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ORIGINAL ARTICLE

Abscisic acid-deficient *sit* tomato mutant responses to cadmium-induced stress

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Abstract There is a very effective cross-talk between signals triggered by reactive oxygen species and hormonal responses in plants, activating proteins/enzymes likely to be involved in stress tolerance. Abscisic acid (ABA) is known as a stress hormone that takes part in the integration of signals. This work aimed to characterize the biochemical response and ultrastructural changes induced by cadmium (Cd) in the Micro-Tom (MT) *sitiens* ABA-deficient mutant (*sit*) and its wild-type (MT) counterpart. MT and *sit* plants were grown over a 96-h period in the presence of Cd (0, 10, and 100 μM $CdCl_2$). The overall results indicated increases in lipid peroxidation, hydrogen peroxide content and in the activities of the key antioxidant enzymes such as catalase, glutathione reductase, and ascorbate peroxidase in both genotypes. On the other hand, no alteration was observed in chlorophyll content, while the activity of another antioxidant enzyme, superoxide dismutase, remained constant or even decreased in the presence of Cd. Roots and shoots of the *sit* mutant and MT were analyzed by light and transmission electron microscopy in order to characterize the structural changes caused by the exposure to this metal. Cd caused a decrease in intercellular spaces in shoots and a decrease in cell size in roots of both genotypes. In leaves, Cd affected organelle shape and internal organization of the thylakoid membranes, whereas noticeable increase in the number of mitochondria and vacuoles in MT and *sit* roots were observed. These results add new information that should help unravel the relative importance of ABA in regulating the cell responses to stressful conditions induced by Cd apart from providing the first characterization of this mutant to oxidative stress.

Keywords Oxidative stress · Phytohormones · Reactive oxygen species · Root and leaf anatomy · *Solanum lycopersicum* · Ultrastructure

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Introduction

Cadmium (Cd) is a non-essential element, being considered the most toxic among the heavy metals, negatively affecting plant metabolism mainly by inducing an oxidative stress condition (Cuypers et al. 2010; Dourado et al. 2013, 2015; Gratão et al. 2015). Cd is naturally present in the environment in trace concentrations, or can be introduced through anthropogenic activities, such as the use of fertilizers, pesticides, and industrial and domestic effluents, being absorbed by plants from contaminated soil or water (Al-Khateeb and Al-Qwasemeh 2014; Su et al. 2014). Recently, the use of nanoparticles containing heavy metal elements in commercial products and industrial applications has significantly increased and raised concerns on their adverse effects on human and environmental health (Arruda et al. 2015). Moreover, Cd has high mobility in the soil–plant system affecting biochemical mechanisms by reducing the redox balance control, causing plasma membrane rupture, which culminates in loss of its function (Gallego et al. 2012; Iannone et al. 2015). This metal can also induce serious disturbances in physiological processes such as

photosynthesis, respiration, plant-water relations, mineral uptake, among others (López-Chuken and Young 2010; Gill et al. 2012), leading to reduced plant growth or even cell death (Shekhawat et al. 2010; Hu et al. 2013; Moradi and Ehsanzadeh 2015).

Structural changes have also been associated with Cd accumulation in plant tissues (Vitória et al. 2003, 2006; Maksimović et al. 2007; Gratão et al. 2009; Lux et al. 2011; Mondal et al. 2013). Structural disorganization in leaves, reduced intercellular spaces, altered thylakoid structure, wilted epidermis, thinner cell walls, cytoplasm condensation due to increase in matrix density, mitochondrial cristae reduction, high level of chromatin condensation, nuclear envelope disruption, mitotic index decrease, and chromosomal aberrations have been observed for a wide range of plant species (Djebali et al. 2005; Vitória et al. 2006; Gratão et al. 2009; Ali et al. 2013a, b).

The molecular oxygen (O_2) naturally present in cells promotes the production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•), which are continuously produced by cells in plants and other organisms under normal or stressful conditions (Peters et al. 2014). Metals can enhance ROS production, which react with several cellular compounds, leading to damage to the cell system and even cell death (Gratão et al. 2015).

The cell redox balance may be maintained through the action of antioxidant mechanisms, which are capable, depending on the level of the stress, of preventing uncontrolled oxidation through the removal of ROS (Azevedo et al. 2011; Hippler et al. 2015). These mechanisms may involve enzymatic and/or nonenzymatic systems. The enzymatic mechanisms of ROS scavenging include the action of a number of enzymes, such as superoxide dismutase (SOD), which catalyzes $O_2^{\bullet -}$ into H_2O_2 (Gratão et al. 2008a). Subsequently, H_2O_2 may be detoxified into H_2O by ascorbate peroxidase (APX) and catalase (CAT), among other peroxidases (Wu et al. 2015). The nonenzymatic antioxidants include ascorbic acid and glutathione, as well as vitamins, flavonoids, alkaloids, and carotenoids (Gratão et al. 2005; Alcântara et al. 2015). Reduced glutathione (GSH) is oxidized to glutathione (GSSG), whereas ascorbate is oxidized into monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Through the ascorbate–glutathione cycle, GSSG, MDHA, and DHA may be reduced and form GSH and ascorbate again (Nogueirol et al. 2015).

It is also known that oxidative stress is highly controlled by plant hormones and there are many studies showing the interaction between ROS and/or antioxidants and plant hormones (Gratão et al. 2012; Monteiro et al. 2011, 2012; Carvalho et al. 2013; Cai et al. 2015; Schellingen et al. 2015; Soares et al. 2016). Although these multiple stress responses are essential for plant survival under heavy metal-stress conditions, the exact role of phytohormones in these responses is still not clear, especially the cross-talk among ROS, phytohormones and antioxidant systems (Asgher et al. 2015).

MT plants feature genetic variations and natural hormonal mutations, such as the deficiency in functional enzyme activity at the final step in abscisic acid (ABA) biosynthesis observed in the hormonal mutant *sit* (Taylor et al. 1988; Mäkelä et al. 2003; Harrison et al. 2011). ABA is often referred as a stress hormone due to its main involvement in biotic stress responses (Harrison et al. 2011). Therefore, plants can constantly adjust ABA levels in response to physiological and environmental changes, especially from root signaling (Cutler et al. 2010). ABA can also be accumulated in roots when subjected to external stresses such as drought, salinity, and nutrient deficiency (or excess), serving as a long distance signal to regulate many adaptive responses in plants, such as compatible solutes accumulation, root modification, stomatal closure, and induction of the antioxidative stress system (Hartung et al. 2005). Therefore, it is not surprising that ABA is also involved in the stress response to heavy metals contamination. For example, Shi et al. (2015) has recently shown that ABA was able to alleviate zinc toxicity in poplar (*Populus* × *canescens; syn. Populus tremula* × *P. alba*). ABA-signaling genes, such as *PYLs*, were generally sensitive during copper stress in cucumber (*Cucumis sativus*) (Wang et al. 2014). ABA has also been shown as a component of stress response induced by Cd. In fact, ABA induced tolerance in wheat (*Triticum aestivum*) seedlings subjected to Cd stress (Han et al. 2012), while potato (*Solanum tuberosum*) exposed to Cd exhibited increased ABA content (Stroiński et al. 2013).

