Role of exogenous salicylic acid in alleviating cadmium-induced toxicity in Kentucky bluegrass

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ABSTRACT

To understand the role of salicylic acid (SA) in alleviating cadmium (Cd) toxicity in Kentucky bluegrass (Poa pratensis L.), we investigated the changes of biochemical and physiological indexes in five-week-old Kentucky bluegrass seedlings exposed to 0, 5, 10 or 50 μM Cd with or without 500 μM SA for 7 d. Results showed that, compared to the Cd treatment applied alone, 500 μM SA pretreatment significantly decreased Cd accumulations and increased the chlorophyll level, growth and nutrient elements content (K, Ca, Mg and Fe) in plants, accompanying with the reduction in malondialdehyde and hydrogen peroxide contents. Furthermore, SA pretreatment enhanced remarkably the superoxide dismutase, ascorbate peroxidase and peroxidase activity in the Cd-stressed plants, but decreased catalase activity. Overall, SA might regulate the antioxidant defense activities, reduce Cd uptake and stimulate nutrient elements absorption in Cd-treated with Kentucky bluegrass, thereby improving its resistance to Cd stress.

1. Introduction

Cadmium (Cd) is a widespread non-redox heavy metal which enters the soil environment largely through diverse anthropogenic activities, constitute one of the major environmental contaminants that restrict plant productivity and harm human health (Sharma and Dietz, 2009). Excess Cd interferes with electron transport chains or blocks antioxidant enzymes structures, leading to oxidative damage, membrane leakage and finally cell death (Schützendübel et al., 2001). Plants have evolved many adaptive strategies to cope with Cd stress, including phytochelation and intracellular sequestration as well as induction of antioxidant machinery and stress proteins (Brune and Dietz, 1995; Vazquez et al., 2006). In addition to these, other defense mechanisms that plants have also developed to cope with damage caused by Cd toxicity are correlated with some stress signaling molecules, such as salicylic acid (SA) (Rivas-San Vicente and Plasencia, 2011; You et al., 2011).

SA is a naturally occurring plant hormone that influences various physiological and biochemical functions in plants, and acts as an important signaling element involved in the plants response to biotic and abiotic stress (Durner et al., 1997). SA is known to be involved in plant protection against heavy metal stress. Metwally et al. (2003) concluded that SA alleviated Cd toxicity to barley roots not at the level of antioxidant defense, but by affecting other mechanisms of Cd detoxification. In soybean, SA does not decrease Cd uptake, although it does change Cd distribution in plant organs (Drazic and Mihailovic, 2005). Further study showed that the effect of SA on the alleviation Cd toxicity is probably attributed to indirect...
maintenance of ionic homeostasis in alfalfa (Dražić et al., 2006). However, Guo et al. (2009) reported that SA-induced Cd tolerance in rice is due to SA enhancement of antioxidant defense activities and to SA regulation of Cd uptake, transport, and distribution in plant organs. Similarly, Shi et al. (2009) suggested that the beneficial effects of SA in alleviating Cd toxicity can be attributed to the SA-induced reduction of Cd uptake and enhancement of antioxidant enzyme activities. Recently, Belkhadi et al. (2010) found that SA alleviated Cd toxicity in flax plants related to stabilize membrane integrity and increase mineral nutrients absorption. Taken together, SA has a broad but divergent effect on Cd stress acclimation and damage development in different species (Belkhadi et al., 2010). However, whether SA involves in alleviating Cd toxicity in Kentucky bluegrass has not been explored.

To reveal the role of SA in the resistance to Cd stress in Kentucky bluegrass, we investigated the growth parameters, chlorophyll levels, malondialdehyde and hydrogen peroxide contents, Cd accumulations, nutrient elements absorption and antioxidative system under 0, 5, 10 or 50 μM Cd with or without 500 μM SA for 7 d. Our results indicated that SA-mediated mitigation of Cd toxicity in Kentucky bluegrass plants can be related to the regulation of antioxidative system, the reduction of Cd uptake and the enhancement of nutrient elements absorption.

