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BASIC AREA - Article

The role of phytochromes in cadmium stress responses in tomato

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ABSTRACT: It is well known that phytochromes mediate a wide range of photomorphogenic processes in plants. In addition, many studies have demonstrated the involvement of phytochromes as part of abiotic stress signaling responses. However, little is known about cadmium (Cd) stress regulation by phytochromes. Thus, in this study, we used the phyA (*far red-insensitive; fri*), phyB1 (*temporary red-insensitive; tri*) and phyB2 (*phyB2*) tomato (*Solanum lycopersicum* L.) mutants to investigate the roles of these three phytochromes on Cd stress responses. The plants were grown over a 21-d period in the presence of Cd. We evaluated plant growth, Cd and chlorophyll content and anatomical changes in the leaves. The results indicated that all genotypes were affected by Cd and showed reduced growth of the shoots and roots, as well as reduced chlorophyll content. The accumulation of Cd was similar for all genotypes, and a higher Cd content was found in roots. Anatomical analysis of the vascular bundles revealed that *fri* and *tri* seem to be more disrupted by Cd. Overall, these results indicate that phytochromes do not determine Cd stress tolerance in tomato plants.

Key words: abiotic stress, heavy metal, mutants, plant anatomy, *Solanum lycopersicum*. L.

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INTRODUCTION

Plants possess photoreceptors that compose sophisticated light-sensing mechanisms that are localized inside and outside the chloroplasts and the nucleus (Hiltbrunner et al. 2005). Among photoreceptors, the phytochrome family stands out as sensors for red (R; ~ 660 nm) and far-red (FR; ~ 730 nm) light (Essen et al. 2008; Yang et al. 2008). The phytochrome holoprotein consists of an apoprotein with a covalently linked linear tetrapyrrole chromophore, and this complex regulates the expression of a large number of light-responsive genes and thus influences many photomorphogenic events (Neff et al. 2000; Quail 2002a,b; Franklin and Quail 2010). In plants, a small family of PHY genes encodes phytochromes. For example, five phytochrome genes are known in *Arabidopsis thaliana* (*PHYA* to *PHYE*) and in tomato (*PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF*), and three *PHY* genes are known in rice (*Oryza sativa*) (*PHYA* to *PHYC*) (Bae and Choi 2008).

The involvement of phytochromes in controlling plant growth and development is well documented (Franklin 2016; Wit et al. 2016). Additionally, these photoreceptors have been shown to act as key modulators of both biotic and abiotic stresses (Carvalho et al. 2011a; Zao et al. 2014; D'Amico-Damião et al. 2015). These reports have led to a series of studies exploring the molecular and biochemical basis by which phytochromes mediate stresses, such as drought (D'Amico-Damião et al. 2015), salinity (Balestrasse et al. 2008a), high light (Boccalandro et al. 2001) or heavy metals (Cui et al. 2011). For instance, studies of *A. thaliana* under cold conditions demonstrated that light signaling by phytochrome B is involved in plant responses to cold via the expression of C-repeat binding factor (*CBF*) genes (Kim et al. 2002) and to biotic stress via the activation of lipoxygenase (Zao et al. 2014). In addition, rice phytochrome B-deficient mutant (*phy*B) plants were more tolerant to drought than the wild type. These plants exhibited reduced stomatal density and length, resulting in a decreased transpiration rate that enhanced drought tolerance (Liu et al. 2012). Similarly, the phyB-deficient mutant of tomato exhibited increased drought tolerance after five days without irrigation (D'Amico-Damião et al. 2015).

Another relevant type of abiotic stress experienced by plants under certain conditions is heavy metals. The increase in the cellular heavy metals are one of the main classes of abiotic stress agents for living organisms because of their high level of bioaccumulation and toxicity (Verkleij et al. 2009; Nagajyoti et al. 2010). The increase in heavy metal concentrations in the cellular environment can disturb several signaling pathways and cause irreversible damage to biological systems (Verkleij et al. 2009; Rossato et al. 2011). Among heavy metals, Cd represents one of the most damaging metals to plants. At high concentrations, this heavy metal can generate reactive oxygen species (ROS) such as superoxide radicals (O_2^{-}), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^{-}), which can disrupt the plant defense system (Gratão et al. 2012; Gratão et al. 2015).