In this study, we tried to further explore the interaction between ABA and Cd in tomato plants, which is the most important vegetable crop species in the world. The Micro Tom *sitiens* ABA-deficient mutant (*sit*) and its wild-type (MT) counterpart were used to characterize the biochemical and ultrastructural changes induced by Cd toxicity. As far as we know, the response of this mutant to heavy metals-induced stress has never been reported.

Materials and methods

Seeds of tomato (*Solanum lycopersicum* L.) cv Micro-Tom (MT) and MT *sitiens* ABA-deficient mutant (*sit*) were sown in trays containing vermiculite supplemented with Hoagland and Arnon (1950) nutrient solution at 20 % ionic strength. After 30 days the tomato plants were transferred to 12-L trays containing Hoagland's nutrient solution at 10 %. This system is similar to a greenhouse hydroponic system used by tomato producers and was previously reported by Piotto et al. (2014) in a system for the isolation of Cd-resistant mutants. The nutrient concentrations were increased daily for 3 days to allow plant adaptation. On the fourth day, before Cd addition, the nutrient solution was changed to 50 % ionic strength.

Chlorophyll measurements were carried out in MT and *sit* mutant leaves. Roots and shoots were then collected, rinsed in distilled water and stored at −80 °C for further biochemical analysis (T0). Subsequently, the solution was supplemented with 0, 10, and 100 μM $CdCl_2$. These concentrations were chosen based on previous results with MT from our research group (Gratão et al. 2009, 2012; Monteiro et al. 2011). Ninety-six hours after Cd addition, leaves were used for chlorophyll measurements and then, roots and shoots were collected, rinsed in distilled water and stored at −80 °C for further biochemical analysis.

Dry weight and Cd concentration determination

Ninety-six hours after Cd addition to the nutrient solution, plants were collected, separated into shoots and roots, and dried in a forced-air oven at 65 °C for 72 h, when the dry weight was recorded. Cd concentration in roots and shoots was determined by digestion with a mixture of nitric and perchloric acids as described by Malavolta et al. (1997), followed by a quantitative analysis using a flame atomic absorption spectroscopy with a Perkin Elmer spectrometer, model 310. Cd concentrations were expressed in milligram of Cd per gram dry weight.

Chlorophyll content determination

A Minolta SPAD-502 meter, which measures leaf transmittance at two wavelengths, red (660 nm, approximately) and near infrared (940 nm, approximately), was used to determine the leaf chlorophyll content. SPAD readings were taken weekly on the terminal leaflet of the fourth leaf from the base of the shoot. The SPAD sensor was randomly placed on the leaf mesophyll tissue, avoiding the veins (Monteiro et al. 2011).

Lipid peroxidation

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substance (TBARS). This method was modified as follows: 200 mg of roots and shoots were grounded in a mortar under liquid nitrogen and homogenized in a solution containing 0.1 % (*v*/*v*) trichloroacetic acid (TCA) and 20 % (*w*/*v*) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 10,000×*g* for 10 min, and then 250 μL of the supernatant were added to a solution composed of 1 mL of 0.5 % (*v*/*v*) 2-thiobarbituric acid (TBA) plus 20 % (*v*/*v*) TCA. The mixture was incubated in a dry bath at 95 °C for 30 min. The homogenate was placed in an ice bath, maintained for 10 min, and centrifuged at 10,000×*g* for 10 min to separate the residues formed during the heating process. Malondialdehyde (MDA) was monitored by measurements at 535 and 600 nm and the concentration calculated using an extinction coefficient of 1.55×10^{-5} mol^{-1} cm^{-1}. MDA content was expressed in nmol g^{-1} fresh weight (Arruda et al. 2013).

Hydrogen peroxide content determination

Samples of roots and shoots from each treatment were macerated in liquid nitrogen and homogenized in 0.1 % (*w*/*v*) TCA. The homogenate was centrifuged at 10,000×*g* for 20 min at 4 °C, and then 200 μL of 100 mM potassium phosphate buffer (pH 7.0) and 800 μL of 1 M potassium iodide (KI) were added. The reaction medium was maintained in the dark in an ice bath for 1 h. The absorbance was then read at 390 nm. The hydrogen peroxide (H_2O_2) content was determined using H_2O_2 as a standard (Rendón et al. 2013).

Antioxidant enzymes activities determination

For enzyme extraction and protein determination, roots and shoots samples were homogenized in buffer volume/fresh weight (2:1) in a mortar with a pestle in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DTT, and 4 % (*w*/*v*) insoluble PVPP. The homogenate was centrifuged at 10,000×*g* for 30 min, and the supernatant was stored in separate aliquots at −80 °C. All steps were carried out at 4 °C unless stated otherwise (Gratão et al. 2012). Protein concentration for all samples was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Catalase (CAT, EC 1.11.1.6)

CAT activity determination (μmol min^{-1} mg^{-1} protein) was assayed at 25 °C in a reaction mixture containing 1 mL 100 mM potassium phosphate buffer (pH 7.5) and 25 μL H_2O_2 (30 % solution). The reaction was initiated by the addition of 25 μL of protein extract, and the activity determined by following the decomposition of H_2O_2 as changes in absorbance at 240 nm, over 1 min (Monteiro et al. 2011).

Glutathione reductase (GR, EC 1.6.4.2)

GR activity determination (μmol min^{-1} mg^{-1} protein) was carried out spectrophotometrically at 30 °C in a mixture consisting of 1 mL 100 mM potassium phosphate buffer (pH 7.5), 0.5 mL 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) 3 mM, 0.1 mL NADPH 2 mM, 0.1 mL GSSG

20 mM, and 50 μL of protein extract. The reduction rate of oxidized glutathione was followed by monitoring the change in absorbance at 412 nm over 1 min (Carvalho et al. 2013).

Ascorbate peroxidase (APX, EC 1.11.1.11)

APX activity (μmol min^{-1} mg^{-1} protein) was determined by the addition of 40 μL protein extract to 1 mL of a solution containing 50 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H_2O_2. The oxidation rate was monitored at 290 nm, at 30 °C, over a period of 1 min (Dourado et al. 2015).

Superoxide dismutase (SOD, EC 1.15.1.1) non-denaturing PAGE assay activity

Polyacrylamide gel electrophoresis (PAGE) analysis was carried out under non-denaturing conditions in 12 % polyacrylamide gels, followed by SOD activity staining as described by Vitória et al. (2001), with equal amounts of protein (40 μg) being loaded onto each gel lane. Electrophoresis buffers and gels were prepared as described by Vitória et al. (2001), except that SDS was excluded. To perform SOD activity staining, after non-denaturing-PAGE separation, the gel was rinsed in distilled water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM riboflavin, 1 mM EDTA, 0.1 mM nitrobluetetrazolium, and 0.3 % N,N,N′,N′-tetramethylethylenediamine. After 30 min, the gels were rinsed with distilled water and then illuminated in water until the achromatic bands of SOD activity were visible on a purple-stained gel. The SOD isoenzymes were distinguished by their sensitivity to inhibition by 2 mM potassium cyanide and 5 mM hydrogen peroxide. One unit of bovine liver SOD (Sigma, St. Louis, MO, USA) was used as a positive control of activity (Barbosa et al. 2012).

