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Effect of nitrogen deficiency on antioxidant status and Cd toxicity in rice seedlings

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Abstract Effect of nitrogen (N) deficiency on antioxidant status and Cd toxicity in rice seedlings was investigated. N deficiency resulted in a reduction of shoot growth but not root growth. The contents of N-containing compounds such as nitrate, chlorophyll, and protein decreased in leaves of rice seedlings grown under N deficiency. Accumulation of abscisic acid and H_2O_2 in leaves was induced by N deficiency. The content of ascorbate and the activities of ascorbate peroxidase, glutathione reductase, and catalase in N-deficient leaves were lower than their respective control leaves. However, glutathione content was not affected and superoxide dismutase activity was increased by N deficiency. Cd toxicity in N-deficient seedlings was more pronounced than that in N-sufficient ones. Pretreatment with ascorbate or L-galactono-1,4-lactone, a biosynthetic precursor of ascorbate resulted in a reduction of Cd toxicity enhanced by N deficiency. N deficiency also resulted in an enhancement of Cd uptake in rice seedlings. The possible mechanism of Cd toxicity enhanced by N deficiency is discussed.

Keywords Antioxidant system · Cadmium · Nitrogen deficiency · Rice

Abbreviations

ABA	Abscisic acid
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
DAB	3,3-Diaminobenzidine
DHA	Dehydroascorbate
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
FW	Fresh weight
GalL	L-galactono-1,4-lactone
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
ROS	Reactive oxygen species
MDA	Malondialdehyde
SOD	Superoxide dismutase

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Introduction

Cadmium (Cd) is a widespread nonessential heavy metal, classified as a human carcinogen, and the uptake and accumulation of Cd in plants represent the main entry pathway into humans and mammals. In plants, Cd causes severe physiological and morphological effects such as chlorosis and growth reduction. Cd is a non-redox metal unable to participate in Fenton-type reactions, but it causes oxidative stress by generating reactive oxygen species (ROS) (Garnier et al. 2006). ROS react with lipids, proteins, pigments and nucleic acids and cause lipid peroxidation, membrane damage and inactivation of enzymes, thus resulting toxic effects. To minimize and/or to protect against the toxic effects of these damaging ROS, plants have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction in order to maintain cellular redox homeostasis. Plants use enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) as well as non-enzymatic

antioxidants like ascorbate (AsA) and glutathione (GSH) to scavenge ROS (Noctor and Foyer 1998).

Crop plants have a fundamental dependence on inorganic nitrogenous fertilizers, principally in the forms of NO_3^- and NH_4^+. The lack of nitrogen (N) is a key limiting factor of crop yields on agricultural soils. As N fertilizer becomes more expensive and as farmers reduce usage of N fertilizer because of the negative environmental impacts, it will be important to gain a better understanding of how crop plants can be designed to grow more efficiently in soils with lower N input (Good et al. 2004).

It has been reported that shortage of N in soils rapidly slows plant shoot growth, while root growth is often less inhibited (Marschner 1995). The involvement of abscisic acid (ABA) in this growth allocation in favor of root growth has been suggested (Vysotskaya et al. 2008). N limitation is associated with decreased enzyme activities that are required for energy metabolism such as photosynthesis and respiration (Marschner 1995). The decline in the ratio of Rubisco activity to electron transport activity under N deficiency has been described previously (Evans and Terashima 1987). As a result, surplus electron flow under N limitation could lead to enhanced oxygen photoreduction in the chloroplast via the Mehler reaction resulting in the production of ROS. In order to detoxify ROS, increases in the activities/content of antioxidants are expected in leaves of N-limited plants. All enzymatic and most non-enzymatic antioxidants contain N except AsA. Thus it also seems plausible that N-deficient plants possess a lower chloroplast antioxidant capacity. The changes in the antioxidant system in response to N deficiency have been studied in spinach (Logan et al. 1999), Arabidopsis (Koprivova et al. 2000; Kandlbinder et al. 2004), barley (Finkemeier et al. 2003), maize (Tewari et al. 2004), and mulberry (Tewari et al. 2007). However, little appears to be known about the changes in antioxidant status in rice plants under N deficiency.

Crops grown in the field may encounter several abiotic stresses, rather than a single stress. For this reason, the importance of focusing the research programs on the responses of crops to a combination of two different stresses has been emphasized (Mittler 2006). Frequently, heavy metal-contaminated sites are low in N availability (Finkemeier et al. 2003). Thus the relationship between N nutrition and Cd toxicity is of ecological importance. Despite the importance of the N-Cd relationship for survival of plants in the presence of Cd, the information about the effect of N deficiency on Cd toxicity at the physiological level is rather limited. In Taiwan, inappropriate disposal of industrial waste has given rise to widespread Cd contamination of irrigated water (higher than 10 mg L^{-1}) (Chen and Lee 1997). Moreover, farmers are encouraged to reduce usage of N fertilizer. It is not known whether Cd toxicity of rice seedlings is influenced by N deficiency. The present study was undertaken with the objective to examine the effect of N deficiency on antioxidant status and Cd toxicity in rice seedlings.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter papers at 37°C in the dark. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a beaker containing half-strength Kimura B nutrient solution with sufficient N supply (control) or deficient N supply (–N). Nutrient solution for the control contains the following macro- and micro-elements: 182.3 μM $(NH_4)_2SO_4$, 91.6 μM KNO_3, 273.9 μM $MgSO_4 \cdot 7\ H_2O$, 91.1 μM KH_2PO_4, 182.5 μM $Ca(NO_3)_2$, 30.6 μM Fe-citrate, 0.25 μM H_3BO_3, 0.2 μM $MnSO_4 \cdot H_2O$, 0.2 μM $ZnSO_4 \cdot 7\ H_2O$, 0.05 μM $CuSO_4 \cdot 5H_2O$ and 0.07 μM H_2MoO_4. Chloride salts substituted for nitrate salts, and Na_2SO_4 for $(NH_4)_2SO_4$, respectively, under –N conditions. The nutrient solutions (pH 4.7) were replaced every 3 days. The hydroponically cultivated seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30/25°C day/night and 90% relative humidity. When the third leaves of control or –N seedlings were fully grown, the second leaves of control and –N seedlings were excised to determine the contents of chlorophyll, protein, nitrate, ABA, H_2O_2, and antioxidants (AsA and GSH), and the activities of antioxidative enzymes (SOD, APX, GR, and CAT).