Shen et al. (2011) demonstrated that the overexpression of *BnHO-1*, which is involved in the biosynthesis of heme oxygenase (HO), a key enzyme in the biosynthesis of the chromophore, provided tolerance to mercury (Hg) in *Brassica napus*, indicating that phytochromes are involved in metal-induced signaling pathways. Additionally, Jin et al. (2013) reported involvement of HO-1 in the alleviation of Cd toxicity in root tissues of alfalfa (*Medicago sativa*). Likewise, soybean (*Glycine max* L.) and alfalfa plants exposed to Cd-stressful conditions exhibited the upregulation of heme oxygenase-1 (HO-1) (Balestrasse et al. 2008b; Cui et al. 2011).

However, direct evidence for the involvement of phytochromes with plant responses to heavy metals are still missing. Therefore, in order to investigate whether phyA and phyB regulate events in Cd-stressful conditions, we assessed phenotypical and anatomical changes in *fri*, *tri* and *phyB2* tomato mutants exposed to elevated cadmium concentrations.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of the tomato (*S. lycopersicum* L.) mutants *fri* (*far red-insensitive*), *tri* (*temporary red-insensitive*) and *phyB2*, and their wild-type counterpart (WT) (cv. Moneymarker, MM) were germinated in boxes containing a mixture of 1:1 (by volume) commercial potting mix (Plantmax HT Eucatex, Brazil) and vermiculite. To avoid premature plant death by cadmium, seven-day-old plants were transferred to hydroponic conditions with Hoagland's nutrient solution initially containing 50 μM·$CdCl_2$. Fourteen days after →

sowing, the plants received 100 μM·$CdCl_2$ for seven more days for a total of a 21-day period. Control plants were grown in Hoagland's solution without $CdCl_2$. The nutrient solution with or without $CdCl_2$ was changed weekly, and the pH was maintained at approximately 5.6. The plants were maintained in a greenhouse with an average mean temperature of 27 °C under a 12 h photoperiod.

Cadmium content

Assays for the amount of Cd in the plant tissues were performed following digestion with a mixture of nitric and perchloric acids, according to Malavolta et al. (2011). Cd concentration was measured using flame atomic absorption spectroscopy with a Perkin Elmer spectrometer model 310. Root to shoot Cd transport of tomato genotypes was assessed using a Cd translocation index (TI), which was calculated as the percentage of the total Cd accumulation that was translocated to the shoots (Usman et al. 2012).

Growth analysis

The leaf area was measured using an Image Analysis System (Delta-T Devices, Cambridge, UK). Root area, diameter, length and density were all recorded using a scanner (Hewlett Packard 5c), and the image of each plant was analyzed by Delta-T Scan software. Subsequently, the plants were weighted for fresh mass. Next, roots and shoots were oven-dried at 60 °C for 72 h. The dry weight of roots and shoots was determined using an analytical balance (Adventurer; Ohaus, Shangai, P. R. China).

Anatomical analysis

A series of anatomical analysis were performed by sampling a middle portion of the leaf blade (3 × 3 mm) from selected leaves at the third node in four replications. The samples were fixed in FAA 50 (formaldehyde + acetic acid + 50% alcohol) for approximately 48 h and stored in 70% alcohol, according to Johansen methods (1940).

Permanent slides were prepared using the traditional methods of ethyl dehydration (85, 95 and 100%) for two hours at each concentration and were embedded in paraffin (Johansen 1940). The material was cut using a precision microtome (Leica RM2065) into 10 mm thick slices with disposable steel razors, yielding a transverse leaf series. The sections were stained with 0.05% toluidine blue (O'Brien et al. 1964) and assembled using synthetic Canada balsam.

The leaf anatomical structures were observed using 10 × and 40 × magnification of a binocular optical microscope (Bel photonics, Bio2 SSI model) and measured using a micrometric objective. The photomicrographs were taken with a trinocular optical microscope (Bel photonics Solaris) coupled with a USB digital camera (5.0 MP Bel). The integrity of the leaf tissue, the thickness of the leaf and palisade, the spongy parenchyma tissues and the diameter of the vascular bundles were evaluated.