Light and transmission electron microscopy

For histological characterization samples were processed for light (LM) and transmission electron microscopy (TEM). Root tips and leaf blade were collected after 20 days of supplementation with 0, 10, and 100 μM $CdCl_2$. Samples were immediately fixed in a modified Karnovsky solution (Karnovsky 1965) (2 % glutaraldehyde, 2 % paraformaldehyde, and 5 mM $CaCl_2$ in 0.05 M sodium cacodylate buffer, pH 7.2) for 48 h. The samples were then rinsed in cacodylate buffer (0.1 M) and post fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2, at room temperature, for 1 h. The samples were dehydrated in a graded acetone series and embedded in Spurr epoxy resin (EMS, Electron Microscopy Sciences, Hatfield, PA, USA), for 48 h. Semi-thin sections (120–200 nm) were collected in glass slides, stained with toluidine blue (2 % in water) for 5 min, rinsed in distilled water, and air-dried. The sections were permanently mounted in Entellan®, observed and documented using an upright light microscope (LMD 7000, Leica, Germany). Ultra-thin sections (60–90 nm) of leaves and roots were collected on copper grids (300 mesh), and stained with uranyl acetate (2.5 %), followed by lead citrate (0.1 %) (Reynolds 1963). Sections were observed at 80 kV under a transmission electron microscope (JEM 1400 JEOL, Tokyo, Japan), and the images digitalized.

Statistical analysis

The experimental design was completely randomized and the results expressed as the mean and standard error (±SE) of three independent replicates of each extract for plant growth, Cd accumulation, dry weight, lipid peroxidation, H_2O_2 content, chlorophyll content, CAT, GR and APX activities. The statistical analysis was performed using the SASM-Agri software version 8.2. Tukey test was used for multiple means comparison, followed by individual ANOVA for each character, at a 0.05 level of significance.

Results

After 96-h of exposure to Cd, MT plants cultivated in 10 and 100 μM $CdCl_2$ exhibited differences in plant growth in comparison to the control (0 μM $CdCl_2$) and plants collected before Cd addition (T0). Root biomass was slightly increased in MT at 100 μM $CdCl_2$, when compared to the other treatments and controls (Fig. 1a). Shoot biomass, in both treatments (10 and 100 μM $CdCl_2$), were significantly increased when compared to the control and T0 (Fig. 1b). Mutant (*sit*) plants cultivated in solution with $CdCl_2$ did not exhibit differences in root and shoot growth in comparison to the control without $CdCl_2$ and T0.

Cd accumulation in the plants was proportional to the metal concentration added to the nutrient solution (Fig. 1). At the concentration of 100 μM $CdCl_2$, *sit* roots absorbed higher amounts of Cd when compared to MT roots (Fig. 1c). Similar amounts of Cd were transported to shoots of genotypes grown in both metal concentrations (Fig. 1d). The chlorophyll content in shoots of MT and *sit* grown in the presence or absence of Cd did not differ (Fig. 2).

MDA concentration varied when plants were exposed to Cd, both in shoots and roots of MT and *sit* (Fig. 3). Mutant (*sit*) roots exhibited higher levels of lipid peroxidation when compared to MT roots (Fig. 3a). MDA levels were higher at the

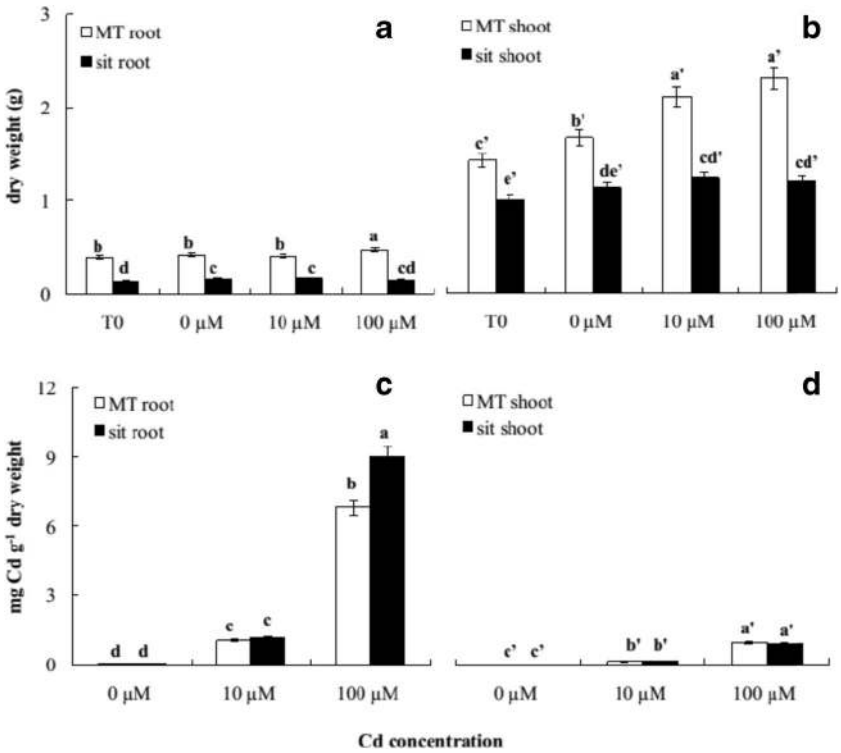

Fig. 1 Dry weight (**a**, **b**) and Cd accumulation (**c, d**) of roots and shoot of Micro-Tom (MT) and *sitiens* (*sit*) plants grown over a 96-h period and treated with 10 or 100 µM $CdCl_2$ or not treated (0 µM). T0 represents plants collected before addition of $CdCl_2$. Values are the means of three replicates ± SE. Different letters indicate significant difference at $p < 0.05$

concentration of 100 µM $CdCl_2$. Shoots of MT and *sit* exhibited similar values of lipid peroxidation (Fig. 3b). Differences in H_2O_2 content were observed in roots and shoots of MT and *sit* plants exposed to Cd (Fig. 3). The concentration of 100 µM $CdCl_2$ induced an increase in H_2O_2 production, mainly in the roots, in both genotypes (Fig. 3c). An increase in H_2O_2 content in shoots of MT and *sit* plants exposed to Cd was also observed (Fig. 3d).

SOD activity in roots and shoots of MT and *sit* plants was determined based on the isoenzymes separation by non-denaturing PAGE (Fig. 4). SOD isoenzymes were detected and characterized as Mn/SOD (SOD I and II) and Fe/SOD (SOD III) (data not shown). Roots and shoots of MT and *sit* exhibited decrease in SOD I activity (Fig. 4a, b). In shoots of MT, SOD II and III activities decreased in the presence of Cd (Fig. 4a, lanes 1, 2, and 3), but in the shoots of the *sit* mutant SOD II and III exhibited similar levels of activity (Fig. 4a, lanes 4, 5, and 6). No alterations were observed in SOD activity in roots of both genotypes grown in nutrient solution with both Cd concentrations (Fig. 4b).

