Abstract: The effect of H$_2$O$_2$ pretreatment on Cd tolerance and translocation of rice seedlings were studied using two rice cultivars (N07-6 and N07-63) differing in Cd tolerance. The contents of malondialdehyde (MDA), reduced glutathione (GSH), non-protein thiols (NPT), phytochelatins (PCs) and the activity of glutathione S-transferase (GST) were compared between the two cultivars exposed to various treatments. The results showed that 50 μmol/L Cd exposure significantly inhibited rice growth, enhanced the production of GSH, NPT, PCs and MDA, and increased the activity of GST, and there were significant differences between the two cultivars. More Cd was transported into the shoot of N07-6. The H$_2$O$_2$ pretreatment alleviated Cd toxicity by further increasing GSH, NPT and PCs contents, as well as the GST activity in roots. The increase degrees of these parameters in N07-63 were higher than those in N07-6, suggesting that the tolerance of N07-63 was enhanced more significantly than N07-6. Hydrogen peroxide reduced Cd translocation to rice shoot but affected the Cd content in root differently. From the above results, it may be speculated that there were remarkable differences in the Cd detoxification and response to the H$_2$O$_2$ pretreatment between the two cultivars.

Key words: cadmium; hydrogen peroxide; pretreatment; rice; tolerance

Cadmium (Cd) is a highly toxic heavy metal, which can cause the accumulation of reactive oxygen species (ROS) by damaging functional groups of biological molecules and electron transport chain (Schützendübel et al, 2001; Schützendübel and Polle, 2002). Cd can be taken up by plants easily, and transported to above-ground tissues. There are a number of mechanisms that plants may adopt in order to resist Cd toxicity. Sulphydryl-containing molecules such as non-protein thiol (NPT), glutathione (GSH), and phytochelatins (PCs) can chelate Cd and form Cd-S complexes (Strasdeit et al, 1991; Zenk, 1996; Mehra and Tripathi, 1999). After the complexes are transported to the vacuole, the content of Cd$^{2+}$ in cytoplasm can be reduced, so it may be possible to enhance Cd tolerance and to control Cd translocation from roots to shoots (Tommasini et al, 1998; Rea, 1999). As a detoxification enzyme in plants, glutathione S-transferase (GST) plays an important role in this process by catalyzing the combination of GSH and toxic substances and reducing oxidative stress caused by Cd (Marrs, 1996).

Hydrogen peroxide (H$_2$O$_2$) is a product of ROS metabolism, and also an important signal molecule in plants. H$_2$O$_2$ is considered to be a signal involved in plant pathogen defense responses and in the adaptation of plants to abiotic stress (Levine et al, 1994; Kovtun et al, 2000). It can regulate the expression of numerous genes. Recent studies have shown that plants pretreated with low concentrations of H$_2$O$_2$ were significantly more tolerant than control plants to abiotic stress (Prasad et al, 1994a, 1994b; Akio et al, 2002; Li and Gong, 2003; Wahid et al, 2007; Li et al, 2007; Ge et al, 2009).

Rice is one of the most important crops in the world. It is also a model plant which has been used to study the mechanism of Cd toxicity and tolerance. In our previous studies, results have shown that H$_2$O$_2$ pretreatment improved the response of antioxidant system in rice, reduced Cd toxicity and translocation from roots to shoots (Hu et al, 2009). However, there was only one rice cultivar investigated. This study therefore aimed to explore the mechanisms of the positive effect of H$_2$O$_2$ on Cd tolerance through the regulation of sulphydryl-containing molecules and glutathione S-transferase; and to explore differences
between two cultivars, N07-63 (Cd tolerant) and N07-6 (Cd sensitive).

