Salicylic Acid Alleviates the Cadmium Toxicity in Barley Seedlings[1]

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Salicylic acid (SA) plays a key role in plant disease resistance and hypersensitive cell death but is also implicated in hardening responses to abiotic stressors. Cadmium (Cd) exposure increased the free SA contents of barley (*Hordeum vulgare*) roots by a factor of about 2. Cultivation of dry barley caryopses presoaked in SA-containing solution for only 6 h or single transient addition of SA at a 0.5 mM concentration to the hydroponics solution partially protected the seedlings from Cd toxicity during the following growth period. Both SA treatments had little effect on growth in the absence of Cd, but increased root and shoot length and fresh and dry weight and inhibited lipid peroxidation in roots, as indicated by malondialdehyde contents, in the presence of Cd. To test whether this protection was due to up-regulation of antioxidant enzymes, activities and transcript levels of the H_2O_2-metabolizing enzymes such as catalase and ascorbate peroxidase were measured in control and SA-treated seedlings in the presence or absence of 25 μM Cd. Cd stress increased the activity of these enzymes by variable extent. SA treatments strongly or completely suppressed the Cd-induced up-regulation of the antioxidant enzyme activities. Slices from leaves treated with SA for 24 h also showed an increased level of tolerance toward high Cd concentrations as indicated by chlorophyll a fluorescence parameters. The results support the conclusion that SA alleviates Cd toxicity not at the level of antioxidant defense but by affecting other mechanisms of Cd detoxification.

Cd is a highly toxic and persistent environmental poison for plants and animals (di Toppi and Gabbrielli, 1999). Cd interferes with many cellular functions mainly by complex formation with side groups of organic compounds such as proteins resulting in inhibition of essential activities. Although the mechanisms of cytoplasmic toxicity are identical in all organisms, different plant species and varieties show a wide range of plasticity in Cd tolerance, reaching from the high degree of sensitivity of most plants on the one hand to the hyperaccumulating phenotype of some tolerant higher plants on the other hand (McGrath et al., 2001). On an expanded concentration scale, even sensitive species vary considerably in their response to Cd. For example pea (*Pisum sativum*) is considerably more sensitive to Cd than barley (*Hordeum vulgare* cv Gerbel), which still grows well at concentrations above 10 μM under nutrient rich conditions. Cd induces genetic and biochemical changes in plant metabolism that are related to general and Cd-specific stress responses (Blinda et al., 1997). Cd tolerance is correlated with intracellular compartmentalization and hence specific transport processes that allow the toxic effects of low Cd levels to decrease at least (Brune et al., 1995; Gonzalez et al., 1999). The activation of the cellular antioxidant metabolism belongs to the general stress responses induced by heavy metals (Dietz et al., 1999). Although an active antioxidative metabolism does not represent a Cd tolerance mechanism in a strict sense, it is beneficial for plant performance under heavy metal stress. Inadequate activities of antioxidant defense systems cause oxidative damage, lipid peroxidation, and membrane leakage in plants exposed to Cu, to Fe, and also to Cd.

Salicylic acid (SA) has been identified as an important signaling element involved in establishing the local and systemic disease resistance response of plants after pathogen attack (Alvarez, 2000; Enyedi et al., 1992; Klessig and Malamy, 1994). After a pathogen attack, SA levels often increase and induce the expression of pathogenesis-related proteins and initiate the development of systemic acquired resistance and hypersensitive response. SA appears to regulate the delicate balance between pro- and antideath functions during hypersensitive response. The molecular events involved in SA signaling are not yet fully understood, although an increasing number of potentially involved components, such as protein phosphatases, MAP kinases, bZIP transcription factors, and ankyrin-repeat-containing proteins (NRP 1), are being identified by molecular approaches (Klessig et al., 2000). The early proposed mode of SA action was related to the inhibition of catalase (CAT) and ascorbate peroxidase (APX), two major H_2O_2 scavenging enzymes. The inhibition might cause the cellular concentrations of H_2O_2 to rise. Subsequently, H_2O_2 may act as second messenger and activating defense-related genes (Chen et al., 1993). But apparently, this mechanism cannot be generalized. Employing a series of increasing SA concentrations fed to excised

[1] This work was supported by the Egyptian Government (personal grant to A.M.) and by the Deutsche Forschungsgemeinschaft (grant no. FOR 387, TP 3).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.102.018457.

Arabidopsis leaves, Rao et al. (1997) detected elevated levels of H_2O_2, increased lipid peroxidation and oxidized proteins, stimulated activities of superoxide dismutase and peroxidase, and slightly decreased activities of CAT and APX in leaves. However, most of the changes were only significant at high concentrations of SA above 1 mM. Under these conditions, SA was a pro-oxidant and phytotoxin. The involvement of SA in the development of oxidative damage during germination was further investigated by comparing Arabidopsis wild-type and mutant plants expressing a bacterial SA-decomposing salicylate hydroxylase. The SA-deficient mutant germinated and grew 5- to 8-fold better than the wild type under salinity and osmotic stress and after application of methylviologen, showing that SA multiplies reactive oxygen species (ROS) generation under stress (Borsani et al., 2001). In a converse manner, SA was reported to mediate some positive acclimation responses to abiotic stressors such as UV, heat, and salinity (Yalpani et al., 1994; Janda et al., 1999; Mishra and Choudhuri, 1999; Tissa et al., 2000).

Apparently, SA has broad but divergent effects on stress acclimation and damage development of plants. Therefore, this study aimed at exploring the interaction of SA and Cd stress by using a single SA-induced priming event, either by presoaking of the caryopses for 6 h or by a 24-h treatment of 3-d-old seedlings. The potential significance of SA for plant growth in a heavy metal-polluted environment was supported by the finding that Cd induced an increase in root SA contents. It is demonstrated that SA application caused partial protection against heavy metal toxicity in barley seedlings. The beneficial role of SA on plants exposed to Cd appeared not to be related to the activation of antioxidants.

RESULTS

Cd Exposure Increases SA Contents of Barley Roots

Cd was administered to hydroponics cultures of barley at a concentration of 25 μM, a concentration that resulted in an inhibition of root growth by about 50% (see below; Brune and Dietz, 1995). As a single short-term SA-priming treatment, dry caryopses were soaked for 6 h with 500 μM SA and then grown for the same time period, either being exposed to Cd or under Cd-free control conditions. SA contents were determined 12 d after soaking of the caryopses (Fig. 1A). About 0.2 μg SA g^{-1} fresh weight was detected in control plants and also in plants grown from SA-presoaked caryopses. Root SA contents was doubled in the Cd-treated plants. Interestingly, SA contents was lower in the plants pretreated with SA.