Chlorophyll content

Leaf chlorophyll content was determined using a Minolta SPAD-502 m, which measures leaf transmittance at two wavelengths: red (approximately 660 nm) and near-infrared (approximately 940 nm). SPAD readings were taken from the terminal leaf of the fourth leaf from the base of the shoot. The SPAD sensor was randomly placed only on the leaf mesophyll tissue, avoiding the veins.

Chlorophyll fluorescence measurement

The fluorescence emission of chlorophyll (Fv/Fm) was measured using a pulse amplitude modulated fluorometer (FMS2, Hansatech Instruments, Pentney King's Lynn, U.K.). The leaves were exposed to darkness for fifteen minutes and were handled with tweezers before fluorescence estimation, as described by Saijo et al. (2000).

Statistical analysis

The experimental design was randomized with twelve plants from three replicate pots, and the results were expressed as the mean and standard error of the mean (± SEM). The means were compared using Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

After 21 days of experiment, Cd accumulation exhibited a similar pattern in all investigated genotypes (Figure 1a). In non-stressful conditions, there was no Cd accumulation (data not shown), whereas after exposure to Cd stress, high →

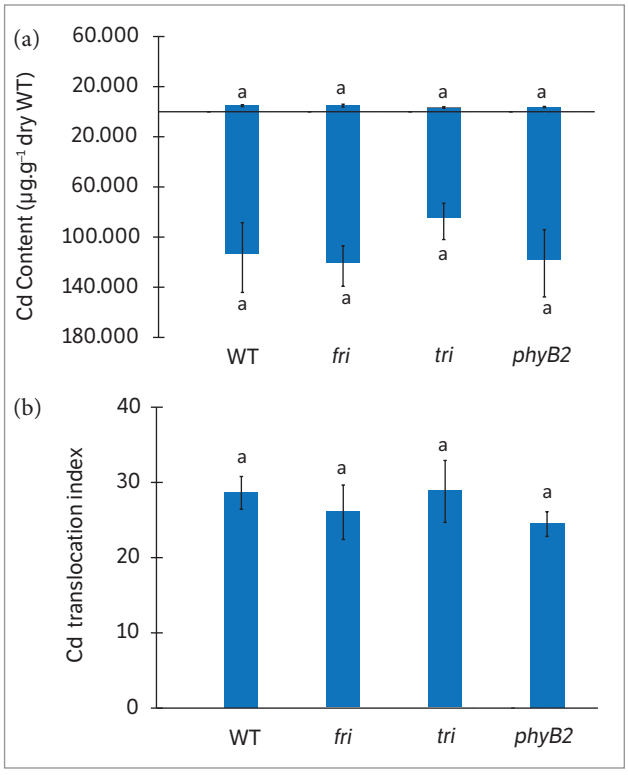

Figure 1. (a) Cadmium measurements in 21 days-old tomato mutants and WT grown with 100 µM·$CdCl_2$ in the coordinate axis, values above and below the 0 corresponds to the cadmium shoot and root content, respectively; (b) Cd translocation index. Data are means ± SEM (n = 3). The same lowercase letters on the bars of each panel indicate non-significant differences at p ≤ 0.05 by Tukey's test.

