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Neighbor signals perceived by phytochrome B increase thermotolerance in Arabidopsis

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.13575

Abstract

Due to the preeminence of reductionist approaches, understanding of plant responses to combined stresses is limited. We speculated that light-quality signals of neighboring vegetation might increase susceptibility to heat shocks because shade reduces tissue temperature and hence the likeness of heat shocks. In contrast, plants of *Arabidopsis thaliana* grown under low red/far-red ratios typical of shade were less damaged by heat stress than plants grown under simulated sunlight. Neighbor signals reduce the activity of phytochrome B (phyB), increasing the abundance of PHYTOCHROME INTERACTING FACTORs (PIFs). The *phyB* mutant showed high tolerance to heat stress even under simulated sunlight and a *pif* multiple mutant showed low tolerance under simulated shade. phyB and red/far-red ratio had no effects on seedlings acclimated with non-stressful warm temperatures before the heat shock. The *phyB* mutant showed reduced expression of several fatty acid desaturase (*FAD*) genes, and less proportion of fully unsaturated fatty acids and electrolyte leakage of membranes exposed to heat shocks. Red-light-activated phyB also reduced thermotolerance of dark-grown seedlings but not via changes in *FADs* expression and membrane stability. We propose that the reduced photosynthetic capacity linked to thermotolerant membranes would be less costly under shade, where the light input limits photosynthesis.

Non acclimated *Arabidopsis thaliana* plants grown under low red/far-red ratios typical of neighboring vegetation are more tolerant to heat stress than plants grown under simulated sunlight. Thermotolerance under low red / far-red ratios is mediated by phyB. Lowering phyB activity reduced the levels of fatty acid desaturase transcripts, the levels of unsaturated fatty acids and increase thermotolerance. In young, etiolated seedlings, thermotolerance is also increased in phyB mutants of dark-grown seedlings but by different mechanisms. The reduced photosynthetic capacity linked to thermotolerant membranes would be less costly under shade environments.

Keywords: Arabidopsis, heat shock, light, membrane stability, phyB.

1 | INTRODUCTION

Although most studies dealing with plant responses to environmental threats consider one source of stress at the time, plants can often be exposed simultaneously to multiple stresses. The impact of different stresses is not necessarily additive and in some cases one stress increases the impact of the other. For example, the damage caused by the combination of drought and salinity or drought and heat results in growth reductions that are more severe than those caused by the sum of the effects of each stress in isolation (Rizhsky, Liang & Mittler, 2002; Ahmed et al., 2013). Similarly, heat stress facilitates pathogen spread causing susceptibility to diseases (Luck et al., 2011; Nicol, Turner, Coyne, Nijs & Hockland, 2011). Conversely, in other cases one stress reduces the impact of the other. For instance, wounding can increase salt tolerance (Capiati, País & Téllez-Iñón, 2006) and ultraviolet radiation, although potentially harmful, can protect plants against herbivorous insects (Rousseaux et al., 2004; Caputo, Rutitzky & Ballaré, 2006). Therefore, plants are likely to have developed physiological and molecular mechanisms of protection against specific combinations of stresses, but these processes can remain hidden in studies involving single stress factors (Pandey, Ramegowda & Senthil-Kumar, 2015).

The shade imposed by neighboring vegetation reduces the photosynthetically active radiation available for the plants within the canopy, and can eventually compromise their survival. Plants respond to the threat associated to neighbors by inducing shade-avoidance responses (such as enhanced stem and petiole elongation), and/or acclimation responses that increase the chances of survival under limiting light (Casal, 2013; Gommers, Visser, Onge, Voesenek & Pierik, 2013). In Arabidopsis, light/shade signals are perceived mainly by phytochrome B (phyB) and cryptochrome 1 (cry1). Phytochromes are a family of five members in Arabidopsis (phyA-phyE). They have two inter-convertible forms: red light transforms the inactive Pr form into the active Pfr form, while far-red light converts Pfr back to the Pr form (Burgie & Vierstra, 2014). Therefore, the activity of phyB increases with the red / far-red ratio of the light, which is high (approx 1.1) in open places and becomes gradually depleted with the proximity of neighboring vegetation reflecting far-red light and under the canopy, which also transmits far-red more efficiently than red light (Casal, 2013; Gommers et al., 2013). There are two canonical cryptochromes in Arabidopsis (cry1-cry2), which increase their activity in response to blue light (Yu, Liu, Klejnot & Lin, 2010). phyB reduces the activity of the bHLH transcription factors PHYTOCHROME INTERACTING FACTOR 3, (PIF3), PIF4, PIF5 and PIF7 by lowering their nuclear abundance and/or DNA

binding capacity (Lorrain, Allen, Duek, Whitelam & Fankhauser, 2008; Park et al., 2012). cry1 also reduces the abundance of PIF4 and PIF5 (de Wit et al., 2016; Pedmale et al., 2016). Therefore, the weaker activity of these photo-sensory receptors under vegetation shade, increases the activity of PIF3, PIF4, PIF5 and PIF7, which promote stem growth and other shade-avoidance responses (Lorrain et al., 2008; Hornitschek et al., 2012; Li et al., 2012; Leivar & Monte, 2014; Huang et al.,2018). The lower activities of phyB and cry1 under shade also lead to stronger nuclear accumulation of CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), which enhances the degradation of LONG HYPOCOTYL IN FAR RED LIGHT 1 (HFR1) (Pacín, Legris & Casal, 2013; Pacín, Semmoloni, Legris, Finlayson & Casal, 2016) a negative regulator of the activity of PIFs.

High temperatures are another source of stress as they inhibit photosynthesis, damage cell membranes and cause cell death (Liu & Huang, 2000; Djanaguiraman, Boyle, Welti, Jagadish & Prasad, 2018). The expected increases in temperature over the following years predict a more severe and frequent incidence of heat stress (Hatfield & Prueger, 2015). Plants posses an inherent basal thermotolerance and also have the ability to acquire thermotolerance by the exposure to a gradual sub-lethal high temperature (heat acclimation) (Hong, Lee & Vierling, 2003). Plants cope with high temperatures by altering their physiological, morphological, biochemical and molecular status during acclimation (Bita & Gerats, 2013). For instance, plants respond to non-stressing high temperatures by increasing stem and petiole elongation and leaf hyponasty; i.e. changes that enhance leaf cooling capacity reducing the probability of stress by further temperature rises (Crawford, McLachlan, Hetherington & Franklin, 2012). Plant cell membranes are direct targets of heat stress, which increase leakage of electrolytes out of the cell (Wahid, Gelani, Ashraf & Foolad, 2007). The degree of unsaturated fatty acids in the membrane is inversely correlated with growth temperatures. A reduced proportion of polyunsaturated fatty acids in the membrane favors seedling growth at elevated temperatures (Falcone, Ogas & Somerville, 2004), but reduces seedling growth in the absence of heat stress (Routaboul, Fischer & Browse, 2000). Heat stress also promotes the immediate expression of heat-shock proteins that act to prevent and restore cell damage and preserve homeostasis (Yángüez, Castro-Sanz, Fernández-Bautista, Oliveros & Castellano, 2013; Wang et al., 2017).

