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Influence of salicylic acid on phytochelatin synthesis in *Zea mays* during Cd stress

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Abstract: Presoaking maize (*Zea mays*) seeds in salicylic acid (SA) reduces damage caused by cadmium. In the present work the possible role of phytochelatins (PCs) in SA-mediated protection against Cd toxicity was investigated. Seeds were presoaked in 0.5 mM SA, and seedlings were grown in hydroponic solution containing 0, 0.01, 0.015, or 0.025 mM Cd. Treatment with Cd increased the PC levels in maize roots, but only slight changes were observed in the leaves. Long-term exposure to Cd decreased the phytochelatin synthase (PCS) activity in the roots and led to an increase in PCS and glutathione reductase (GR) activities in maize leaves. Although presoaking seeds in SA solution before exposure to Cd may reduce the level of heavy metal injury and has an effect on the composition of individual PCs, this protection is not directly connected with the altered regulation of PCs.

Key words: Cadmium, heavy metal, phytochelatins, salicylic acid, *Zea mays*

1. Introduction

The accumulation of heavy metals in the soil is dangerous for all living organisms. In plants, cadmium is one of the most readily absorbed and rapidly translocated heavy metals, which explains why it exerts such strong toxicity even at relatively low concentrations (Seregin & Ivanov, 1998; Pál et al., 2006; Khurana & Kansal, 2012; Tran & Popova, 2013). The level of Cd uptake in higher plants is determined by the Cd concentration in the soil and by its biological availability. Cd is transported symplastically through the root cortex to the stele and from there through the xylem to the shoots, although the phloem is also involved in transportation (Tudoreanu & Phillips, 2004).

Although maize (*Zea mays* L.) is considered to be a relatively Cd-tolerant plant (Pál et al., 2006), many toxic symptoms may result if the Cd concentration exceeds a critical level. These symptoms include the inhibition of growth and photosynthesis, activation or inhibition of enzymes, and disturbances in water and ion metabolism. Cd toxicity is often associated with the oxidative stress caused by excessive formation of free oxygen radicals (Sanita di Toppi & Gabrielli, 1999) and may lead to the modified activity of various antioxidant enzymes (Hegedűs et al., 2001). Antioxidant systems play an important role in protection against various stressors (Baloğlu et al., 2012; Sekmen Esen et al., 2012). Under severe stress conditions, however, antioxidant capacity may not be sufficient to minimise the harmful effects of oxidative injury. In addition to general stress responses, plants synthesise special complex-forming agents called phytochelatins (PCs), which are produced in the cytosol and play a special role in the detoxification of toxic heavy metals (Grill et al., 1985; Namdjoyan et al., 2012). They have the structure [(γ-Glu – Cys)n – Gly], where n is the number of replications of (γ-Glu – Cys) units, generally in the range of 2–11. Metal–PC complexes are transported into the vacuole, thus sequestering the metals from sensitive enzymes. Once in the vacuole, low-molecular-weight (LMW) complexes are converted to high-molecular-weight (HMW) complexes by the incorporation of sulphide, PC, and further Cd ions (Cobbet, 2000). PCs are synthesised by phytochelatin synthase (PCS) from glutathione (GSH) by transferring a g-Glu-Cys moiety from a donor to an acceptor molecule. The reaction involves the transpeptidation of the g-Glu-Cys moiety of GSH initially onto a second GSH molecule to form PC_2 and, in the later stages of incubation, onto a PC molecule to produce an n+1 oligomer (Grill et al., 1989).