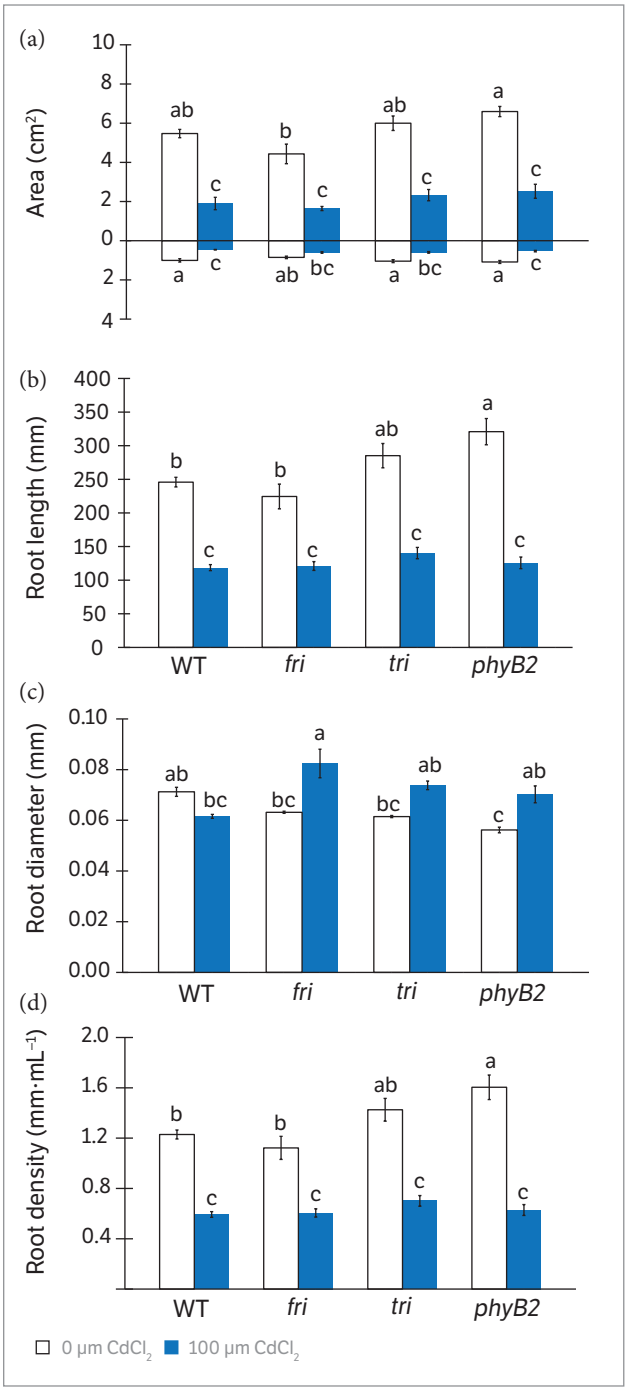

Figure 2. Leaf and root measurements in 21 days-old tomato mutants and WT grown without or with 100 µM·$CdCl_2$. (a) in the coordinate axis, values above and below the 0 corresponds to the leaf and root area, respectively; (b) Root length; (c) diameter and (d) and density. Control plants of each genotype did not receive Cd. Data are means ± SE (n = 3). Different lowercase letters on the bars of each painel indicate significant differences at p ≤ 0.05 by Tukey's test.

accumulation of Cd was observed in both shoots and roots. This accumulation was more pronounced in roots and was not affected by the genotype (Figure 1). In addition, the translocation index was similar for all genotypes (Figure 1b).

The presence of cadmium in the nutrient solution decreased leaf and root area and the length of roots in all genotypes (Figure 2a,b). Although Cd accumulated mainly in roots, we observed an increase in root diameter in *fri* and *phyB2*, whereas WT and *tri* did not show differences between the control and Cd treatments (Figure 2c). In fact, exposure to Cd inhibited root length, area and density in all genotypes (Figure 2).

After treatment with $CdCl_2$, WT, *fri* and *tri* exhibited lower shoot fresh weight, while no significant change in root biomass was detected (Figure 3a). However, only WT and *tri* suffered reduction of shoot dry weight after Cd exposure (Figure 3b). Plants cultivated in $CdCl_2$ did not exhibit a reduction in the root dry weight, but *phyB2,* showed an increase in dry weight after exposure to $CdCl_2$ (Figure 3b). In addition, Cd exposure caused a strong height reduction in WT, *fri* and *tri*, while plant height was unaltered by high Cd in *phyB2* (Figure 3c). However, in the absence of Cd, *phyB2* and *fri* had similar heights as WT, whereas *tri* was taller.