The aim of this study was to investigate whether the low red / far-red ratio signals of neighboring vegetation perceived by phyB affect thermotolerance. There are several reasons that justify the proposed analysis. First, there is ecological convergence of the light and temperature cues. For instance, canopy shade reduces the irradiance and temperature levels

experienced by plants (Legris, Nieto, Sellaro, Prat & Casal, 2017). Second, plant population responses to global warming can be modified by light, as in forest understory, the largest changes in thermophilization of species (the replacement of cold-adapted understory species with warm-adapted species) occurs more intensively when higher light and temperature levels coincide (De Frenne et al., 2015). Third, there is molecular convergence in the plant perception and signaling of light and temperature cues. Noteworthy, phyB functions not only as a light receptor but also as a temperature sensor, which is inactivated by far-red light and also by warm temperatures (Jung et al., 2016; Legris et al., 2016). PIF4 (Koini et al., 2009; Franklin et al., 2011; Lau et al., 2018), HFR1 (Foreman et al., 2011) and COP1 (Kim et al., 2017; Park, Lee, Ha, Kim & Park, 2017), which are described above as components of the signaling network involved in the responses to the degree of shade, play a role in thermomorphogenesis (Casal & Balasubramanian, 2019). Fourth, heat shocks modulate light signaling in etiolated Arabidopsis seedlings (Karayekov, Sellaro, Legris, Yanovsky & Casal, 2013), suggesting that the reciprocal control of thermotolerance by light signals could also occur. Fifth, a recent work has reported enhanced thermotolerance of a *phyB* mutant (Song, Liu, Hu & Wu, 2017). We found that neighbor signals perceived by phyB increase thermotolerance in Arabidopsis at least in part by adjusting membrane function.

2 | MATERIALS AND METHODS

2.1 | Plant Material

The wild-type accessions of *Arabidopsis thaliana* used in this study were Landsberg *erecta* (L*er*) and Columbia (Col), depending on the background of the mutant. The *phyB* (*phyB-5)*(Reed, Nagpal, Poole, Furuya & Chory, 1993) and *cry1 cry2* (*hy4-2.23n, fha-1*) (Casal & Mazzella, 1998) mutants are in the L*er* background. The *phyA-211* (Reed et al., 1993), *phyB-9*,(Reed, Nagatani, Elich, Fagan & Chory, 1994), *hy5-211* (Shin, Park & Choi, 2007), *cry1-1*, *cry2-1*(Guo, Yang, Mockler & Lin, 1998), *pif1-1*, *pif3-3* (Monte et al., 2004), *pif4-101*, *pif5-3* (Lorrain et al., 2008), *pif1 pif3*, *pif4 pif5*, *pif3 pif4*, *pif1 pif3 pif5*, *pif3 pif4 pif5*, and *pif1 pif3 pif4 pif5* (Leivar et al., 2008); *hfr-101* (Duek, Elmer, Van Oosten & Fankhauser, 2004), *cop1-4*, *cop1-6* (McNellis, 1994), *spa1-1*, *spa2-1*, *spa3-1*,*spa4-1*, *spa1 spa2 spa3 and spa1 spa2 spa4* (Laubinger, Fittinghoff & Hoecker, 2004) mutants are in the Col background. Seeds were surface sterilized (4 h of exposure to the fumes produced by 1.25% HCl/NaClO) and

sown on 0,8% w/v agar plates (experiments with etiolated seedlings) or 0,8% w/v agar plates containing one-half-strength Murashige and Skoog basal medium pH 5.7 (MS) (experiments with light-grown seedlings). After 3 d at 4 ˚C in darkness, the seeds were exposed to a red-light pulse for 2 h to promote germination.

2.2 | Light conditions

White light (WL) was provided by fluorescent lamps (Philips, 80 µmol m^{-2} s^{-1}) and red light (12 µmol m^{-2} s^{-1}) was provided by fluorescent lamps (Philips) combined with red, orange and yellow filters (LEE #106, #105 and #101 respectively). For the experiments with light-grown plants the seedlings were grown under continuous light to minimize the effects of circadian rhythms. For the WL treatments supplemented with far-red light (WL+FR), continuous WL was given from above as described and far-red light (30 µmol m^{-2} s^{-1}) was provided continuously from below by incandescent lamps filtered with a red acetate filter and six blue acrylic filters (Paolini 2031, La Casa del Acetato, Buenos Aires, Argentina) and a water filter.

2.3 | Temperature treatments

Plants were grown at 22 ˚C. For heat-shock treatments the boxes containing the seedlings were placed during 45 min in a shaker or 90 min in water bath, both at 45 ˚C in darkness. The protocol for heat acclimation of light-grown seedlings was as described in Silva-Correia , Freitas, Tavares, Lino-Neto & Azevedo (2014). Briefly, 9 day-old WL or WL+FR grown seedlings were exposed during 60 min to 37 ˚C (acclimated) or 22 ˚C (control), returned at 22 ˚C during 120 min for recovering, and then exposed to a heat shock during 45 min to 45 ˚C. For heat acclimation of etiolated seedlings, the boxes containing seedlings were placed during 90 min in a shaker at 35 ˚C in darkness.

2.4 | Scoring of damage

Damage and survival were respectively recorded 5 and 7 d after the heat shock. At the rosette stage, a plant was considered damaged when it showed at least one cotyledon completely bleached. At the seedling stage survival rates were assessed by recording the proportion of seedlings that generated the first pair of leaves after the heat shock.

2.5 | Electrolyte leakage

Approximately 200 mg of the seedlings were harvested after the heat shock challenge, rinsed twice with demineralized water and subsequently floated on 10 ml of demineralized water at room temperature. Electrolyte leakage in the solution was measured 24 h later by using a conductimeter (Corning TDS-60). Data are presented relative to total conductivity obtained after boiling the samples at 100º C for 15 min (Wang et al., 2013).

