J Plant Growth Regul (2008) 27:205–210
DOI 10.1007/s00344-008-9047-6

ORIGINAL ARTICLES

The Effect of Salinity Pretreatment on Cd Accumulation and Cd-Induced Stress in *BADH*-Transgenic and Nontransgenic Rice Seedlings

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Received: 30 December 2007 / Accepted: 20 March 2008 / Published online: 25 April 2008

Abstract The influence of betaine aldehyde dehydrogenase (BADH) and salinity pretreatment on oxidative stress under cadmium (Cd) toxicity was investigated in rice *cv.* Xiushui 11 and its *BADH*-transgenic line Bxiushui 11. The results showed that plants previously treated with 4.25 and 8.5 mM NaCl, respectively, for 5 days each had higher Cd concentrations in both roots and shoots of the two rice genotypes compared with the controls. Malondialdehyde (MDA) content in both leaves and roots was increased by salinity pretreatment and was significantly lower in the salinity-pretreatment plants than in the controls when the plants were consequently exposed to Cd stress. Salinity pretreatment also increased proline content and the activities of superoxide dismutase (SOD) and peroxidase (POD) in both leaves and roots. It can be assumed that salinity pretreatment enhances the defensive ability of plants against oxidative stress through increasing activities of antioxidative enzymes. The *BADH*-transgenic line (Bxiushui 11) had lower Cd and MDA content, higher SOD and POD activities, and higher proline content than its wild type (Xiushui 11). The current results suggest that betaine, a product of *BADH* expression, improves the tolerance of rice plants to Cd stress through increasing the activities of antioxidative enzymes and osmoprotectant content.

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Keywords Betaine aldehyde dehydrogenase · Cadmium stress · Rice *(Oryza sativa* L.) · Salinity · Tolerance

Introduction

Abiotic stresses, like salinity and heavy-metal toxicity, which are known to disturb redox homeostasis in plant cells, can induce a burst of reactive oxygen species (ROS), thus resulting in oxidative stress. The generation of ROS is considered one of the main causes of injuries in plants exposed to abiotic stress (Sanità di Toppi and Gabbrielli 1999; Shah and others 2001; Hegedüs and others 2001; Sandalio and others 2001). Therefore, the ability of a plant to eliminate or reduce ROS is closely associated with its tolerance to abiotic stress. It is well documented that some antioxidants, such as glutathione, ascorbate, and cysteine, and antioxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione peroxidase (GPX), and glutathione reductase (GR), are involved in scavenging ROS from plant cells (Delaunay and others 2000). Plants with higher antioxidant content or antioxidative enzyme activity under abiotic stress commonly show an increased level of stress tolerance. Consequently, it can be assumed that a plant species or genotype with greater tolerance to a certain stress due to increased synthesis of antioxidants or antioxidative enzymes is also expected to be highly tolerant to other abiotic stresses. However, the available results are still contradictory (McBirde 1987).

Another important physiologic strategy employed by higher plants to resist abiotic stress is the adjustment of osmosis through production and subsequent transportation of organic osmoprotectants in plant cells (Tamura and others 2003). Betaine is considered one of the most important

osmoprotectants (McNeil and others 1999), and betaine aldehyde dehydrogenase (BADH) is the key enzyme involved in betaine synthesis. Hayashi and Murata (1998) reported that plants with higher BADH activity had a high degree of resistance to abiotic stress. Accordingly, the plants with higher BADH activity can be expected to show higher tolerance to both salinity and heavy-metal stresses than those with lower BADH activity. In this context, the *BADH*-transgenic line may provide conclusive evidence of the relationship between BADH and abiotic stress tolerance.

Plants may suffer simultaneously from multiple abiotic stresses during growth. It has been suggested that plant injury would be enhanced when they are exposed to several stresses simultaneously (Sepehr and others 2003). It has also been reported that plants previously exposed to a modest degree of stress show an enhanced tolerance to subsequent other stresses (King and Nelson 1987; Mozafar and Oertli 1990). However, the mechanism underlying this phenomenon is not known. In this study we investigated whether betaine and salinity pretreatment could alleviate Cd toxicity in rice.