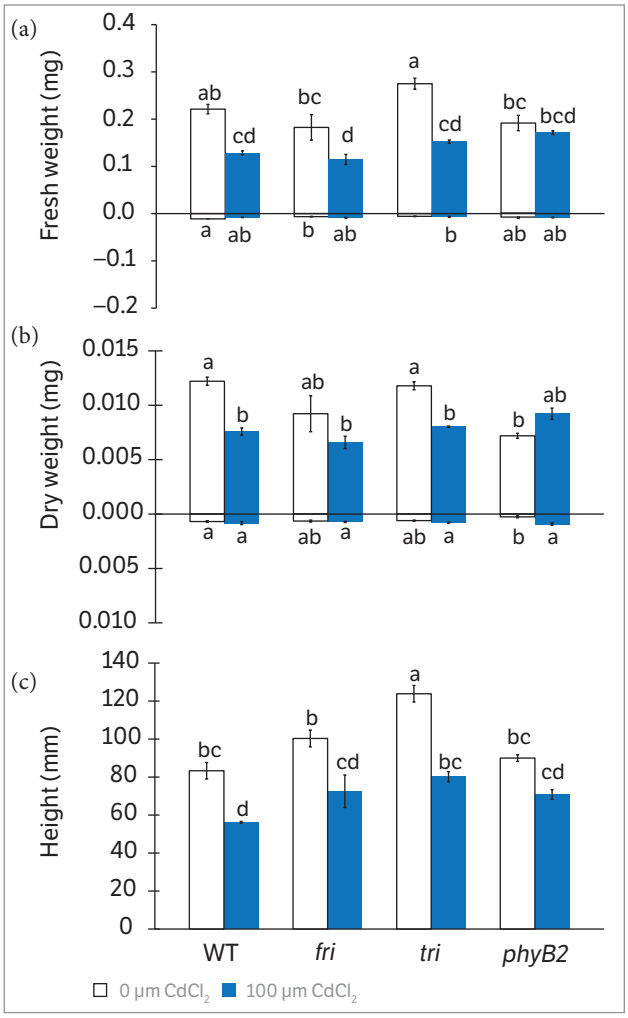

Figure 3. Leaf and root measurements in 21 days-old tomato mutants and WT grown without or with 100 µM·$CdCl_2$. (a) in the coordinate axis, values above and below the 0 corresponds to the shoot and root fresh weight, respectively; (b) values above and below the 0 corresponds to the shoot and root dry weight, respectively and (c) shoot height. Control plants of each genotype did not receive Cd. Data are means ± SE (n = 3). Different lowercase letters on the bars of each panel indicate significant differences at $p \leq 0.05$ by Tukey's test.

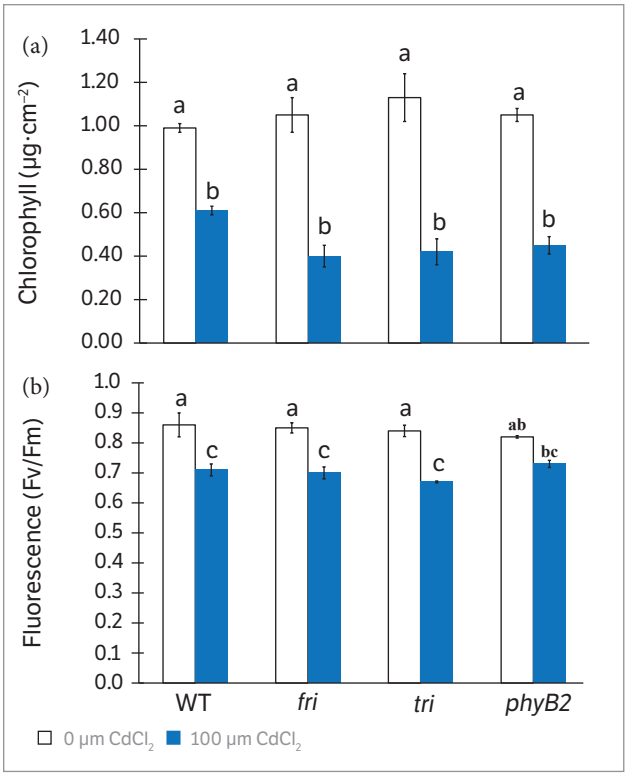

Figure 4. Chlorophyll and fluorescence measurements in 21 days-old tomato mutants and WT grown without or with 100 µM $CdCl_2$. (a) chlorophyll content and (b) fluorescence. Control plants of each genotype did not receive Cd. Data are means ± SE (n = 3). Different lowercase letters on the bars of each panel indicate significant differences at $p \leq 0.05$ by Tukey's test.