Salicylic acid (SA) plays a key role in the signal transduction pathways of various stress responses (Raskin, 1992; Horváth et al., 2007; Hayat et al., 2010). In one of the first papers demonstrating the protective effect of SA against an abiotic stress factor, SA treatment induced tolerance to copper toxicity in cucumber and tobacco (Strobel & Kuc, 1995). The ameliorating effect of SA treatment was also shown on seed germination and seedling growth

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under Pb^{2+} or Hg^{2+} stress in rice (Mishra & Choudhuri, 1997). It was later found that SA induces thermotolerance in mustard seedlings (Dat et al., 1998a, 1998b), provides protection against chilling injury in maize (Janda et al., 1999; Szalai et al., 2000) and winter wheat (Tasgin et al., 2003; Sasheva et al., 2010), and modifies plant responses to salt and osmotic stress (Borsani et al., 2001), ozone or UV light (Sharma et al., 1996), and drought (Senaratna et al., 2000). Although a relatively large number of studies have described the role of SA in stress adaptation processes (using mainly exogenous SA), information on the cross-talk between SA and other signalling pathways is very limited (Liu et al., 2006; Mukherjee et al., 2010).

It has also been shown that presoaking maize seeds in SA before exposure to Cd has a protective effect on photosynthesis and reduces the oxidative damage caused by Cd (Krantev et al., 2008). The aim of the present work was to investigate the possible role of PCs in the SA-mediated protection against Cd toxicity.

2. Materials and methods

2.1. Plant material and Cd treatment

Seeds of maize (*Zea mays*, hybrid Norma) were sterilised and divided into 2 groups. Half the seeds were soaked in 0.5 mM SA solution for 6 h, while the other half were soaked in distilled water. Both groups were then allowed to germinate on moist filter paper in the dark. Three-day-old, dark-grown seedlings were placed in beakers filled with 600 mL of modified Hoagland solution (Pál et al. 2005) containing $Cd(NO_3)_2$ at concentrations of 0, 0.01, 0.015, or 0.025 mM. The nutrient solution was changed 3 times every week. The plants were grown in a Conviron PGR-15 growth chamber (Controlled Environments Ltd., Winnipeg, Canada) at 250 μmol m^{-2} s^{-1} photon flux density with a day/night cycle of 16/8 h at 22/18 °C, respectively. After 14 days of growth the plants were harvested for analysis.

2.2. Measurement of PC and PCS activity

The PC content and PCS activity were measured according to the methods of Chen et al. (1997) with slight modifications. Root or leaf samples weighing 750 mg were ground with 750 μL of extraction buffer consisting of 50 mM Tris-HCl buffer, pH 8.0; 10 mM b-mercaptoethanol (βME); 1 mM phenylmethylsulfonyl fluoride; and 14% (v/v) glycerol. They were then centrifuged for 10 min at 10,000 × *g*. The standard assay mixture for PCS contained 200 μL of the supernatant, 25 μL of 2.4 M Tris-HCl buffer (pH 8.0), 25 μL of 6 mM $CdNO_3$, 25 μL of 120 mM βME, and 25 μL of 120 mM GSH. After incubation for 60 min at 35 °C the reaction was terminated by adding 30 μL of 50% sulfosalicylic acid followed by incubation on ice for 5 min. Sulfosalicylic acid was added to the same mixture without incubation (0 min) to determine the initial PC level (which also represents the in vivo PC level). PCs were analysed by reverse-phase HPLC (Waters W2690 separation module) and determined after postcolumn derivatisation with Ellman's reagent using a UV-VIS diode array detector (W996) at 412 nm. The PCs were quantified using a glutathione calibration curve. LMW and HMW PCs were defined as PC_{2-4} and PC_{5-10}, respectively. PCS activity was calculated using PC produced during the 60-min incubation compared to the initial level. The activities were expressed as nkat/g FW.

2.3. Enzyme assays

For the analysis of antioxidant enzyme activity, 0.5 g of tissue from the third leaves and the roots were homogenised in 2.5 mL of ice-cold Tris buffer (0.5 M, pH 7.5) containing 3 mM $MgCl_2$ and 1 mM EDTA.

The glutathione reductase (GR; EC 1.6.4.2.) activity was determined at 412 nm according to the methods of Smith et al. (1988). The reaction mixture contained 75 mM Na-phosphate buffer (pH 7.5), 0.15 mM diethylenetriamine-pentaacetic acid, 0.75 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM NADPH, 0.5 mM oxidised glutathione, and 50 μL of plant extract in a total volume of 1 mL.