Materials and Methods

Plant Material and Solution Culture

A japonica rice *cv*. Xiushui 11 and its *BADH*-transgenic line Bxiushui 11, provided by Biological Engineering Center, Chinese Rice Research Institute (CRRI, Hangzhou), were used in the study. These two genotypes were seeded on a sandy bed, which was previously washed with dilute sulfuric acid. When the fourth leaves emerged, the seedlings were selected for uniformity and transferred to 4.5-L plastic pots filled with nutrient solution. The composition (mg L^{-1}) of the nutrient solution was as follows: $(NH_4)_2SO_4$, 48.2; $NaH_2PO_4 \bullet 2H_2O$, 49.9; K_2SO_4, 174.3; $CaCl_2$, 111.0; $MgSO_4 \bullet 7H_2O$, 419.0; $MnCl_2 \bullet 4H_2O$, 1.8; $(NH_4)_6Mo_7O_{24} \bullet 2H_2O$, 0.09; H_3BO_3, 1.1; $ZnSO_4 \bullet 7H_2O$, 0.05; $CuSO_4 \bullet 5H_2O$, 0.04. The solution was renewed every 3 days and adjusted to pH 5.5 with KOH or HCl, as required.

Salinity Pretreatment and Cd Stress

Salinity pretreatment was carried out at 7 days after transplantation or initiation of solution culture. For salinity pretreatment, plants were first treated with 4.25 mM NaCl for 5 days, followed by treatment with 8.5 mM NaCl for another 5 days. After salinity pretreatment, Cd treatments were initiated. There were three Cd levels—0.0, 1.0, and 5.0 μM—supplied as $CdSO_4$. The experiment was conducted in a completely random design (CRD) with four replications, with each replication comprising 40 plants.

Estimation of Cd Content

Plant samples (shoots and roots) were collected 10 days after Cd treatment. Roots were immersed in a solution containing 1.0 mM EDTA for 2 h and then rinsed with distilled water thoroughly. Samples were dried in an oven at 100 and 60°C for 1 and 48 h, respectively. Cd concentrations in shoots and roots were measured by inductively coupled argon-plasma emission spectrometry (ICAP 61E trace analyzer; Themo-Jarrell Ashe, Franklin, MA, USA).

Biochemical Estimations

Plants were sampled (leaves and roots) for different biochemical estimations 10 days after salinity pretreatment and 10 or 20 days after Cd treatment. Proline content was determined according to Bates and others (1973). The samples were washed with distilled water and ground with a pestle and mortar under chilled conditions in a buffer specific for each enzyme. The homogenate was filtered through four layers of muslin cloth, centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatant was used for enzyme assay. Malondialdehyde (MDA) content and the activities of superoxide dismutase (SOD) and peroxidase (POD) were measured according to Shah and others (2001). For MDA content, plant tissues (0.2 g FW) were homogenized and extracted in 10 ml of 0.25% (w/v) TBA made with 10% (v/v) trichloroacetic acid (TCA). The extract was heated at 95°C for 30 min and then quickly cooled on ice. After centrifugation at 10,000*g* for 10 min, the absorbance of the supernatant was measured at 532 nm.

For SOD assay, the samples (0.5 g FW) were homogenized in 5 ml extraction buffer consisting of 50 mM phosphate (pH 7.8), 0.1% (w/v) BSA, 0.1% (w/v) ascorbate, and 0.05% (w/v) ß-mercaptoethanol. The assay mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 9.9 mM L-methionine, 57 μM NBT, 0.025% (w/v) Triton X-100, and 0.0044% (w/v) riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm, and an inhibition curve was prepared against different volumes of extract. SOD activity was expressed as U g^{-1} FW and 1 unit (U) was defined as the amount in the volume of extracts that caused inhibition of NBT photoreduction by 50%.