2. Materials and methods

2.1. Germination experiment

Seeds of Kentucky bluegrass were sterilized with sodium hypochlorite solution (5%) for 5 min and rinsed thoroughly with distilled water, then divided into two groups. One half of the seeds were soaked in different SA solutions (10, 50, 125, 250, 500, 1000, 2500 or 5000 μM) for 12 h, while the other half were soaked in distilled water for 12 h. After soaking, the both groups were placed into Petri plates containing sterile filter sheets moistened with either 2 ml of distilled water. Germination percentages were estimated after 7 days at 25 °C in the dark, using radicle protruded through the seed coat as a criterion. Each treatment was repeated three times independently containing 50 seeds in each replicate.

2.2. Plant growth and treatment with Cd

Seeds of Kentucky bluegrass were soaked for 12 h either in 500 μM SA or in distilled water. Seeds were germinated on moistened filter paper for 7 d at 25 °C in the dark. When the plumule emerged, seedlings were selected for uniformity, and subsequently transferred into polyethylene pots filled with 0.5 l modified Hoagland solution (2 mM KNO₃, 1 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.25 mM NH₄H₂PO₄, 11.5 μM Fe·citrate, 11.5 μM H₂BO₃, 1.25 μM MnSO₄, 0.2 μM ZnSO₄, 0.075 μM CuSO₄, and 0.025 μM NH₄MoO₄) for five weeks. The nutrient solution was adjusted to pH 5.5 with 0.1 M HCl/NaOH, and was renewed every 2 d. Plants were grown in a growth chamber at a day/night cycle 16 h/8 h, at 22 °C, and a light intensity of 120 μmol m⁻² s⁻¹ PAR. Five-week-old seedlings were used for following treatment. Plants were treated with the modified Hoagland’s nutrient solution supplemented with 0, 5, 10 or 50 μM CdCl₂. Plants were harvested at 7 d after treatment, the shoots and roots length were measured, and leaf sample was frozen immediately in liquid nitrogen for storage at −80 °C until further use for biochemical analyses. Ten plants for Kentucky bluegrass were pooled in each replicate. The experiment was repeated six times.

2.3. Determination of chlorophyll content in the leaf tissues

Chlorophyll (Chl) contents were estimated using the method modified from Ma et al. (2012). Fresh leaf samples was grinded using little quartz sand with 80% acetone and centrifuged at 12,000 × g for 10 min at 4 °C. The optical density of the solution was recorded at 645 nm and 663 nm for chlorophyll using a spectrophotometer (UV-9100), and chlorophyll content was expressed as mg g⁻¹ FW.

2.4. Determination of lipid peroxidation and H₂O₂ in leaf tissues

The level of lipid peroxidation in terms of malondialdehyde (MDA) formation was measured with the method of Dhindsa et al. (1981) with slight modifications. A 1 ml aliquot of supernatant was mixed with 4 ml of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) solution, and centrifuged at 15,000 × g for 30 min at 4 °C. The test-tubes were heated for 30 min at 95 °C, and then quickly cooled in an ice bath. After cooling and centrifugation to give a clear supernatant the absorbance of the supernatant at 532 nm were read and the value for the non-specific absorption at 600 nm was subtracted. The level of MDA was calculated by using the extinction coefficient for MDA of 155 mM⁻¹ cm⁻¹ (Heath and Packer, 1968). Contents were calculated as nmol g⁻¹ fresh weight (FW).

