drought response (Fig. 1). Variation in the effects of *PHYB* alleles has been detected in other traits such as germination (Heschel et al. 2008). However, allelic effects of *PHYA* have not been detected in previous studies (Heschel et al. 2008).

Table 1 Tests of whether overall phytochrome gene contributions to stomatal conductance (g_{ST} = mmol H_2O m^{-2} s^{-1}) and abscisic acid concentration [ABA = ng/mg FW] were additive

	g_{ST}	[ABA]
Drought treatment		
PHYA	0.414	0.189
PHYE	3.915+	5.188*
PHYA × *PHYE*	2.902	1.874
PHYB	9.148*	0.0578
PHYE	7.178*	0.172
PHYB × *PHYE*	3.876+	6.739*
Moist treatment		
PHYA	0.383	NA
PHYE	0.266	NA
PHYA × *PHYE*	0.334	NA
PHYB	0.973	NA
PHYE	0.624	NA
PHYB × *PHYE*	0.546	NA

* $P < 0.05$, + $P < 0.10$

For each moisture treatment, the upper portion tests for interactions between *PHYA* and *PHYE*, and the lower portion tests for interactions between *PHYB* and *PHYE*. *F*-statistics and *P* values are presented

The relationship between *PHYA* and *PHYE* might be additive, based on ANOVA as well as comparative effects of the monogenic versus double phytochrome gene mutants (Fig. 1; Table 1). In dry conditions, ANOVA indicated that *PHYA* and *PHYE* gene effects might be additive for stomatal conductance (Table 1). An examination of mutant responses to drought (Fig. 1) supports the ANOVA results. Comparatively, monogenic *phyA* and *phyE* mutants both had degrees of stomatal plasticity similar to WT, while the degree of stomatal plasticity was greater than WT for the *phyA/phyE* double mutant (Fig. 1).

ELISA data indicated that phyA, phyB, and phyE might influence ABA levels, providing a potential mechanism for stomatal regulation. Loss of phytochrome function for *phyB*, *phyE*, and *phyA/phyB* mutants resulted in increased foliar ABA concentration in drought conditions relative to WT (Fig. 2). These ABA data suggest that functional phyA, phyB, and phyE may play a role in inhibiting a drought tolerance strategy via decreased ABA levels. The observed phytochrome effect on ABA might be mediated more strongly by the apoprotein because the chromophore mutant (*hy2*-1) did not differ in foliar ABA content from WT (Fig. 2). Also, *PHYB* and *PHYE* effects on [ABA] seem to be nonadditive under drought conditions (Table 1).

Overall, these data indicate that phytochrome may influence plant responses to drought stress, with three phytochrome genes (*PHYA*, *PHYB*, and *PHYE*) having a role in ABA levels as well as stomatal conductance plasticity. These phytochrome gene effects might be both additive (g_{ST}) and nonadditive (ABA), hinting at the complexity surrounding the genetic mechanisms behind plastic responses to drought stress. These potentially additive and nonadditive or pleiotropic effects may allow a plant to fine-tune its drought response. Evolutionarily, populations might differentiate toward particular allelic forms of these phytochromes in response to particular drought stress regimes (e.g. *PHYB* allelic effects on stomatal response); heterogeneous drought conditions might maintain a diversity of allelic forms within a given population in the wild. What seems clear is that the members of the phytochrome gene family in *A. thaliana* are not as redundant as once thought (Pickett and Meeks-Wagner 1995); just as certain genes promote particular germination responses (see Heschel et al. 2008), certain phytochrome genes might have a role in suppressing particular drought responses.

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