Cd treatment

Cd toxicity (chlorosis) in control seedlings was visually observed 6 days after $CdCl_2$ treatment (Chao et al. 2010). Thus, to evaluate Cd toxicity, twelve-day-old control and –N seedlings were transferred to N-sufficient and -deficient nutrient solution with or without 5 μM $CdCl_2$ for 6 days, respectively. The contents of chlorophyll, malondialdehyde (MDA), H_2O_2 and AsA in the second leaves were then determined.

Growth response

At the end of treatment, the seedlings were divided into their separate parts (shoots, adventitious roots, and primary

roots). The length of the shoots and primary roots and the fresh weight (FW) and dry weight (DW) of shoots and roots (adventitious roots plus primary roots) were measured. For DW determination, the shoots and roots were dried at 65°C for 48 h, a time when DW was constant.

Determination of H_2O_2, chlorophyll, malondialdehyde, and nitrate

To visualize H_2O_2 in situ, 3,3-diaminobenzidine (DAB) staining was performed as described previously (Hsu and Kao 2007). The H_2O_2 content was also measured colorimetrically. H_2O_2 was extracted with sodium phosphate buffer (50 mM, pH 6.8) containing 1 mM hydroxylamine, a catalase inhibitor. The H_2O_2 content was measured after reaction with $TiCl_4$ (Tsai et al. 2004). The blank reaction consisted of 50 mM phosphate buffer in the absence of leaf extracts. The absorbance was measured at 410 nm. The amount of H_2O_2 was calculated by using a standard curve prepared with known concentrations of H_2O_2.

The chlorophyll content was determined according to Wintermans and De Mots (1965) after extraction in 96% (v/v) ethanol. For protein determination, leaves were homogenized in a 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976). Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined by the thiobarbituric acid reaction as described by Heath and Packer (1968). For nitrate determination, leaves were homogenized with double distilled water. The homogenate was centrifuged for 25 min at 17,600 *g*. The supernatant was used for determination of nitrate by the method described by Hecht and Mohr (1990). The contents of H_2O_2, chlorophyll, MDA, and nitrate were expressed on the basis of FW.

Determination of Cd

For determination of Cd, samples were dried at 65°C for 2 days. Dried material was ashed at 550°C for 4 days. The ash residue was incubated with 31% HNO_3 and 17.5% H_2O_2 at 72°C for 2 h, and dissolved in distilled water. Cd concentrations were then quantified using an atomic absorption spectrophotometer (Model AA-6800, Shimadzu, Kyoto, Japan) and expressed on the basis of DW.

Determination of AsA, DHA, GSH, and GSSG

AsA and DHA contents in 5% (w/v) trichloroacetic acid were determined as described by Law et al. (1983). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA. The Fe^{2+} then forms complexes with bipyridyl, giving a pink color that absorbs at 525 nm. GSH and GSSG contents in 3% sulfosalicylic acid extract were determined by the method of Smith (1985). The content of GSH was spectrophotometrically determined with an enzyme-recycling assay at 412 nm. The assay is based on sequential oxidation of GSH by 5, 5-dithiobis-(2-nitrobenzoic acid) and reduction by NADPH in the presence of known amount of GR. The contents of AsA, DHA, GSH and GSSG were expressed on the basis of FW.

Enzyme extraction and assays

Leaf samples were excised and immediately used for enzyme extraction. All operations were carried out at 4°C. For extraction of enzymes, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of APX activity, 2 mM AsA was added to the extraction buffer. The homogenate was centrifuged at 12,000 *g*. SOD activity was determined according to Paoletti et al. (1986). This method, originally used for animal material (liver), has been used to determine SOD activity in rice (Dey and Kar 1995), maize (Bennicelli et al. 1998), cucumber (Piacentini et al. 2001), wheat (Goggin and Colmer 2005), and potato (Agrawal et al. 2008). The reaction mixture (2.73 mL) contained 100 mM triethanolamine-diethanolamine buffer (pH 7.4), 7.5 mM NADH, EDTA/$MnCl_2$ (100 mM/50 mM, pH 7.4), 10 mM 2-mercaptoethanol, and enzyme extract (0.2 mL). The reaction was started by the addition of NADH. The reaction was allowed to proceed for 10 min. The absorbance was measured at 340 nm. One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NADH oxidation observed in blank sample. CAT activity was assayed according to Kato and Shimizu (1987). The decrease in H_2O_2 was followed as the decline in the absorbance at 240 nm, and the activity was calculated using the extinction coefficient (40 $mM^{-1}cm^{-1}$ at 240 nm) for H_2O_2. One unit of CAT was defined as the amount of enzyme which degraded 1 µmol H_2O_2 per min. APX activity was determined according to Nakano and Asada (1981). The decrease in AsA concentration was followed as a decline in the absorbance at 290 nm and activity was calculated using the extinction coefficient (2.8 $mM^{-1}cm^{-1}$ at 290 nm) for AsA. One unit of activity for APX was defined as the amount of enzyme that degraded 1 µmol of AsA per min. GR activity was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreased 1 optical density min^{-1} at 340 nm. Enzyme activities were expressed on the basis of FW.