For POD assay, the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2, and the enzyme extract. The increase in the absorbance due to oxidation of guaiacol (E = 25.5 mM^{-1} cm^{-1}) was measured at 470 nm, and the enzyme activity was calculated in terms of μmol of guaiacol oxidized min^{-1} mg^{-1} protein at 25 ± 2°C. Protein content in leaves was determined according to Bradford (1976), with bovine serum albumin (BSA) as the standard protein.

Statistical Analysis

The data were analyzed by two-way ANOVA for the salinity-pretreatment experiment and by three-way ANOVA for the Cd stress experiment. The mean values were compared by the *post-hoc* least-significant-difference test (LSD) at $P \leq 0.05$. The statistical software Data Processing System (DPS) (Tang and Feng 1997) was used for data analysis.

Results

Cd Content

In the present study, Cd concentration in both shoots and roots increased with increasing Cd level; however, it was significantly higher in roots than in shoots. Irrespective of plant tissues and levels of Cd stress applied, Cd concentration was constantly lower in the *BADH*-transgenic line Bxiushui 11 than in its wild-type Xiushui 11 (Table 1). The effect of salinity pretreatment on Cd concentration in plants varied with Cd level in nutrient solution and plant tissue. On the whole, Cd concentration increased in both shoots and roots when the plants were pretreated with 8.5 mM NaCl in comparison with the controls (without salinity pretreatment). There were significant differences between the controls and salinity-pretreatment plants in shoot Cd concentrations with treatment of 5.0 μM Cd, and in root Cd concentration in both treatments of 1.0 and 5.0 μM Cd. However, no significant difference was found in shoot Cd concentrations in the 1.0 μM Cd treatment.

Table 1 Cd Concentrations (mg kg^{-1} dry mass) in Shoots and Roots under Different Cd Levels in Relation to Salinity Pretreatment in Rice

Salinity pretreatment	Cd (μM)	Shoot		Root	
		Xiushui 11	Bxiushui 11	Xiushui 11	Bxiushui 11
Control	0.0	0.01	0.03	0.06	0.03
	1.0	3.38	1.66	90.95	83.70
	5.0	21.20	18.90	229.33	205.88
NaCl	0.0	0.02	0.03	0.04	0.05
	1.0	4.56	1.99	115.37	108.92
	5.0	32.61	26.22	275.23	236.84
$LSD_{0.05}$		1.29		13.10	

Malondialdehyde (MDA) Content

MDA content increased significantly in leaves and roots when the plants were exposed to salinity pretreatment for 10 days compared with the controls without salinity pretreatment (Table 2). Subsequent Cd stress caused a significant increase in MDA content irrespective of plant tissues and rice genotypes. Under Cd stress (1.0 and 5.0 μM Cd treatments), MDA content was lower in the *BADH*-transgenic line Bxiushui 11 than in Xiushui 11, suggesting that the *BADH* gene is responsible for alleviating oxidative stress. The Cd effect on MDA content in relation to salinity pretreatment exhibited a similar trend at 10 and 20 days after Cd stress (Table 3). Thus, the plants pretreated with salinity had constantly lower MDA content than did the controls at the same Cd level irrespective of plant tissues and rice genotypes.

Superoxide Dismutase (SOD) Activity

After 10 days of salinity pretreatment, there was a significant increment in both shoot and root SOD activities compared with the controls (Table 2). Moreover, the activity was significantly higher in the *BADH*-transgenic line Bxiushui

Table 2 Effect of Salinity Pretreatment on MDA and Proline Contents and Activities of Superoxide Dismutase (SOD) and Peroxidase (POD) in Rice Prior to Initiation of Cd Stress