CAT activity in roots and shoots of MT and *sit* was determined spectrophotometrically (Fig. 5). At T0, roots of *sit* and shoots of MT exhibited high activity of this enzyme (Fig. 5a, b). Roots of MT and *sit* exhibited small differences in CAT activity when exposed to 0 and 10 µM $CdCl_2$, however, the highest CAT activity encountered was observed in roots at the highest metal concentration for both genotypes (Fig. 5a). Shoots of MT and *sit* exhibited high CAT activity increase when exposed to 10 and 100 µM $CdCl_2$ (Fig. 5b).

Cd induced increase in GR activity in roots of MT and *sit* (Fig. 5). The highest activity was observed in MT roots exposed to 10 µM $CdCl_2$ (Fig. 5c). The shoot of s*it* exhibited increased GR activity when exposed to the concentration of 100 µM $CdCl_2$ (Fig. 5d). The highest APX activities (Fig. 5) were observed in roots and shoot of both, MT and s*it* plants, in the presence of Cd. Roots exhibited increased APX activity at 100 and 10 µM $CdCl_2$, for MT and *sit*, respectively (Fig. 5e). The enzyme activity in shoot increased with Cd, irrespective of the genotypes analyzed (Fig. 5f).

Anatomical and ultrastructural analyses of roots and leaves of MT and *sit* exposed to 0, 10, and 100 µM $CdCl_2$ were

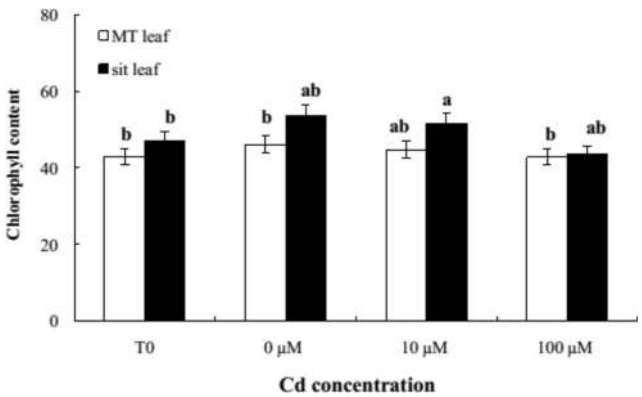

Fig. 2 Chlorophyll content measured (SPAD units) in leaves of Micro Tom (MT) and *sitiens* (*sit*) in plants grown over a 96-h period in the presence of 10 and 100 µM $CdCl_2$ or not treated (0 µM). T0 represents plants collected before addition of $CdCl_2$. Values are the means of three replicates ± SE. Different letters indicate significant difference at $p < 0.05$

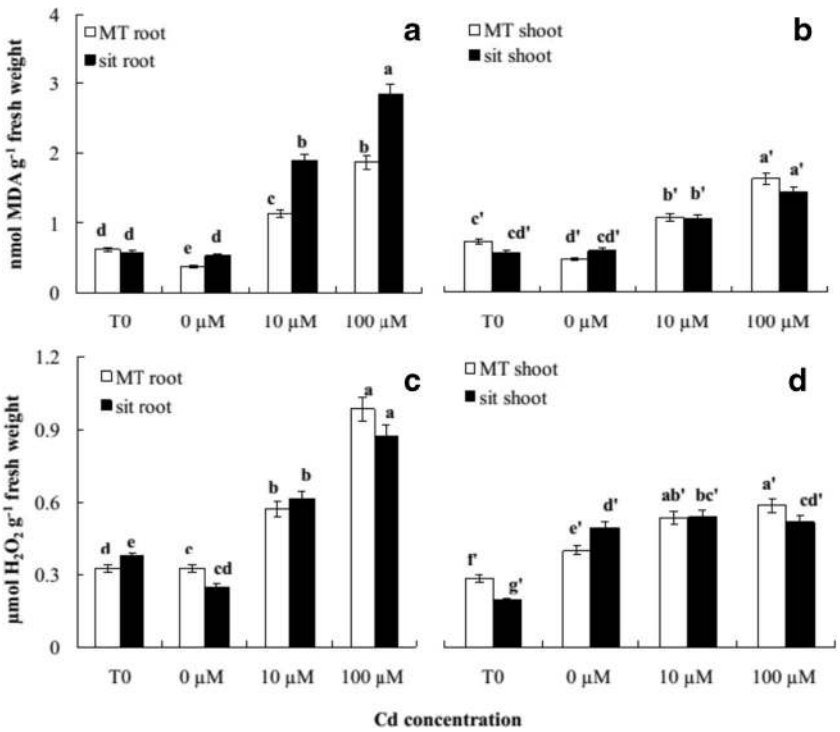

Fig. 3 Lipid peroxidation measured as malondialdehyde (MDA) content (nmol MDA g^{-1} fresh weight) in roots (**a**) and shoots (**b**) of Micro-Tom (MT) and *sitiens* (*sit*) plants and hydrogen peroxide (H_2O_2) content (µmol H_2O_2 g^{-1} fresh weight) in roots (**c**) and shoots (**d**) of MT and *sit* plants grown over a 96-h period in the presence of 10 and 100 µM $CdCl_2$ or not treated (0 µM). T0 represents plants collected before addition of $CdCl_2$. Values are the means of three replicates ± SE. Different letters indicate significant difference at $p < 0.05$

performed in plants grown in nutrient solution, and exposed to Cd over a period of 20-days. Histological sections observed under light microscopy (LM) revealed anatomical differences between MT and *sit* exposed to 10 and 100 µM $CdCl_2$ (Figs. 6 and 7). A decrease in intercellular spaces and leaf area in the leaves in the presence of Cd for both genotypes, and an increase in cell size, especially in the palisade parenchyma, were observed when compared to the control (Fig. 6b, c, e, f). In the roots, MT and *sit* exhibited cell size reduction in the presence of the metal in both concentrations tested (Fig. 7b, c, e, f). Ultrastructural analysis of root samples of plants grown in 10 and 100 µM $CdCl_2$ revealed an increased number of mitochondria (Fig. 8, white arrows). Ultrastructural changes in mitochondria shape (Fig. 8b, c, e, f, k, l) and increase in vacuole number (more in roots of MT than in *sit* roots) (Fig. 8e, f) were also detected. No alterations were observed in the nuclear membrane and cell wall (Fig. 8b, e, k, l). In leaves of MT and *sit* plants exposed to 10 and 100 µM $CdCl_2$, disorganization in the internal structures of chloroplasts was observed (Fig. 9b, c, e, f). Thylakoid membrane and grana were