2.6 |Quantitative real time PCR (qPCR)

Total RNA was extracted using Trizol Reagent (Life Technologies Inc., USA) and treated with RQ1 RNase-free DNase I (Promega, USA). 3 µg of total RNA were reverse-transcribed in a 25 µl reaction using MMLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions, using oligo (dT) primers. cDNA were diluted 1:40 before qPCR. qPCR reactions were performed in a DNA Engine Opticon 2 System (MJ Research, USA) using the 5x HOT FIREPol EvaGreen® qPCR Mix Plus (NO ROX) kit (Solis BioDyne, Estonia). The primers for *FAD2* (AT3G12120) were 5'-CCTTCCTCCTCGTCCCTTAC-3' and 5'-CTCTTTCGAGGGATCCAGTG-3' and for *FAD6* (AT4G30950) were 5'-CCGTGGTATCTGCTACCGTT-3' and 5'- TAGGAAGGCGAGAGTACCCA-3' (Shen et al., 2010); primers for *FAD5* (AT3G15850) were 5'-AACAACTGGTGGGTAGCAGC-3' and 5'-ACCGATGGCTTGAAGGAAC-3' (Luo et al., 2010); primers for *FAD7* (AT3G11170) were 5'- TGTTTGGCCTCTCTATTGGC-3' and 5'-AAGGGTATGCAAGCATCACG -3'; primers for *FAD8* (AT5G05580) were 5'-GAGGCTGAACAGTGTGGCT-3' and 5'-

CTTGTAGATGCTTTCAGGCAA-3'. *ACTIN 8* (AT1G49240) was used as normalization control (Mazzella et al., 2005). The conditions for PCR were optimized with respect to primer concentrations, primer annealing temperatures and duration of steps. Cycling conditions were 95°C for 15 min followed by 38 cycles of 15 s at 94°C, 12 s at 60°C, 12 s at 72°C. PCR for each gene fragment was performed alongside standard dilution curves of cDNA pool. All gene fragments were amplified in duplicate from the same RNA preparation, and the mean value was considered for each replicate. Data are means of three 3 independent experiments.

2.7 | Lipid Extraction and Fatty Acid Analysis

For each sample, total lipids were extracted with methanol/chloroform mix (2/1 v/v) as described (Bligh & Dyer, 1959). Lipid extracts were dried, weighted, suspended in 2 mL of a fresh solution of 10% KOH in ethanol and saponified for 60 min at 80 °C using stoppered glass tubes. Two ml of hexane were added and fatty acids were extracted by shaking. The upper organic phase (non-saponified) was discarded. The aqueous layer was acidified with 1.5 ml of concentrated HCl and fatty acids were extracted twice with 1.5 ml hexane. Extracts containing total free fatty acids were dried under a nitrogen stream, dissolved in 1.5 ml BF3 (10 % in methanol) and 1.5 ml benzene, and esterified by heating to 100 °C and shaking for 1 h. Fatty acid methyl ester (FAME) were extracted twice with hexane and washed with distilled water. After washing, the organic phase was evaporated under a nitrogen stream, re-dissolved in hexane, and analysed by GLC. One µl of FAME solution was injected into an Omegawax X250 (Supelco Inc., Bellefonte, PA, USA) capillary column (30 m × 0.25 mm; 0.25-mm film) in a Hewlett Packard HP-6890 (Santa Clara, CA, USA) chromatograph equipped with a flame ionization detector. The column temperature was programmed for a linear increase of 3 °C min^{-1} from 175 to 230 °C. The cromatographic peaks of FAME were identified by comparing their retention times with standards under the same conditions.

3 | RESULTS

3.1 | Neighbor signals increase the tolerance to a heat shock

In order to study whether neighbor signals affect thermotolerance, Arabidopsis seedlings grown for 9 d under either WL or WL+FR (high, or low red / far-red ratios, respectively) were exposed to a heat shock (45 ºC for 45 min) and plant damage was recorded after 5 d of recovery at 22 ºC under WL or WL+FR according to the previous growth condition (see protocol in Figure S1a). Wild-type (WT) seedlings either of the L*er* or Col background showed less frequent damage (enhanced thermotolerance) when grown under WL+FR simulating the presence of neighboring vegetation than under WL (Figure 1a,b,d,e).

3.2 phyB activity reduces the tolerance to a heat shock

In principle, the action of supplementary far-red light (i.e., WL+FR compared to WL) could be mediated by different perception and signaling steps as low red / far-red ratios reduce the proportion of active phyB but increases phyA activity (Franklin, 2003; Rausenberger et al., 2011; Trupkin, Legris, Buchovsky, Tolava Rivero & Casal, 2014) and the relative excitation of photosystems I and II (Anderson, Chow & Park, 1995). Compared to the WT, the *phyA* mutant showed no difference under WL and an enhanced response to WL+FR (Figure 1a, d). The *phyA phyB* double mutant and the *phyA* mutant showed similar thermotolerance under WL+FR and this high thermotolerance was already observed in seedlings of *phyA phyB* grown under WL (Figure 1a,d). These results indicate that in the WT, WL+FR increases thermotolerance by lowering phyB activity. Furthermore, the enhanced phyA activity caused by supplementary far-red light only slightly counteracted the promotion of thermotolerance caused by lowering phyB activity. Since the response of *phyA phyB* to WL+FR compared to WL was not significant, we obtained no evidence for a role of changes in photosystem balance (Figure 1a).

If the above conclusion is correct, the *phyB* mutation should be enough by itself to increase thermotolerance under WL (i.e. in the absence of neighbor signals). This expectation was met by the data obtained with two independent alleles (Figure 1c,d,e). Under WL, the damage of the *cry1 cry2* double mutant was similar to that observed for the WT (Figure 1c,f).

3.3 | Enhanced thermotolerance under low red / far-red ratios requires PIFs

Since phyB negatively regulates PIFs, the reduced activity of phyB under low red / far-red ratios increases the activity of PIFs (Lorrain et al., 2008; Li et al., 2012). Under WL, thermotolerance of the *pif1 pif3 pif4 pif5* quadruple mutant was similar to that of the WT;

however, thermotolerance did not increase in *pif1 pif3 pif4 pif5* in response to WL+FR (Figure 1b,e). This suggests that in the WT, the increased thermotolerance under low red / far-red ratios is mediated by increased activity of PIFs. HFR1 is a negative regulator of the effects of PIFs on growth (Hornitschek, Lorrain, Zoete, Michielin & Fankhauser, 2009) but the *hfr1* mutation did not affect thermotolerance (Figure 1b,e).

3.4 | phyB decreases thermostability of the plasma membranes

To test whether the increased damage observed in the WT compared to the *phyB* mutant is associated with changes in the functional integrity of plasma membranes, we evaluated electrolyte leakage in seedlings grown under WL, immediately after exposure to a heat shock of 45 min at 45 ºC, compared to the seedlings that remained at 22 ºC as controls (Figure S1a). Both in the L*er* and Col backgrounds, the WT, *phyB* mutants and *cry1 cry2* mutants showed similar levels of electrolyte leakage in the absence of a heat shock (22 ºC) (Figure 2). After exposure to the heat shock, the WT and the *cry1 cry2* double mutant increased the leakage of electrolytes, more than the *phyB* mutant (Figure 2), indicating that phyB enhances the heat damage of the plasma membrane.

3.5 | phyB increases polyunsaturated fatty acids

Differences in membrane thermostability can result from changes in the lipid profile (Falcone et al., 2004). We therefore investigated fatty acid composition in seedlings harvested immediately prior the time when they had to be exposed to the heat shock in the above experiments (Figure S1a). Compared to the WT, the *phyB* mutant showed a significant reduction in total unsaturated fatty acids (16:3 and 18:3), a significant increase in partially unsaturated (18:1 and 18:2) but no differences in saturated (14:0 and 16:0) fatty acids (Figure 3a,b). The *cry1 cry2* double mutant showed an overall level of total unsaturated fatty acids similar to the WT (Figure 3b), an increment only in 18:2 and a slight reduction in 16:3 (Figure 3a).