Salinity pretreatment	Genotype	MDA content (μg g^{-1} FW)	SOD activity (U g^{-1} FW)	POD activity (μmol guaiacol reduced min^{-1} mg^{-1} protein)	Proline content (μg g^{-1} FW)
(a) Leaf					
Control	Xiushui 11	109.9	2.81	12.07	11.70
	Bxiushui 11	82.6	7.21	12.86	14.98
NaCl	Xiushui 11	136.6	5.68	14.99	15.24
	Bxiushui 11	96.0	11.69	18.81	18.81
$LSD_{0.05}$		10.2	1.69	1.27	2.01
(b) Root					
Control	Xiushui 11	34.4	3.76	10.27	10.70
	Bxiushui 11	16.6	9.65	12.55	13.06
NaCl[c]	Xiushui 11	62.6	5.68	14.74	15.33
	Bxiushui 11	24.2	12.85	15.78	16.19
$LSD_{0.05}$		5.5	1.03	3.71	3.44

Table 3 Effect of Cd Stress in Relation to Salinity Pretreatment on MDA Content (μg g^{-1} FW) in Rice

Salinity pretreatment	Cd (μM)	Leaf		Root	
		Xiushui 11	Bxiushui 11	Xiushui 11	Bxiushui 11
(a) 10 days after Cd stress					
Control	0.0	111.68	96.34	39.82	32.0
	1.0	127.41	105.70	73.18	35.14
	5.0	157.63	121.75	98.77	45.23
NaCl	0.0	86.86	91.25	53.58	22.89
	1.0	115.99	94.25	60.57	23.93
	5.0	145.38	115.04	70.62	26.81
$LSD_{0.05}$		10.45		2.31	
(b) 20 days after Cd stress					
Control	0.0	107.22	74.67	58.26	24.27
	1.0	121.98	83.18	66.17	25.89
	5.0	134.26	94.42	97.95	36.87
NaCl	0.0	70.79	64.21	41.35	15.60
	1.0	86.02	81.16	59.82	21.52
	5.0	111.80	87.34	81.63	30.93
$LSD_{0.05}$		10.32		5.73	

11 than in Xiushui 11 irrespective of plant tissues and salinity pretreatment. With increasing Cd level, SOD activities in both leaves and roots increased in the two genotypes. Leaf SOD activity was consistently higher in Bxiushui 11 than in Xiushui 11 irrespective of the level and duration of Cd stress (Table 4). However, root SOD activity, although higher in Bxiushui 11 than in Xiushui 11 at 10 days after Cd stress, was significantly lower in the *BADH*-transgenic line than in its wild type at 20 days after Cd stress.

Table 4 Effect of Cd Stress in Relation to Salinity Pretreatment on Superoxide Dismutase Activities (U g^{-1} FW) in Rice

Salinity pretreatment	Cd (μM)	Leaf		Root	
		Xiushui 11	Bxiushui 11	Xiushui 11	Bxiushui 11
(a) 10 days after Cd stress					
Control	0.0	5.27	13.81	5.09	10.25
	1.0	5.67	15.08	5.44	14.22
	5.0	6.99	15.85	6.09	14.75
NaCl	0.0	2.01	8.34	2.01	5.74
	1.0	3.57	10.63	3.57	7.96
	5.0	4.69	12.70	4.69	8.52
$LSD_{0.05}$		0.84		0.77	
(b) 20 days after Cd stress					
Control	0.0	6.18	14.11	5.94	3.90
	1.0	7.20	17.13	6.28	5.05
	5.0	7.89	19.25	7.01	5.83
NaCl	0.0	5.74	9.46	9.19	8.76
	1.0	7.96	13.20	14.75	11.26
	5.0	8.52	15.72	17.06	13.71
$LSD_{0.05}$		0.94		0.79	

Peroxidase (POD) Activity

POD activities in both leaves and roots were also markedly affected by salinity pretreatment (Table 2) and subsequent Cd stress (Table 5). There was no significant difference between the two genotypes in both leaf and root POD activities under normal conditions. Salinity pretreatment caused a significant increase in POD activity irrespective of plant tissues and genotypes, except for roots of Bxiushui 11. Moreover, the difference between the two genotypes in leaf MDA content became significant, with the *BADH*-transgenic line being higher than its wild type. A similar trend in POD activity was observed at 10 and 20 days after Cd stress. The effect of salinity pretreatment on POD activity under Cd stress varied with genotype, plant tissue, and the time of Cd stress. Significantly higher POD activity was detected in salinity-pretreated plants than in the controls for leaves of Xiushui 11 and roots of Bxiushui 11 after 10 days of Cd stress, and in roots of both genotypes after 20 days of Cd stress. In addition, a significant genotypic difference in POD activity was detected when plants were