Determination of ABA

For extraction of ABA leaves were homogenized with a pestle and mortar in extraction solution (80% methanol containing 2% glacial acetic acid). To remove plant pigments and other non-polar compounds which could interfere in the immunoassay extracts were first passed through a polyvinylpyrrolidone column and C18 (Sep-Pak Vac) cartridges (Waters, Milford, MA, USA). The eluates were concentrated to dryness by vacuum evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantified by ELISA. The ABA immunoassay detection kit (Phytodek) was purchased from Agdia (Elkhart, IN, USA) and is specific for (+)-ABA. By evaluating [^{3}H]ABA recovery, [^{3}H]ABA loss was <3% by the method described here. The content of ABA was expressed on the basis of FW.

The *Rab16A* is an ABA responsive rice gene (Mundy and Chua 1988; Hong et al. 2009). Thus, the ABA level was also judged by the expression of *OsRab16A*. Semi-quantitative RT-PCR analysis was used to examine the expression of *OsRab16A*. Total RNA was isolated from the second leaves of seedlings with use of TRIzol reagent (Invitrogen, CA, USA), according to supplier's recommendations. To prevent DNA contamination, RNA was treated with Turbo DNase I (Ambion, TX, USA) for 30 min at 37°C before RT-PCR analysis. Moreover, the control PCR amplifications involved use of RNA as a template after the DNase I treatment to verify the complete elimination of contaminated DNA. The reverse-transcription reactions involved 200 ng of total RNA by use of the SuperScript III first-strand synthesis RT-PCR system (Invitrogen, CA, USA) according to the manufacturer's protocol.

The gene-specific primer was designed from the 3′UTR of the rice *Rab16A* gene (Hong et al. 2009). The sequences used and the predicted amplicon are listed in Table 1. The RT-PCR program initially started with 50°C for 30 min; 94°C denaturation for 5 min, followed by 94°C for 30 s, 22 to 32 cycles of 50°C for 30 s, and 68°C for 30 s. The PCRs were optimized for a number of cycles to ensure product intensity within the linear phase of amplification. All tests were repeated at least three times, and one of the repeats is shown in the figures. For all treatments, three replicates of RT-PCR were conducted with three batches of total RNA samples isolated independently. PCR products were resolved by elecrophoresis in 3% agarose gel and stained with ethidium bromide. The gel images were digitally captured with use of a SynGene gel documentation system and analyzed with use of Genetools (Syngene, MD, USA). The rice *OsActin* gene was used for normalization.

Statistical analysis

Statistical differences between measurements ($n = 3$, 4, or 40) for different treatments were analyzed following Student's *t*-test or Duncan's multiple range test. A $P < 0.05$ was considered statistically significant.

Results

Effect of N deficiency on growth response

Effect of N deficiency on growth response was shown in Table 2. Shoot length but not root length was significantly reduced by N deficiency. N deficiency also resulted in a decline in the FW and DW of shoots. However, the FW and DW of roots were not affected by N deficiency.

Table 2 Length, fresh weight (FW), and dry weight (DW) of roots and shoots of rice seedlings grown under N-sufficient (control and -deficient (–N) conditions

Parameter	Control	–N
Shoot length (cm)	13.2 ± 0.1	11.9 ± 0.1*
Root length (cm)	5.0 ± 0.2	5.0 ± 0.3
Shoot FW (mg seedling^{-1})	58.9 ± 0.9	44.9 ± 0.6*
Root FW (mg seedling^{-1})	41.3 ± 1.5	38.0 ± 1.2
Shoot DW (mg seedling^{-1})	12.2 ± 0.2	10.3 ± 0.2*
Root DW (mg seedling^{-1})	5.9 ± 0.1	5.8 ± 0.1

The seedlings were cultivated for 12 days in a Phytotron with natural sunlight at 30°C (day)/25°C (night) and 90% relative humidity. *Asterisks* represent values that are significantly different between control and –N treatments at $P < 0.05$

Table 1 Primers used in semi-quantitative RT-PCR assay

Gene	TIGR Locus Name	Primer	Sequence (5′ to 3′)	Products (bp)
OsRab16A	LOC_Os11g26790.1	*Rab16A-5′*	CACAGTACAAACAACACGCAGACA	105
		Rab16A-3′	CCGAGCGCAATAAAAGGAAA	
OsActin	LOC_Os03g50885.1	*Actin-5′*	ATGCTCTCCCCCATGCTATC	465
		Actin-3′	TCTTCCTTGCTCATCCTGTC	

Effect of N deficiency on N-containing compounds

Chlorophyll, protein and nitrate are compounds containing N. Deficiency of N significantly decreased the content of chlorophyll, protein, and nitrate in the second leaves (Fig. 1a, c, d). Chlorosis was also visually observed in N-deficient leaves (Fig. 1b).