The activities were expressed in nkat/g FW.

2.4. Statistical analysis

The experiments were repeated 3 times and representative data are shown. The results were the means of 5 measurements. The data were statistically evaluated using the standard deviation and t-test methods.

3. Results and discussion

Our earlier results showed that maize plants grown with 0.01, 0.015, and 0.025 mM $CdCl_2$ exhibited a significant growth inhibition, as measured by shoot fresh weight and root dry weight accumulation and shoot and root length, and that Cd treatment led to significantly reduced chlorophyll content (Krantev et al., 2008). The rate of CO_2 assimilation also decreased, especially due to a reduction in the activity of the carboxylating enzymes phosphoenolpyruvate carboxylase and ribulose 1,5-bisphosphate carboxylase (Drażkiewicz et al., 2003; Krantev et al., 2008). Pretreatment of seeds with 0.5 mM SA for 6 h alleviated the negative effect of Cd on plant growth and photosynthetic parameters (Krantev et al., 2008); however, the exact mechanism of the protection is still poorly understood. Our previous experiment also showed that Cd content in the root tissue of plants without SA treatment increased 13, 15, and 18 times in samples from plants treated with 0.01, 0.015, and 0.025 mM Cd, respectively (Krantev et al., 2008). Although other results indicate that phenols may have an important role in the uptake of Cd (Kovacik et al., 2011), the SA treatment used in this experiment did not cause a significant change in the Cd level of the plants in the previous study (Krantev et al., 2008).

3.1. PC content

The exposure of maize plants to 0.01, 0.015, or 0.025 mM Cd for 14 days did not increase the total PC level in the leaves (Figure 1); however, the pattern of individual PCs exhibited changes (Table). Long exposure to Cd initiated the synthesis of HMW PCs. Total PC content was lower in SA pretreated plants at 0 and 0.01 mM Cd concentrations compared to the leaves of nontreated plants; however, it was higher at a high Cd concentration (0.025 mM). These differences could be attributed to the different HMW PC levels (Figure 2).

Cd treatment significantly increased the total PC level in maize roots (Figure 3). This increase was mainly due to the elevated level of HMW PCs; however, a slight but statistically significant increase could also be seen in the LMW PC level (Figure 4; Table). In plants pretreated with SA before exposure to Cd, the same pattern was observed.

3.2. PCS activity

PCS expression is constitutive, and the enzyme is activated through metal ions and/or metal–GS complexes (Clemens, 2006). Little is known about the tissue specificity of PCS expression and/or PC biosynthesis. In the only study on tissue-specific PCS expression to date, activity was detected in the roots and stems of tomato plants but not in the leaves or fruits (Chen et al., 1997). PCS activity increased in leaves treated with Cd (Figure 5a). SA pretreatment increased the PCS activity in the leaves of non-Cd–treated plants. In contrast to the leaves, PCS activity substantially decreased in the roots of maize plants after exposure to Cd (Figure 5b). The activity of PCS is affected by several factors, such as the concentration of reduced glutathione and Cd and PCs levels. Our data are in agreement with other findings that demonstrated that at constant glutathione concentration, PCS activity exhibited a transient increase, with increasing Cd concentration up to a threshold value, and then decreased sharply (Ogawa et al., 2011). This may also explain the Cd-concentration–dependent increase in PCS activity in the leaves, where the Cd concentration is relatively low, and reduced activity in the roots, where the Cd concentration is high (Szalai et al., 2005). With in vitro reactions, PC biosynthesis continued until the activating metal ions were chelated, either by the PCs formed or by the addition of a metal chelator such as EDTA (Loeffler et al., 1989). This provides a mechanism for the autoregulation of PC biosynthesis in which the product of the reaction chelates the activating metal, thereby terminating the reaction. Furthermore, in the present work slightly increased PC levels in the roots of Cd-treated plants were found, suggesting that a feedback mechanism may also exist to regulate the activity of PCS enzyme in the cells.