MATERIALS AND METHODS

Rice materials and experimental design

Seeds of rice (Oryza sativa L. cv. N07-6, N07-63) were sterilized with 3% NaClO for 10 min, rinsed thoroughly with distilled water, and germinated in the dark. Seedlings were maintained in the following growth condition: 12 h light/12 h dark cycle [(65.2 µmol/(m²·s)], with 32±1°C (light) and 27±1°C (dark). When the second leaf emerged, seedlings were transferred into a greenhouse with solution cultured. After the third leaf had fully expanded, seedlings were transplanted to plastic containers. Four treatments with three replicates were applied, including a control (neither H₂O₂ pretreatment nor Cd-stressed), Cd-stressed only, H₂O₂-treated only and H₂O₂&Cd stressed (pretreatment with H₂O₂ and then Cd-stressed). The nutrient solution was renewed twice a week.

Rice seedlings were divided into two parts and harvested one week later. One part was dried for determination of Cd content and other growth parameters, and the rest were stored at -70°C for biochemical analysis.

Determination of Cd content

Rice seedlings were soaked in 5 mmol/L prechilled EDTA-Na₂ for 15 min to remove adsorbed metals on the root surfaces, and rinsed with deionized water, and dried with paper. Samples were oven-dried at 90°C for 15 min, and then kept at 70°C for 24 h. A 0.1 g dry sample was weighed and digested using a mixture of HNO₃ and HClO₄ (4:1). Cd content was determined by an inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Perkin Elmer Optimal 2100 DV).

NPT and GSH assays

Fresh tissues (1.0 g) were homogenized in 8 mL of ice-cold 5% sulfosalicylic acid solution. The homogenate was centrifuged at 10 000×g (4°C) for 15 min. The supernatant was made up to 10 mL, and stored at -70°C as crude extract for NPT and GSH determination.

The content of NPT was determined by measuring the absorbance at 412 nm following the reaction between NPT and 5,5-dithio-2,2-dinitrobenzoic acid (DTNB) (Rama and Prasad, 1998). GSH was quantified by the fluorescence spectroscopy method (Hissin and Hilf, 1976). A 0.5-mL supernatant was diluted 4 folds with phosphate buffer (pH 8.0) and the pH was adjusted to 8.0 with NaOH. The final assay mixture contained 0.5 mL of diluted supernatant, 1.4 mL of buffer and 0.1 mL of 0.1% O-phthalaldehyde (OPT). After thorough mixing and incubation at room temperature for 15–20 min, the fluorescence intensity was monitored at 420 nm after excitation at 350 nm (20°C).

Estimation of PCs content

The amount of PCs was estimated by subtracting the amount of GSH form that of NPT (Bhargava et al, 2005).

MDA assays

A 0.5-g fresh sample was homogenized in 10% TCA and was centrifuged at 10 000×g for 15 min. Then the supernatant was made up to 5 mL. Two milliliter of supernatant was mixed with the same volume of 0.5% TBA solution. The mixture was heated at 95°C for 15 min and then quickly cooled in ice. The absorbance of the reaction mixture was monitored at 532 nm, 600 nm and 450 nm (Kong and Xi, 2008).

GST assays

A 0.5-g fresh sample was homogenized in 4 mL ice-cold extraction buffer [0.2 mol/L Tris-HCl (pH 7.8), 1 mmol/L EDTA and 5 mmol/L DTT)] and centrifuged at 12 000×g for 15 min. Then the supernatant was made up to 5 mL. GST activity was determined by recording the increase in absorbance at 340 nm for 4 min [25°C, E=9.6 mmol/(L·cm)] according to Habig et al (1974). The reaction mixture (3 mL) included 100 mmol/L potassium phosphate buffer (pH 6.5), 0.1 mmol/L 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 mmol/L GSH and 0.1 mL enzyme extract. One unit (U) of GST was
calculated as 1 µmol/L GSH reduced in 1 min. The activity of GST was expressed as U/g protein. Total protein content was determined by the Bradford method (Bradford, 1975), using bovine serum albumin as a standard.

**Statistical analysis**

All data in the tables were expressed as mean ± standard deviation (SD) of three replicates. Statistical significance of the means was compared by SPSS16.0 at the 5% probability level using one-way ANOVA. Statistical significance between different cultivars was evaluated by Paired-Samples T-test.