Table 5 Effect of Cd Stress in Relation to Salinity Pretreatment on Peroxidase Activities (μmol guaiacol reduced min^{-1} mg^{-1} protein) in Rice

Salinity pretreatment	Cd (μM)	Leaf		Root	
		Xiushui 11	Bxiushui 11	Xiushui 11	Bxiushui 11
(a) 10 days after Cd treatment					
Control	0.0	12.91	18.76	8.43	14.99
	1.0	13.86	19.28	16.61	15.23
	5.0	15.13	24.05	20.58	16.52
NaCl	0.0	14.64	16.43	8.45	22.45
	1.0	18.32	21.42	16.11	29.05
	5.0	20.80	21.61	20.54	36.21
$LSD_{0.05}$		1.86		1.42	
(b) 20 days after Cd treatment					
Control	0.0	17.91	17.43	13.36	14.61
	1.0	20.12	26.47	15.47	15.22
	5.0	21.91	22.04	19.05	21.28
NaCl	0.0	17.30	16.68	17.40	28.16
	1.0	19.44	21.73	21.63	33.37
	5.0	21.73	23.80	24.06	36.36
$LSD_{0.05}$		1.11		1.98	

exposed to Cd stress, with Bxiushui 11 having consistently higher activity than Xiushui 11 (Table 5).

Proline Content

Salinity pretreatment increased proline content in leaves (Table 2). In response to salinity pretreatment, leaf proline content was higher in the *BADH*-transgenic line Bxiushui 11 than in Xiushui 11, whereas there was no significant difference between the two genotypes in root proline content. When the plants were exposed to Cd stress, proline content increased significantly (Table 6) and the plants pretreated with salinity had significantly higher proline content than the controls, irrespective of plant tissue and genotype. It can also be seen from Tables 2 and 6 that proline content was constantly higher in Bxiushui 11 than in Xiushui 11 under normal conditions (without Cd stress) and under Cd stress.

Discussion

Oxidative stress is one of the most important factors that cause damage to plants exposed to many abiotic stresses (Shah and Dubey 1997; Prasad and others 1999; Lin and Kao 2000; Shah and others 2001). It is well documented that MDA is a product of cell membrane lipid peroxidation (Shah and others 2001; Hegedüs and others 2001), and its content in vivo can indicate the extent of oxidative stress in plants and cell membrane homeostasis. Betaine is a key organic osmoprotectant for osmotic homeostasis in plant cells, and its synthesis is partly dependent on BADH (Wood and others 1996; McNeil and others 1999). In *BADH*-transgenic rice, salt tolerance has been reported to be significantly enhanced in comparison with its wild type (McNeil and others 1999). The present results showed that Xiushui 11 had consistently higher MDA content than its *BADH*-transgenic line Bxiushui 11 when both plants were exposed to salt or Cd stress, indicating that betaine may alleviate oxidative stress.