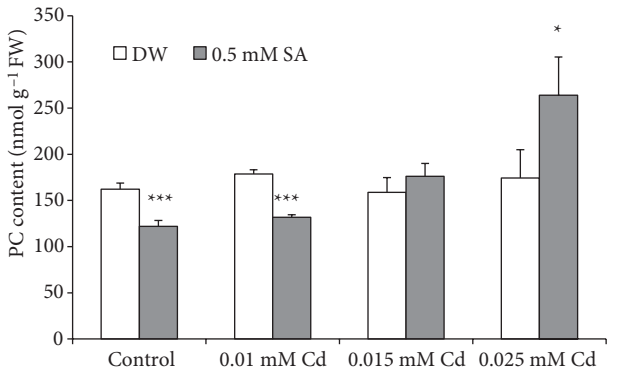

Figure 1. Changes in the total phytochelatin (PC) content in the leaves of young maize plants after soaking seeds in distilled water (DW) or 0.5 mM SA solution during Cd stress. *, ***: significant difference between the DW- and SA-treated plants at $P < 0.05$ and $P < 0.001$, respectively.

Based on the reasonable assumption that most PCS-expressing organisms, or at least most PCS-expressing cells and organs, are never exposed to toxic metals, 3 possible explanations for the distribution and constitutive expression of PCS proteins were proposed by Clemens (2009): 1) the PC pathway has an important role in essential metal homeostasis; (ii) PCSs serve other, as yet unknown, essential functions (Clemens, 2006); and 3) Cd and As are essential elements, and the PC pathway is part of the homeostatic network for these elements. The third hypothesis has not yet been supported by experimental data for higher plants, but in the diatom *Thalassiosira weissflogii* a carboanhydrase was described that is expressed under conditions of low CO_2 and Zn deficiency. This enzyme uses Cd as a cofactor in place of Zn (Xu et al., 2008).

3.3. Activity of antioxidant enzymes

A variety of abiotic stresses, including heavy metals, cause molecular damage to plant cells either directly or indirectly through a burst of reactive oxygen species (ROS) (Cuipers et al., 2002; Zhang et al., 2005). Although Cd does not generate ROS directly, it generates oxidative stress via interference with the antioxidant defence system (Sanita di Toppi & Gabrielli, 1999). A high level of SA in maize plants may act directly as a preformed antioxidant to scavenge ROS and/or indirectly modify the redox balance by activating antioxidant responses (Horváth et al., 2007). In our previous experiment the activities of catalase, ascorbate peroxidase, guaiacol peroxidase, glutathione-S-transferase, and superoxide dismutase were measured; in each of these, the activity did not change during Cd stress (Krantev et al., 2008), while in the present study 0.01, 0.015, or 0.025 mM Cd in the nutrient solution led to a significant increase in the GR activity in maize leaves (Figure 6a). The highest value was detected in plants treated with 0.01 mM Cd. Higher Cd concentrations gradually reduced the GR activity. Presoaking seeds in SA did not itself significantly affect the GR activity in control plants; however, it reduced

Table. Pattern of phytochelatins (PC_{2-10}) in the leaves and roots of young maize plants after soaking seeds in distilled water (DW) or 0.5 mM SA solution during Cd stress. Means ± SD; *: significant difference between the DW- and SA-treated plants at least at $P < 0.05$; nd: not detected (below the detection limit).