Hydrogen peroxide (H₂O₂) was measured according to a method modified by Velikova et al. (2000) with minor modifications. 200 mg of leaf tissues were frozen in liquid N₂ and homogenized in an ice bath with 10 ml of 0.1% trichloroacetic acid. After centrifugation for 20 min (12,000 × g, 4 °C), one milliliter of the supernatant was mixed with 1 ml of 10 mM sodium phosphate buffer (pH 7.0) plus 2 ml of 1 M KI. The photometric absorption of the reaction solution supernatant was read at 390 nm. Contents were calculated as nmol g⁻¹ FW.
2.5. Assay of antioxidant enzymes

For the analysis of antioxidant enzyme activity, 200 mg fresh leaf sample was homogenized in 4 ml phosphate buffer (50 mM, pH 7.0) containing 1% (w/v) soluble polyvinylpyrrolidone and 0.2 mM ascorbic acid. The homogenate was centrifuged at 15,000 × g for 10 min at 4 °C, and the supernatant was then used for the enzyme assays. The activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11) were determined according to the methods described previously (Jiang and Huang, 2001). The peroxidase (POD, EC 1.11.1.7) activity was based on the determination of guaiacol oxidation (ε = 26.6 mM/cm) at 470 nm by H2O2 (Chance and Maehly, 1955). Protein content was determined using bovine serum as a standard (Bradford, 1976). Enzyme activities were expressed on the basis of per unit protein weight.

2.6. Determination of K, Ca, Mg, Fe and Cd content

Harvested plants were washed thoroughly with running distilled water, divided into shoots and roots, and oven dried at 80 °C for 3 d till constant weight. Dried plant tissues were weighted, ground and was wet digested in H2SO4/HNO3 mixture (1/5, v/v) for 24 h, and then it was treated with HNO3/HClO4 mixture (5/1, v/v). K, Ca, Mg, Fe and Cd in the digest were measured by atomic absorption spectrophotometer Perkin–Elmer, AAnalyst 300 (Germany), respectively.

2.7. Statistical analysis

All the data are presented as means with standard errors (SE). Statistical analyses, Student’s t test were performed by statistical software (Ver.13.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Seeds germination of plant

As shown in Fig. 1, the germination percentage of seeds increased from 10 to 500 µM and peaked at 500 µM SA, then declined sharply from 1000 to 5000 µM SA. These suggested that 500 µM SA promoted seed germination of Kentucky bluegrass.

3.2. Plant growth and chlorophyll content

The length or dry weight (DW) of shoots and roots was decreased significantly with the increasing concentrations of Cd and 50 µM Cd caused the significant reduction compared to the control (Table 1). However, pretreatment of SA enhanced the length or DW of shoots and roots in plants exposed to 5–50 µM Cd compared to the Cd alone (Table 1).

Chl contents in leaves showed the toxic nature of Cd in the plant system. The Chl content showed a reduction trend with the increase of Cd concentrations in both Cd alone and SA pretreatment (Table 2). However, the pretreatment of SA on plants
grown with 5–50 μM Cd led to 27–46% reduction of toxic effect on the Chl levels compared to Cd treatments alone, respectively (Table 2).

3.3. Lipid peroxidation and H₂O₂ accumulation

It has been known that Cd can induce oxidative stress, and further damage to the leaf membranes was investigated by monitoring MDA and H₂O₂ content. Under Cd alone or SA pretreatment conditions, both MDA and H₂O₂ content showed an increasing trend with the increasing concentrations of Cd (Table 2). However, SA pretreatment decreased the level of MDA and H₂O₂ significantly, respectively, compared with the Cd concentrations applied alone (Table 2).

3.4. SOD, POD, APX and CAT activity

The addition of 5, 10 and 50 μM Cd caused the disturbances in the activity of the antioxidative enzymes. The SOD activity was increased with the increasing Cd levels in both Cd alone (former) and SA pretreatment plus Cd (latter) conditions, and it was at a lower level than that of the former (Fig. 2b). Similar pattern was also observed in the changes of POD activity (Fig. 2b). In contrast to SOD, APX activity was reduced by the former or latter, while SA pretreatment APX activity of the latter increased by 19.2, 29.1, or 30.1% relative to the former (Fig. 2c). Furthermore, CAT activity showed a relatively small change in activity in both the former and latter, but CAT activity of latter decreased by 17.7, 22.9 or 11.2% compared to the former (Fig. 2d).

