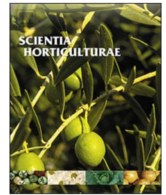
Scientia Horticulturae 222 (2017) 126–135

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti



Phytochromes are key regulators of abiotic stress responses in tomato

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ARTICLE INFO

Keywords:
Abiotic stress
Mutants
Phytochromes
Photomorphogenesis
Tomato

ABSTRACT

Phytochromes are the best characterized and most frequently studied plant photoreceptors. A plethora of studies have revealed important roles for phytochromes in plant development, and more recently, evidence indicates that these photoreceptors also modulate responses to a multitude of abiotic and biotic stresses. Thus, the present work aimed to investigate whether tomato phytochromes phyA, phyB1 and phyB2 are involved with responses to low water potential (polyethylene glycol 6000 at Ψ_w of −0.3 MPa), high salinity (100 mM NaCl), cadmium contamination (65 mM $CdCl_2$), high temperature (42 °C for six hours during three days) and ultraviolet B radiation (UV-B – 280–320 nm for eight hours during three days) stresses. For this purpose, seedlings of tomato mutants impacted by phytochrome A (*fri*), phytochrome B1 (*tri*) and phytochrome B2 (*phyB2*) were subjected to abiotic stresses and evaluated for their growth, pigment and osmoprotectant accumulation and lipid peroxidation. Under the conditions of this study, the results did not shown large variations of phyA mutant when compared to the wild genotype. However, the tomato phytochromes B1 and B2 mainly act as negative regulators of growth, pigment maintenance and osmoprotectant accumulation during responses to the different abiotic stresses.

1. Introduction

Light is one of the most important environmental signals regulating plant development. Fluctuations in light quality and quantity can deeply modify how, when and where a plant will grow; therefore, light is a crucial signal for a species to thrive in its environment. Thus, it is not surprising that plants have evolved different receptors to perceive light signals. Among these types of photoreceptors, phytochromes are, so far, the best characterized and more frequently studied (Carvalho et al., 2011a). Phytochromes are dimeric proteins (~130 kDa) covalently linked to the phytochromobilin, a linear tetrapyrrole that acts as a chromophore and recognizes specific light signals to interconvert the phytochrome from its inactive state, which perceives red light wavelengths (Pr), to the active state, which perceives far-red light wavelengths (Pfr) (Chen et al., 2005). Tomato (*Solanum lycopersicum* L.) is an important crop species in which the characterization of the molecular functions and modes of action of phytochromes have been constantly studied. The tomato plant harbors the following five phytochrome types: phyA, phyB1, phyB2, phyE and phyF (Pratt et al., 1997).

Since its discovery, a plethora of studies have revealed important roles of phytochromes in plant development, from seed germination to flowering (Carvalho et al., 2010; Toledo-Ortiz et al., 2010). Initial evidence for the involvement of plant photoreceptors as mediators of stress responses date back from the 1970s (Williams et al., 1972), but the topic has only recently been gaining more interest. The knowledge of how phytochromes work at the molecular level, the identification of transcription factor families whose action is regulated by phytochromes (Castilon, 2007; Zheng et al., 2014) and the increased availability of phytochrome mutants in different plant species provide ideal tools for studying the participation of these photoreceptors in biotic and abiotic stress responses.

With respect to the multitude of abiotic and biotic stresses modulated by phytochromes, they have been shown to be important components of plant signaling pathways involved in responses to insect herbivory (Ballaré, 2009; Howe and Jander, 2008), temperature fluctuations (Auge et al., 2012; Donohue et al., 2008; Foreman et al., 2011), harmful light radiation (e.g., ultraviolet B (UV-B)) (Boccalandro et al., 2001; Kreslavski et al., 2013, 2015; Mani and Guruprasad, 2015), salt stress (Balestrasse et al., 2008a; Datta et al., 2008), water stress (D'amico-Damião et al., 2015; Kidokoro et al., 2009) and even heavy

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http://dx.doi.org/10.1016/j.scienta.2017.04.035
Received 17 January 2017; Received in revised form 28 April 2017; Accepted 30 April 2017
Available online 13 May 2017
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metal intoxication (Cui et al., 2011). Because those environmental conditions can greatly affect plant productivity, the studies on phytochrome regulation of stress responses have become a hot spot of research.

Photomorphogenetic mutants have constantly been employed to evaluate the role of phytochromes in stress responses (Carvalho et al., 2011b; D'amico-Damião et al., 2015). For example, Indorf et al. (2007) found that phyA and phyB *Arabidopsis thaliana* mutants showed a reduced expression of *STO,* a gene involved in salt stress responses, when treated with red light, suggesting that the phytochrome family contributes to salt stress responses. In rice modified with maize phytochrome interacting factor 3, it was shown to improve drought and salt stress tolerance (Gao et al., 2015). In fact, phyB seems to be a fundamental part of abiotic stress responses. Studies with rice *phyB* mutants showed that the presence of the *phyB* genotype reduced water loss per unit of leaf area, conferring better tolerance to water stress (Liu et al., 2012). Arabidopsis phytochrome B mutants indicate that this photoreceptor is involved in responses to high temperature (Njimona et al., 2014) and prolonged UV-B radiation (Boccalandro et al., 2001; Huang et al., 2012; Li et al., 2013; Rusaczonek et al., 2015).

However, although the control of stress response by phytochromes is an old topic (Williams et al., 1972; Thomsen et al., 1992; Cockburn et al., 1996), recently, evidence has emerged revealing new roles of these molecules. Transgenic plants overexpressing the chromophore biosynthesis enzymes have been associated with increased tolerance to mercury toxicity (Shen et al., 2011). Transgenic plants expressing the hyperactive mutant S599A-PhyA showed improved tolerance to zinc (Gurunani et al., 2016), while phyA mutants in tomato showed altered response to nutritional stress (Carvalho et al., 2016). These studies suggest a complex, multifaceted control of the stress response by phytochromes, raising questions about how and which phytochromes modulate different abiotic stresses. Thus, the present work aimed to investigate if tomato phytochromes phyA, phyB1 and phyB2 are involved in responses to low water potential, high salinity, cadmium contamination, high temperature and UV-B radiation stresses. For this purpose, seedlings of tomato mutants impacted in phytochrome A (*fri*), phytochrome B1 (*tri*) and phytochrome B2 (*phyB2*) were subjected to abiotic stresses and their growth, pigment and osmoprotectant accumulation and lipid peroxidation were evaluated.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds from the tomato photomorphogenic mutants *far-red light insensitive* (*fri*), *temporarily red light insensitive* (*tri*) and *phyB2,* defective for the genes encoding the PHYA, PHYB1 and PHYB2 apoproteins, respectively, were obtained from the "Tomato Genetics Resource Center" (TGCR; Davis − California). All mutants were present in the Moneymaker cultivar (Van Tuinen et al., 1995a,b; Kerckhoffs et al., 1999), which was used as a wild type (WT) parent for all experiments. To avoid fungal contamination, seeds were pre-treated with a 5% sodium hypochlorite solution for 10 mins and then thoroughly washed with water before use. Germination was performed in plastic boxes containing two layers of filter paper embedded in water, under 25 °C in the dark. For all experiments described, plants were grown in a chamber under 12 h white light (60 μmol $m^{-2} s^{-2}$) at 25 °C.