**RESULTS**

### Plant growth

As shown in Table 1, Cd exposure inhibited the growth of the two cultivars. Shoot dry weight and shoot length of N07-6 were reduced more than N07-63 following Cd exposure, and there was a significant difference between the two cultivars. Root length and dry weight followed the similar trends of growth in shoots, but there was no significant difference between N07-6 and N07-63. Growth inhibition was considerably alleviated when 100 µmol/L H2O2 was applied before Cd stress, and there were significant differences between the shoot lengths of the two cultivars when compared to the control. Under the H2O2 and Cd treatment, N07-63 grew better than N07-6. The H2O2 treatment alone did not affect seedling growth except shoot length.

### Cd content

Cd content of seedlings increased sharply ($P<0.05$) in the Cd-stressed only and the H2O2&Cd stressed groups compared to the control (Table 2). There was a higher accumulation of Cd in the N07-63 roots than N07-6, but the Cd content in shoots was lower in N07-63. So the S/R ratio (shoot Cd content/ root Cd content×100%) of N07-6 was higher than N07-63 by 25.34% (Fig. 1). In comparison of the Cd treatment, the pretreatment of H2O2 significantly

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**Table 1. Growth of the two rice cultivars under different treatments.**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Shoot dry weight (mg/plant)</th>
<th>Root dry weight (mg/plant)</th>
<th>Shoot length (cm)</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CK</td>
<td>H2O2</td>
<td>Cd</td>
<td>H2O2+Cd</td>
<td></td>
</tr>
<tr>
<td>N07-6</td>
<td>28.62±3.70 a</td>
<td>26.75±5.13 (-6.56) a</td>
<td>13.56±1.02 (-52.63) b</td>
<td>18.96±1.00 (-33.75) b</td>
<td></td>
</tr>
<tr>
<td>N07-63</td>
<td>21.26±2.36 a</td>
<td>21.44±1.71 (+0.85) a</td>
<td>18.33±2.08 (-13.78) a</td>
<td>19.33±0.58 (-9.08) a</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>N07-6</td>
<td>8.15±0.27 a</td>
<td>8.22±0.77 (+0.84) a</td>
<td>7.04±0.59 (-13.67) b</td>
<td>7.56±0.77 (-7.34) b</td>
<td></td>
</tr>
<tr>
<td>N07-63</td>
<td>6.44±0.77 a</td>
<td>6.00±0.67 (-6.90) a</td>
<td>5.69±0.76 (-11.78) a</td>
<td>6.43±0.61 (-0.23) a</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>*</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>N07-6</td>
<td>19.01±0.79 a</td>
<td>17.84±0.27 (-6.15) b</td>
<td>12.63±0.14 (-33.57) d</td>
<td>14.43±0.38 (-24.12) c</td>
<td></td>
</tr>
<tr>
<td>N07-63</td>
<td>16.89±0.35 a</td>
<td>15.42±0.62 (-8.17) b</td>
<td>14.27±0.20 (-15.50) c</td>
<td>15.65±0.39 (-7.30) b</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

The same letters after the data of the same cultivar indicate no significant differences among treatments ($P>0.05$). Data in parentheses denote the percentage of increase/decrease for each treatment compared with CK (control). * Significant difference between two cultivars in the same treatment ($P<0.05$); ns, No significant difference.
increased N07-63 root Cd content, and decreased that of N07-6 seedlings. The S/R ratios of the two cultivars were declined by 7.89% (N07-6) and 18.44% (N07-63), respectively, and there was a significant difference between them.

**MDA content**

Under the Cd stress, MDA content in shoots of N07-6 was considerably higher than that of the control by 27% (P<0.05) (Table 3). For the MDA content in roots, there was no significant difference between the control and Cd treatment only. The H2O2&Cd treatment brought a substantial decline of MDA levels, especially for N07-63. The MDA content in N07-63 was significantly lower than N07-6 (P<0.05). H2O2 treatment alone had little impact on MDA content.