In additional experiments we used the same protocol but in seedlings of the Col background and incorporating the WL+FR condition (Figure S1a). Compared to the controls grown under WL, WT plants grown under WL+FR showed reduced abundance of fully unsaturated fatty acids and increased abundance of saturated fatty acids, without changes in

the abundance of partially unsaturated fatty acids (Figure 3c). As expected, the *phyB* mutant under WL resembled the WT under WL+FR (Figure 3c). Neither the *phyB* mutant nor the *pif1 pif3 pif4 pif5* quadruple mutant responded to the red / far-red ratio (Figure 3c). Taken together, these observations are consistent with those obtained in L*er* (although with quantitative differences) and support the view that low red / far-red ratios reduce the activity of phyB leading to a shift in the fatty acid composition from unsaturated to saturated pools.

3.6 | phyB increases *FAD* expression

In Arabidopsis, seven fatty acid desaturase (FAD) enzymes are involved in the different steps leading to the generation of trienoic acids (Shanklin & Cahoon, 1998). Two of them, FAD2 and FAD3, localize to the endoplasmic reticulum, whereas the other five, FAD4, FAD5, FAD6, FAD7 and FAD8, localize to the chloroplast (Wallis & Browse, 2002). Given the observed changes in fatty acid composition, we evaluated whether the *phyB* mutation affects the expression of five *FAD* genes by real time PCR, in seedlings grown under WL and harvested immediately prior the time corresponding to the heat shock (Figure S1a). The expression of *FAD2*, *FAD5*, *FAD6*, *FAD7* and *FAD8* was significantly lower in the *phyB* mutant than in the WT (Figure 4a).

At least the expression of *FAD2* and *FAD5* was reduced in WT seedlings grown under WL+FR compared to WL (Figure 4b). The response of *FAD* genes to WL+FR appears to require prolonged exposures to low red / fad-red ratios because published data indicate that short-term treatments have no effects (*FAD* expression mean ± standard error. *FAD2*: WL= 12,30± 0.08, WL+FR= 12,30± 0.08; *FAD5*: WL= 11,76± 0.03, WL+FR= 11,91± 0.05; *FAD6*: WL= 11,33± 0.01, WL+FR= 11,69± 0.02; *FAD7/8*: WL= 11,00± 0.10, WL+FR= 11,36± 0.05, data from Leivar *et al.* 2012).

3.7 | Light reduces thermotolerance in etiolated seedlings

We decided to investigate whether light-activation of phyB also affects thermotolerance in dark-grown etiolated seedlings. We exposed 4-day-old etiolated seedlings to a heat shock of 90 min at 45 ºC followed by a 7 d recovery period before scoring survival. Prior to the heat shock the seedlings were grown for 2 d under four different conditions that resulted from the combination of a daily mild heat shock of 90 min at 35 ºC to acclimate the seedlings to high

temperatures and 6 h of WL (protocol in Figure S1b): the controls (no light and no acclimation treatments), the acclimated seedlings, the light-treated seedlings, and the acclimated and light-treated seedlings. Although for simplicity we refer here to etiolated seedlings, those exposed to light pretreatments were partially de-etiolated.

Whilst no acclimation was necessary to see a basal level of thermotolerance in light-grown seedlings (Figure 1), heat shock treatments given to non acclimated etiolated seedlings (Control in Figure S1b) were lethal for all genotypes (Survival rates for all genotypes without acclimation and without heat shock: 100% ±100%; without acclimation and exposed to heat shock (Control and Light pre-treated): 0% ± 0%). Thus, in etiolated seedlings acclimation under non lethal warm temperatures was necessary. The seedlings that were acclimated to elevated temperatures survived the heat shock but exposure to light during the acclimation period (Acclimated +WL) significantly reduced subsequent seedling survival (Figure 5a).

3.8 | In etiolated seedlings, light reduction of induced thermotolerance requires phyB, PIFs, and COP1

Survival of temperature acclimated seedlings was increased in the *phyB* mutant background (*phyB* and *phyA phyB* mutants) and *cry1 cry2* double mutants with or without light treatment, while *phyA* showed the same response as the WT (Figure 5a). The effects of mutations in photosensory receptor genes in dark-grown seedlings are not entirely unexpected because all the seedlings (including dark controls) were exposed to light to induce germination and this has already been shown to establish enough photoreceptor activity for some responses (e.g. Mazzella et al., 2005). The *cry1* single mutant showed WT survival rates and *cry2* mutant show reduced survival, particularly when exposed to light, but the *cry1 cry2* double mutant showed increased survival (Figure 5a). This pattern indicates genetic redundancy between cry1 and cry2 (Mockler, Guo, Yang, Duong & Lin, 1999; Mazzella & Casal, 2001).

If the survival response to light compared to darkness is as least partially mediated by phyB, one would expect the *phyB* mutation to reduce the effect. Although this was not the case, the response was significantly reduced in the *phyA phyB* mutant (Figure 5c), suggesting that phyA activity would be enough to saturate the response in the *phyB* single mutant. In order to test this interpretation, we used red light instead of white light, to avoid phyA activation by blue light (Neff and Chory, 1998; Casal and Mazzella, 1998), while still

activating phyB. Following the expectations, plant survival was reduced in the WT exposed to red light (Acclimated+Red-light) and this effect was absent in the *phyB* mutant (Figure 5b). The plant survival response to red light was not significantly different from zero in *phyB* but retained a WT magnitude in *phyA* (Figure 5d).

Compared to the WT, in darkness, plant survival was reduced in the *hy5*, *pif1 pif3 pif5*, *pif3 pif4 pif5*, *pif1 pif3 pif4 pif5*, *cop1-4*, *cop1-6*, and *spa1 spa2 spa4* mutants (Figure 5b). Noteworthy, the *cop1*, *pif1 pif3 pif5*, *pif3 pif4 pif5* and *pif1 pif3 pif4 pif5* mutants showed an inverted response to light, which actually increased rather than reduced plant survival in these genotypes (Figure 5d).

3.9 | phyB and PIFs do not affect membrane thermotolerance in etiolated seedlings

To investigate if the mechanisms that lead to a phyB-mediated reduction in heat tolerance in etiolated seedlings were similar to those described above for fully de-etiolated seedlings, we analyzed electrolyte leakage and the expression of *FAD* genes in etiolated seedlings. The 45 ºC heat shock increased leakage compared to the seedlings that did not receive this treatment, but no effects of the acclimation or light pre-treatments and of the *phyB* and *pif1 pif3 pif4 pif5* mutations were observed (Figure S2a), despite the large effects of these variants on seedling survival (Figure 5). No consistent effects of light or of the *phyB* or *pif1 pif3 pif4 pif5* mutations were observed on the expression of *FAD* genes (Figure S2b). Taken together, these observations indicate that although phyB reduces thermotolerance in light-grown as well as in etiolated seedlings, the mechanisms are different.