2.2. Induction of low water potential, high salt concentration and cadmium contamination stresses

Three days after germination, 25 seedlings with 2 mm radicles were transferred to 1 L plastic pots containing two layers of filter paper embedded in 15 mL of different solutions prepared to induce the studied stressful condition. For low water potential, a solution of polyethylene glycol 6000 (PEG) was prepared to achieve a Ψ_w of −0.3 MPa, as described by Vilela et al. (1992). Solutions of 100 mM NaCl and 65 mM $CdCl_2$ were prepared to induce high salt concentration and cadmium contamination stresses, respectively. As a control condition, a set of pots was filled with 15 mL of distilled water. Each of the described conditions consisted of three pots that were maintained for seven days in a growth chamber adjusted to the same conditions aforementioned in Section 2.1.

2.3. Induction of high-temperature stress and prolonged UV-B light exposure

Four days after germination, 25 seedlings were transferred to 1 L plastic pots containing two layers of filter paper embedded in 15 mL of distilled water and placed inside a control chamber, set at 25 °C, with a 12 h photoperiod and 60 μmol $m^{-2} s^{-2}$ light intensity (Alexieva et al., 2001). A set of three pots was kept in this chamber as a control for the experiment. For high temperature stress, a set of three pots was moved to a chamber maintained at 42 °C for six hours and then returned to the control chamber. This procedure was repeated on the 6th and 7th days after germination, always returning the plates to the control chamber after treatment with high temperature. Plates were then kept in the control chamber for three extra days (10 days total) until further measurements. For prolonged UV-B light exposure, a set of three pots was moved to a chamber containing mercury lamps set to specifically emit UV-B light wavelength (8W-T5, BRAVO, peak at 305 nm − 310 nm) added to white lamp (20W-T10, NSK). Plates were kept under the UV-B light for eight hours and then returned to the control chamber. This procedure was also repeated on the 6th and 7th days after germination, always returning the plates to the control chamber after treatment with high temperature. Plates were then kept in the control chamber for three extra days (10 days total) until further measurements. Fig. 1 illustrates the strategy used for induction of high temperature (Fig. 1A) and UV-B light (Fig. 1B) stresses.

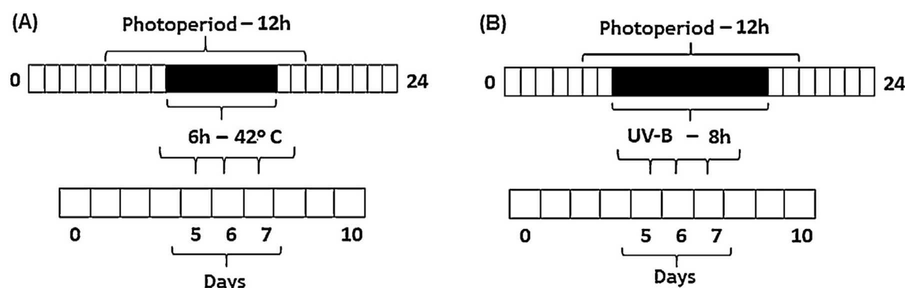

Fig. 1. Methodology used for high temperature (A) and prolonged UV-B light (B) stresses. Seedlings were kept in a control chamber (25 °C, 12 h photoperiod, see Methods) for an initial period of 4 days. For high temperature stress, seedlings were then transferred to a chamber set at 42 °C for six hours during the 5th, 6th and 7th days after germination, always returning the plates to the control chamber after treatment. For prolonged UV-B light exposure, seedlings were moved to a UV-B (305–310 nm) chamber for eight hours during the 5th, 6th and 7th days after germination, always returning the plates to the control chamber after treatment. For both treatments, plates were kept at the control chamber for three extra days (10 days total) until measurements were taken.

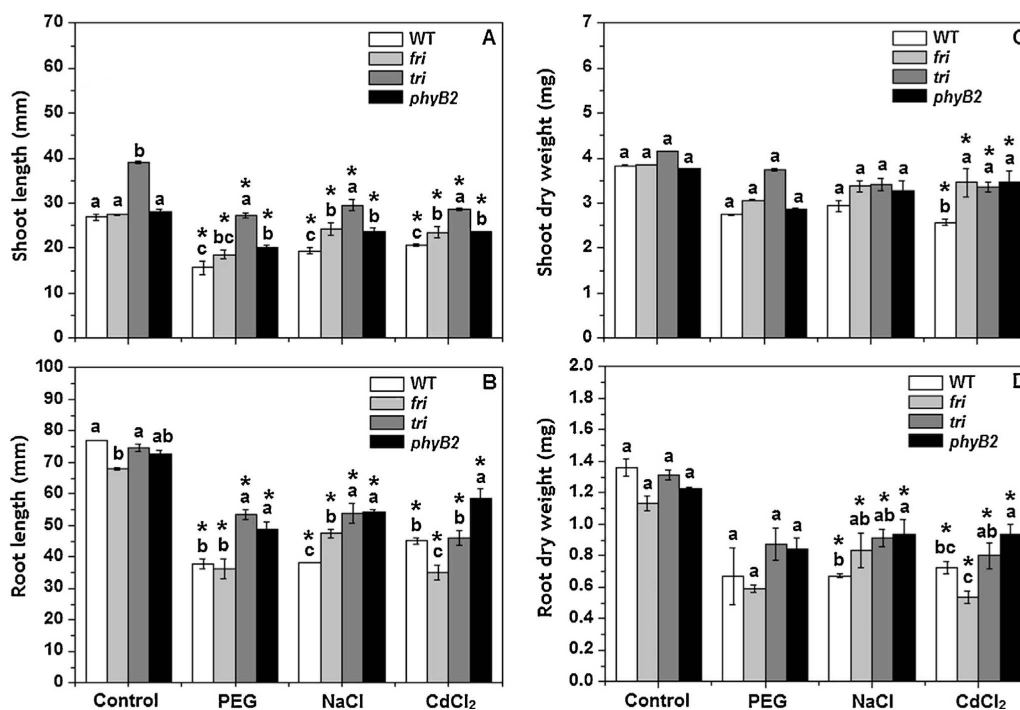

Fig. 2. Shoot and root length (A and B) and biomass (C and D) of wild type (WT) and tomato phytochrome mutants (*fri*, *tri* and *phyB2*) grown for seven days in different solutions were prepared to determine the effects of low water potential (PEG at Ψ_w of −0.3 MPa), high salt stress (100 mM NaCl) or cadmium contamination stresses (65 mM $CdCl_2$, see methods). As a control condition, a set of plants was grown in water only. Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

2.4. Analysis of growth parameters

At the end of each stress induction, ten randomly chosen seedlings were used to evaluate seedling length and biomass. Hypocotyl root and shoot length were measured using a digital caliper. The same roots and shoots were then dried for 48 h at 40 °C for quantification of biomass accumulation, which was performed using an analytic scale model AA 200 from the Denver Instrument Company.