ABA accumulation is induced by N deficiency

When ELISA was used to determine ABA, we found that deficiency of N resulted in an increase in ABA levels in the second leaves (Fig. 2a). Rab16A (initially called Rab1) mRNA is known to increase in rice embryos, leaves, roots, and callus-derived suspension cells on treatment with ABA (Mundy and Chua 1988). Our previous work also demonstrated that increasing the ABA concentration from 1 to 12 μM progressively increased the expression of *OsRab16A* in rice roots (Hong et al. 2009). Thus, ABA content reported in this study was also judged by the transcripts of *OsRab16A*. Figure 2b shows that expression of *OsRab16A* was increased in N-deficient leaves.

H_2O_2 accumulation is induced by N deficiency

In the present study, H_2O_2 production was first visualized by a histochemical method with DAB that is based on the formation by H_2O_2 of a brown polymerization product. N deficiency induced an accumulation of DAB-H_2O_2 reaction product in the second leaves (Fig. 3a). When H_2O_2 was measured colorimetrically, we also observed that N deficiency increased H_2O_2 content in the second leaves of rice seedlings (Fig. 3b).

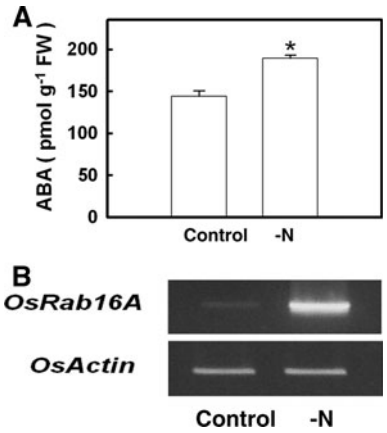

Fig. 2 ABA contents (**a**) and *OsRab16A* transcript levels (**b**) in the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions for 12 days. *Bars* show means ± SE ($n = 4$). *Asterisks* represent values that are significantly different between control and –N treatments at $P < 0.05$

Effect of N deficiency on antioxidant status

When rice seedlings were grown under conditions of N deficiency for 12 days, it was observed that N deficiency had no effect on GSH, GSSG, and the GSH/GSSG in the second leaves of seedlings (Fig. 4d–f). In contrast, N deficiency resulted in a reduction of AsA content and the AsA/DHA ratio in the second leaves of seedlings (Fig. 4a, c). However, N deficiency increased DHA content (Fig. 4b). In the present study, we also investigated the effect of N deficiency on the activities of antioxidative enzymes such as SOD, APX, GR, and CAT. It was observed that N deficiency increased SOD activity (Fig. 5a), but decreased APX, GR, and CAT activities (Fig. 5c, b, d).

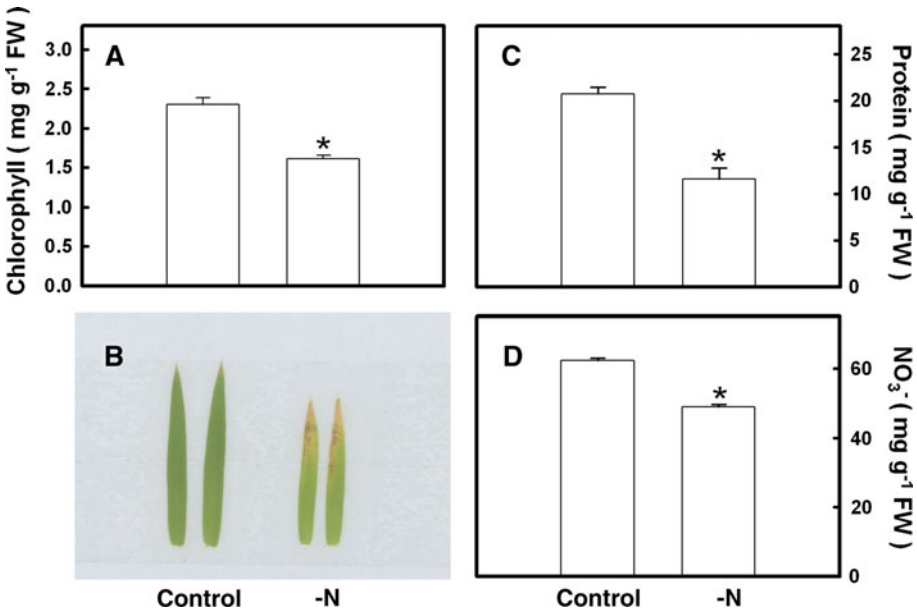

Fig. 1 The contents of chlorophyll (**a**), protein (**c**), and nitrate (**d**) in and chlorosis (**b**) of the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions for 12 days. Bars show means ± SE ($n = 4$). *Asterisks* represent values that are significantly different between control and –N treatments at $P < 0.05$