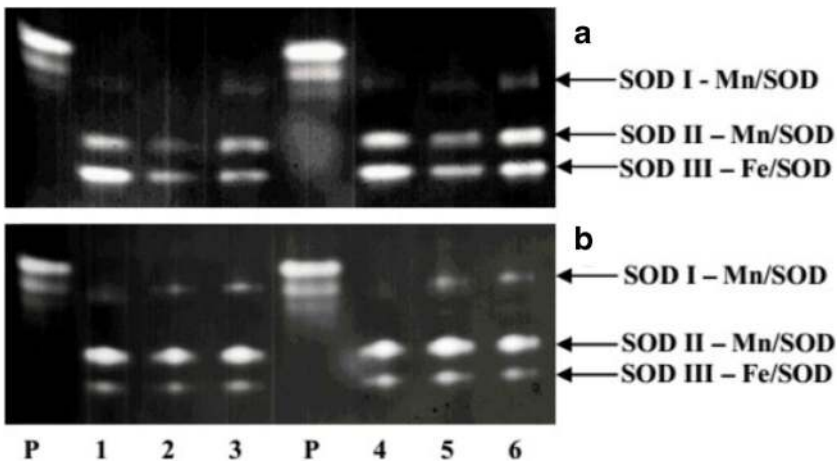

Fig. 4 Superoxide dismutase (SOD) activity staining following non-denaturing polyacrylamide gel electrophoresis of shoots (**a**) and roots (**b**) extracts isolated from Micro-Tom (MT) and *sitiens* (*sit*) plants grown over a 96-h period in the presence of 10 and 100 µM $CdCl_2$ or not treated (0 µM). The lanes listed are: (*P*) bovine SOD standard, (*1*) MT 0 µM $CdCl_2$, (*2*) MT 10 µM $CdCl_2$, (*3*) MT 100 µM $CdCl_2$, (*4*) *sit* 0 µM $CdCl_2$, (*5*) *sit* 10 µM $CdCl_2$, (*6*) *sit* 100 µM $CdCl_2$

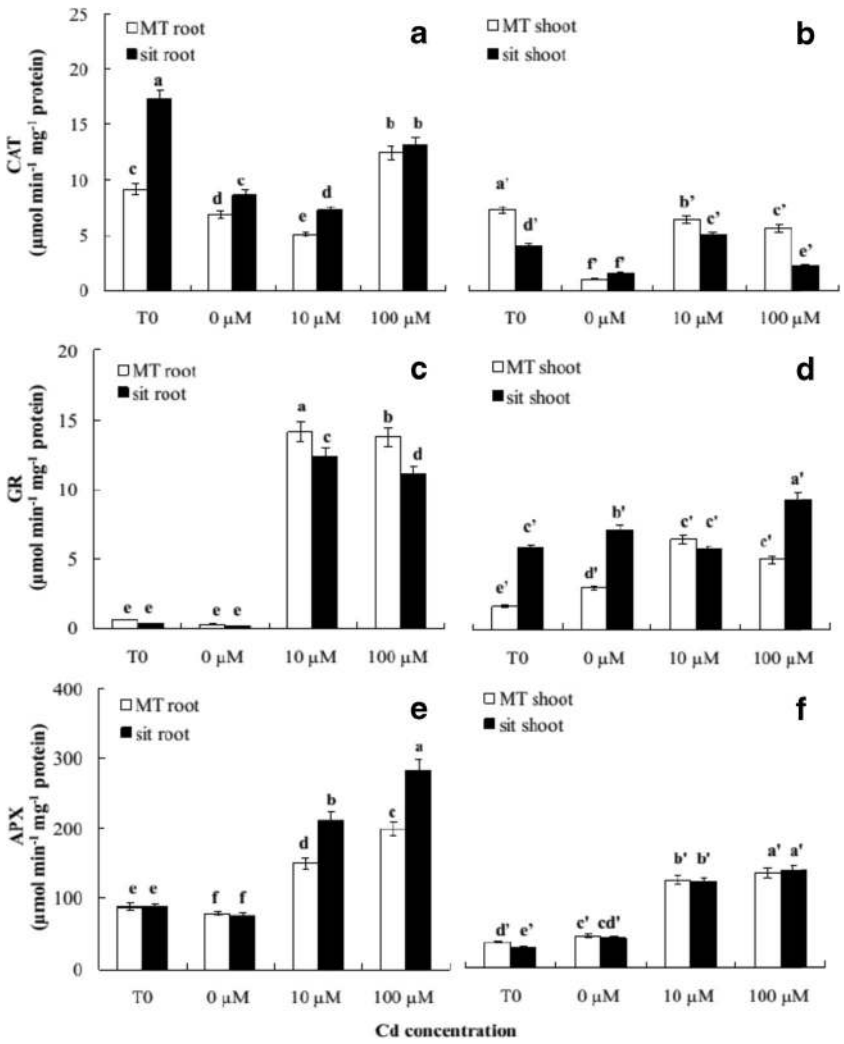

Fig. 5 Total activity (μmol min^{-1} mg^{-1} protein) of Catalase (CAT) in roots (**a**) and shoots (**b**) of Micro-Tom (MT) and *sitiens (sit)* plants; glutathione reductase (GR) in roots (**c**) and shoots (**d**) of MT and *sit* plants; and ascorbate peroxidase (APX) in roots (**e**) and shoots (**f**) of MT and *sit* plants grown over a 96-h period in the presence of 10 and 100 μM $CdCl_2$ or not treated (0 μM). Values are the means of three replicates ± SE. Different letters indicate significant difference at $p < 0.05$

disorganized, and the chloroplasts were malformed, whereas in the control leaves the chloroplasts had a normal shape and contained well-compartmentalized grana and organized thylakoids (Fig. 9a, d).

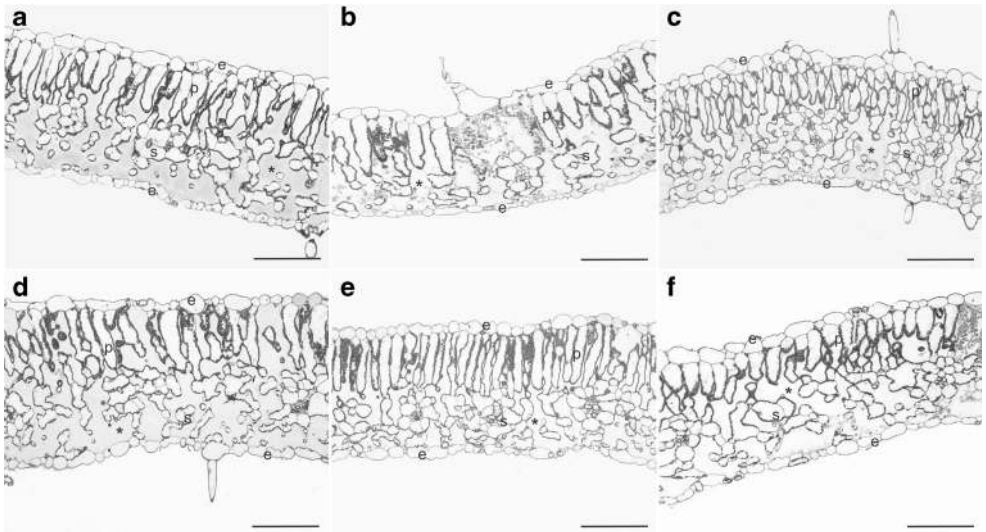

Fig. 6 Leaf cross-sections of Micro-Tom (MT) and *sit* mutant grown in nutrient solution with 10 and 100 μM $CdCl_2$, or not treated (0 μM), observed by light microscopy. **a–c** Leaves of MT: 0 μM $CdCl_2$ (**a**), 10 μM $CdCl_2$ (**b**), 100 μM $CdCl_2$ (**c**). **d–f** Leaves of *sit*: 0 μM $CdCl_2$ (**d**), 10 μM $CdCl_2$ (**e**), 100 μM $CdCl_2$ (**f**). *e*, epidermis; *p*, palisade parenchyma; *s*: spongy parenchyma; asterisks indicate intercellular space. *Scale bar* = 100 μm