2.5. Pigment content

Quantification of chlorophyll and carotenoids was performed using 25 mg of cotyledons added to Eppendorf tubes and extracted in 2 mL of acetone. Tubes were kept at 4 °C in the dark for 24 h under constant agitation. Cotyledons were then removed, and the remaining extract was evaluated according to Lichtenthaler (1987). Anthocyanins levels were measured using 35 mg of hypocotyls added to tubes containing a solution of hydrogen chloride:water:chloroform (24:18:48, v/v/v). Tubes were kept at 4 °C in the dark for 24 h. The hypocotyls were then removed, and the remaining extract was evaluated according to Peters et al. (1989). Pigments were quantified at the end of the experiments (10 days). All extraction processes were performed in triplicate, and the total pigment content was expressed as µg of pigment per g of fresh weight.

2.6. Quantification of osmoprotectants (glycine betaine and proline)

Proline content was determined following the protocol described by Bates et al. (1973). For this purpose, 250 mg of plant material was macerated on 5 mL of 3% (w/v) sulfosalicylic acid. Aliquots of 1 mL were used for proline quantification by adding 1 mL of acid ninhydrin and 99,5% (v/v) glacial acetic acid (1 mL). The mixture was agitated and further incubated at 100 °C for 60 mins. Samples were then cooled on ice and 2 mL of toluene was added for phase separation. The fraction containing the chromophore group was collected, and its absorbance was determined at 520 nm using a spectrophotometer (Beckman Colter DU 640 − USA). Proline concentration was determined through a standard curve, and the result was expressed in µmol proline g^{-1} fresh weight.

Glycine betaine content was determined as described by Grieve and Grattan (1983), where 10 mL of deionized water was added to 250 mg of plant material and left under constant agitation for 24 h at 25 °C. The obtained extract was mixed with H_2SO_4 in a 1:1 proportion. A total of 0.5 mL of the obtained solution was collected in test tubes and incubated on ice for 60 mins. Then, 0.2 mL of potassium diiodine was added following agitation and incubation for 16 h at 4 °C. Samples were then centrifuged at 10.000 rpm for 15 mins at 0 °C, and the pellet was collected and dissolved in 3 mL of 1.2-dichloroethane. Samples were left at room temperature for 150 mins after the absorbance was determined at 365 nm in a spectrophotometer. The glycine betaine concentration was determined through a standard curve, and the result was expressed as mg glycine betaine g^{-1} fresh weight.

2.7. Lipid peroxidation

Quantification of lipid peroxidation was performed as described in Heath and Packer (1968), via analysis of the 2-thiobarbituric acid reactive substances present in the form of malondialdehyde (MDA). Samples containing 250 mg of plant material were mixed with 3 mL of 0.1% trichloroacetic acid (w/v) containing 20% of polyvinylpolypyrrolidone (PVPP). After homogenization, the extract was centrifuged at 10.000 rpm at 15 °C for 5 mins. A total of 250 µL of the obtained supernatant was added to a 1 mL solution containing 20% trichloroacetic acid (w/v) and 0.5% 2-thiobarbituric acid (w/v). Samples were kept at 95 °C for 30 mins and then cooled on ice, following centrifugation at 10.000 rpm at 15 °C for 10 mins. Reads were performed on a spectrophotometer at 535 and 600 nm, and the results were expressed in nmol of MDA g^{-1} fresh weight.

2.8. Statistical analysis

All experiments were repeated at least three times. For all the experiments three replicates (pots) were used, with 25 seedlings (subreplicates), of which a minimum of nine seedlings were selected randomly to compose the averages. Statistical inferences were performed with ANOVA followed by a Tukey test ($P < 0.05$) using the program Assistant (www.assistat.com).

3. Results

3.1. Plant growth

We observed that shoot and root length of tomato seedlings were reduced by PEG, NaCl and $CdCl_2$ treatments in all genotypes studied as compared with plants grown on control pots (supplied with only water) (Fig. 2A and B). Interestingly, however, tomato phytochrome mutants responded differently to the stresses applied as compared with WT and other phytochrome mutant plants growing in similar conditions. The *PHYA* mutant *fri*, for example, appeared to be less sensitive to high salt concentration, as it roots and shoots were longer than WT plants growing in NaCl. The involvement of *PHYA* in a stress response can also be organ specific as observed for *fri* hypocotyls (but not roots) with $CdCl_2$, which were longer than those of WT. On the other hand, the *PHYB1* mutant *tri* developed longer hypocotyls than WT, *fri* and *phyB2* mutants in control pots (as observed by Van Tuinen et al., 1995a) and all other conditions analyzed. However, its roots were longer than WT only for the PEG and NaCl treatments. Finally, *phyB2* was the only one that showed less sensitivity to all three stresses analyzed in both shoot and roots organs compared with WT plants. These data suggest that tomato phytochromes are negative regulators of low water potential, salt and cadmium stresses.

Statistical analysis suggests that biomass accumulation is less impacted by low water potential, salt and $CdCl_2$ stresses as compared with the root and shoot size (Fig. 2C and D). PEG and NaCl treatments did not affect dry shoot weight in any of the genotypes studied when compared to control conditions (Fig. 2C). However, in pots where $CdCl_2$ was added, all genotypes showed reduced shoot biomass accumulation. As observed for the shoot length in the same treatment, all phytochrome mutants appeared to be less sensitive to this type of stress, showing higher biomass accumulation in hypocotyls than WT plants in this same condition. Treatment with NaCl and $CdCl_2$ reduced root dry weight in all genotypes studied but in a genotype-dependent manner. The *phyB2* mutant was the only one less sensitive to these conditions when compared to WT plants (Fig. 2D). Therefore, these results indicate that different tomato phytochromes can regulate different stress responses in an organ-dependent manner.