3.5. Cd accumulation in plant

As shown in Fig. 3, the Cd concentration in shoots and roots increased significantly with the increasing concentrations of Cd, while Cd concentration in roots was always significantly higher than that in shoots under 5–50 μM Cd treatments. As described above, the similar trends were also observed under SA pretreatment plus Cd. However, pretreatment with SA plus Cd decreased Cd accumulation in shoots and roots remarkably, respectively, compared to corresponding the Cd treatments applied alone.

3.6. K, Ca, Mg and Fe content in plant

As shown in Table 3, Cd applied alone caused a reduction in nutrient (K, Ca, Mg, and Fe) content in both shoots and roots, the effect being most marked at 50 μM Cd. Nutrient contents in shoots was more than that in roots under 5–50 μM Cd treatments. Similarly, the change trends of all nutrient contents were also found under SA pretreatment plus Cd. In presence of 5–50 μM Cd, however, SA-mediated induced a significant increase of all nutrient contents in both shoots and roots.

### Table 1

<table>
<thead>
<tr>
<th>Cd (μM)</th>
<th>SA (μM)</th>
<th>Shoots length (cm)</th>
<th>Roots length (cm)</th>
<th>Shoots DW (mg plant⁻¹)</th>
<th>Roots DW (mg plant⁻¹)</th>
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<td>0</td>
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<td>15.91 ± 0.14</td>
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<td>0</td>
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<td>16.20 ± 0.21</td>
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<td>8.86 ± 0.22*</td>
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<td>2.35 ± 0.04*</td>
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<td>0.96 ± 0.07</td>
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<td>7.49 ± 0.47*</td>
<td>1.93 ± 0.06*</td>
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<td>6.86 ± 0.06*</td>
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<td>1.67 ± 0.06*</td>
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### Table 2

<table>
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<tr>
<th>Cd (μM)</th>
<th>SA (μM)</th>
<th>Total chlorophyll content (mg g⁻¹ FW)</th>
<th>MDA content (nmol g⁻¹ FW)</th>
<th>H₂O₂ content (nmol g⁻¹ FW)</th>
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<tbody>
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<td>0</td>
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<td>18.20 ± 1.41</td>
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<td>40.28 ± 1.03</td>
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<td>SA +</td>
<td>1.26 ± 0.01*</td>
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<td>21.31 ± 1.81*</td>
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<td>38.51 ± 2.20</td>
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<td>10</td>
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<td>1.05 ± 0.03*</td>
<td>38.83 ± 1.48</td>
<td>29.81 ± 1.46*</td>
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<tr>
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<td>SA +</td>
<td>0.92 ± 0.03*</td>
<td>46.80 ± 1.82*</td>
<td>40.52 ± 2.31*</td>
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</table>
4. Discussion

4.1. SA pretreatment affected seeds germination in Kentucky bluegrass

We carried out the germination assays using different concentrations of SA to determine how SA affects seed germination in Kentucky bluegrass. In this study, we found that different concentrations of SA have both promotive and inhibitory effects on germination, depending on the concentration applied and the assay conditions. For example, the germination percentage of seeds increased from 0 to 500 μM and peaked at 500 μM SA, however, above SA concentrations of 500 μM, the process of seed germination was remarkably impeded compared to 0 μM SA, especially suppressed at 5 mM SA (Fig. 1). Similar results have been reported for over 1 mM SA delay or even inhibit germination in Arabidopsis thaliana (Rajjou et al., 2006);
higher doses SA (>0.25 mM) inhibit seed germination in barley (Xie et al., 2007), while maize embryo germination, for which high doses of SA, in the range of 3–5 mM, completely inhibited germination (Guan and Scandalios, 1995). This is presumably because higher concentrations of SA induced an oxidative stress, thus inhibiting seeds germination (Rao et al., 1997; Yuan and Lin, 2008; Lee et al., 2010). Therefore, we choose 500 μM SA to carry out all subsequent experiments, a concentration that promotes seed germination significantly and is beneficial for establishment of the seedling.