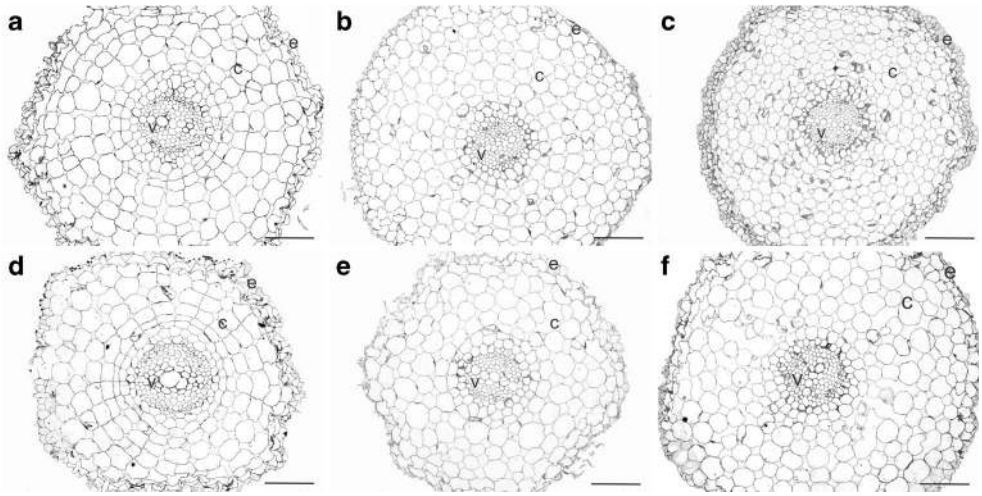

Fig. 7 Micro-Tom (MT) and *sit* mutant roots of plants grown in nutrient solution with 0, 10, or 100 μM $CdCl_2$, observed in cross-sections by light microscopy. **a–c** MT: 0 μM $CdCl_2$ (**a**); 10 μM $CdCl_2$ (**b**); 100 μM $CdCl_2$ (**c**); **d–f** *sit*: 0 μM $CdCl_2$ (**d**); 10 μM $CdCl_2$ (**e**); 100 μM $CdCl_2$ (**f**). *e*, epidermis; *c*, cortex; *v*, vascular cylinder. *Scale bar* = 100 μm

Discussion

Although research carried out in recent years has shown that all hormonal classes have important roles in abiotic stress response, ABA is a fundamental molecule that strongly regulates the outcome of the interaction between plants and a harmful environment. It has been shown that the mechanisms by which ABA regulates stress responses involve many molecular, biochemical and morpho-anatomical changes (Hong et al. 2013; Kim 2014). In other words, ABA is part of the stress response, which involves multiple and complex events, especially when different abiotic stresses are considered. Thus, in order to further elucidate the roles of ABA during Cd stress response in tomato, the ABA-deficient *sit* mutant was used and exposed to concentrations of 0, 10, and 100 μM $CdCl_2$ over a 96-h period in a hydroponic system and biochemical, physiological and anatomical responses were analyzed. Moreover, the *sit* mutant has not yet been fully characterized and has the potential to help unraveling the roles of ABA on abiotic stresses.

In tomato, ABA plays a multifaceted role when water, salinity, nutrient, and temperature stresses occur, including adjustment mechanisms of stress tolerance (Hermans et al. 2010; An et al. 2014; Osakabe et al. 2014). Under Cd stress, the ABA-deficient *sit* mutant exhibited multiple stress responses, which appears to be dependent upon the plant tissue or Cd concentration. Indeed, while the wild-type (MT) counterpart exhibited enhanced root dry weight at 100 μM $CdCl_2$, and shoot dry weight when grown in both Cd concentrations chosen for the experiments, the dry weight in roots and shoots of the *sit* mutant did not differ from the control and was considerably lower than in MT (Fig. 1a, b). These results may be, at least in part, explained by the natural reduced growth shown by *sit* due to its ABA deficiency (Carvalho et al. 2011). However, it is interesting to note that *sit* accumulated higher Cd amounts in the root when compared to MT (Fig. 1c), whereas in the shoot there was no difference between MT and the mutant (Fig. 1d), indicating different rates of Cd translocation among plant organs between the two genotypes. Thus, Cd accumulation in *sit* may not explain the reduced growth of roots, but it seems that there is a signaling between root and shoot, which is dependent upon ABA since a progressive increase in dry weight was not observed in *sit*. In fact, this may be reinforced by the similar Cd accumulation in MT and *sit* shoots.

Considering the increase in dry weight observed in MT from 0 to 100 μM $CdCl_2$, it is not surprising that small concentrations of toxic elements appear to stimulate growth. In fact, the growth stimulus in the presence of Cd has previously been observed in in vitro cell culture of sugarcane (Fornazier et al. 2002), mycelium of *Aspergillus nidulans* (Guelfi et al. 2003), coffee suspension cells (Gomes-Junior et al. 2006), tobacco BY-2 cells (Gratão et al. 2008a), and also in tomato plants (Gratão et al. 2008b; Zhu et al. 2011). This hormetic mechanism by which Cd induces growth is still poorly understood, but the absence of dry weight increase observed in the *sit* mutant, which is ABA deficient, allows us to suggest that this mechanism also appears to be dependent on ABA.