**NPT, GSH and PCs contents**

Addition of Cd stress to the nutrient solution caused a high production of GSH in roots of N07-6 and N07-63 by 38.01% and 56.28%, respectively. Cd stress and H2O2 pretreatment increased GSH content in roots further, and that of N07-63 was produced more significantly. Similarly, Cd exposure instigated root NPT synthesis, and the effect was stronger in the H2O2 pretreatment. NPT contents in roots of the two cultivars significantly increased (P<0.05) under Cd exposure, and H2O2&Cd enhanced NPT production in N07-6 and N07-63 by 25.35% and 31.78%, respectively. However, NPT contents in rice shoots were decreased separately by 16.18% (N07-6) and 5.58% (N07-63) (Table 4).

Compared to GSH, Cd exposure significantly induced PCs synthesis in rice roots (P<0.05). H2O2 &Cd treatment increased PCs content in roots by 27.38% (N07-6) and 31.82% (N07-63) compared to the Cd stress. The rates of increase in roots of N07-63 under Cd exposure without and with H2O2 pretreatment were higher than those of N07-6 by 29.92% and 48.80%, respectively. H2O2&Cd treatment decreased PCs content in the rice shoots compared to the Cd stress alone (Table 4).

**GST activity**

GST activity in roots was higher than that in shoots because of more Cd accumulation in roots. The GST activity in the roots of the two cultivars was stimulated by Cd stress, especially for N07-63, which...
increased by 26.80% than N07-6 (P<0.05) (Table 5). The enzyme responded more efficiently under H2O2 pretreatment due to the Cd stress. In contrast, H2O2 stress only did not affect the GST activity. Nevertheless, the GST activity in shoots did not change under the Cd stress or the H2O2 treatment compared to the control.

**DISCUSSION**

This study investigated the role of H2O2 in the acclimation of Cd stress, and compared the difference of its effects on the two rice cultivars, N07-6 (Cd sensitive) and N07-63 (Cd tolerant). Results demonstrated that Cd exposure inhibited rice growth, and the effect on N07-6 was more obvious. The H2O2 pretreatment alleviated Cd toxicity, and the tolerance of N07-63 was enhanced higher than that of N07-6. This was shown from the growth indicators (Table 1) and MDA content (Table 3). The difference of the tolerance induced by the H2O2 application between N07-6 and N07-63 may refer to Cd absorption, translocation and detoxification of rice plants.

Under the Cd exposure, N07-63 accumulated more Cd in roots but translocated less Cd to shoots than N07-6 (Table 2). This indicates that for N07-63,

### Table 4. Contents of GSH, PCs and NPT of the two rice cultivars under different treatments. µg/g

| Cultivar | Treatment | Glutathione content in shoots | | | Glutathione content in roots | | | Non-protein thiols content in shoots | | | Non-protein thiols content in roots | | | Phytochelatins content in shoots | | | Phytochelatins content in roots | | |
|----------|-----------|-----------------------------|---|---|-----------------------------|---|---|-----------------------------|---|---|-----------------------------|---|---|
|          |           | CK                           | H2O2 | Cd | H2O2+Cd                      | CK                           | H2O2 | Cd | H2O2+Cd                      | CK                           | H2O2 | Cd | H2O2+Cd                      | CK                           | H2O2 | Cd | H2O2+Cd                      |
| N07-6    |           | 9.28±2.03 b                  | 8.81±1.42 (-5.02) b | 12.14±3.73 (+30.86) b | 16.01±3.14 (+72.61) a | 14.66±2.09 b                  | 13.91±0.80 (-5.10) b | 20.23±1.85 (+38.01) a | 24.77±2.07 (+68.98) a | 20.25±3.44 b                  | 25.25±2.81 (+24.70) b | 38.16±2.31 (+88.47) a | 36.03±3.35 (+77.96) a | 37.64±4.85 b                  | 30.88±3.24 (-17.96) c | 49.15±1.43 (+30.57) a | 41.20±3.69 (+9.44) b |
| N07-63   |           | 9.53±0.85 b                  | 11.81±0.80 (+23.90) b | 16.52±2.16 (+73.35) a | 16.01±2.94 (+68.00) a | 16.22±1.84 c                  | 17.03±2.30 (+5.00) c | 25.35±4.04 (+56.28) b | 33.38±3.55 (+105.81) a |
| Difference|            | ns                          | * ns | ns | ns                          | ns                          | * ns | ns | ns                          | ns                          | ns | ns | ns                          | ns                          |

The same letters after the data of the same cultivar indicate no significant differences among treatments (P>0.05). Data in parentheses denote the percentage of increase/decrease for each treatment compared with CK (control).