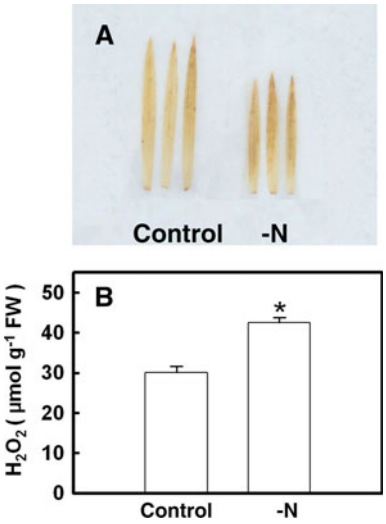

Fig. 3 DAB-H_2O_2 reaction product (**a**) and H_2O_2 content (**b**) in the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions for 12 days. A: Representative photographs of DAB-H_2O_2 reaction products are shown. *Bars* in B show means ± SE ($n = 4$). *Asterisk* represents values that are significantly different between control and –N treatments at $P < 0.05$

N deficiency enhances Cd toxicity and Cd uptake

Effects of N deficiency on the contents of chlorophyll, AsA, MDA, and H_2O_2 were examined. We observed that Cd-decreased chlorophyll and AsA contents (Fig. 6a, d) and Cd-increased MDA and H_2O_2 contents (Fig. 6b, c) in N-deficient leaves were more pronounced than their respective control leaves.

We also examined the effect of N deficiency on the uptake of Cd. It was observed that shoot and roots of N-deficient seedlings had higher Cd concentration than those of N-sufficient seedlings (Fig. 7a, b). Irrespective of the N supply, Cd concentration in roots (Fig. 7b) was significantly higher than that in shoots (Fig. 7a).

Effect of AsA or L-galactono-1,4-lactone (GalL) pretreatment on Cd toxicity of N-deficient seedlings

AsA content was increased in the second leaves of N-deficient seedlings pretreated with 0.5 mM AsA or 0.5 GalL, the putative biosynthesis precursor of AsA (Smirnoff and Wheeler 2000) for 6 h (Fig. 8a). To test if AsA plays an important role in regulating Cd-induced toxicity of N-deficient seedlings, AsA or GalL-pretreated seedlings were then transferred into nutrient solution with or without $CdCl_2$ for another 6 days. We observed that pretreatment of N-deficient seedlings with AsA or GalL exhibited a reduction of Cd-decreased chlorophyll content and Cd-increased MDA content in the second leaves (Fig. 8b, c).

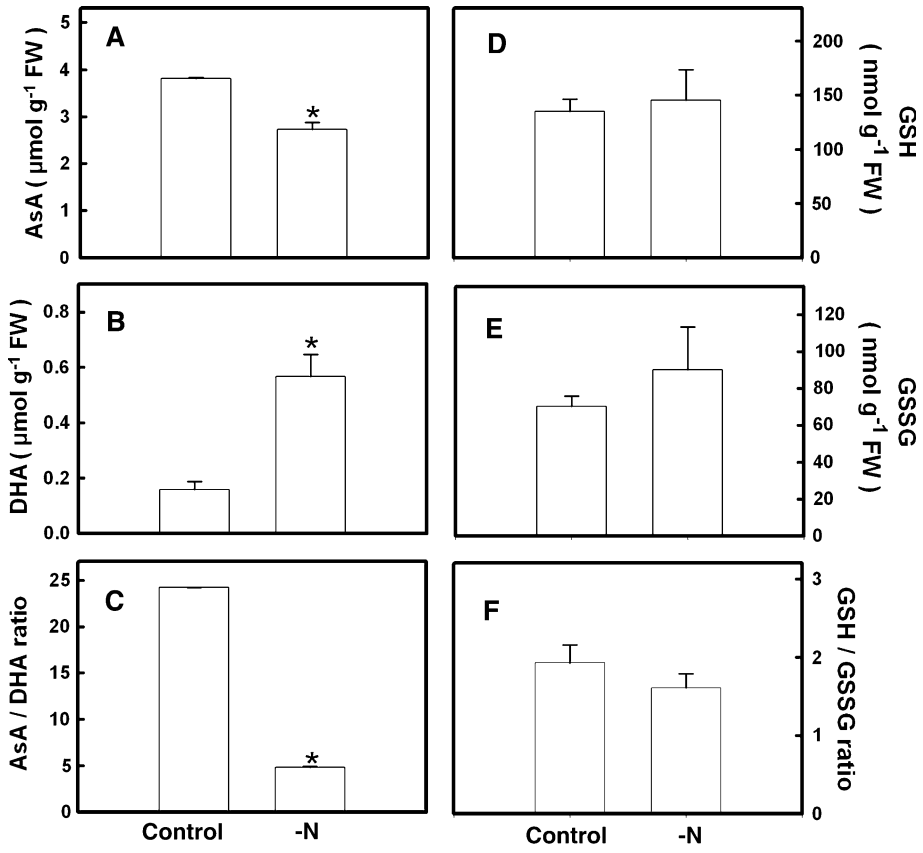

Fig. 4 The contents of AsA (**a**), DHA (**b**), GSH (**d**), GSSG (**e**) and the ratios of AsA/DHA (**c**) and GSH/GSSG (**f**) in the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions for 12 days. *Bars* show means ± SE ($n = 4$). *Asterisks* represent values that are significantly different between control and –N treatments at $P < 0.05$