SA Treatment Decreases Cd Toxicity in Barley Seedlings

The basic experiment compared the growth performance of barley seedlings upon Cd exposure with or without previous treatment with SA. Figure 1B exemplifies the time course for one SA presoaking experiment. Root fresh weight increased with an increment of about 7 mg d^{-1} under control growth conditions. The Cd treatment decreased root growth to 3.2 mg d^{-1}. Pretreatment with SA resulted in a growth rate in the presence of 25 μM Cd of 5.1 mg d^{-1}. The beneficial effect of SA was less pronounced on shoot growth (not shown). In the standard experiment, the analyses were then performed with 12-d-old seedlings (Fig. 2). Presoaking had a slightly inhibitory effect on the accumulation of fresh and dry weight of both roots and shoots, respectively (Fig. 2, A–D). Cd exposure reduced root length and root and shoot fresh weight by about 50%, and shoot length and root and shoot dry weight by about 35%. SA pretreatment decreased Cd toxicity. The beneficial effect of SA was seen with all growth parameters and was shown to be statistically significant except for shoot dry weight (not shown). The same positive effect of SA on growth in the presence of Cd was seen in the second type of experiment where 3-d-old seedlings were treated with 500 μM SA added to the hydroponics culture medium for 24 h, 3 d after im-

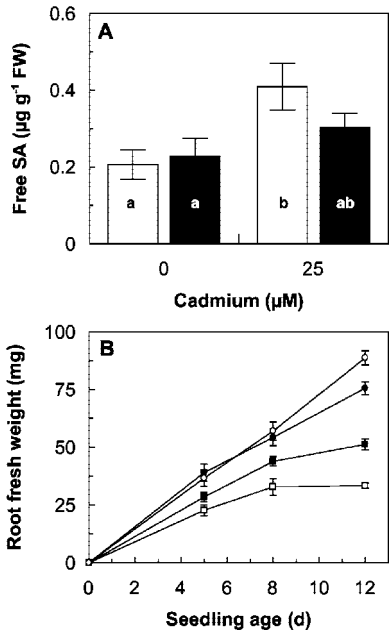


Figure 1. SA contents of 12-d-old barley seedlings (A) and time-dependent effect of SA on growth during a presoaking experiment (B). A, Dry caryopses were soaked in 500 μM SA (black bars) or water (white bars) for 6 h and were grown for 12 d without (left pair of bars) or with (right pair of bars) Cd in the hydroponics medium. The data are means ± SE from $n = 4$ from two experiments. Different letters indicate significant difference at $P = 0.05$ (LSD). B, Development of barley seedlings in the absence (○, ●) or presence (□, ■) of 25 μM $CdCl_2$. Dry caryopses were soaked in 500 μM SA (■, ●) or water (□, ○) for 6 h. Plants were harvested, and root and shoot fresh weight was determined at the time points as indicated. The data are means (± SE) of 15 plants from one experiment, except d 12, which is the mean of 135 plants from three independent experiments.

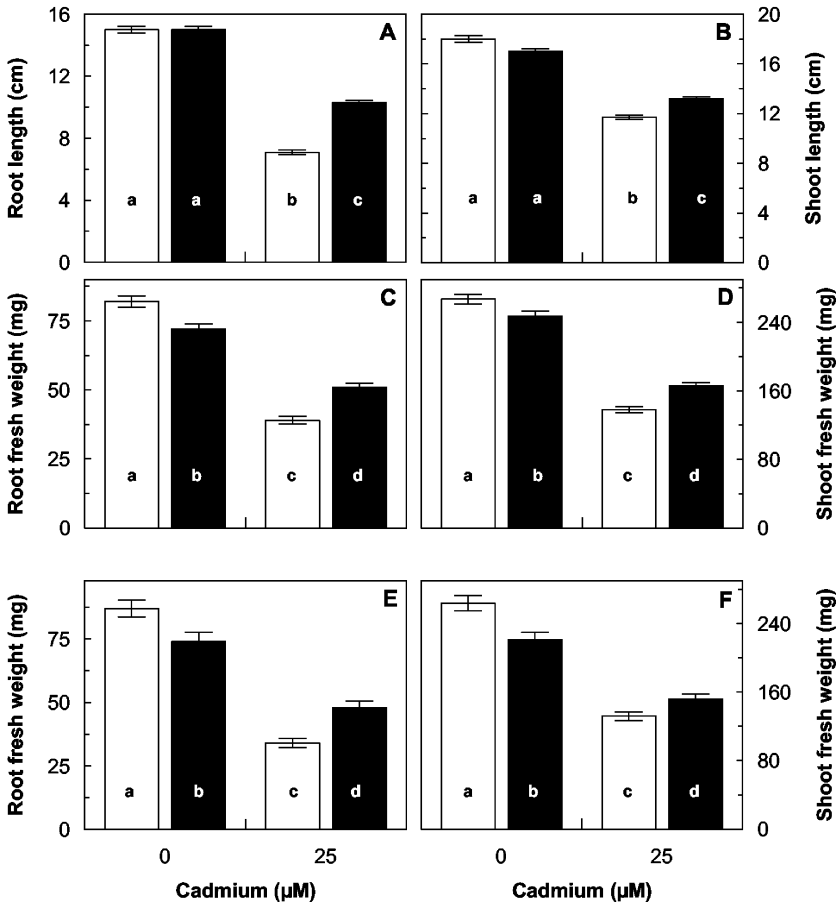


Figure 2. Growth parameters of 12-d-old barley seedlings from a SA presoaking experiment (A–D) or a pretreatment experiment (E and F). Dry caryopses were soaked in 500 μM SA (black bars) or water (white bars) for 6 h, and were grown for 12 d without (left pair of bars) or with (right pair of bars) Cd in the hydroponics medium. The data of root (A) and shoot (B) length as well as root (C) and shoot (D) fresh weight are means ± SD from three independent experiments with a total of 135 plants. For the pretreatment experiment, barley was germinated in water for 2 d, transferred to hydroponics medium, and grown for 10 more d. The SA treatment was performed at d 4 for 24 h by adding 500 μM SA to the hydroponics medium. Afterward, plant growth was continued in normal hydroponics medium. The data on root (E) and shoot (F) fresh weight are means ± SE from 45 plants in two independent experiments. Different letters indicate significant differences at $P = 0.05$ (ANOVA, post-hoc LSD).

bibition (Fig. 2, E and F). Also in this experiment, root and shoot growth were inhibited in the presence of SA in control plants.