3.10 | Heat acclimation abolishes phyB effects on thermotolerance of light-grown seedlings

The above results show that light-activated phyB reduced thermotolerance in non-acclimated light-grown and in acclimated etiolated seedlings; we therefore tested the effect of acclimation on light-grown seedlings. We used two different protocols for this purpose.

We first applied to 9 day-old light-grown seedlings the same protocol used to acclimate etiolated seedlings (a heat acclimation of 35 ºC during 90 min for two consecutive days, finished with a heat shock just after the heat acclimation treatment, Figure S1b). Worthy of note is the finding that the warm pre-treatments that served to acclimate etiolated seedlings actually caused the opposite effect, i.e. reduced survival, in light grown-seedlings

exposed to a heat shock (plant survival % under WL, mean ±SE of 10 replicates, non-pre-treated seedlings: WT= 32 ±7; *phyB*= 56 ±8; *pif1 pif3 pif4 pif5* 40 ±8; pre-treated seedlings: WT= 12 ±6; *phyB*= 28 ±12; *pif1 pif3 pif4 pif5:* 13 ±8; the effect of the pre-treatment is significant at $P < 0,05$). A likely interpretation is that the warm temperatures immediately before the heat shock increased the heat stress in seedlings of this developmental context. This result further supports the idea that thermotolerance involves different mechanisms in etiolated and de-etiolated seedlings.

Although informative, the first protocol did not answer the question of whether acclimation affects the phyB-mediated effect in light-grown seedlings. We then shifted to a protocol used to acclimatize early light-grown seedlings (Silva-Correia et al., 2014). Nine day-old WL or WL+FR light grown seedlings were exposed during 60 min to 37 ºC, then returned for 120 min to 22 ºC under the same light regime for recovery, and then the seedlings received a heat shock of 45 min during 45 ºC. Plant survival was evaluated 5 days later (Figure 1Sc). This protocol effectively increased thermotolerance in response to a heat shock in WT seedlings grown under WL. In addition, acclimated seedlings showed no effect of phyB or low red / far-red ratio treatments on thermotolerance (plant survival of acclimated plants %, mean ±SE of 6 replicates, plants grown under WL: WT= 80 ±14; *phyB*: 91 ±4; *pif1 pif3 pif4 pif5:* 86 ± 9; plants grown under WL+FR: WT= 91 ±4; *phyB*= 92 ±5; *pif1 pif3 pif4 pif5:* 87 ±7; as a reference, plant survival of non-acclimated WT plants grown under WL: 34± 6).

4 | DISCUSSION

We show that non acclimated *Arabidopsis* rosettes grown under low red / far red ratios, typical of places with close neighbors, are more tolerant to a heat shock than those grown under high red / far-red ratios, typical of un-shaded spots (Figure 1). The *phyB* mutant showed constitutively high thermotolerance, unaffected by the red / far-red ratio (Figure 1). Thus, the increased thermotolerance under low red / far-red ratios is mediated by a reduction in phyB activity. Although low blue light and blue / green ratios are also typical of shade and reduce cry1 and cry2 activity (Sellaro et al., 2010), the *cry1* and/or *cry2* mutations failed to enhance thermotolerance (Figure 1c,f). The acquisition of thermotolerance under low red / far-red ratios requires PIFs because this response is lost in the *pif1 pif3 pif4 pif5* quadruple mutant (Figure 1b). Consistently, the activity of PIFs increases when that of phyB is low due either to a loss-of-function mutation or to low red / far ratios (Leivar & Quail, 2011).

Compared to the WT, the *phyB* mutants showed reduced electrolyte leakage after exposure to a heat shock (Fig. 2), implicating a differential functional response of the plasma membrane (Wang, 1988). Reactive oxygen species induced by heat stress promote peroxidation of unsaturated fatty acids, damaging the membranes (Djanaguiraman, Prasad & Seppanen, 2010). Therefore, lowering the levels of unsaturated fatty acids can reduce the targets of oxidative damage and increase thermotolerance (Khan, Anjum, Sofof, Kizek & Baier, 2016). Compared to the WT under WL, lowering phyB activity either by exposing the plants to low red / far-red ratios or by using *phyB* mutants caused a shift in the composition of fatty acids consistent with increased thermotolerance. In effect, low red / far-red (Figure 3c) or *phyB* (Figure 3b,c) reduced the levels of unsaturated fatty acids while increasing the levels of partially unsaturated or fully saturated fatty acids. Lipid composition was unaffected by red/ far red ratio in the *pif1 pif3 pif4 pif5* mutant (Fig. 3c), which is consistent with a role of PIFs downstream phyB in the response to the light cue. Compared to the WT under WL, low red / far-red ratios (Figure 4b) or the *phyB* mutation (Figure 4a) reduced the transcript levels of selected *FAD* genes before exposure to a heat shock, and this could account for the observed shifts in fatty acid composition. Therefore, the weaker membrane damage observed in response to reduced phyB activity (Figure 2) would be the result of lower expression of selected *FAD* genes (Figure 4) and the consequently low levels of polyunsaturated fatty acids (Figure 3). Photosynthetic reactions can increase the oxidative stress caused by heat stress (Djanaguiraman et al., 2010) and low phyB activity can reduce photosynthesis (Boccalandro et al., 2009). However, the latter is unlikely to be the cause of enhanced thermotolerance because the *cry1 cry2* mutant also shows reduced rates of photosynthesis (Boccalandro et al., 2012) and no enhanced thermotolerance (Fig. 1).

Acclimation by exposure to mild warm temperatures increased subsequent thermotolerance of WT plants grown under high red / far-red ratios, largely eliminating the effects of low red / far-red ratios or of the *phyB* mutations (see Results). This indicates that thermotolerance can be nearly saturated by cues either from neighbors or from warm temperatures. The low red / far-red ratios unequivocally work via phyB (Fig. 1). At this developmental context, the warm temperature cue inducing acclimation could also be partially perceived by a reduction in phyB activity (Jung et al., 2016; Legris et al., 2016) because both warm temperatures and the phyB mutation increase thermotolerance. However, other possibilities involving later convergence of the signals cannot be excluded.

In young, etiolated seedlings, light-activated phyB also reduced thermotolerance (Figure 5). However, several pieces of evidence point to substantial mechanistic differences

between both cases. In contrast to light-grown plants, in etiolated seedlings no obvious effects of phyB on membrane stability (electrolyte leakage) or *FAD* gene expression were observed (Figure S2). Furthermore, warm temperature acclimation was dispensable for the survival of light-grown seedlings and absolutely required for etiolated seedlings.