Next, we evaluated the participation of phytochromes in growth responses to high temperature and prolonged UV-B light exposure. As observed for the low water potential, salt and cadmium stresses, treatment with high temperature (42 °C) or UV-B light also caused a pronounced reduction in seedling shoot and root length on all genotypes analyzed when compared to seedlings grown at 25 °C under fluorescent light (Control condition – Fig. 3A and B, see methods). Moreover, results also showed that responses to those two environmental signals are dependent on the genotype and organ studied. For example, besides presenting slightly longer shoots than WT when growing under the UV-B light, *fri* seedlings behaved as WT plants in all other conditions analyzed. On the other hand, high temperature and UV-B exposure are less detrimental to the root and shoot growth of phytochrome B mutants *tri* and *phyB2* as compared with WT or *fri*. Seedlings of *tri* showed longer shoots than WT and *fri* at 42 °C, whereas the roots but not the shoots of *phyB2* were longer than WT and *fri* in the same condition. Finally, the seedlings of *tri* and *phyB2* developed longer roots and shoots than WT plants when grown under prolonged UV-B light exposure. These data suggest that the two tomato phytochrome B mutants are important modulators of plant responses to high temperature and UV-B light stress.

Biomass accumulation results indicated that exposure to 42 °C is not sufficient to cause loss of seedling shoot or root dry weight in any of the genotypes studied. These data are evidenced by the statistical analysis comparing plants growing at high temperature and those growing in control conditions (Fig. 3C and D). On the other hand, exposure to UV-B light caused a reduction in shoot and root dry weight in WT and all phytochrome mutants. In agreement with results obtained for seedling length, biomass accumulation of *tri* and *phyB2* mutants was less affected by UV-B treatment than WT and *tri* plants, suggesting that the removal of phytochrome B1 and B2 has led to reduced sensitivity to light stress. Thus, these results highlight the importance of tomato phytochromes B1 and B2 as negative regulators of high temperature and UV-B light stress responses.

3.2. Pigment accumulation

When exposed to PEG and NaCl, all genotypes showed a decrease in total chlorophyll and carotenoid levels as compared with control plants (Fig. 4A and B), whereas treatment with $CdCl_2$ only affected carotenoid but not chlorophyll content. Interestingly, even though none of the phytochrome mutants studied differed from the WT in the levels of these two pigments under control conditions, the phytochrome B mutants *tri* and *phy2* showed a higher content of total chlorophyll and carotenoids as compared with WT plants grown on filter paper containing PEG or a high concentration of NaCl (Fig. 4A and B). Moreover, phytochrome B1 is also involved in carotenoid accumulation in response to Cd stress, as evident from the *tri* mutant, which showed higher levels of these pigments than the WT seedlings in this condition. The role of phytochrome A appeared to be less prominent than phytochromes B1 and B2, as the *fri* mutant showed higher accumulation of carotenoids only after PEG and $CdCl_2$ stresses, as compared with WT seedlings. These results support the hypothesis that phytochrome B1 and B2, and, to a lesser extent, phytochrome A, are key regulators of abiotic stress responses in tomato.

Temperature and UV-B radiation negatively affected total chlorophyll levels in WT and phytochrome mutants, as compared with the control conditions (Fig. 5A). Once more, this effect seemed to be dependent on phytochromes B1 and B2. The phytochrome B1 mutant *tri*, for example, showed higher levels of chlorophyll A + B than WT plants when grown at 42 °C and under UV-B light. Carotenoid content was reduced in all genotypes only when plants were treated with high-temperature stress, whereas UV-B radiation caused an increase in the concentration of the pigments (Fig. 5B). Again, we observed that both, *tri* and *phyB2* accumulated more pigments when subjected to high temperature or UV-B stress when compared to WT.

Finally, we checked whether anthocyanin levels were differentially affected by these two abiotic stresses. It was observed that high temperature and prolonged UV-B exposure led to a major increase in anthocyanin content in all genotypes (Fig. 5C). Phytochrome B mutants differed from WT as anthocyanin levels were lower in *tri* and *phyB2* grown at 42 °C and lower in *tri* grown under the UV-B light. These collective results indicated that phytochromes B1 and B2 are negative regulators of plant pigment accumulation in response to abiotic stresses in tomato.

3.3. Accumulation of osmoprotectants

Treatment with abiotic stresses that cause low water potential (PEG) and high salt concentration (NaCl) led to an increase in the levels of the osmoprotectants amino acid proline and glycine betaine in all genotypes studied (Fig. 6A and B). Proline levels in the phytochrome A mutant *fri* did not differ from WT plants grown in any of the conditions studied. On the other hand, statistical analysis indicates that glycine betaine content is higher in *fri* plants than WT subjected to PEG stress

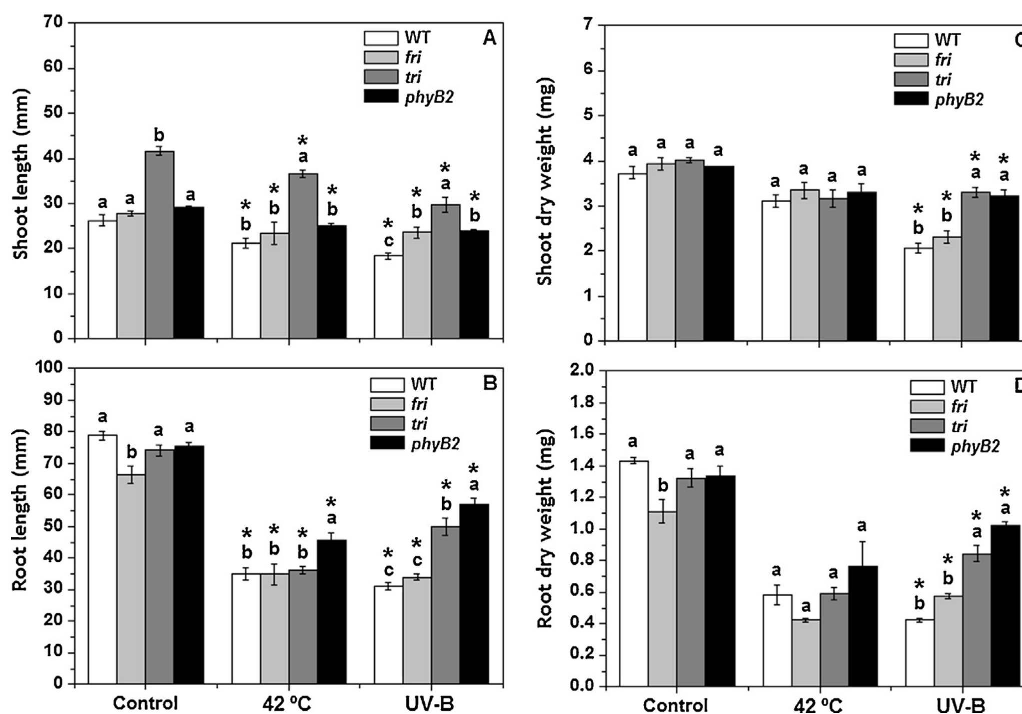

Fig. 3. Shoot and root length (A and B) and biomass (C and D) of wild type (WT) and tomato phytochrome mutants (*fri*, *tri* and *phyB2*) treated with high temperature (42 °C for six hours during three days) or prolonged UV-B light exposure (UV-B − 305–310 nm for eight hours during three days). As a control condition, a set of plants was grown at 25 °C, under white light. Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