Element Contents of Roots and Shoots

Cd contents of root and shoot tissue were very low in the absence of Cd in the growth medium and more than 100-fold increased in samples from plants treated with 25 μM Cd (Table I). Cd contents were the same in control and SA-treated plants. Cd reduced root contents of Mn, K, and P and shoot contents of Mn, Ca, and K. SA treatment did not affect element composition in the absence of Cd except S contents. The Cd-induced changes were mostly unaltered after SA treatment. In roots of Cd-exposed plants, four

Table I. *Element contents of roots and leaves of 10-d-old barley subjected to a SA presoaking experiment*

Dry caryopses were soaked in 500 μM SA or water for 6 h and were grown for 12 d without or with 25 μM Cd in the hydroponics medium. Means of $n = 6$ ± SE from three independent experiments. Different letters mean significance of difference between the treatments ($P < 0.05$, ANOVA; post-hoc test LSD)

Element	Root				Shoot			
	−Cd		+Cd		−Cd		+Cd	
	−SA	+SA	−SA	+SA	−SA	+SA	−SA	+SA
	μmol g^{-1} dry wt							
Cd	0.039 ± 0.01^a	0.027 ± 0.01^a	7.07 ± 0.59^b	7.88 ± 0.71^b	0.007 ± 0.00^a	0.003 ± 0.00^a	1.07 ± 0.02^b	1.06 ± 0.05^b
S	116 ± 5.02^a	136 ± 7.60^b	93 ± 4.13^c	109 ± 3.93^{ac}	115 ± 1.60^a	112 ± 1.27^a	128 ± 1.94^b	126 ± 1.77^b
Mn	9.46 ± 1.82^a	11.55 ± 1.32^a	3.81 ± 0.23^b	2.82 ± 0.48^b	1.29 ± 0.12^a	1.31 ± 0.10^a	0.88 ± 0.04^b	0.81 ± 0.06^b
Zn	1.52 ± 0.12^{ab}	1.46 ± 0.12^{ab}	1.14 ± 0.07^a	1.59 ± 0.18^b	0.77 ± 0.07^a	0.75 ± 0.08^a	0.70 ± 0.04^a	0.77 ± 0.07^a
Mg	460 ± 79.4^a	563 ± 87.8^a	681 ± 71.1^a	526 ± 57.7^a	135 ± 6.51^a	130 ± 2.44^a	137 ± 0.61^a	140 ± 4.79^a
Ca	57 ± 4.48^a	62 ± 3.20^a	66 ± 9.09^a	68 ± 5.41^a	70 ± 3.86^a	73 ± 3.98^a	55 ± 2.25^b	53 ± 5.72^b
K	1,171 ± 33.1^a	1,269 ± 56.8^a	989 ± 27.4^b	1,029 ± 18.1^b	2,150 ± 36.3^a	2,161 ± 24.1^a	1,587 ± 30.6^b	1,689 ± 34.8^b
Fe	96 ± 4.01^a	105 ± 8.74^a	142 ± 10.66^b	94 ± 3.19^a	3.0 ± 0.60^a	1.9 ± 0.15^b	2.2 ± 0.12^{ab}	2.4 ± 0.28^{ab}
P	228 ± 13.05^a	259 ± 22.18^a	173 ± 9.89^b	168 ± 6.79^b	286 ± 8.88^a	299 ± 11.5^a	266 ± 2.64^a	279 ± 5.43^a

differences in element contents appeared to be related to SA pretreatment. Mn and Fe contents were lower and Zn and S levels increased after SA treatment.

Partial Protection to Cd Toxicity Is Seen after SA Pre- or Post-Treatment in Short-Term Experiments with Leaves

The SA-mediated protection was investigated using infiltrated leaf slices of 10-d-old barley. The leaf slices were either pretreated with SA for 24 h followed by a 24-h exposure to 500 μM Cd (Fig. 3A) or first treated with 500 μM Cd for 24 h with subsequent SA treatment (Fig. 3B). Leaf slices were chosen to ensure controlled SA application avoiding transpiration-dependent effects. Chlorophyll a fluorescence was employed as a noninvasive parameter of functional photosynthesis. It is noteworthy that the high concentration of Cd only resulted in a 50% decrease of photosynthetic yield during the 24-h treatment, signifying a low net uptake of Cd. In both experiments, the photosynthetic yield of PSII declined significantly slower in the leaf slices treated with SA either before or after the Cd exposure. An experiment was designed to investigate the role of uptake and vacuolar compartmentalization of Cd in the mechanism of SA-induced alleviation of Cd toxicity. After a 24-h period with or without 500 μM SA in the feeding solution of cut leaves, mesophyll protoplasts were isolated and exposed to 25 μM Cd for 4 h. Intact mesophyll protoplasts were re-isolated and either analyzed directly or used for the isolation of mesophyll vacuoplast. Vacuoplasts are obtained from mesophyll protoplast by ultracentrifugation on a Percoll-density gradient. All dense-cell constituents are lost from the vacuoplasts, which contain the intact vacuole, a small fraction of the cytoplasm, and part of the plasma membrane (Lörz et al., 1976). Their element contents mainly reflect the element composition of the vacuole. The Cd/Ca and Ca/P ratios of protoplasts and vacuoplasts prepared from leaves were indistinguishable between control and SA-treated samples (Table II). Element ratios were calculated to circumvent the problem of tissue-demanding marker enzyme determination. It should be noted that the yield of intact protoplasts was low after both treatments.