In tomato, low phyB activity due either to far-red light or to the loss-of function *phyB* mutation enhance cold tolerance by increasing *CBF1* transcript levels (Wang et al., 2016). In *Oryza sativa* the *phyB* mutant shows reduced membrane lipid peroxidation and increased membrane integrity in response to cold stress (He et al., 2016). Taken together with the enhanced tolerance to heat stress reported here and elsewhere (Song et al., 2017), these observations indicate that the *phyB* mutant is less susceptible to both temperature extremes. It appears that phytochrome mutants are less affected by growth-restricting abiotic stresses at the cost of reduced growth in the absence of stress (Yang et al., 2016; Wies, Mantese, Casal, & Maddonni, 2019).

Since radiation load is one of the main controls of the temperature of plant tissues (Legris et al., 2017), heat stress would be more likely to affect plants fully exposed to sunlight than plants shaded by neighboring vegetation. Furthermore, heat stress can be more severe if combined with high light, due to enhanced photo-oxidative stress (Foyer, Descourvières, Kunert & Descourvieres, 1994). Therefore, at first glance the observation that tolerance to heat stress is higher in plants grown under low red / far-red ratios typical of shade is counterintuitive. However, a more complete picture is obtained if not only the probabilities of heat stress but also the costs of thermotolerance are taken into account. In fact, photosynthesis is favored by membranes relatively rich in unsaturated fatty acid (McConn & Browse, 1998), which are more susceptible to heat stress (Routaboul, Skidmore, Wallis & Browse, 2012). Therefore, for a plant grown under the high red / far-red ratios of unshaded places, membranes relatively rich in unsaturated fatty acids might help to take advantage of the high light available for photosynthesis. This would come at the cost of lower basal thermotolerance and the need to rely on anticipatory warm temperature cues to induce acclimation before the impending occurrence of severe heat stress. Under the low red / far-red ratios typical of shade, photosynthesis is already limited by light availability. Under these conditions, reducing the level of unsaturated fatty acids would not come at a significant cost because photosynthesis is already reduced, and would offer the reassuring advantage of a higher basal thermotolerance.

6 ACKNOWLEDGMENTS

This work was supported by *Agencia Nacional de Promoción Científica y Tecnológica*, Argentina (PICT 2014-545 to MAM, and PICT-2016-1459 to JJC); and CONICET Argentina (PIP 2013-2015 num 455 to MAM). We thank Dr. Romina Fox for technical support with real time PCRs.

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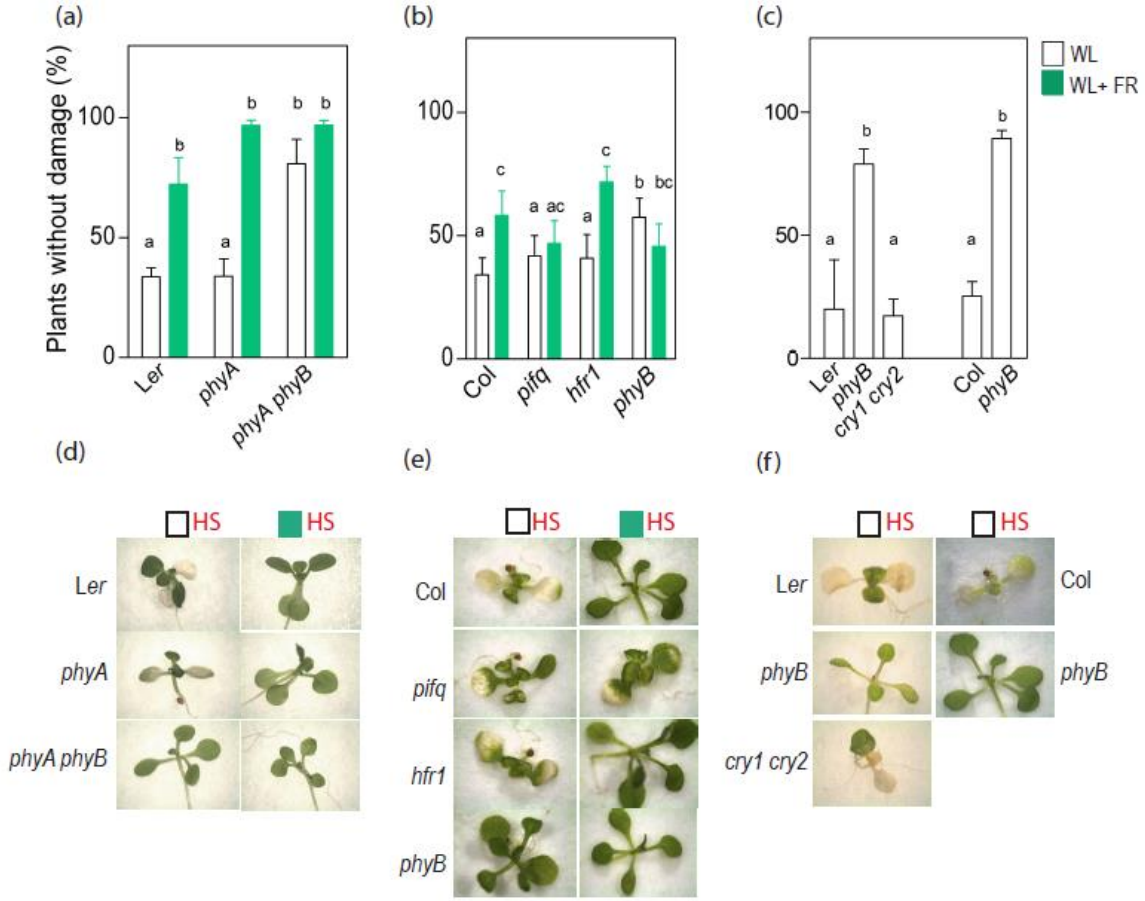

Figure 1. **Low red / far-red ratios increase tolerance to a heat shock**. Rosettes of the WT and different mutants were grown under WL or WL+FR and exposed to a heat shock (HS) during 45 min at 45 ºC (protocol in Figure S1a). (a), (b), (c) Percentage of plants without damage counted 5 d after the heat shock. Data are means of at least 6 independent replicates ±SE (each replicate is average of ten plants). Different letters indicate significant differences (at least $p < 0.05$) in ANOVA followed by Bonferroni post-tests. (d), (e), (f) Representative photographs after the heat shock treatments. *pifq*: *pif1 pif3 pif4 pif5* quadruple mutant.