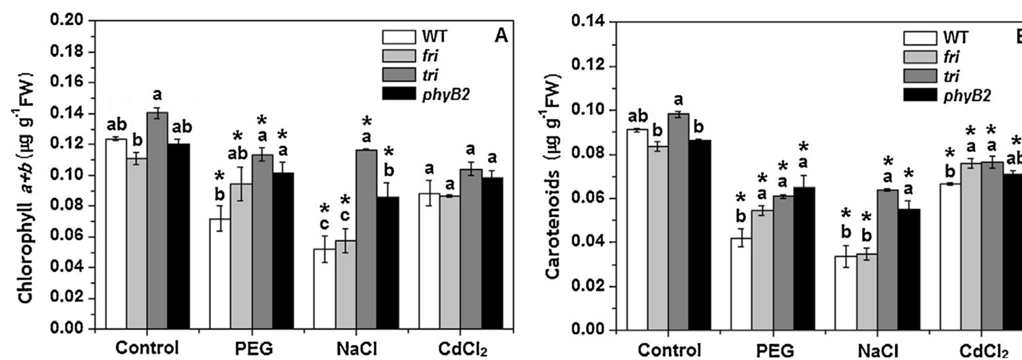

Fig. 4. Total chlorophyll (a + b) (A) and carotenoid (B) content in WT and tomato photomorphogenetic mutants treated with low water potential (PEG at Ψ_w of −0.3 MPa), high salt stress (100 mM NaCl) or cadmium contamination stresses (65 mM $CdCl_2$). Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

but lower under NaCl stress, suggesting that phytochrome A can act as a positive or negative regulator of osmoprotectant accumulation in response to a specific environmental condition. Furthermore, we observed that tomato phytochromes B1 and B2 act as positive regulators of osmoprotectant content in response to abiotic stresses, as *tri* and *phyB2* plants accumulated a higher content of proline and glycine betaine than WT plants when grown under PEG and NaCl.

3.4. Lipid peroxidation

When treated with a variety of abiotic stresses (PEG, NaCl, $CdCl_2$, high temperature and UV-B light), the seedlings of WT and the three phytochrome mutants showed an increase in lipid peroxidation (Fig. 7A and B), as evaluated by the malondialdehyde (MDA) quantification method (Heath and Packer, 1968). The specific analysis of each stress suggested the involvement of tomato phytochrome A in the regulation of lipid peroxidation is dependent on the type of abiotic stress inflicted. Under low water potential stress (PEG), phytochrome A acts as a negative regulator of lipid peroxidation, as the *fri* mutant showed higher levels of MDA than WT plants (Fig. 7A). Treatment with NaCl and UV-B triggers the opposite phenotype in this mutant; MDA levels were lower than those of WT (Fig. 7A and B), suggesting that under these type of stresses, phytochrome A acted as a positive regulator of lipid peroxidation. Moreover, lipid peroxidation responses to high temperature and Cd treatments were not affected in *fri*.

In a different fashion, tomato phytochromes B1 and B2 exerted a negative function in lipid peroxidation under all stresses studied (Fig. 7A and B). The mutants, *tri* and *phyB2,* always showed lower MDA levels than WT seedlings growing in the same condition independent of the stressful condition analyzed. This observation was more evident in the PEG treatment, where phytochrome B mutants showed approximately 75% of the total MDA quantified in the WT plants (Fig. 7A). These results, once again, highlighted the function of phytochromes B1 and B2 as negative regulators of abiotic stress responses in tomato.

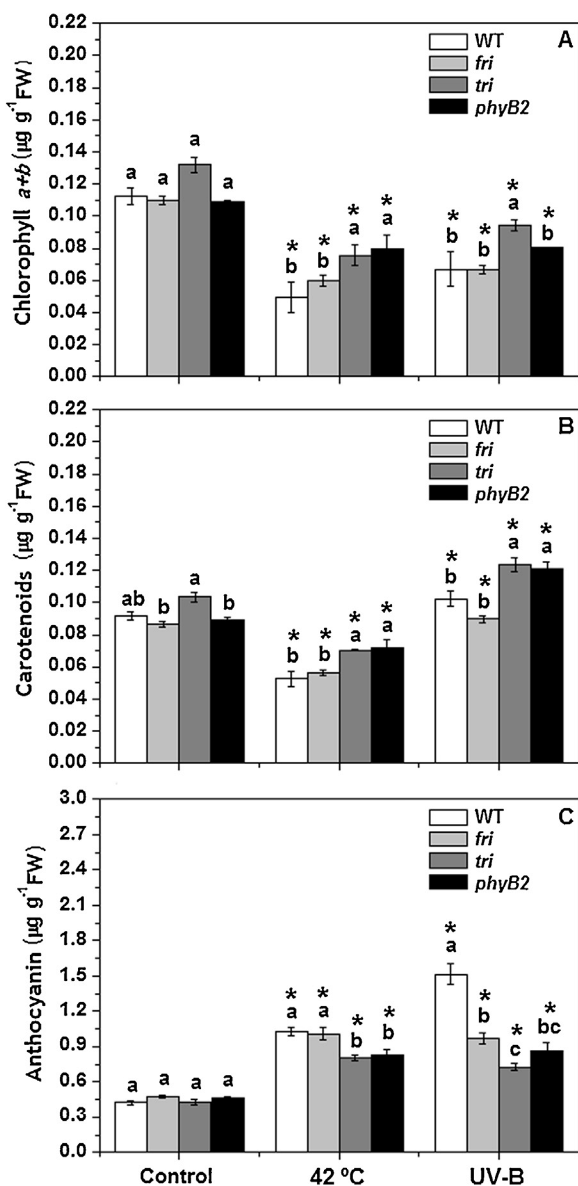

Fig. 5. Total chlorophyll (a + b) (A), carotenoid (B) and anthocyanin (C) content in WT and tomato photomorphogenetic mutants treated with high temperature (42 °C for six hours during three days) or prolonged UV-B light exposure (UV-B − 305–310 nm for eight hours during three days). Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

4. Discussion

To our knowledge, the present work is the first to provide a broad overview of how specific phytochromes regulate tomato responses to multiple abiotic stresses. A summary of the results obtained is shown in Table 1.