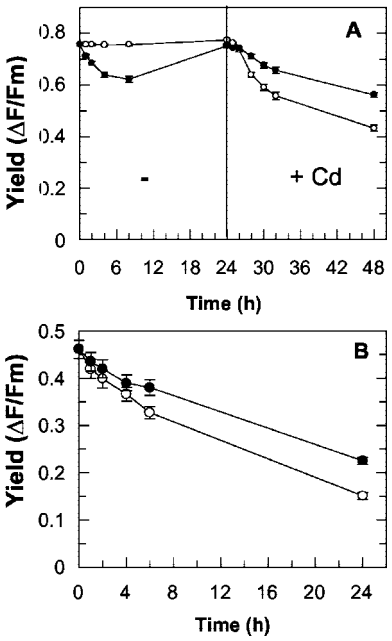


Figure 3. Cd toxicity on fluorescence yield of photosystem II in leaf strips as affected by pre- or post-treatment with SA. A, Slices of primary leaves from 10-d-old barley (1 mm width) were incubated for 24 h in 500 μM SA (●) or water (○), followed by 24-h exposure to 500 μM $CdCl_2$ (pretreatment). Quantum yield of photosystem II (ΦPSII) was measured with a PAM chlorophyll fluorimeter. B, Leaf slices were treated with 500 μM $CdCl_2$ for 24 h, followed by a 24-h measuring period in water (○) or 500 μM SA (●). The data are means ± SD from four independent experiments with 48 determinations.

Table II. *Element contents of mesophyll protoplasts and vacuoplasts isolated from Cd- and SA-treated leaves*

Barley leaves were supplied with 500 μM SA or water for 24 h via the cut leaf sheath. Mesophyll protoplasts were isolated and suspended in sorbit medium supplemented with 25 μM $CdCl_2$. Vacuoplasts were prepared after 4 h. Mesophyll protoplasts and mesophyll vacuoplasts were analyzed for element composition. The table provides the data on element ratios from three independent experiments ± SD.

Element Ratios	Mesophyll Protoplasts		Mesophyll Vacuoplasts	
	Control	SA-Pretreated	Control	SA-Pretreated
Cd/Ca ($\times 10^{-4}$)	17.3 ± 2.8	17.9 ± 0.8	55.1 ± 2.4	57.4 ± 2.8
Cd/P ($\times 10^{-4}$)	8.7 ± 1.6	7.9 ± 1.2	6.1 ± 0.9	5.8 ± 1.0

SA Decreases Cd Toxicity-Induced Lipid Peroxidation Despite Accumulation of Similar Amounts of Cd

Figure 4, A and B, compares malondialdehyde (MDA) and Pro contents of roots from barley plants subjected to toxic Cd with or without soaking of the caryopses in SA. MDA contents indicate lipid peroxidation and increased by about 50% upon Cd exposure in roots of the SA-free controls, but by less than 10% in barley seedlings previously exposed to SA. The effect of SA on lipid peroxidation was not caused by decreased accumulation of Cd in roots and shoots (Table I). Concentrations of the stress metabolite Pro decreased upon presoaking with SA and increased upon Cd exposure in both the control and the SA treatments. The Cd-induced increase in Pro contents was insignificant in the SA-pretreated plants.

Cd Effects on Non-Protein Thiol Contents Were Slightly Changed by SA Pretreatment

Cd binding to sulfhydryl groups of phytochelatins (PCs) is a fundamental mechanism of Cd detoxifica-

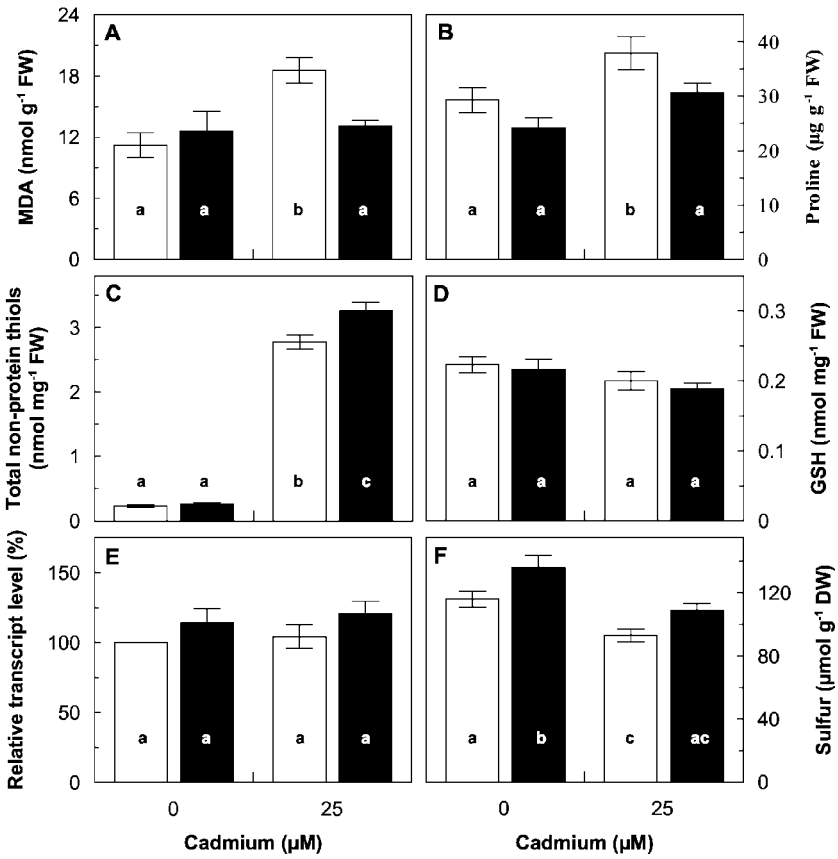


Figure 4. Root MDA (A), Pro (B), non-protein thiols (C), total glutathione contents (D), relative transcript levels of PCS (E), and total S (F) of 12-d-old barley subjected to a SA presoaking-experiment. Dry caryopses were soaked in SA (black bars) or water (white bars) for 6 h and were grown for 12 d without (left pair of bars) or with (right pair of bars) Cd in the hydroponics medium. The data are means ± SE from six to nine determinations of three to four independent experiments. Different letters indicate significant differences at $P = 0.05$ (ANOVA, post-hoc LSD).

tion. PCs are synthesized from glutathione, and their amount can be estimated from the difference of non-protein thiols and glutathione. Therefore, contents of S and thiol compounds and transcript levels of PC synthase (PCS) were measured in the four treatments of a standard SA-presoaking experiment (Fig. 4, C–F). Total S was slightly increased upon SA pretreatment and decreased after Cd administration. Glutathione concentrations were indistinguishable between the treatments, as were the PCS transcript amounts. Total non-protein thiols increased 10-fold upon Cd exposure, and the Cd response was enhanced by 20% after the SA pretreatment.