		PC content (nmol g^{-1} FW)							
		Leaf				Root			
		Control	0.01 mM Cd	0.015 mM Cd	0.025 mM Cd	Control	0.01 mM Cd	0.015 mM Cd	0.025 mM Cd
PC_2	DW	8.7 ± 0.9	8.9 ± 0.2	10.4 ± 0.2	11.3 ± 2.7	11.3 ± 0.9	14.6 ± 1.5	18.4 ± 0.9	15.2 ± 0.9
	0.5 mM SA	9.3 ± 1.4	10.5 ± 0.3*	10.2 ± 0.8	11.3 ± 0.5	11.4 ± 0.3	15.0 ± 1.0	12.8 ± 2.9*	13.0 ± 0.8*
PC_3	DW	8.5 ± 0.9	9.1 ± 0.2	10.1 ± 0.2	11.6 ± 2.7	11.2 ± 0.9	14.8 ± 1.5	18.2 ± 0.9	15.0 ± 0.9
	0.5 mM SA	9.8 ± 1.4	10.6 ± 0.3	10.7 ± 0.8	12.3 ± 0.5	11.0 ± 0.3	15.4 ± 1.0	12.6 ± 2.9*	13.2 ± 0.8*
PC_4	DW	nd	nd*	nd	13.5 ± 2.2	nd	10.4 ± 3.7	12.2 ± 6.3	nd
	0.5 mM SA	nd	nd	nd	nd*	nd	nd*	nd*	28.3 ± 7.5*
PC_5	DW	nd	nd	nd	nd	nd	nd	nd	31.6 ± 4.7
	0.5 mM SA	nd	nd	54.9 ± 18.3*	58.7 ± 11.2*	nd	19.8 ± 0.2*	29.0 ± 4.6*	66.7 ± 11.3*
PC_6	DW	nd	61.4 ± 7.0	59.3 ± 11.0	55.1 ± 12.0	nd	38.9 ± 10.8	nd	66.0 ± 9.2
	0.5 mM SA	46.8 ± 21.2*	39.0 ± 13.4	nd*	nd*	8.5 ± 3.0*	59.7 ± 3.0*	59.2 ± 13.8*	43.1 ± 3.8*
PC_7	DW	45.1 ± 9.5	nd	nd	nd	3.7 ± 1.3	83.5 ± 17.0	15.8 ± 11.9	70.9 ± 10.3
	0.5 mM SA	19.0 ± 2.5*	30.4 ± 2.3*	19.4 ± 3.8*	24.9 ± 13.6*	19.3 ± 2.8*	67.4 ± 10.9	40.0 ± 3.2*	42.8 ± 10.3*
PC_8	DW	21.3 ± 6.4	46.7 ± 4.1	26.2 ± 3.7	49.6 ± 20.1	10.1 ± 9.0	86.4 ± 11.9	65.6 ± 19.1	61.2 ± 2.8
	0.5 mM SA	nd*	nd*	nd*	50.5 ± 13.0	nd	64.2 ± 8.9	25.5 ± 0.3*	25.6 ± 6.3*
PC_9	DW	12.2 ± 0.3	nd	nd	nd	1.8 ± 0.1	67.5 ± 7.9	48.9 ± 11.5	nd
	0.5 mM SA	37.4 ± 14.7	41.4 ± 11.1*	56.3 ± 13.2*	30.1 ±19.9*	12.4 ± 0.9*	22.7 ± 1.1*	25.4 ± 2.0*	13.1 ± 1.8*
PC_{10}	DW	65.9 ± 7.3	52.7 ± 9.4	52.5 ± 24.7	33.5 ± 9.1	5.3 ± 4.3	21.8 ± 4.8	30.9 ± 2.1	26.3 ± 4.0
	0.5 mM SA	nd*	nd*	25.0 ± 3.2	77.0 ± 26.9	nd	6.6 ± 4.9*	10.6 ± 2.9*	6.11 ± 1.5*

the Cd-induced increase in GR at 0.01 and 0.025 mM Cd concentrations. In our earlier experiments decreased levels of malondialdehyde and proline and a reduced rate of electrolyte leakage were observed in maize plants pretreated with SA, possibly confirming the protective role of SA against oxidative stress caused by Cd (Krantev et al., 2008). In contrast to the leaves, changes in GR were not statistically significant in the root (Figure 6b). In the other treatments the differences were not significant.