The supply of $CdCl_2$ significantly decreased chlorophyll content in all genotypes, especially in the phytochrome mutants (Figure 4a). Additionally, there was a clear reduction of chlorophyll fluorescence in WT, *fri* and *tri,* while no significant change was observed in Cd-treated *phyB2* plants (Figure 4b).

All genotypes showed leaf blades with unseriated adaxial and abaxial epidermis, palisade parenchyma presenting one layer of cells, spongy parenchyma thicker than the palisade parenchyma and evident intercellular spaces. The vascular bundles were trapezoidal and surrounded by parenchyma cells in both the adaxial and abaxial sides of the leaf (Figures 5a-f).

The *fri* and *phyB2* plants (without the addition of $CdCl_2$) had a large amount of parenchyma cells above the vascular bundles, which was not observed in these same genotypes when exposed to Cd, indicating a reduction in the number of cells of this tissue in this area. After the other genotypes were analyzed, we observed that there was no difference in the parenchymal cell density both above and below the vascular bundles. The *tri* genotype usually had a prominent apical cell above the parenchyma of the vascular bundles (Figure 5).

When we looked at the amount of parenchyma cells located below the vascular bundles in the midrib of the analyzed leaves, we also noticed a reduction in the number of these cells in the *fri* and *tri* genotypes supplied with Cd. The vascular bundles of the *fri* genotype showed a smaller diameter in plants undergoing Cd stress, while only small differences could be observed in the diameter of vascular bundles for the remaining genotypes (Table 1). There was a decrease in the number of xylem cells in all genotypes except *phyB2* in Cd stress conditions. The significant decrease in the number of xylem cells in *fri* plants, as well as the large →