SA Pretreatment Lowered the Cd-Dependent Increase in Antioxidant and Defense Enzymes

CAT and APX detoxify H_2O_2 in peroxisomes, cytosol, and chloroplasts, respectively. Their activities were measured as representative enzymes involved in antioxidant metabolism and increased upon Cd exposure (Fig. 5). The response pattern to SA pretreatment and to Cd in SA-presoaked plants was opposite for both enzymes; whereas CAT activity dropped to 60% in SA-treated plants, APX activity increased slightly by about 20%. CAT activity of SA-pretreated plants was enhanced upon Cd administration. Despite the increase, the absolute activity in Cd-treated SA plants was in the range of the untreated control. In a converse manner, APX activity was decreased in Cd-treated SA seedlings. Guaiacol-dependent peroxidase and chitinase activities were chosen as indicators of defense and stress response and revealed congruent changes in response to the four experimental conditions. They were slightly increased in SA-presoaked seedlings and more stimulated upon Cd exposure of control plants, but unaffected or decreased by Cd in SA plants.

Expressional Patterns Reflect Distinct Responses to Cd and SA

Transcript levels of six genes related to antioxidant defense were quantified by semiquantitative reverse transcriptase (RT)-PCR (Fig. 6). No pronounced changes were observed for the transcript amounts of *gr* and *dhar*. *Cat*, *apx*, and *gpx* mRNA levels exhibited parallel changes, i.e. no effects after SA treatment, up-regulation in the presence of Cd, and a suppression of Cd-induced up-regulation of transcript amounts in the SA presoaked samples. A distinct pattern was seen for the transcript of GS that was present at elevated amounts in the SA-presoaked control and down-regulated in the presence of Cd in the nutrient solution.

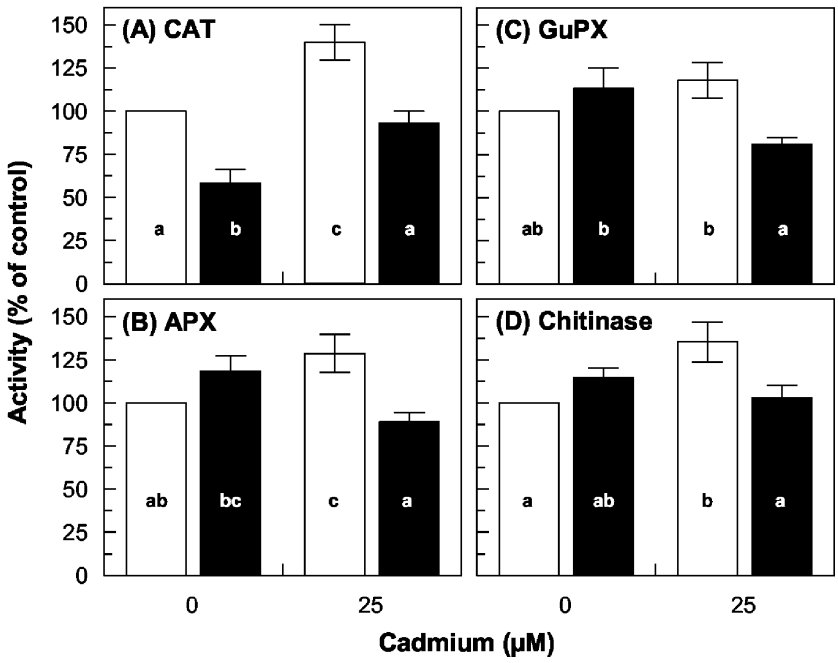


Figure 5. Root activities of CAT (A), APX (B), guaiacol-dependent peroxidase (C), and chitinase (D) in 12-d-old barley subjected to a SA presoaking-experiment. Dry caryopses were soaked in 500 μM SA (black bars) or water (white bars) for 6 h and were grown for 12 d without (left pair of bars) or with (right pair of bars) Cd in the hydroponics medium. The data are means ± SE from nine determinations from three independent experiments. Different letters indicate significant differences at $P = 0.05$ (ANOVA, post-hoc LSD).

DISCUSSION

The experiments described here analyze the beneficial effect of SA on plants exposed to toxic Cd concentrations both in short- and long-term experiments. Free Cd in plasmatic compartments is highly toxic by disturbing cell metabolism and regulation (Van Assche and Clijsters, 1990). As a consequence, ROS are liberated and lipid peroxides formed that are deleterious to cells (Dietz et al., 1999). Oxidative stress is indicated by the increased MDA contents of Cd-treated controls (Fig. 4). The Cd-induced increase in MDA was not seen in SA-treated plants. Growth,