Leaf chlorophyll breakdown is an important catabolic process, which commonly occurs in plants grown under stress, and thus can be used as an important stress indicator (Monteiro et al. 2011). However, we observed that the chlorophyll content was not altered in MT and *sit* in the presence of Cd (Fig. 2). Accordingly, the presence of Cd also did not negatively impact the growth of MT and *sit* plants (Fig. 1a, b). This could have occurred by an insufficient period of exposure to the metal, although two concentrations of Cd were used, which were high enough to accumulate Cd in the roots and shoots, at least at 100 μM $CdCl_2$, and to induce oxidative stress changes. As a matter of fact, an induction of lipid peroxidation, measured by changes in MDA levels, can be observed in roots (Fig. 3a) and shoots (Fig. 3b) from 10 to 100 μM $CdCl_2$ in both MT and *sit*,

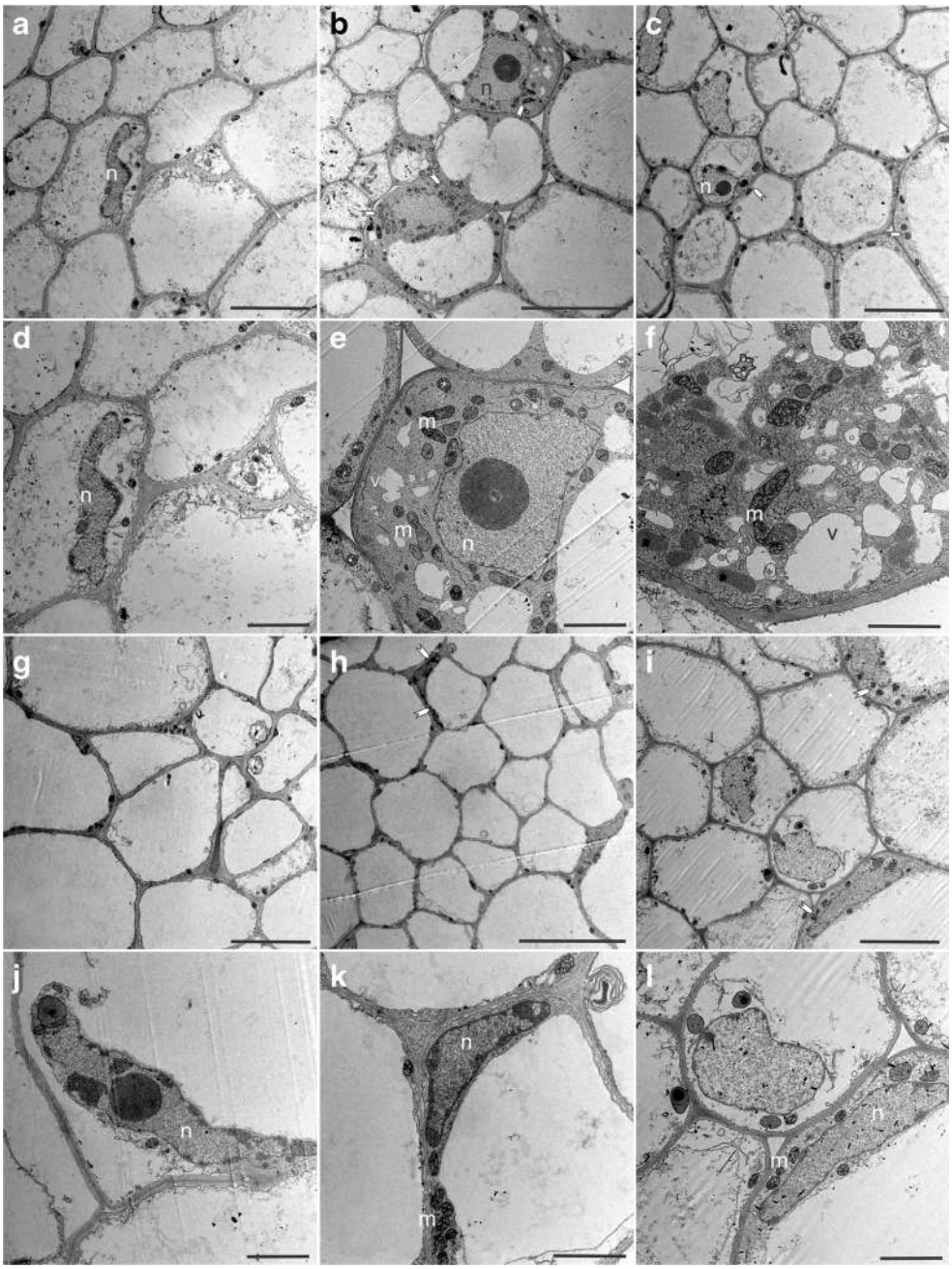

Fig. 8 Ultrastructure effect of cadmium on root tip cells of Micro-Tom (MT) and *sit* mutant plants grown in nutrient solution with and without $CdCl_2$. **a–f** plants of MT; **g–l** plants of *sit*. **a**, **d**, **g**, **j** = 0 μM $CdCl_2$; **b**, **e**, **h**, **k** = 10 μM $CdCl_2$ and **c**, **f**, **i**, **l** = 100 μM $CdCl_2$. Note the increase in number of mitochondria (**b**, **c**, **e**, **f**, **i**, **l**) and increase of vacuoles (**e**, **f**). *Arrows* indicate mitochondria, *n*, nucleus; *c*, cell wall; *m*, mitochondria; *v*, vacuole. *Scale bars*: **a**, **c**, **g**, **i** = 500 μm; **b**, **h** = 1000 μm; **d**, **e**, **j**, **k** = 5 μm; **f**, **k** = 2.5 μm

being more pronounced in the roots of the mutant. These results indicate that although Cd treatments did not alter growth (Fig. 1a, b) or chlorophyll content (Fig. 2) in these genotypes, the accumulation of Cd in the roots, and in a lesser extent in shoots, induced lipid peroxidation in these organs. Moreover, an enhanced MDA accumulation in the roots of *sit* treated with $CdCl_2$ was clearly observed, suggesting that ABA deficiency amplifies the stress response to Cd. In other words, the enhanced MDA in *sit* could be attributed to a negative effect of ABA on lipid peroxidation, which seems to be true only for metal stress response, because the natural ABA deficiency in the mutant did not alter MDA under control conditions (without Cd) (Fig. 3), as previously reported in tomato (Monteiro et al. 2012).

In the presence of Cd an increase in H_2O_2 accumulation in roots of MT and *sit* (Fig. 3c, d) was observed, whereas the increase in shoots seems to be associated to time since the increase in H_2O_2 occurred between treatments without Cd (0 μM $CdCl_2$), when compared to T0. The enhanced MDA content may be in part due to the accumulation of H_2O_2, but that is not dependent on ABA since the H_2O_2 content in the mutant did not differ from MT.