When studying seedling growth in response to stress, we observed that phyB mutants showed longer hypocotyls and roots than WT plants growing under a low water potential or high salt concentration (Fig. 2A and B). The increase in hypocotyl length observed in *tri* mutant refers to an etiolated phenotype, classically observed in sun plants when exposed to a shadow condition. The control of shading is recognizably influenced by the phyB event (Van Tuinen et al., 1995a,b), and the biologically active isomer is photostable. This event is supported by the increase in stem length or hypocotyl length, without any proportional gain in biomass, as observed in this study. It is known that the action of this phytochrome is dependent on the Phytochrome-interacting Factors (PIFs), which represent a family of transcriptional basic helix-loop-helix type factors (bHLH) that repress photomorphogenesis. Especially PIF4 promotes hypocotyl elongation and inhibits the action of the active isomer of phyB (Huq and Quail 2002; Foreman et al., 2011). Since the mutants *tri* and *phyB2* are deficient in phyB, PIF4 action is not sufficiently inhibited to the point to curb the excessive growth of the hypocotyl, even in the presence of light.

Furthermore, the roots of both *phyB1* and *phyB2* mutants showed more increases in root growth on water deficit and salt stress, wherein *phyB2* showed more biomass accumulation than WT seedlings in the presence of NaCl (Fig. 2D). Although there is no literature for the genotype response to this stresses in tomato, it is possible that PIF3, well known as a part of drought and high salinity response, may be involved (Gao et al., 2015). In maize, *ZmPIF3* transgenic plants were more tolerant to dehydration and salt stresses including growth parameters (Gao et al., 2015).

The low availability of water in the soil, as well as salt presence, decreases the external hydric potential and interferes with the efficiency of water usage by plants so that the cellular content and vital processes might be impaired (Golldack et al., 2014). As a strategy for the maintenance of cellular contents due to osmotic adjustment, there is an accumulation of osmoregulator substances, such as proline and glycine betaine (Wani et al., 2013; Kishor and Sreenivasulu, 2014). Therefore, the higher content of these molecules in *tri* and *phyB2* tomato mutants under water stress (Fig. 6A and B) support the hypothesis that the phytochrome B participates in the production or transport pathways of proline and glycine betaine in low hydric potential conditions (González et al., 2012). Interestingly, under high salinity treatment, glycine betaine accumulation was less pronounced when compared to proline. Therefore, only the phyB2 mutant differed positively from the non-mutated genotype in glycine betaine accumulation (Fig. 6B). In fact, phyB seemed to be a fundamental part of compatible osmolyte accumulation during a hydric deficit. It was observed that, under hydric stress, *phy*B1 rice mutant plants showed a greater accumulation of proline (Liu et al., 2012), while *phyB1 Arabidopsis* mutants showed a greater accumulation of total osmolytes (González et al., 2012).

We observed that the chlorophyll content remained higher in the cotyledons of *phyB* mutants under hydric deficit and high salinity (Fig. 4A). This result may be related to two possible causes: the osmolytes that assisted in the maintenance of cellular homeostasis decreased the deleterious effects of the stresses imposed on the photosynthetic apparatus (Raza et al., 2007; Wani et al., 2013). Moreover, in conditions of hydric stress, there may be a reduction in leaf area and increased stomatal density in the *phyB* mutants, probably as a result of the highest concentration of chlorophyll per unit area (Liu et al., 2012). In addition to the chlorophylls, the carotenoids accumulated in greater amount in the *phyB* mutant seedlings subjected to NaCl stress and in all mutants in seedlings subjected to low osmotic potential (Fig. 4B), whereby carotenoids acts in the photoprotection of chlorophylls and their synthesis is known to be regulated by PIFs (Toledo-Ortiz et al., 2010). The production of carotenoids is regulated by a dynamic repression-activation module formed by PHYTOCHROME-INTERACTING FACTOR1 (PIF1) and LONG HYPOCOTYL5 (HY5). These transcription factors directly and oppositely control the expression of the gene encoding PHYTOENE SYNTHASE (PSY), the first and main rate-determining enzyme of the carotenoid pathway (Bou-Torrent et al., 2015). Interestingly, under a water deficit, *fri* showed an increase in carotenoid (Fig. 4B) and glycine betaine content (Fig. 6B). However, in this same condition, lipid peroxidation in *fri* exceeded the values obtained in WT (Fig. 7A). Although phyA has been correlated to some factors generating complex metabolic responses including water deficit (Chen et al., 2014), in this study, the mutation in this gene did not cause large variations when compared to the wild genotype.

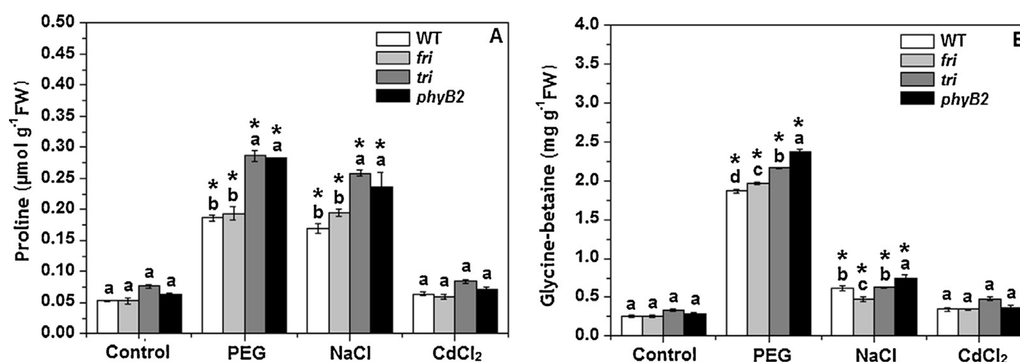

Fig. 6. Proline (A) and glycine betaine (B) content in WT and tomato photomorphogenetic mutants treated with low water potential (PEG at Ψ_w of −0.3 MPa), high salt stress (100 mM NaCl) or cadmium contamination stresses (65 mM $CdCl_2$). Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

Moreover, during stress, a complex antioxidant system is trigged in plants to cope with damage because reactive oxygen species (ROS) are strongly induced. These stressors are normally produced in chloroplasts, mitochondria, and peroxisomes, and the balance between production and removal may be disturbed due to various abiotic stresses (Moller and Sweetlove, 2010). These molecules have the ability to cause oxidative damage to proteins, nucleic acids, and lipids, including the lipid constituents of cell membranes (Apel and Hirt, 2004; O'Brien et al., 2012). The change in composition of the chloroplast membranes and consequent chlorophyll degradation (Baier and Dietz, 2005; Sevengor et al., 2011; Hishida et al., 2014) causes lower levels of this pigment in water deficit and salt stress conditions in the genotypes with higher MDA content, and those that denote greater lipid peroxidation, such as WT and *fri* (Figs. 4 A and 7 A). On the other hand, the osmoregulator substances, mainly glycine betaine (Chen and Murata, 2011) accumulated in greater amounts in *tri* and *phyB2* mutants and may be responsible for maintaining the integrity of the membranes of chlorophyll molecules and the removal of reactive oxygen species (ROS) (Anjum et al., 2014).