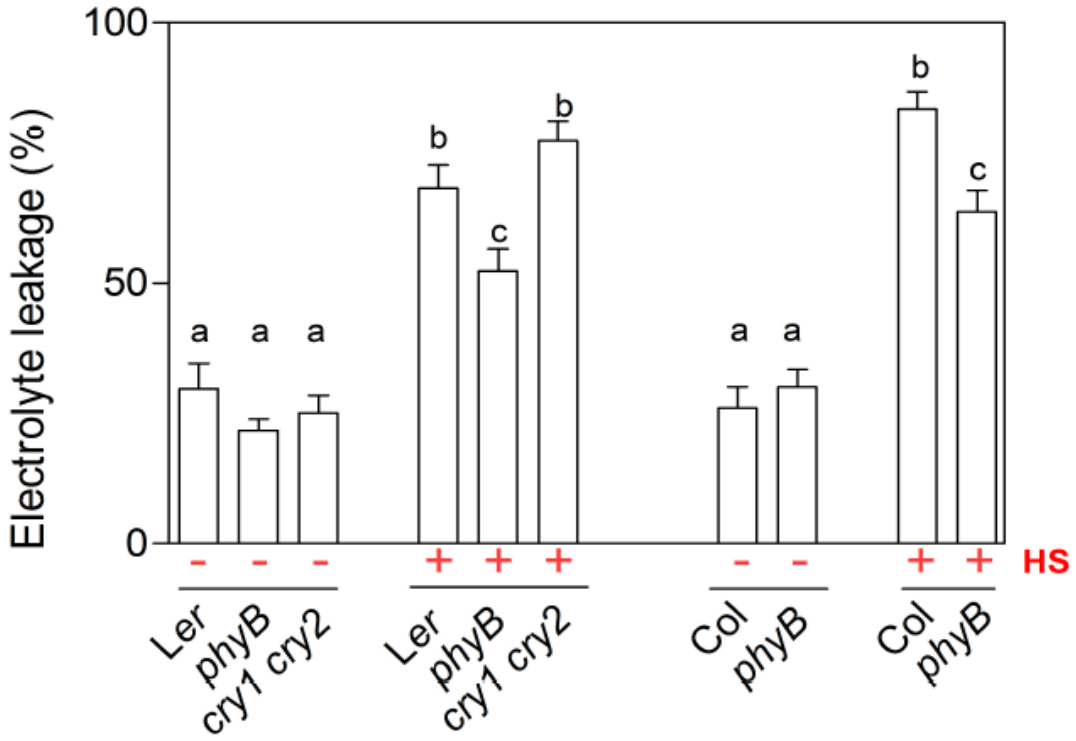

Figure 2. phyB increases electrolyte leakage after a heat shock. Rosettes of the WT and different mutants were grown under WL, either exposed or not exposed to a heat shock (HS) during 45 min at 45 ºC and immediately harvested for the measurement of electrolyte leakage (protocol in Figure S1a). Data are the means of at least three independent replicates ±SE (each replicate is average of at least twenty plants). Different letters indicate significant differences (at least $p < 0.05$) in ANOVA followed by Bonferroni post-tests. Electrolyte leakage measurements are expressed as a percentage of the leakage achieved after boiling the plants.

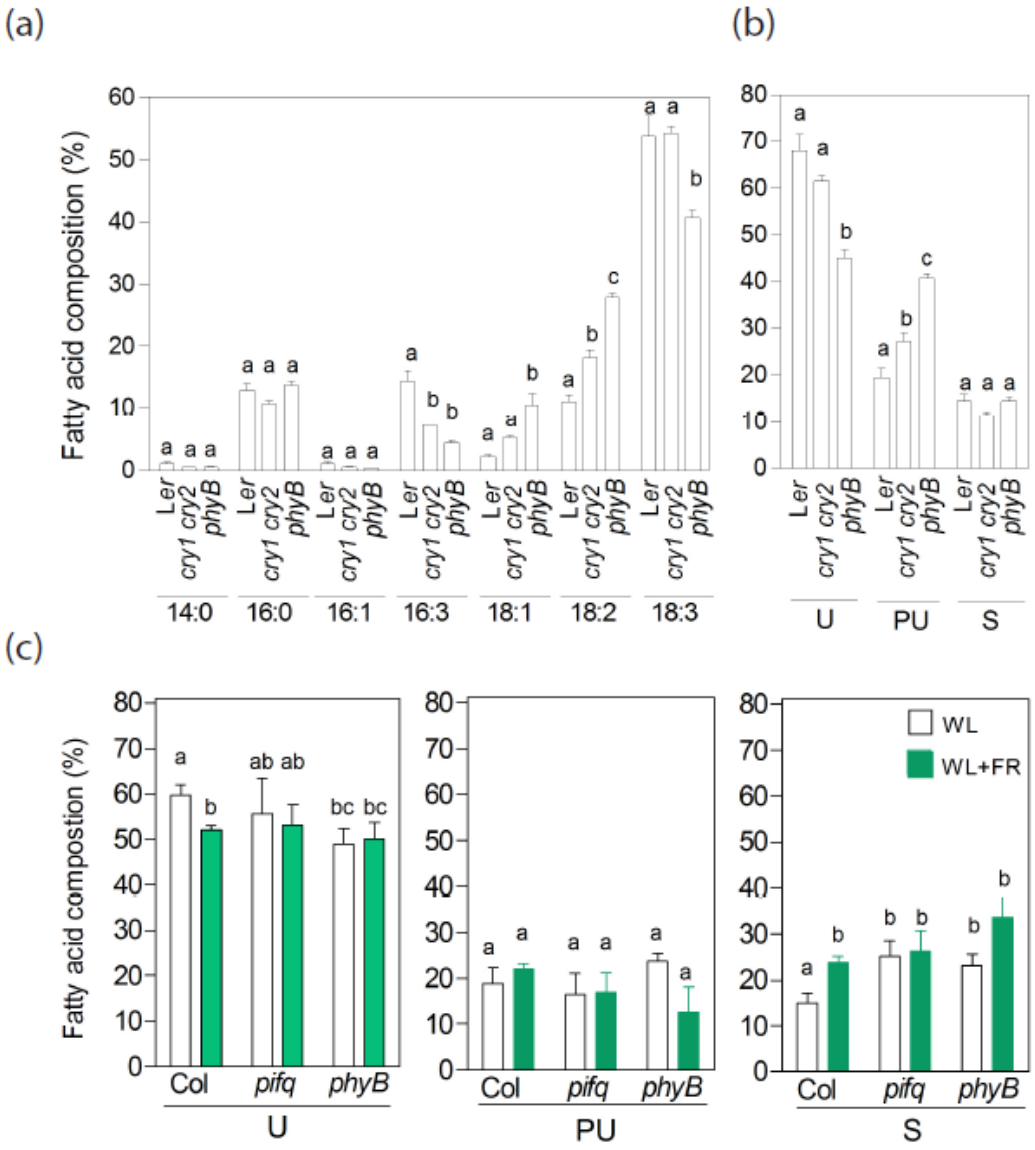

Figure 3. phyB increases polyunsaturated fatty acids. Rosettes of the WT and different mutants were grown under WL or WL+FR, and harvested at the time they would have been exposed to a heat shock (protocol in Figure S1a). (a) Total triacylglycerol fatty acid composition in WL grown plants. (b,c) Saturated (S) (14:0 and 16:0), partially unsaturated (PU) (16:1, 18:2 and 18:1) and totally unsaturated (U) (16:3 and 18:3) fatty acid composition in WL (b) or WL+FR grown plants (c). Data are the means of at least three independent replicates ±SE (each replicate is average of ten plants). Different letters indicate significant differences (at least $p<0.05$) in ANOVA followed by Bonferroni post-test.