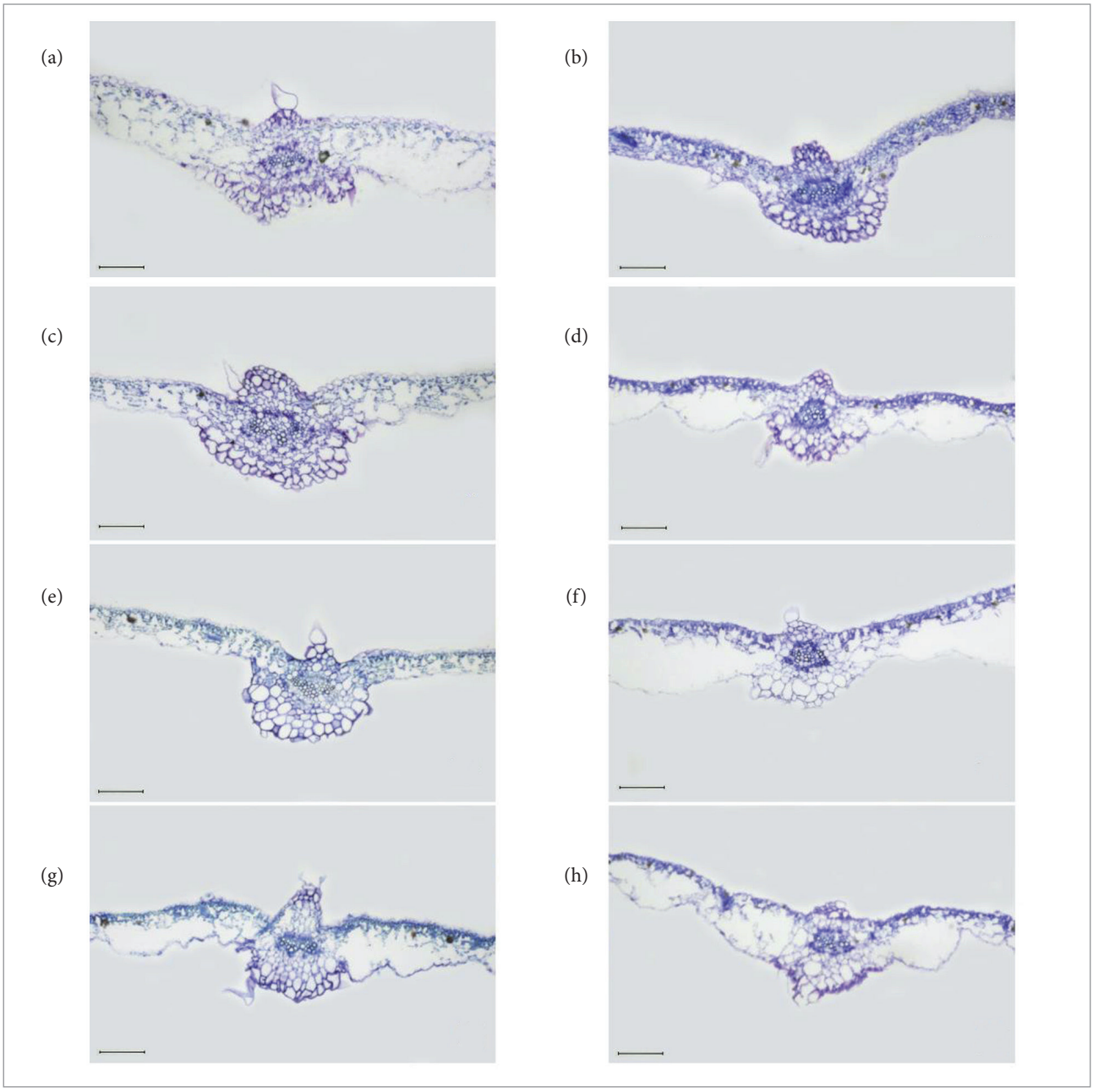

Figure 5. Anatomical analysis in 21 days-old tomato mutants and WT grown without or with 100 µM·$CdCl_2$ (a) WT without Cd; (b) WT with Cd; (c) *fri* without Cd; (d) *fri* with Cd; (e) *tri* without Cd; (f) *tri* with Cd; (g) *phyB2* without Cd and (h) *phyB2* with Cd. Scales = 30 µm.

number of tiny cells of xylem in *tri* and *fri* before Cd contact, were clearly noted (Table 1).

It is increasingly evident that phytochromes play a key role in abiotic stress responses (Zilli et al. 2008; Carvalho et al. 2011a; Xie et al. 2011). In this study, we used the phyA (*fri*), phyB1 (*tri*), and phyB2 (*phyB2*) mutants of tomato to evaluate whether some of these phytochromes were involved in plant responses to Cd stress conditions. D'Amico-Damião et al. (2015) reported that phytochrome B mutants grow better under drought conditions. Thus, better plant development of these genotypes may be expected in Cd conditions.

However, all plants except *phyB2* exhibited reduced shoot fresh and dry weight and reduced height in the presence of Cd (Figures 2a-c). Although this observation could suggest that *phyB2* was tolerant to Cd stress, these values were similar to those of the WT plants in the same conditions, suggesting that *phyB2* plants grow slower in non-stressful conditions. →

Table 1. Anatomical comparison among 21 days-old tomato mutants and WT grown without or with 100 µM·$CdCl_2$. *Signs ++, +- and -- indicating, respectively, greater, middle and less integrity, diameter or amount of cells from 21 days-old tomato mutants and WT grown without or with 100 µM·$CdCl_2$.

Characteristics	$CdCl_2$ (µM)	WT	*fri*	*tri*	*phyB2*
Parenchymal integrity	0	−−*	+−	++	−−
	100	++	−−	−−	−−
Parenchyma above the vascular bundles	0	+−	++	+−	++
	100	+−	+−	+−	+−
Parenchyma below the vascular bundles	0	++	++	++	+−
	100	++	+−	+−	+−
Diameter of the vascular bundles	0	+−	++	+−	+−
	100	+−	−−	+−	+−
Number of xylem cells	0	++	++	++	+−
	100	+−	−−	+−	+−

On the other hand, Cd induced a larger root diameter in all photomorphogenic mutants (Figure 1c), especially *fri,* which had a larger root diameter than WT, but there was no increase in root dry weight (Figure 2b). These results can be explained by the impairment of root elongation and phytochrome involvement in root development. In fact, it has been observed that phytochromes triggered the expression of *ELONGATION HYPOCOTYLS 5* (*HY5*) and, in turn, the HY5 protein acted as a transcription factor and promoted root growth in response to light (Lee et al. 2016). In addition, it was verified that plants treated with Cd exhibited an enlargement of the roots and a reduction in root elongation (Fusconi et al. 2007; Maksimovic et al. 2007). Changes in root development in response to Cd exposure could be due to negative effects on the root cell microtubule cytoskeleton, which plays a crucial role during root development (Fusconi et al. 2007). However, more studies are needed to clarify these issues.