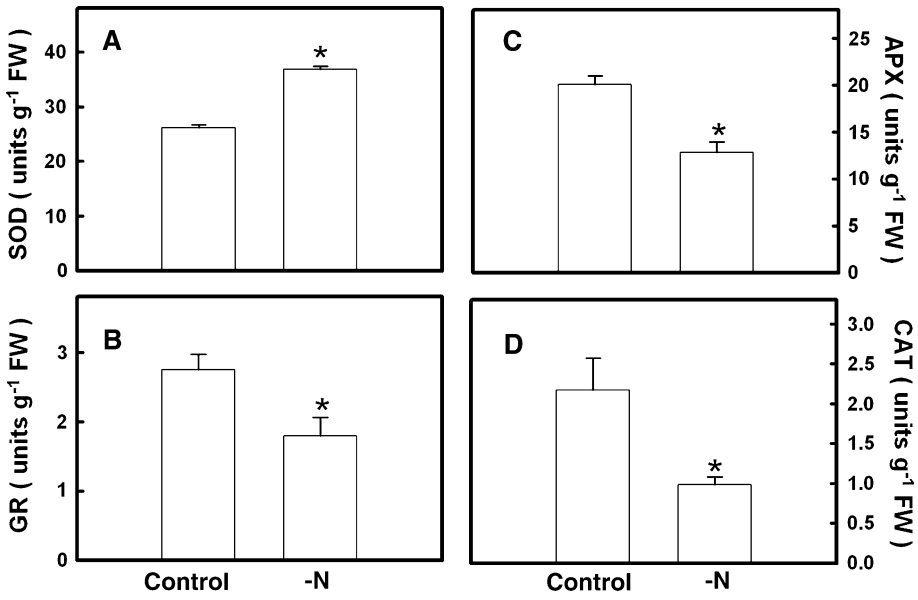

Fig. 5 The activities of SOD (**a**), GR (**b**), APX (**c**), and CAT (**d**) in the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions for 12 days. Bars show means ± SE ($n = 4$). *Asterisks* represent values that are significantly different between control and –N treatments at $P < 0.05$

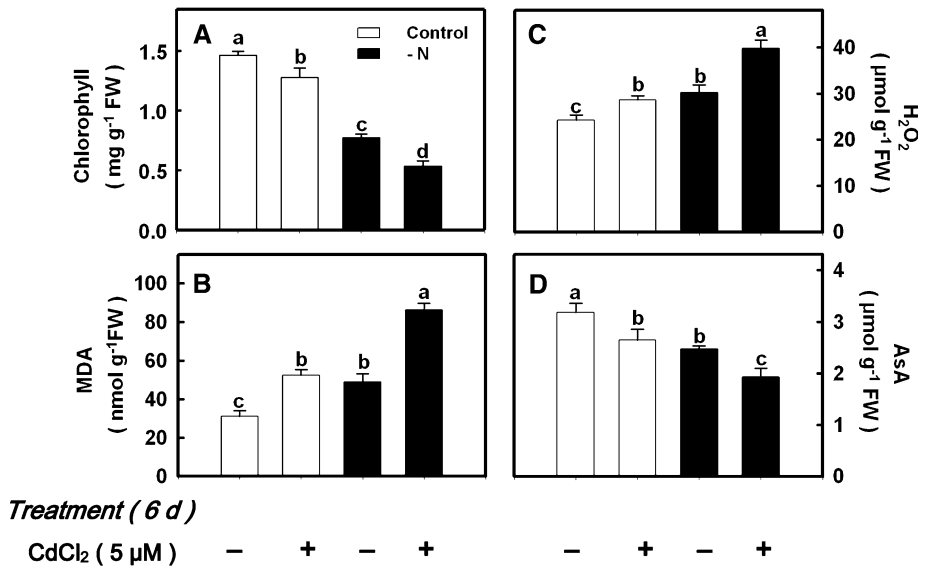

Fig. 6 Effect of $CdCl_2$ on the contents of chlorophyll (**a**), MDA (**b**), H_2O_2 (**c**), and AsA (**d**) in the second leaves of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions. Rice seedlings were first grown under control and –N conditions for 12 days. Control and –N seedlings were then transferred to the control and –N nutrient solution with or without 5 μM $CdCl_2$ for another 6 days, respectively. The second leaves were used to determine chlorophyll, MDA, H_2O_2, and AsA All measurements were expressed on the basis of initial FW. *Bars* show means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$

Discussion

In this study, rice seedlings were grown under N-sufficient and -deficient nutrient solutions for 12 days, by which time the third leaves of seedlings were fully grown. The most general symptom of N deficiency or Cd toxicity is leaf chlorosis (Marschner 1995; Das et al. 1997). When rice seedlings were grown under N-deficient conditions or treated with $CdCl_2$, chlorosis was first visually observed in the second leaves (Chao et al. 2010, Fig. 1b). For this reason, the second leaves of rice seedlings were used to perform all measurements except growth analysis and Cd uptake.

Nitrate and ammonium are the major sources of inorganic N taken up by the roots of higher plants. Chlorophyll and protein are N-based macromolecules. N deficiency decreased the contents of chlorophyll, protein and nitrate in the second leaves (Fig. 1a, c, d). It appears that analyses of chlorophyll, protein and nitrate are accurate tool to diagnose N deficiency.