4.2. SA pretreatment altered antioxidant defensive response in Kentucky bluegrass plants exposed to Cd stress

Cd does not generate ROS (reactive oxygen species) directly, but it induces oxidative stress by interference with the antioxidant defense system, leading to oxidative damage, membrane leakage and finally cell death (Sanità di Toppi and Gabrielli, 1999). In the present study, all the growth parameters and total chlorophyll content of Kentucky bluegrass plants were decreased significantly as Cd concentration increased, accompanying with the significant increase in the lipid peroxidation (MDA) and ROS (H2O2) contents (Tables 1 and 2). Cd toxicity in plants was linked to liberate ROS and form lipid peroxides, leading to generate oxidative stress by interference ROS producing and/or ROS-removing system, thus destroying biological membranes under Cd stress (Apel and Hirt, 2004). To repair the Cd-induced inhibitory effects of ROS, plants employ ROS-detoxifying antioxidant defense mechanisms (Gill et al., 2011). SOD catalyzes the conversion of superoxide anion (O2-) to O2 and H2O2 (Sudhakar et al., 2001), its activity reflects the O2 radical-eliminating ability of plants (Ekmecki et al., 2008). We observed that under Cd alone or SA pretreatment plus Cd, SOD activities were increased proportionally with the increase of Cd concentrations, but the SOD activity was at SA pretreatment plus Cd higher than that of Cd alone (Fig. 2a), indicating enhanced the activation of the antioxidative system to scavenge the accumulation of O2. Meanwhile, POD, CAT and APX are major scavengers of H2O2. We here found that significant enhancement of POD activity was observed under Cd alone, which further showed high efficiency under SA pretreatment (Fig. 2b). In contrast to POD, APX and CAT activities showed a reduction trend in both under Cd alone and SA pretreatment plus Cd, but its were at SA pretreatment plus Cd lower than that of Cd alone (Fig. 2c and d). Similar results were also reported in other plants exposed to SA pretreatment plus Cd (Krantev et al., 2008). Notably, CAT activity showed a relatively small change in activity under SA pretreatment plus Cd (Fig. 2d). POD catalyzes H2O2-dependent oxidation of substrate, while CAT eliminates H2O2 by breaking it down directly to form water and oxygen. CAT is less efficient than POD in H2O2 scavenging because of its low-substrate affinity, and so as long as the stress is not too strong for the plant defense capacity. Siedlecka and Krupa (2002) also found that and the main response to heavy metal is an increase in SOD and POD activities along with a decrease in CAT. Another likely explanation is that CAT is downregulated at the level of steady-state mRNA by SA (Dorey et al., 1998). In addition, H2O2 generated by plant cells under stress treatments is believed to be scavenged mainly by the ascorbate glutathione cycle, in which APX reduces H2O2 to H2O using ascorbate as the electron donor (Mittler, 2002). Therefore, the levels of ROS and the extent of oxidative damage depend largely upon the whole antioxidant defense system and the cooperation or coordination among the ROS-scavenging enzymes (Li et al., 2003). Moreover, leaf lipid peroxidation and ROS in Kentucky bluegrass plants was shown to increase significantly with Cd stress, but this oxidative damage was alleviated by SA pretreatment (Table 2). As previously described by Guo et al. (2007) in Cd-treated rice plants, SA pretreatment elevated enzymatic antioxidants in shoots, hence leading to alleviation of the oxidative damage as indicated by the lowered MDA and H2O2 levels. Belkadi et al. (2010) found that decreased oxidative damage can be ascribed to the role of SA in stabilize membrane integrity to improve flax plants resistance to Cd stress. Taken together, the protective role of SA against Cd stress was related to the reduction in lipid peroxidation and ROS accumulation by regulating the antioxidant systems, which resulted in lesser inhibition of total chlorophyll content. On the other hand, SA as signaling molecules plays an important role in regulating photosynthesis and respiration of plant cells, thereby improving the Cd stress tolerance (Duan et al., 2010; Wang et al., 2013).