Additionally, *tri* was taller than WT in both control and stressful conditions, but similar shoot dry weight was verified, indicating no difference in biomass accumulation (Figure 2). Indeed, it was demonstrated that *phyB1* plays a key role in stem elongation and shade avoidance responses (Schrager-Lavelle et al. 2016). Thus, *tri*, a *phyB1*-deficient mutant, exhibited a pronounced elongation of the stem due to impaired light perception (Figure 2c).

The Cd accumulation and translocation index were similar in all genotypes (Figure 1). Despite this similarity, *fri* plants showed a reduced number of xylem cells and a reduced diameter of the vascular bundles compared to WT plants after Cd exposure (Table 1). The same result was observed by Auge et al. (2012), whose hand-cut stem cross-sections showed a lower xylem vessel number and transversal area in *fri* plants under high evaporative demand. This reduction in xylem caused a reduction in the water supply to the leaves (Auge et al. 2012) and was expected to affect the transport of Cd from the roots to the shoot. In fact, Cd enters into the root system through the symplast or apoplast and eventually reaches the xylem, where it can be translocated to shoots (Lux et al. 2011). Furthermore, Cd translocation in the roots is impaired by the barriers in the exo- and endoderm (Redjala et al. 2011). Therefore, the destabilization of the root tissue facilitates the diffusion of Cd and its loading in the xylem, thus affecting Cd transport from the roots to the shoot (Lux et al. 2011). In addition, Cd accumulation in the shoot causes severe damage to plant physiological processes such as photosynthesis, water relations, mineral metabolism and leaf morphology (Lopez-Chuken and Young 2010; Gill et al. 2012; Asgher et al. 2015). In our study, we observed Cd in the shoots of all genotypes (Figure 1), and there was also a clear disruption in the xylem and parenchymal tissues (Figure 5 and Table 1), as well as a reduction in chlorophyll content and fluorescence, independent of genotype (Figure 4), which were most likely due to instability in the chloroplast membrane due to the Cd accumulation.

In order to assess how Cd exposure affects plant growth and development, the anatomical structure of leaves was investigated. So, Cd accumulation and the loss of phytochrome function in the mutants induced clear cell disorganization caused by stress conditions. In addition, we observed increased cell and vascular bundle disorganization in all genotypes, but mainly in mutants (Figure 5 and Table 1). More recently, it has been demonstrated that phytochromes play a pivotal role in cell division and cell number in the leaf blade (Tsukaya et al. 2002; Nishihama et al. 2015). For example, the phyB mutant of *Arabidopsis* exhibited lower leaf size and cell number in the leaf mesophyll compared to the WT (Tsukaya et al. 2002). In addition, several studies have proven that Cd has prejudicial effects on cell ultrastructure and leaf anatomy (Ali et al. 2015; Plusquin et al. 2015; Vaculik et al. 2015). For instance, in Cd stress conditions, ethylene- and auxin-insensitive tomato mutants showed reduced mesophyll cell size, smaller palisade parenchyma cells and disorganization of the chloroplasts (Gratão et al. 2009).

In fact, our results could indicate the control of these responses by phytochromes. Clear anatomical differences were observed between the tested phytochrome mutants. For instance, *fri* and *phyB2* exhibited more parenchyma cells above the vascular bundles, while *tri* had a prominent apical cell in the parenchyma above the vascular bundles. However, under Cd stress conditions, both parenchymal integrity and parenchyma cells above or below the vascular bundles were severely reduced in *fri* compared to WT plants (Figure 5 and Table 1).

CONCLUSION

Despite mounting evidence that phytochromes are involved in plant responses to several abiotic stresses (Carvalho et al. 2011b; Auge et al. 2012), none of the three phytochrome mutants assessed in the present study exhibited higher tolerance to Cd. Thus, taken together, these results indicate that phytochromes do not integrate the pathway of Cd stress response in tomato plants.

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