Regarding Cd stress response, it is well known that Cd is not essential but is highly accumulated by plants, causing phytotoxicity and inhibition of plant growth (Azevedo et al., 2012). This inhibition mainly results from its deleterious effects in photosystems (Gill et al., 2012) and chlorophyll synthesis because Cd competes with Fe by carriers, reducing the accumulation of this pigment in the plant (Nazar et al., 2012). However, as far as we know, the only evidence for phytochrome control of heavy metal stress is from the heme oxygenase family (HO), which is required for phytochrome chromophore biosynthesis and is involved in metal stress response (Balestrasse 2008a,b; Cui et al., 2011, 2012; Shen et al., 2011). However, some detailed studies need to be refined. HO is considered an essential enzyme for the synthesis of the chromophores present in all phytochromes (Muramoto et al., 2002; He and He, 2014) in addition to being an important molecule that acts in plant responses to oxidative damage caused by various abiotic stresses, such as high salinity (Xie et al., 2011; Zilli et al., 2008) and heavy metal contamination (Balestrasse et al., 2008b; Han et al., 2008). Some studies have shown that the presence of $CdCl_2$ induced HO expression in soybean and alfalfa respectively prevents and mitigates the oxidative effects of heavy metals (Balestrasse et al., 2008b; Cui et al., 2012).

In Cd treatment, we observed that the largest hypocotyl length of *tri* confirmed that the *phyB1* mutation has a differential response to stress. Additionally, only *tri* showed a higher biomass when compared to WT (Fig. 2A and B). However, high levels of Cd accumulation in the roots caused an inhibitory effect in this organ in most plants, which indicates that phyB2 mutation provided greater length and accumulation of biomass in the roots as compared with WT. Therefore, it is suggested that phyB2 is involved in regulation of root growth (Fig. 2B), and there is biomass gain in plants exposed to $CdCl_2$ (Fig. 2D). In this study, we observed that the lack of phyA caused a reduction in root growth and dry biomass, which could support the hypothesis of participation of this phytochrome in response to metal contamination. Recently, the physiological and biochemical changes derived from the overexpression of Serine 599 Alanine-PhyA (S599A-PhyA) in the transgenic turfgrass plants caused significant improvement in terms of overall growth than the non-transgenic (NT) control plants when grown under heavy metal toxicity (Gururani et al., 2016).

Some studies suggested that osmoregulator molecules can also act in defense mechanisms that alleviate Cd toxicity in plants because this type of stress is often related to cell water loss (Gratão et al., 2008;

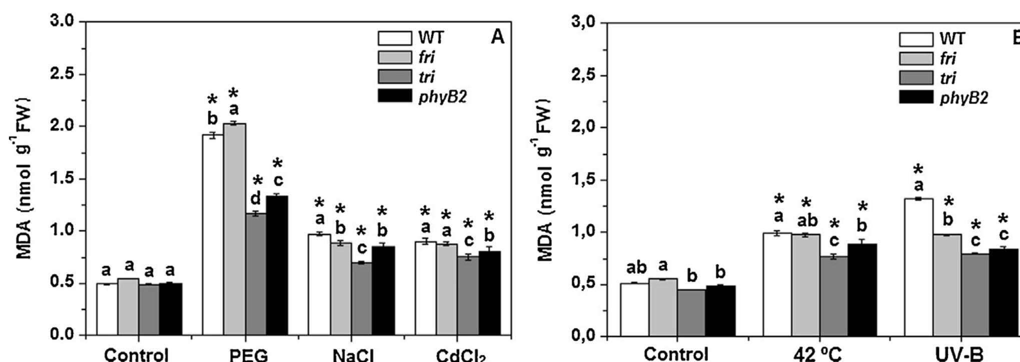

Fig. 7. Malondialdehyde (MDA) content as an index of lipid peroxidation in WT and tomato photomorphogenetic mutants treated with low water potential (PEG at Ψ_w of −0.3 MPa), high salt stress (100 mM NaCl) or cadmium contamination stresses (65 mM $CdCl_2$) (A), high temperature (42 °C for six hours during three days) or prolonged UV-B light exposure (UV-B − 305–310 nm for eight hours during three days) (B). Bars represent the average ± S.E. Letters on top of bars indicate significant differences between genotypes inside the same growth condition according to Tukey's test ($P < 0.05$). Asterisks (*) on top of bars indicate significant differences between the control and a specific stressful condition according to Tukey's test ($P < 0.05$).

Table 1
Summary of the results obtained in the present work compared to WT (square symbol). Arrows represent higher (↓) or lower (↑) values for a specific mutant when compared to the WT plants growing in the same condition. Values that do not differ from WT are represented by (−). Non-associated analysis to stress type (x).

Conditions	Genotypes	Analysis									
		Growth		Dry weight		Pigment content			Organic osmolytes		Stress indicator
		Shoot	Root	Shoot	Root	Chlorophyll	Carotenoids	Anthocyanin	Proline	Glycine betaine	MDA
Control	WT	■	■	■	■	■	■	■	■	■	■
	fri	–	↓	–	–	–	–	–	–	–	–
	tri	↑	–	–	–	–	–	–	–	–	–
	phyB2	–	–	–	–	–	–	–	–	–	–
PEG	WT	■	■	■	■	■	■	x	■	■	■
	fri	↑	–	–	–	–	↑	x	–	↑	↑
	tri	↑	↑	–	–	↑	↑	x	↑	↑	↓
	phyB2	↑	↑	–	–	↑	↑	x	↑	↑	↓
NaCl	WT	■	■	■	■	■	■	x	■	■	■
	fri	↑	↑	–	–	–	–	x	–	↓	↓
	tri	↑	↑	–	–	↑	↑	x	↑	–	↓
	phyB2	↑	↑	–	↑	↑	↑	x	↑	↑	↓
$CdCl_2$	WT	■	■	■	■	■	■	x	■	■	■
	fri	↑	↓	↑	–	–	↑	x	–	–	–
	tri	↑	–	↑	–	–	↑	x	–	–	↓
	phyB2	↑	↑	↑	↑	–	–	x	–	–	↑
42 °C	WT	■	■	■	■	■	■	■	x	x	■
	fri	–	–	–	–	–	–	–	x	x	–
	tri	↑	–	–	–	↑	↑	↓	x	x	↓
	phyB2	–	↑	–	–	↑	↑	↓	x	x	↓
UV-B	WT	■	■	■	■	■	■	■	x	x	■
	fri	↑	–	–	–	–	–	↓	x	x	↓
	tri	↑	↑	↑	↑	↑	↑	↓	x	x	↓
	phyB2	↑	↑	↑	↑	–	↑	↓	x	x	↓