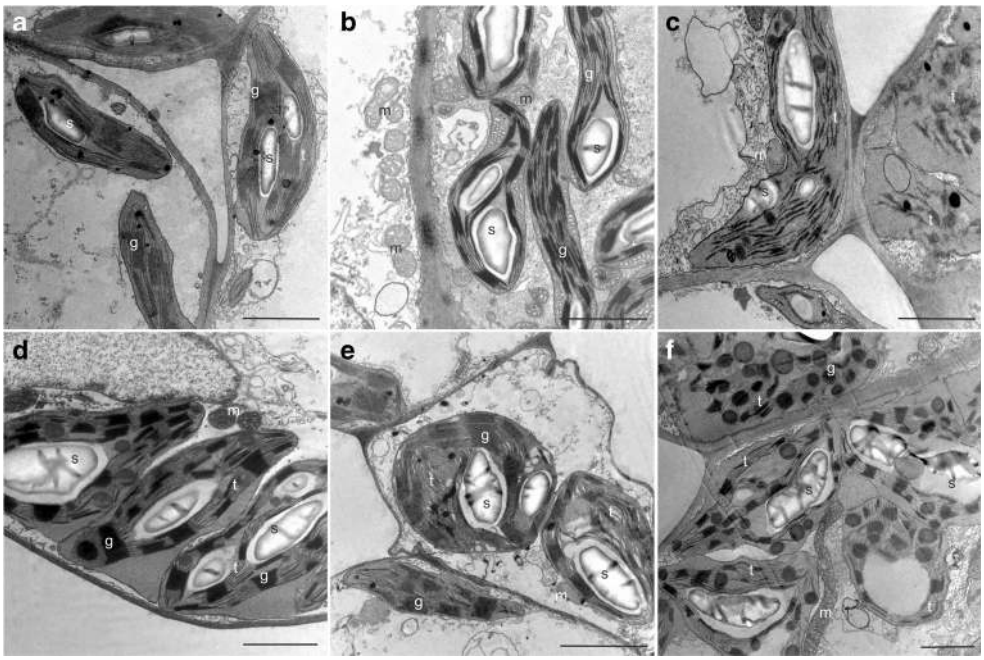

Fig. 9 Effect of cadmium on chloroplast ultrastructure of Micro-Tom (MT) and *sit* mutant plants grown in nutrient solution with 0, 10 and 100 μM $CdCl_2$. **a–c** plants of MT: 0 μM $CdCl_2$ (**a**), 10 μM $CdCl_2$ (**b**), 100 μM $CdCl_2$ (**c**); **d–f** plants of *sit*: 0 μM $CdCl_2$ (**d**), 10 μM $CdCl_2$ (**e**), 100 μM $CdCl_2$ (**f**). *c*, chloroplast; *g*, grana; *s*, starch grain; *m*, mitochondria, *t*, thylakoids. *Scale bars*: **a**, **b**, **e** = 2 μm; **c**, **d**, **f** = 5 μm

Regarding enzyme peroxidase activity, which can reduce H_2O_2, the data showing that a higher activity of CAT and APX, and reduction of H_2O_2 and MDA in roots of MT and *sit* grown from T0 to 10 μM $CdCl_2$, are taken together. When comparing time and $CdCl_2$ concentration, the enhanced CAT activity in roots (Fig. 5a) can be associated to reduced MDA and H_2O_2 (Fig. 3a). However, at 100 μM $CdCl_2$, this was not the case, most likely because a burst of oxidative stress was triggered in the roots, inducing strong lipid peroxidation and antioxidant enzyme activities such as for GR (Fig. 5c) and APX (Fig. 5e). Moreover, compared to MT, the exposure to Cd did not alter SOD total activity in roots (Fig. 4a, b), indicating that the alteration in H_2O_2 does not appear to be associated with the level of this enzyme by catalyzing the dismutation of superoxide into H_2O_2.

Although *sit* naturally did not exhibit changes in lipid peroxidation when compared to MT (Fig.3a, b), this mutant revealed a multifaceted antioxidant enzyme activity pattern, which was depended upon organ, enzyme, time, and metal concentration (Fig. 5). When compared to MT, *sit* exhibited enhanced CAT activity with and without Cd (Fig. 5a), whereas GR (Fig. 5d) and APX (Fig. 5e) activities of shoots and roots, respectively, were enhanced in the Cd treatments. On the other hand, *sit* exhibited reduced CAT activity in T0 and 100 μM $CdCl_2$ in shoots, and GR activity in 10 and 100 μM $CdCl_2$ (Fig. 5c). In general, the high antioxidant activity in *sit* could be associated to a constitutive ABA deficiency, which results in a phenotypically wilt plant, stunted and epinastic (Tal 1966). Thus, under stressful conditions *sit* plants exhibit more sensitivity, such as previously shown to salinity (Mäkelä et al. 2003) and water deficit (Thompson et al. 2004), and in this work the mutant was shown to alter the antioxidant response also to the metal treatment. Therefore, it seems clear that a complex biochemical network involves ABA-signaling in metal stress.

In addition, leaves and roots anatomical and ultrastructural changes induced by Cd were also observed. The most evident response was the reduction in *sit* and MT of leaf intercellular space, cell area and size (Fig. 6b, c, e, f), as well as structural abnormalities in vacuoles (Fig. 8e, f) and mitochondria (Fig. 8b, c, e, f, k, l) in roots, and in chloroplasts (Fig. 9b, c, e), an expected outcome also previously reported in response to Cd stress (Daud et al. 2009; Gratão et al. 2009). On the other hand, root cell size was reduced in both MT and *sit* (Fig. 7b, c, e, f). Hence, even showing a strong accumulation of Cd in 100 μM $CdCl_2$ (Fig. 1c) and increasing lipid peroxidation (Fig. 3a), *sit* does not show a reduction in root cell size (Fig. 7). Yet, these results do not confer more Cd tolerance to *sit* because MT and the mutant did not present reduced growth from 0 to 100 μM $CdCl_2$.

In this work, we initially hypothesized that ABA is part of the Cd stress signaling in tomato, and to test that we submitted the ABA-deficient mutant *sit* to 0, 10, and 100 μM $CdCl_2$. The results suggest a confirmation of our hypothesis, with a clear important role of ABA on Cd stress signaling in tomato. In fact, the mutant accumulated more Cd in the roots when compared to its wild-type counterpart MT. In other words, ABA production seems to be related to a decrease in Cd accumulation in this plant species. Wang et al. (2016) using two *Solanum photeinocarpum* ecotypes (mining and farmland) applied exogenous ABA, which resulted in increased Cd content in both ecotypes. Although the interaction between ABA and Cd seems dependent on the plant species, exogenous ABA application can act differently from ABA production

under Cd treatment. This observation is also supported by previous reports in which ABA content rapidly increased in the leaves and roots in the rice Cd-tolerant cultivar (cv. Tainung 67, TNG67), but not in the Cd-sensitive cultivar (cv. Taichung Native 1, TN1) (Hsu and Kao 2003). Additionally, a molecular mechanism of interaction between Cd and ABA has been clarified in *Arabidopsis*, with a decrease in Cd accumulation in the presence of exogenous ABA, by inhibiting the *IRON-REGULATED TRANSPORTER 1* (*IRT1*) (Fan et al. 2014), a broad-spectrum transporter in roots, which is involved in the absorption of several other divalent cations, including Cd (Vert et al. 2002). Additionally, we have been able to show that the mechanisms by which ABA interacts with Cd is followed by evident histological and biochemical alterations. Although we found some evidence that Cd stress response is mediated by ABA in tomato, the use of an ABA-deficient mutant to study the role of this hormone in plants grown under stress can be complex because of their natural adverse water relations and altered growth (Thompson et al. 2004). Ongoing research is being carried out, in which *sit* and other hormonal mutants such as *Never ripe* and *diageotropica* have been subjected to combined stresses such as water deficit and metal to further elucidate the role of ABA signaling, and the involvement of other hormones in response to stress.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests or conflict of interest.

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