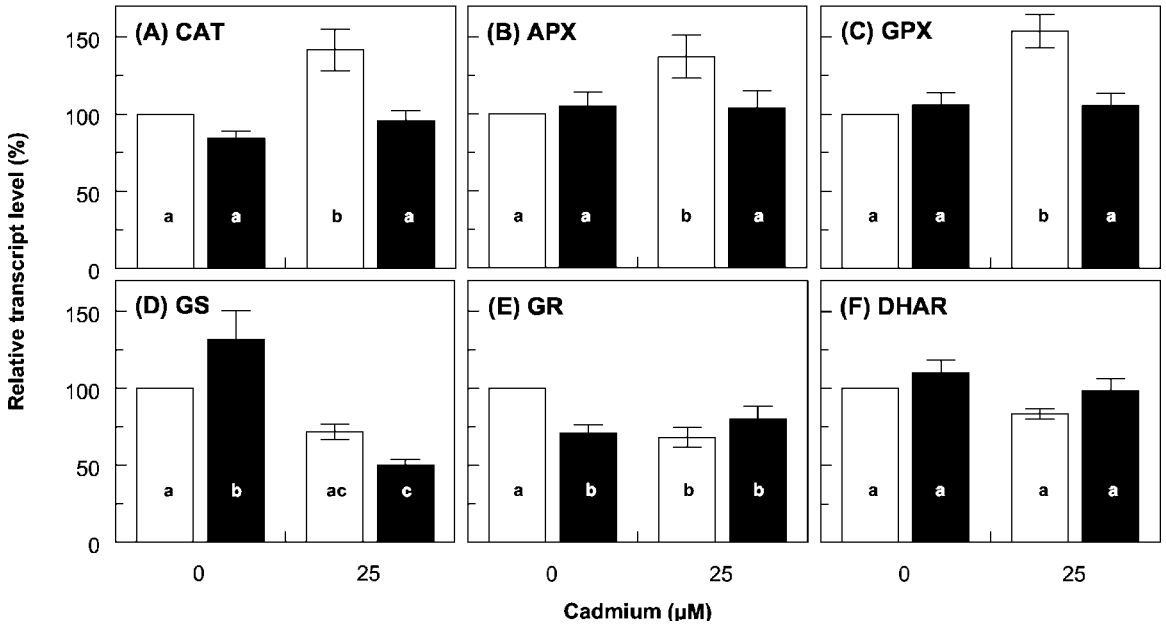


Figure 6. Root levels of transcripts encoding enzymes of redox homeostasis and antioxidant defense in 12-d-old barley subjected to a SA presoaking-experiment. Dry caryopses were soaked in 500 μM SA (black bars) or water (white bars) for 6 h, and grown for 12 d without (left pair of bars) or with (right pair of bars) Cd in the hydroponics. Transcripts were amplified by gene-specific RT-PCR, digitized, and densitometrically analyzed. A, CAT; B, APX; C, glutathione peroxidase (GPX); D, glutathione synthase (GS); E, glutathione reductase (GR); and F, dehydroascorbate reductase. The data are means ± SE from eight to 12 determinations of four experiments. Different letters indicate significant differences at $P = 0.05$ (ANOVA, post-hoc LSD).

photosynthetic parameters, and activities of antioxidant enzymes confirmed the positive SA effect under Cd stress. Cd sequestration and chelation constitute the two principle mechanisms employed to avoid free Cd in plasmatic compartments and to tolerate exposure to elevated Cd levels in the soil (Clemens, 2001). Alternatively, repair of damage may alleviate Cd toxicity. Therefore, the following discussion will center around these mechanisms as being possibly involved in the expression of the beneficial effects of SA on Cd-stressed plants.

SA and Cd Compartmentalization

A moderate resistance to heavy metals can be realized by selective Cd exclusion, lowered uptake, or active efflux from the roots, i.e. by mechanisms leading to lower cytoplasmic Cd contents (Hall, 2002). However, Cd tissue contents were unaltered, both at the whole-plant and organ level, in mesophyll cells and vacuoplasts, ruling out the involvement of differential transport of Cd between plant organs and across the plasma membrane as a physiological cause for the beneficial effect of SA. Members of the ABC transporter family are known to be involved in vacuolar sequestration of heavy metals (Rea et al., 1998). Transcript levels of some Arabidopsis ABC transporters are modified in response to SA (L. Bovet and E. Martinoia, unpublished data). Such transporters might facilitate vacuolar sequestration of Cd in the SA-treated plants. However, Cd distribution was also unaltered between the vacuolar compartment and the rest of the cells as shown by element analysis of the vacuoplasts.

SA content was increased in Cd-stressed plants (Fig. 1). Interestingly the SA content was lower in plants grown from SA-presoaked caryopses. The result also excludes the possibility that formation of stable SA-Cd complexes has lowered Cd toxicity after SA pretreatment. Such complexes may form at mild acidity (Svoboda and Jech, 1994; Gao et al., 1994). Likewise, Cd-SA complex formation in the hydroponics solution is an unlikely cause for the beneficial effect of SA because the exposure to Cd started 3 d after the 6-h SA pretreatment in the presoaking experiment and 24 h afterward in the pretreatment experiment. However, complex formation might have played a role in the short-term experiments with leaf slices.

The beneficial effect of SA was particularly reflected in corresponding changes of a variety of biochemical parameters even at d 12 after SA treatment. Contents of Pro and MDA were lower in the Cd-exposed SA-pretreated plants than in the Cd-treated controls. Pro accumulates in plants under unfavorable growth conditions including drought, salt, and heavy metal stress. In a comparative study with *Silene vulgaris*, Schat et al. (1997) showed that Pro accumulation was higher in non-tolerant than in tolerant plants at identical internal metal loads. A partial relation was established between the Pro accumulation and a heavy metal-induced water deficit due to root growth inhibition. In any case, Pro accumulation appeared to be a suitable indicator of the heavy metal stress experienced by the plants and indicates partial relief from Cd stress after SA treatment in this study. The same conclusion can be drawn from root MDA contents, which indicate oxidative damage to membranes (Dietz et al., 1999), and from activities of guaiacol-dependent peroxidase and chitinase, which can be considered as general stress and defense markers. Because the beneficial effect could not be attributed to modified compartmentalization, increased activities of defense mechanisms such as antioxidant enzymes could be involved in lowering Cd toxicity.

Stimulated Antioxidant Defense Appears Not to Be the Reason for SA-Induced Alleviation of Cd Toxicity

Activities and transcript levels of CAT and APX and mRNA amounts of GPX were increased in response to Cd. The increase was absent in SA-pretreated plants. In plants, APX isoforms are associated with at least four subcellular locations, i.e. thylakoids, stroma, mitochondrion, and cytosol. Root APX activity as measured here mainly reflects the cytosolic isoforms. Total APX activity is higher in root extracts than in leaves and is known to respond to Cd exposure (Dixit et al., 2001). The Cd response was fully suppressed by SA. CAT activity and expressional level decreased upon SA pretreatment. This result concurs with the observation of Ding et al. (2002) that CAT expression was decreased in tomato (*Lycopersicon esculentum*) fruits during the first 3 d after treatment with 10 μM SA for 16 h. Afterward, CAT mRNA levels were higher in treated fruits than in the untreated ones. In these experiments, SA treatment decreased chilling injury of the tomato fruits. The authors hypothesized that inhibition of CAT increases cellular ROS concentrations during the first period of 3 d and triggers activation of defense responses, which allow tolerance of chilling stress. Enzyme activities were not determined in that study (Ding et al., 2002). This and other investigations suggest a critical balance between pro-oxidant and antioxidant activities as basis for the beneficial effect of SA under abiotic stresses such as UV, heat, and salt (Yalpani et al., 1994; Janda et al., 1999; Mishra and Choudhuri, 1999; Tissa et al., 2000). In long-term experiments, a high level of oxidative stress is reflected by concomitant stimulation of certain antioxidant and stress enzymes such as APX, CAT, peroxidases, and chitinases. The pattern of changes of antioxidant enzymes in the presence of Cd indicates that the level of Cd-induced oxidative stress is lower in the SA-treated plants than in the control plants despite lower activities of antioxidant enzymes. It

has to be concluded that stimulated antioxidant defense is not the reason for SA-induced alleviation of Cd toxicity.