Commonly, MDA content is used to indicate the degree of lipid peroxidation, which will be enhanced when plants are exposed to some kinds of abiotic stresses, including salinity and Cd toxicity (Chaoui and others 1997; Wu and others 2003). In the current study, salinity pretreatment caused a dramatic increase of MDA content in both shoots and roots, indicating the occurrence of oxidative stress. However, in the subsequent Cd treatment, the plants pretreated with salinity had lower MDA content than the controls, suggesting alleviation of oxidative stress due to Cd toxicity. Obviously the alleviation is attributed to induced enhancement of antioxidative enzymes, including SOD and POD, in the salinity-pretreated plants (Tables 4 and 5). It may be suggested that the moderate salinity stress induced acclimation of rice plants to the subsequent Cd stress. Meanwhile, it was found that the acclimation, resulting in higher tolerance to Cd stress as reflected by low MDA content and high antioxidative enzyme activity, increased the Cd concentration in plant tissues. Hence, it should be carefully considered if the crop cultivars with high tolerance to salinity accumulate more Cd when they are planted in Cd-contaminated soils. Guo and others (2004) reported that barley genotypes with high tolerance to aluminum tend to have higher Cd concentrations. Moreover, the current results showed that MDA content and Cd concentrations in both leaves and roots were much lower in the *BADH*-transgenic line Bxiushui 11 than in its wild-type Xiushui 11. Hence, it can be assumed that betaine may protect the cell membrane from Cd- or salinity-induced injuries, probably through alleviating oxidative stress.

SOD and POD are two key enzymes for scavenging reactive oxygen species (ROS), thus protecting the cell membrane against the damage caused by oxidative stress (Himelblau and others 1998; Sanità di Toppi and Gabbrielli 1999; Schutzendubel and Polle 2000; Ranieri and others 2001). Plants under abiotic stress have evolved a defense system against oxidative stress by increasing SOD and POD activities (Shah and others 2001; Hegedüs and others 2001; Sandalio and others 2001; Mittova and others 2004). In the current study, SOD and POD activities were much higher in the *BADH*-transgenic line Bxiushui 11 than in Xiushui 11, indicating that the expression of the *BADH* gene may stimulate the synthesis and/or activation of these two enzymes. It was also found that salinity pretreatment increased SOD and POD activities in both rice genotypes and caused significantly reduced MDA content under subsequent Cd stress. Meanwhile, under Cd stress, SOD activities in leaves and roots were lower in salinity-pretreated plants than in control plants, indicating that salinity-pretreated plants suffer from slight antioxidative stress. A higher SOD activity in the roots of salinity-pretreated plants may be attributed to a limited duration of aftereffect

Table 6 Effect of Cd Stress in Relation to Salinity Pretreatment on Proline Content (μg g^{-1} FW) in Rice

Salinity pretreatment	Cd (μM)	Leaf		Root	
		Xiushui 11	Bxiushui 11	Xiushui 11	Bxiushui 11
Control	0.0	52.64	63.45	53.19	66.32
	1.0	87.31	90.44	85.67	93.56
	5.0	99.11	107.36	109.58	117.98
NaCl	0.0	69.47	78.52	72.35	79.41
	1.0	97.53	103.61	99.41	113.87
	5.0	108.46	120.57	118.01	125.49
$LSD_{0.05}$		9.40		7.68	

produced by the salinity pretreatment. It was reported that plants previously exposed to a moderate abiotic stress might develop a tolerant response and enhance tolerance to subsequent abiotic stress, although different mechanisms might be involved (Kuznetsov and others 1999; Cuartero and others 2006; Cayuela and others 2007). The current results showed that enhanced tolerance to Cd stress in salinity-pretreated plants could be related to increased activities of antioxidant enzymes.

It is generally thought that proline accumulates when plants are exposed to salinity or other abiotic stresses (McNeil and others 1999; Matysik and Bhalu 2002; Siripornadulsil and others 2001). In this study, no significant difference was observed in root proline content between the transgenic line and its wild type at the end of salinity pretreatment. However, under Cd stress, the two genotypes showed a significant difference in proline content, with the content in the *BABH*-transgenic line Bxiushui 11 higher than in the wild-type Xiushui 11. Hence, it can be assumed that proline accumulation might not be a direct consequence of the response to salt stress, and BADH may enhance its synthesis, leading to a favorable osmotic adjustment.

Acknowledgments The authors are grateful to the Zhejiang Bureau of Science and Technology (2005C12024), the National Natural Science Foundation (30600379), and the Zhejiang Natural Science Foundation (Z304104) for their financial support of this research program.

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