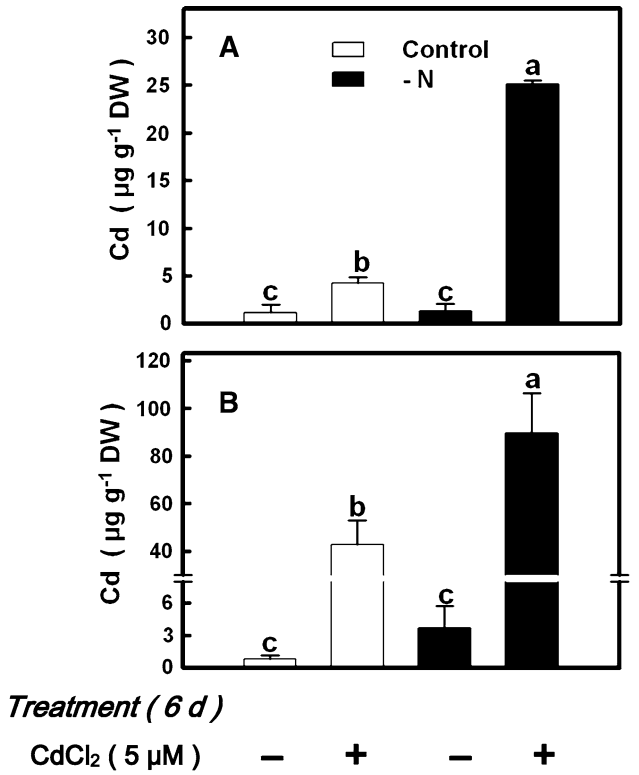

Fig. 7 Effect of $CdCl_2$ on the concentration of Cd in shoots (**a**) and roots (**b**) of rice seedlings grown under N-sufficient (control) and -deficient (–N) conditions. Rice seedlings were first grown under control and –N conditions for 12 days. Control and –N seedlings were then transferred to the control and –N nutrient solution with or without 5 μM $CdCl_2$ for another 6 days, respectively. *Bars* show means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$

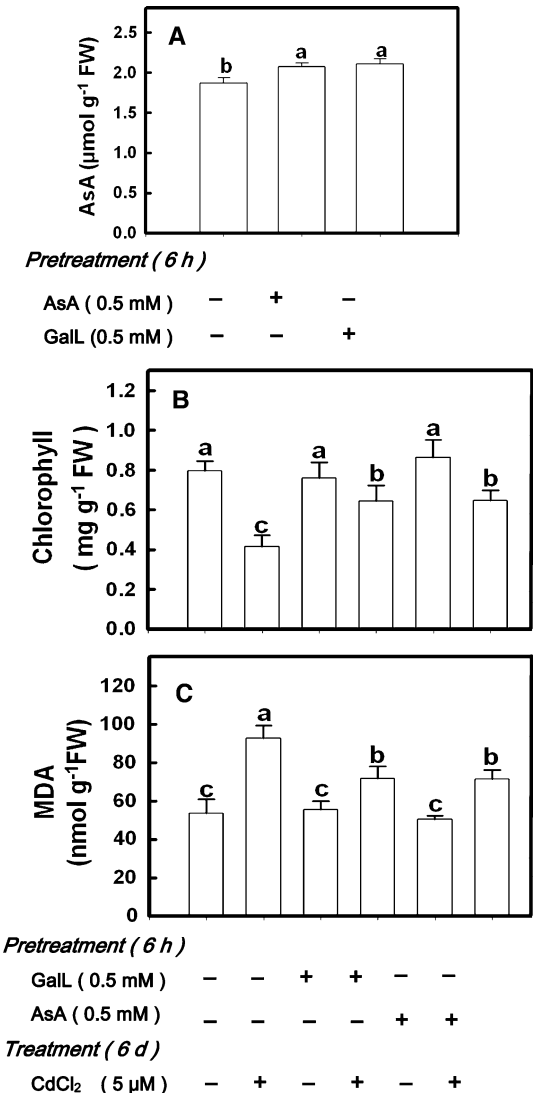

Fig. 8 Effect of AsA or GalL pretreatment on the content of AsA in the second leaves of rice seedling grown under –N conditions (**a**) and the effect of $CdCl_2$ on the contents of chlorophyll (**b**) and MDA (**c**) in the second leaves of rice seedlings grown under –N conditions. A: Twelve-day-old N-deficient seedlings were pretreated with 0.5 mM AsA or 0.5 mM GalL for 6 h. AsA content was determined 6 h after 0.5 mM AsA or 0.5 mM GalL pretreatment. B and C: Twelve-day-old N-deficient seedlings were pretreated with or without 0.5 mM AsA or 0.5 mM GalL for 6 h and then transferred to –N nutrient solution with or without 5 μM $CdCl_2$ for 6 days, respectively. The contents of chlorophyll and MDA were determined 6 days after 5 μM $CdCl_2$ treatment and expressed on the basis of initial FW. Bars show means ± SE ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$

It has been shown that ABA content of leaves increases in the mineral-deprived plants (Mizarhi and Richmond 1972; Vysotskaya et al. 2008). The accumulation of ABA in response to N deficiency was also observed in rice leaves (Fig. 2). The content of ABA in plants increases upon their exposure to drought (Zabadal 1974). O'Toole and Cruz (1980) reported the linear relationship between leaf rolling and leaf water potential and concluded that leaf rolling in rice could be used as an estimate of water deficit. In the hydroponically grown N-deficient rice seedlings of the present study, no leaf rolling was visually observed (Fig. 1b). It is most likely that ABA accumulated in rice leaves is directly due to N deficiency. N deficiency resulted in a growth reduction in shoots but not in roots of rice seedlings (Table 2). Thus, ABA accumulation induced by N deficiency is probably involved in the reduction of shoot growth.