In another study it was shown that pretreatment of barley seedlings with SA prevented the lipid peroxidation

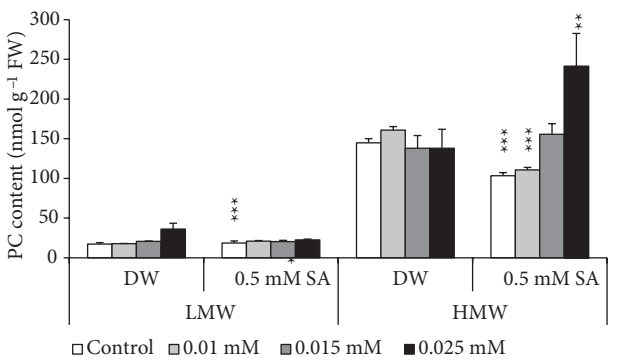

Figure 2. Pattern of low-molecular-weight (LMW; PC_{2-4}) and high-molecular-weight (HMW; PC_{5-10}) phytochelatins in the leaves of young maize plants after soaking seeds in distilled water (DW) or 0.5 mM SA solution during Cd stress. **, ***: significant difference between the DW- and SA-treated plants at $P < 0.01$ and $P < 0.001$, respectively.

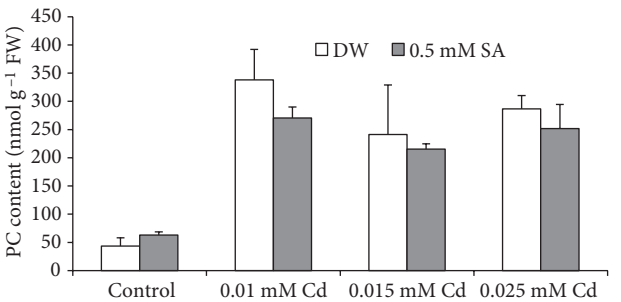

Figure 3. Changes in the total phytochelatin (PC) content in the roots of young maize plants after soaking seeds in distilled water (DW) or 0.5 mM SA solution during Cd stress.

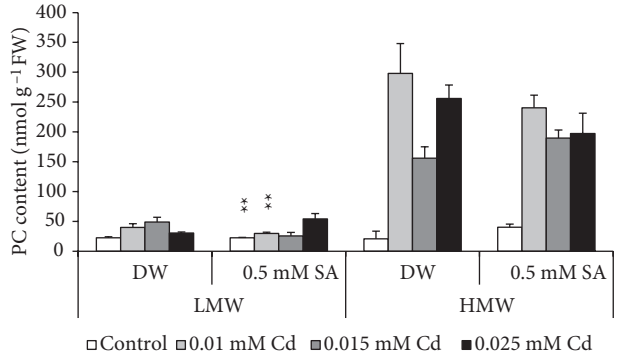

Figure 4. Pattern of low-molecular-weight (LMW; PC_{2-4}) and high-molecular-weight (HMW; PC_{5-10}) phytochelatins in the roots of young maize plants after soaking seeds in distilled water (DW) or 0.5 mM SA solution during Cd stress. **: significant difference between the DW- and SA-treated plants at $P < 0.01$.

caused by Cd. On the other hand, antioxidant enzyme activities increased in Cd-stressed seedlings; however, pretreatment with SA suppressed this effect (Metwally et al., 2003). In maize plants, treatment with Cd decreased the ascorbate peroxidase activity but substantially increased the activity of superoxide dismutase. Pretreatment with SA caused an increase in both ascorbate peroxidase and superoxide dismutase activity but caused a strong reduction in catalase activity (Krantev et al., 2008). These data suggest that SA may protect cells against Cd-induced oxidative damage. In the present experiments exposure to