Islam et al., 2009; Siripornadulsil et al., 2002; Sharma and Dietz, 2006). Moreover, osmoregulatory molecules such as proline can act as antioxidants (Zheng et al., 2015; Zouari et al., 2016). The present study was unable to determine a role for proline or glycine betaine accumulation in the studied phytochromes among genotypes in the presence of Cd upon comparison with the control condition (Fig. 6A and B). Although there was no change in osmolytes, we observed an increase in the MDA content in WT and *fri* (Fig. 7A), indicating that these genotypes showed severe lipid peroxidation in comparison with *phyB* mutants. Thus, alterations in the proteomics of Chlamydomonas algae may be caused by contamination with several metals (Zn, Cu, Fe, Co, Ni, As, Cd, and Cr), resulting in the upregulation of phytochrome B (Cid et al., 2010). However, to date, there have been no studies directly correlating the role of phytochromes in responses to stresses caused by metal contamination. Thus, the fact that *phyB* mutants have less MDA content and increased root dry weight in the presence of Cd as compared with WT indicates that the phytochrome probably regulates the response to Cd. This point raises questions about how phyB mediates enzymes responses during metal stress.

One of the most obvious environmental factors related to light is temperature. Taken together, light and temperature are two major stimuli that provide immediate cues regarding energy availability, day length, proximity of other species and seasonal changes (Lorenzo et al., 2016), and both are involved with phytochrome control (Foreman et al., 2011; Koini et al., 2009). In tomato, temperatures above 35 °C severely affect germination and seedling growth (Wahid et al., 2007). In this study, root growth was more severely affected than shoot growth in all genotypes in comparison with the control. In shoot, *tri* showed better hypocotyl growth than WT, while the root growth was superior in *phyB2* than in WT; however, none of them showed differences in dry weight. This absence of mass gain could be related to decreased photosynthetic activity since one of the organelle membranes most affected by high temperatures is the thylakoids, which are considered extremely labile cellular structures (Vacha et al., 2007). Besides, both chlorophylls, as other pigments belonging to the photosynthetic apparatus, as the carotenoids, may have its content affected by temperature increase (Grant et al., 2015). As expected, there was a decrease in the total chlorophyll and carotenoid content after high-temperature treatment as compared with the control condition, whereas WT and *fri* showed significantly lower values than those *phyB* mutants. Furthermore, an increase in temperature weakens plant metabolism and makes plants more susceptible to other abiotic factors, such as light incidence (Suzuki et al., 2014). Therefore, as a defense mechanism, there is an accumulation of flavonoids, which filter the excess radiation. As a result of the temperature increase, there was an increased anthocyanin content, which was higher in WT and *fri* (Fig. 5C). In this case, the highest accumulation of anthocyanins occurred in WT, followed by *fri* (Fig. 5C). It is well known that high temperature triggers oxidative stress, which disrupts biological membranes and leads to the loss of function. For this reason, the low MDA accumulation observed in phyB mutants could be related to maintenance of chloroplast membranes and consequently maintenance of pigment at high temperature.

It has been proposed that high radiation stress, such as UV-B radiation, can also be perceived by phytochromes (Boccalandro et al., 2001). In this study, UV-B radiation caused a substantial impact on seedling growth. Root growth was equally affected by the high radiation in both WT and *fri* (Fig. 3B); the response was also observed in biomass, in which *phyB* mutants stood out positively when compared to WT (Fig. 3D). Similar to the tolerance of *phyB* mutants to stress, as already discussed, UV-B radiation caused less deleterious effects on the growth of these genotypes as compared with WT. Mani and Guruprasad (2015) observed that when excluding the UV-B and UV-A spectra of sunlight using filters, WT had a greater biomass than *Arabidopsis phyB* and *phyAB* mutants, indicating the relationship between the UV receiver and the phytochrome B during growth under UV-B radiation. Moreover, the influence of negative control of phytochromes B1 and B2 in response to UV-B radiation in both length and root biomass (Fig. 3B and D), chlorophyll content (Fig. 5A), carotenoids (Fig. 5B), anthocyanins (Fig. 6B), and MDA (Fig. 7B) was clear, while the phyA mutant did not show significant differences compared with WT.

Considering that biomass gain depends on proper functioning of the

photosynthetic apparatus in conjunction with cellular homeostasis and that UV-B radiation is accompanied by the ROS formation, causing damage to membranes and macromolecules (Mittler et al., 2012; Questa et al., 2013), this stress treatment was expected to induce lipid peroxidation. WT and *fri* genotypes accumulated highest MDA amounts under UV-B radiation (Fig. 7B). Thus, it is likely that mutation in *phyB* genes triggers antioxidant responses to minimize the effects of ROS, as observed by Boccalandro et al. (2001); however, the mechanism of this phenomenon is not known. Under UV-B, only *tri* showed higher chlorophyll content compared with WT (Fig. 5A). The carotenoid content in WT and *fri* showed significantly lower values than *phy*B mutants, but this pigment increased under UV-B as compared with control (Fig. 5A and B). Despite chlorophyll damage induced by the high radiation excess, the synthesis of protective pigments such as anthocyanins is improved (Becatti et al., 2009). Interestingly, anthocyanin content was higher in WT than the other genotypes, suggesting a role for phyA, phyB1 and phyB2 in flavonoid accumulation. Rusaczonek et al. (2016) assumed that phyB of Arabidopsis is a positive regulator of PSII photochemistry, ROS signaling, and has a positive involvement not only in chlorophyll biosynthesis but also in other photosynthetic pigments under UV-C exposure. In another study about the role of phytochrome in responses triggered by UV-B, it was observed that phyB mutation inhibited the opening of cotyledons, while the mutation in phyA did not alter this variable (Boccalandro et al., 2001) suggesting that active phyB enhances de-etiolation response in Arabidopsis. Xie et al. (2012) demonstrated severe oxidative damage in a *hy1* mutant of Arabidopsis, which is defective in chromophore biosynthesis, and thus, severely deficient in phytochrome activities, was connected to a decrease in chlorophyll content and carotenoid/flavonoid metabolism and the downregulation of antioxidant defenses.

In the present paper, for the first time, we demonstrated the multifaceted role of the phytochromes on abiotic stress response in tomato using phyA, phyB1 and phyB2 mutants. It is clear that phyB(s) plays a fundamental role in the signaling pathway that mediates stress responses. Although phyB mutants' tolerance is apparently triggered by the modification of the oxidative stress system, it is necessary to address how and which molecular and biochemistry pathways induce the tolerance. In other words, this work will instigate intensive research in the next few years to provide evidence for the manipulation of phytochromes in agriculture.

Acknowledgments

We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granted to M.A.G.

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