CONCLUSIONS

SA exerted a significant beneficial effect on Cd-exposed plants. Increased antioxidant defense appears not to be involved in the alleviation of Cd toxicity in SA-treated plants. Also, total Cd in root and leaf tissue was unaltered in SA-preteated plants. Three hypothetical explanations may account for the positive SA effect on Cd-challenged barley and are discussed in the following. (a) The SA-induced responses may run through distinct phases. In tomato, SA treatment caused hardening against chilling. The expressional pattern of PR proteins and CAT changed with time subsequent to the SA treatment (Ding et al., 2002). Here, it is shown that SA still alleviated toxicity effects during long-term Cd exposure 12 d after SA administration. This may be a manifestation of the beneficial effect of SA during earlier growth periods, which prevented cumulative damage development in response to Cd. (b) SA may activate Cd tolerance mechanisms different from Cd distribution and antioxidant defense. One mechanisms is avoidance of damage and includes any mechanisms of Cd binding resulting in lowered plasmatic free Cd. PC concentrations were slightly increased in the SA-pretreated roots. Thus PCs or other low molecular mass metabolites and proteins could be involved in Cd binding, for example metallothioneins (Wang et al., 1992; Rauser, 1999). (c) Alternatively, SA could enhance repair processes. As mentioned above, SA stimulates expression of certain ABC transporters. Such transporters have been implicated in the vacuolar sequestering of the products of Cd action rather than Cd itself (Rea et al., 1998). A detailed metabolic analysis of SA-treated plants under Cd stress and the use of Cd-sensitive microelectrodes may be appropriate approaches to evaluate the hypotheses.

MATERIALS AND METHODS

Plant Material and Experimental Design

For the SA-presoaking experiment, barley (*Hordeum vulgare* cv Gerbel) grains were soaked for 6 h either in 0.5 mM SA (sodium salt) or in water as a control. The grains were then germinated on vermiculite for 2 d. Small-rooted caryopses were placed in polyethylene pots (2.5 g pot^{-1}) filled with 1.6 L of nutrient solution containing 1.5 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.25 mM $(NH_4)H_2PO_4$, 11.9 μM iron-tartrate, 11.5 μM H_3BO_3, 1.25 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.075 μM $CuSO_4$, and 0.025 μM $(NH_4)Mo_7O_{24}$. The nutrient solution was buffered to pH 5.5 with MES/KOH, aerated, and changed twice per week. $CdCl_2$ was added at a concentration of 25 μM. Plants were grown in a growth chamber at a day/night cycle of 16 h/8 h, at 22°C/20°C, respectively, a relative humidity between 50% and 60% and a light intensity of 100 μmol quanta m^{-2} s^{-1}. After 10 d of growth in hydroponics, i.e. 12 d after soaking the caryopses, the plants were harvested, growth parameters determined, and material was frozen at −80°C for biochemical analysis. For the "pretreatment experiment," the plants were germinated in moist vermiculite and transferred to hydroponics, and on the 3rd d, one-half of the pots were supplemented with 0.5 mM SA for 1 d. The nutrient solution was then replaced, and Cd was added to each second pot. After 8 d, the plants were harvested for analysis.

Determination of Element Composition

Dried leaves and roots and mesophyll and vacuoplast suspensions were macerated in 10% (v/v) HNO_3 at 165°C under pressure. Clear extracts were analyzed with an inductively coupled plasma atomic emission spectrometer (Jobin Yvon JY 70, Instruments S.A., Longjumea, France) as described before (Brune and Dietz, 1995).

Quantification of Free SA in Plant Samples

SA was determined using the method described by Siegrist et al. (2000) with minor modifications. After extraction of tissue equivalent to 1 g fresh weight in 5 mL of methanol, the extracts were cleared by centrifugation. The pellet was re-extracted with 5 mL of methanol. Both methanol extracts were vacuum-dried, and the pellets dissolved in 300 μL of 0.02 M KPO_4, pH 7.6. SA was determined with an HPLC system equipped with fluorescence detection. The mobile phase consisted of 0.02 M KPO_4 buffer, pH 6.1, and methanol at a ratio of 4:1. The samples were passed through a microfilter, and 10-μL aliquots were loaded on a Hypersil BDS-C18 column (250 mm, diameter 4.6 mm, 1.5 mL min^{-1}; Agilent, Agilent Technologies, Waldbronn, Germany) at 40°C. Elution of SA was monitored by fluorescence emission at 410 nm after excitation at 210 nm. Authentic SA was used for calibration, and specificity of the identified peak was proven using a digestion reaction with salicylate hydroxylase from *Pseudomonas* sp. (Sigma Chemicals, Taufkirchen, Germany).

Lipid Peroxidation, Non-Protein Thiols, Glutathione, and Pro Contents

The level of lipid peroxidation in the plant tissue was quantified by determination of MDA, a breakdown product of lipid peroxidation. MDA content was determined with thiobarbituric acid reaction. In brief, 0.25 g of tissue was homogenized in 5 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was spun at 10,000*g* for 5 min. To a 1-mL aliquot of the supernatant, 4 mL of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid was added. The mixture was heated at 95°C for 15 min and cooled immediately, and the absorption of the supernatant read at 532 nm. The value was corrected for the nonspecific absorption at 600 nm. The concentration of MDA was calculated using the extinction coefficient of 155 $mmol^{-1}$ L^{-1} cm^{-1} (Zaho et al., 1994). Contents of Pro and non-protein thiols were measured using colorimetric procedures described by Schat et al. (1997) and Ellman (1959), respectively.