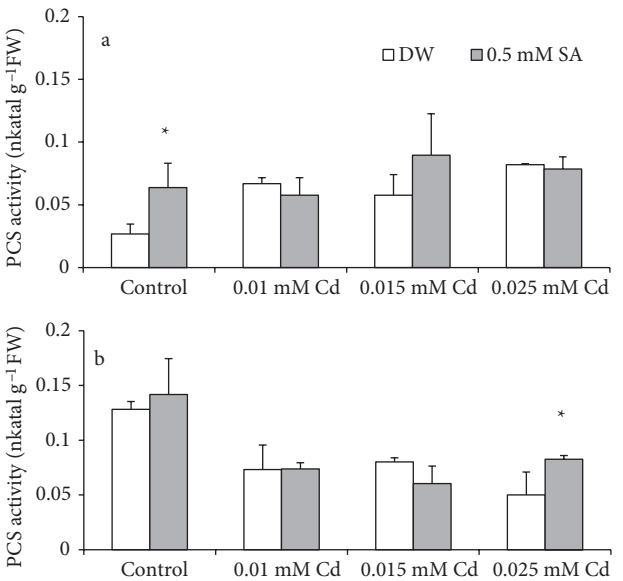

Figure 5. Changes in the phytochelatin synthase (PCS) activity in the leaves (a) and roots (b) of young maize plants after soaking seeds in distilled water (DW) or in 0.5 mM SA solution during Cd stress. *: significant difference between the DW- and SA-treated plants at $P < 0.05$.

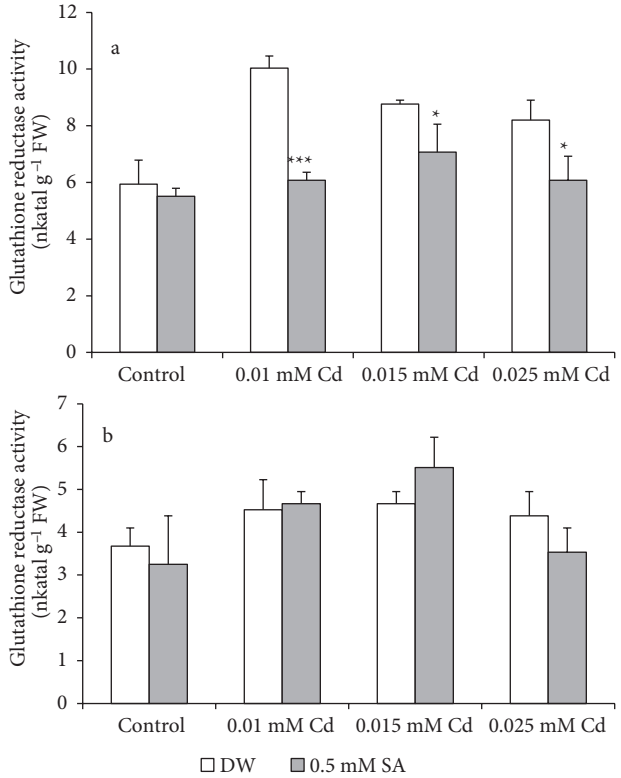

Figure 6. Changes in the glutathione reductase activity in the leaves (a) and roots (b) of young maize plants after soaking seeds in distilled water (DW) or in 0.5 mM SA solution during Cd stress. *, ***: significant difference between the DW- and SA-treated plants at $P < 0.05$ and $P < 0.001$, respectively.

Cd did not significantly change GR activity in the leaves of plants pretreated with SA. This can be explained by assuming that the lower level of oxidative damage did not necessitate the induction of this enzyme in these plants.

4. Conclusions

The exposure of maize plants to Cd may increase the level of PC in the roots, and due to the reduction of substrate and/or feedback regulation processes, this may lead to reduced PCS enzyme activity. Although the presoaking of seeds in SA solution before exposure to Cd may reduce the level of heavy metal injury and has an effect on the pattern of the individual PCs, this protection is not directly connected with the altered regulation of PCs. The low activity of GR in the leaves of SA-treated plants may also indicate a lower level of oxidative stress.

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