Table 3

Effect of SA (without SA) or SA+ (SA pretreatment) plus different concentrations of Cd on K, Ca, Mg and Fe content of Kentucky bluegrass at 7 d. Values are means ± SE. Each value is a mean of six replicates (n = 6). Asterisk (*) denotes (t test) significant difference (P < 0.05).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cd (μM)</th>
<th>SA (μM)</th>
<th>K (mg g-1DW)</th>
<th>Ca (mg g-1DW)</th>
<th>Mg (mg g-1DW)</th>
<th>Fe (mg g-1DW)</th>
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<td>Shoot</td>
<td>0</td>
<td>SA–</td>
<td>98.2 ± 10.81</td>
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<td></td>
<td></td>
<td>SA+</td>
<td>100.3 ± 11.62</td>
<td>24.21 ± 4.68</td>
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<td></td>
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<td>29.86 ± 3.98*</td>
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4.3. SA pretreatment reduced Cd uptake and stimulated the nutrient elements absorption in Kentucky bluegrass plants exposed to Cd stress

Mechanisms of metal tolerance can involve both ion transporters that transport the metal out of the cytoplasm (either into an internal compartment or out of the cell), and the synthesis of metal-binding ligands that can detoxify the metal in the cytoplasm (Clemens, 2001). ATP-Binding Cassette (ABC) transporters is involved in sequestering metals (Cd^{2+}) into vacuole to alleviate Cd toxicity (Rea et al., 1998; Yamaguchi et al., 2002; Huang et al., 2012; Wang et al., 2013). Notably, the expression of ABC transporter gene (GmPDR12) is strong and rapidly induced in Glycine max after SA treatment (Eichhorn et al., 2006). Our results showed that SA pretreatment decreased Cd uptake in shoot and root subjected to Cd treatments compared to corresponding the Cd treatments applied alone (Fig. 3). This could be one of the important causes for SA-induced Cd tolerance in Kentucky bluegrass plants. Such SA-mitigated Cd toxicity has been shown in other plant species, including rice (Panda and Patra, 2007), maize (Krantev et al., 2008) and flax (Belkhadi et al., 2010). The effects of SA on Cd accumulation may be therefore attributed to stimulate the transcriptional activation of certain ABC transporters by SA (Eichhorn et al., 2006).

Interestingly, we also found that pretreatment with SA induces a significant increase of internal K, Ca, Mg and Fe concentrations in both shoots and roots under Cd stress (Table 3). Until now only a few experimental data in the literature showed that SA influence nutrient absorption under heavy metal toxicity (Drazic and Mihailovic, 2005; Metwally et al., 2003; Belkhadi et al., 2010). Indeed, it is well known that H+-ATPase in plasma membrane plays an important role in the transport of multiple ions (Palmgren and Harper, 1999), and there are investigations indicating that SA-mediated could induce H+-ATPase activity (Gordon et al., 2004), which might be responsible for SA increasing absorption of K, Ca, Mg and Fe under Cd toxicity. Integrated the findings, we conclude that, SA might play an important role in reducing Cd uptake and maintaining ionic homeostasis in Kentucky bluegrass plants subjected to Cd stress, thereby contributing to improving the resistance to Cd stress.

In conclusion, SA may attenuate Cd toxicity in Kentucky bluegrass plants exposed to Cd stress, which probably includes the regulation of the antioxidant system, the reduction of Cd uptake and the improvement of mineral nutrient absorption. This suggests that SA could be used as a potential growth regulator to improve the plant growth under Cd stress. This may be at some extent helpful in understanding the role of SA in Kentucky bluegrass plants resistance to Cd stress.

Acknowledgments

This research was financially supported by the Scientific Innovation Ability Construction Project of Beijing Academy of Agriculture and Forestry Sciences (BAAFS) (Grant No. KJCK201101003), the International Cooperation and Exchange Project of China (Grant No. 2008DFR30200) and the National Basic Research Program of China (973 Program, Grant No. 2010CB951502). We are grateful to the anonymous reviewers and editors for their valuable suggestions and comments on the initial version of the manuscript.

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