An increase in H_2O_2 content in roots has been reported upon exposure of plants to N-deficient conditions (Shin et al. 2005; Schachtman and Shin 2007; Zhao et al. 2007). In the present study, an increase in H_2O_2 content was observed in leaves (Fig. 3) but not in roots (data not shown) under N deficiency conditions. SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H_2O_2. APX and CAT are responsible for scavenging or eliminating H_2O_2. We observed that in N-deficient leaves SOD activity was greater while APX and CAT activities were lower than their respective control leaves (Fig. 5).

This may result in a higher H_2O_2 content in N-deficient leaves.

A role for plasma membrane NADPH oxidase in the production of the H_2O_2 has been a recent focus in ROS signaling research (Sagi and Flurher 2006). Cakmak (2005) demonstrated that the activity of NADPH oxidase increases in potassium-deficient bean leaves. Thus, the possibility that NADPH oxidase represents another source for production of H_2O_2 in N-deficient rice leaves cannot be excluded.

Accumulating evidence indicates that one mechanism of ABA action is associated with ROS production in plant cells. It has been shown that ABA can increase the production of H_2O_2 (Hung and Kao 2004; Hu et al. 2006). Whether ABA is a key inducer of H_2O_2 production in leaves of rice seedlings under conditions of N deficiency remains to be investigated.

In previous work, we have shown that the decline in chlorophyll and AsA contents, the increase in MDA (an indicator of lipid peroxidation) content and the accumulation of H_2O_2 in the second leaves of rice seedlings are associated with Cd toxicity (Hsu and Kao 2007; Chao et al. 2010). Thus, in the present study we evaluated Cd toxicity by the decrease in chlorophyll and AsA contents and the increase in MDA and H_2O_2 contents in the second leaves of rice seedlings. Based on these criteria, we demonstrated that N deficiency enhanced Cd toxicity (Fig. 6).

Several lines of evidence have revealed that oxidative stress is a major component of Cd stress (Cho and Seo 2005; Garnier et al. 2006; Hsu and Kao 2007). Thus the capacity of the antioxidant system seems to play an important role in Cd stress. APX and GR, which are, respectively, the first and last enzymes in the AsA-GSH cycle, are responsible for H_2O_2 detoxification (Noctor and Foyer 1998). We observed that N-deficient rice leaves have lower activities of APX, GR, and CAT than N-sufficient leaves (Fig. 5b–d). It appears that the decrease in APX, GR, and CAT may be responsible for Cd toxicity enhanced by N deficiency. Decreased activities of APX, GR, and CAT have also been demonstrated in other N-deficient plants (Logan et al. 1999; Finkemeier et al. 2003; Kandlbinder et al. 2004).

AsA is the most abundant antioxidant in plants. Our recent work demonstrated that the decline of AsA content is associated with the Cd toxicity of rice seedlings (Chao et al. 2010). N deficiency significantly decreased AsA content and the AsA/DHA ratio in the second leaves of rice seedlings (Fig. 4a, c). Pretreatment of AsA or GalL, a biosynthetic precursor of AsA, caused a reduction in Cd toxicity in N-deficient rice seedling (Fig. 8b, c). All these results suggest that the decline of AsA content is involved in Cd toxicity enhanced by N deficiency. GSH is another important antioxidant. However, we observed that N deficiency had no effect on GSH content (Fig. 4d). A similar result has also been reported in N-deficient Arabidopsis (Koprivova et al. 2000).

Irrespective of the N supply, Cd treatment resulted in an increase in Cd concentration in rice roots and shoots (Fig. 7a, b). The Cd concentration was lower in shoots than that in roots (Fig. 7a, b), indicating that a higher proportion of the Cd taken up by rice remained in the roots. This is in agreement with a number of earlier reports (Jalil et al. 1994; Wu et al. 2004; Chao et al. 2010). In the present study, we observed that Cd concentration was higher in N-deficient shoots and roots than their respective control shoots and roots (Fig. 7a, b). This would explain why N deficiency enhanced Cd toxicity in rice seedlings (Fig. 6).

The uptake of Cd is shown to be mediated through activation of some divalent cation transporters. Iron-regulated transporter (IRT1) is a major Fe transporter (Eide et al. 1996). A role for OSIRT1 in Cd transport has been previously investigated in yeast (Nakanishi et al. 2006). Recent experiments using transgenic rice plants overexpressing *OsIRT1* confirmed the conclusion from those earlier yeast experiments that OsIRT1 does indeed transport Cd (Lee and An 2009). The *IRT1* metal transporter gene has been shown to be induced by iron deficiency (Connolly et al. 2002; Bereczky et al. 2003; Nakanishi et al. 2006; Graziano and Lamattina 2007). It is not known whether N deficiency induces iron deficiency and activates the expression of *OsIRT1* in rice roots. Future research on the effect of N deficiency on the expression of *OsIRT1* in rice roots is likely to be highly rewarding.

In summary, the data presented here show that N deficiency enhanced Cd toxicity in rice seedlings. It appears that the stress combinations could have a significant impact on agricultural production. To develop sustainable practices, enhancing our knowledge of the molecular, physiological, and metabolic responses of plants to a combination of nutrient deficiency and Cd stress is thus needed.

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