For the determination of SH group contents, plant tissue (100 mg fresh weight) was homogenized in 0.1 M HCl/1 mM EDTA solution. The homogenate was spun at 12,000*g* for 5′. The supernatant was collected and stored at −80°C until the assay was performed, or it was used immediately. Total non-protein SH contents were measured as described by Noctor and Foyer (1998). Supernatant (200 μL) was mixed with 700 μL of assay buffer containing 120 mM sodium phosphate, pH 7.8, and 6 mM EDTA, and the absorption at 412 nm was measured after 2 min following the addition of 100 μL of 6 mM 5′-dithiobis-2-nitrobencoic acid to a 1-mL sample. The absorption at 412 nm was corrected for the absorption of appropriate controls. Total glutathione was analyzed using GR as described by Noctor and Foyer (1998).

Enzyme Assays

Roots equivalent to about 100 mg fresh weight were homogenized in 1 mL of HEPES/KOH buffer (pH 7.5) using a precooled mortar and pestle. The homogenate was spun at 10,000*g* and 4°C for 10 min. The supernatant was used for the enzyme assays. CAT activity was determined by measuring the rate of H_2O_2 conversion to O_2 at room temperature using an O_2 electrode (Dat et al., 1998). APX activity was measured in the presence of 0.25 mM ascorbic acid and 0.5 mM H_2O_2 by monitoring the decrease in absorption at 290 nm (Janda et al., 1999). Peroxidase activity was determined according to Adam et al. (1995). The assay contained 1.5 mL of 100 mM

sodium acetate buffer (pH 5.5), 1 mL of 1 mM guaiacol, 10 μL of tissue extract, and 190 μL of water. The reaction was started by addition of 300 μL of 1.3 mM H_2O_2. The increase in absorption was recorded at 470 nm. Chitinase activity was measured using the substrate carboxy-methyl chitin remazol brilliant violet (CM-chitin-RBV, Blue Substrates, Göttingen, Germany) according to the method described by Wirth and Wolf (1990).

Protoplast and Vacuoplast Isolation

Mesophyll protoplasts were prepared from barley leaves as described by Brune et al. (1995). Vacuoplasts were obtained from mesophyll protoplasts by ultracentrifugation on a Percoll gradient (Lörz et al., 1976).

Chlorophyll a Fluorescence Parameters

Primary leaves were cut at their base from 10-d-old seedlings grown in soil culture, recut under water, and placed in water or 0.5 mM SA in the growth chamber. After 24 h, the leaves were cut in slices of 1 mm width and vacuum-infiltrated with water. The leaf slices were distributed among the wells of a microtiter plate, and $CdCl_2$ was added at final concentrations of 500 μM. Chlorophyll a fluorescence transients were determined with the chlorophyll fluorimeter (MINI-PAM, Waltz, Effeltrich, Germany). Fluorescence yield (ΦPSII) was calculated as $\Phi PSII = (F_m' - F)/F_m'$, where F_m' is the fluorescence sampled from the slices after application of a saturating light pulse of high quantum flux density (5,000 μE) and F represents the fluorescence in the steady state of photosynthesis.

Transcript Quantification

Root tissue was homogenized with mortar and pestle in liquid nitrogen. RNA was extracted using Trizol Reagent (Invitrogen, Karlsruhe, Germany) followed by chloroform extraction, isopropanol precipitation, and spectrophotometric quantification. cDNA was synthesized from DNase-treated RNA with Superscript reverse transcriptase (Invitrogen). The reaction mix contained 1.5 μL of oligo(dT) primer (0.5 $\mu g\ \mu L^{-1}$), 6 μL of first-strand buffer (5× concentrated), 3 μL of dithiothreitol (100 mM), 1.5 μL of dNTPs (10 mM each), 1.5 μL of RNasin, 4 μL of water, and 1.5 μL of Superscript (300 units). After incubation at 42°C for 50 min, the reaction was terminated by heating to 70°C for 15′. cDNA products were standardized for semiquantitative RT-PCR using β-actin primers as reference. For each transcript, sequence-specific 5′ and 3′ primers were designed with melting temperatures between 52°C to 60°C. Cycle numbers were optimized for each template using root cDNA from control plants to assure that the amplification reaction was tested in the exponential phase. The following primers were designed for the gene-specific transcript amplification: dehydroascorbate reductase (EMBL-ACC, AL503912), forward (fw)-5′-GCTGGAGGAGAAGAAGGTGC-3′, and reverse (rv)-5′-GACGCTGGTCAGTGTTTCAG-3′; GR (EMBL-ACC, AL503318), fw-5′-CTGCGTCCCCAAGAAGATAC-3′ and rv-5′-CGGGTAGCTCCTCCAAACTT-3′; GPX (EMBL-ACC, AJ238745), fw-5′-GACTTCACCGTCAAGGATGC-3′ and rv-5′-ATCCTTCTCAATGCTCATGG-3′;GS(EMBL,EMBL-ACC,AL499828),fw-5′-CAAGAACCATCCGAGATCAG-3′ and rv-5′-CCTCTTTCTTGTTCAGTTCC-3′; PCS (EMBL-ACC, AL510072), fw-5′-CACCACCGATCTCAATCTTG-3′ and rv-5′-AAGATCTTATTTCAACGGCG-3′; actin (EST, EMBL-ACC, AL450706), fw-5′-GTGATCTCCTTGCTCATACG-3′ and rv-5′-GGAACTGGAATGGTCAAGG-3′; CAT (EMBL, EMBL-ACC, U20777), fw-5′-CAAGACCTGGCCAGAGGA-3′ and rv-5′-GACGCATCGCACTGTGAC-3′; and APX (EMBL-ACC, AJ006358; Hess and Börner, 1998), fw-5′-CCTCATCGCCGAGAAGAA-3′ and rv-5′-T-GTCCAGGGTCCCTCAAA-3′.

Cloning of PCR Products

PCR products were ligated into pCR2.1-TOPO vector (Invitrogen). The products were transformed into TOP10-*E. coli* cells. Plasmid DNA was isolated and sequenced (MWG Biotec, Eberswalde, Germany; Finkemeier et al., 2002).

Statistics

Data were analyzed with the STATISTICA software. Significance of difference was tested at $P = 0.05$ using ANOVA, post-hoc LSD.

ACKNOWLEDGMENT

We thank Petra Witte-Brüggemann for excellent technical assistance in conducting the HPLC analyses for SA.

Received November 28, 2002; returned for revision January 7, 2003; accepted February 4, 2003.

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