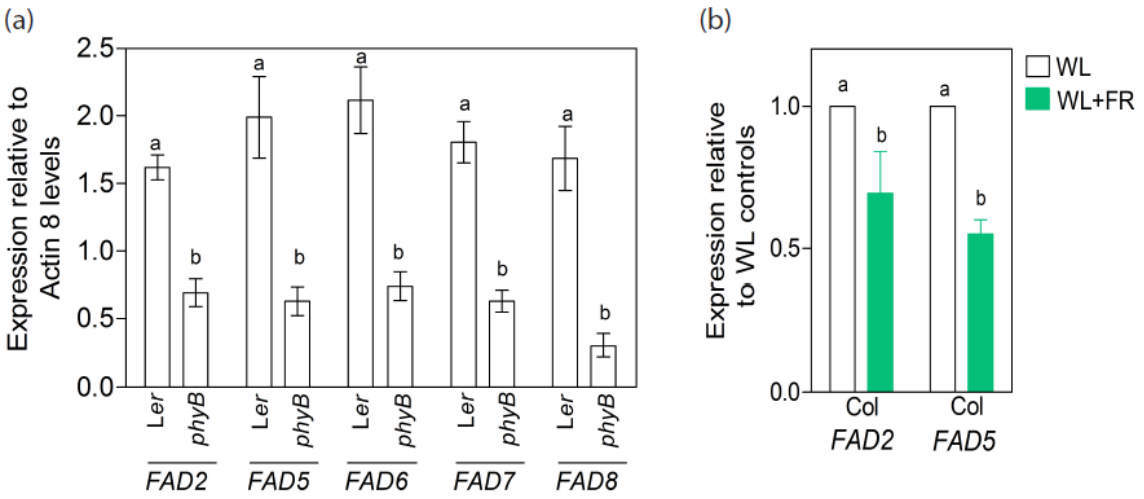

Figure 4. phyB increases the expression of *FAD* genes. Rosettes of the WT and different mutants were grown under WL or WL+FR, and harvested at the time they would have been exposed to a heat shock (HS, protocol in Figure S1a). (a) Effect of the *phyB* mutation under WL. (b) Effect of WL+FR compared to WL. Data are the means of at least three independent replicates ±SE. Different letters indicates significant differences (at least $p<0.05$) in ANOVA followed by Bonferroni post-tests.

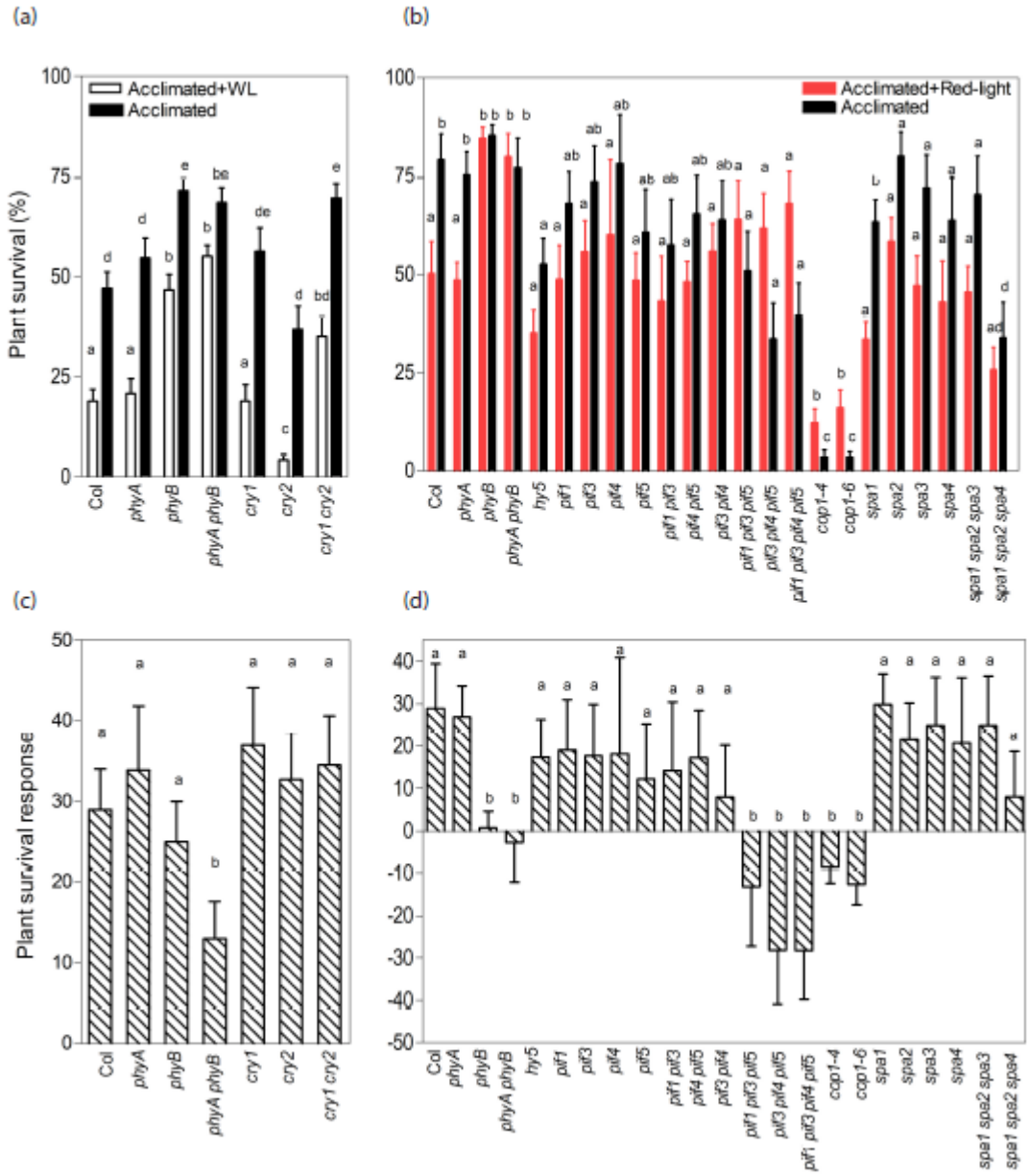

Figure 5. phyB reduces tolerance to a heat shock in etiolated seedlings. Etiolated seedlings were given a heat acclimation pre-treatment (35 ºC) and either grown in complete darkness (Acclimated) or exposed to light periods before a heat shock of 45 ºC during 90 min followed by a recovery period (protocol in Figure S1b). Survival rates in response to WL (Acclimated+WL) (a), or red light (Acclimated+Red-light) (b). (c), (d) Difference in plant survival between seedlings acclimated by warm temperatures either exposed or not exposed to light. Data are the means of at least six independent replicates ±SE (each replicate is average of ten plants). Different letters indicate significant differences (at least $p<0.05$) in ANOVA followed by Bonferroni post-tests.