* Significant difference between two cultivars in the same treatment (P<0.05); ns, No significant difference.

### Table 5. GST activities of the two rice cultivars under different treatments. U/g

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Different treatment</th>
<th>Glutathione S-transferase activities of shoots</th>
<th></th>
<th></th>
<th>Glutathione S-transferase activities of roots</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CK</td>
<td>H2O2</td>
<td>Cd</td>
<td>H2O2+Cd</td>
<td>CK</td>
<td>H2O2</td>
</tr>
<tr>
<td>N07-6</td>
<td></td>
<td>80.77±30.77 a</td>
<td>94.99±16.34 (+17.60) a</td>
<td>103.28±47.90 (+27.87) a</td>
<td>96.05±29.78 (+18.91) a</td>
<td>479.70±100.84 c</td>
<td>614.60±80.39 (+28.10) bc</td>
</tr>
<tr>
<td>N07-63</td>
<td></td>
<td>75.65±10.44 a</td>
<td>68.40±4.98 (-9.58) a</td>
<td>74.29±2.98 (-1.79) a</td>
<td>61.94±9.19 (-18.12) a</td>
<td>340.58±63.59 c</td>
<td>432.04±9.53 (+26.85) bc</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

The same letters after the data of the same cultivar indicate no significant differences among treatments (P>0.05). Data in parentheses denote the percentage of increase/decrease for each treatment compared with CK (control).

* Significant difference between two cultivars in the same treatment (P<0.05); ns, No significant difference.
Cd toxicity was mitigated by reducing the translocation of Cd from roots to shoots. After the H$_2$O$_2$ pretreatment, Cd translocation declined in both cultivars, but this trend was more pronounced for N07-63 than N07-6 (Table 2). More Cd accumulation but less stress in the roots of N07-63 demonstrated that H$_2$O$_2$ strengthened the sequestration and detoxification of Cd. This agreed with the findings of the previous research (Metwally et al, 2003). However, Cd accumulation and translocation in roots of N07-6 under H$_2$O$_2$&Cd treatment was declined. This might be related to the development of secondary cell wall promoted by exogenous H$_2$O$_2$ (Miao et al, 2001; Pnuel et al, 2003). The secondary cell wall in higher plants is made up of cellulose, hemicelluloses and lignin, which may strengthen mechanical intensity of cell wall through a highly cross-linked way and presumably repress Cd absorption to root cells (Song et al, 2008).

Cd ions entering root cells may be complexed to sulfhydryl-containing molecule, such as GSH and PCs and subsequently transported to vacuole. Under the Cd exposure, GSH, PCs and NPT levels in rice roots increased significantly (Table 4). This type of stress response has been observed in various plants. For example, in wheat, the addition of Cd produced considerably more PCs compared to the control (Sun et al, 2005). In this study, the synthesis of PCs in N07-63 was more evident than in N07-6 upon Cd treatment. At the same time, Cd stress stimulated the increase of GSH level in rice roots, which was also reported in previous studies (Dominguez-Solís et al, 2001; Guo et al, 2009). However, the variation of GSH was lower than that of PCs. This might be due to the fact that GSH is the substrate of PCs (Lou and Shen, 2001). The H$_2$O$_2$ pretreatment further enhanced the production of GSH, PCs and NPT (Table 4). Similarly, as Guo et al (2009) reported, H$_2$O$_2$ induced in rice roots due to a salicylic acid (SA) pretreatment positively correlated to the increase of GSH and NPT contents. This confirmed that H$_2$O$_2$ addition may alleviate Cd toxicity by promoting the production of sulfhydryl-containing molecule. Cd or H$_2$O$_2$&Cd treatments increased NPT, GSH and PCs levels, and resulted in the greater Cd retention in rice roots. Therefore, the increased tolerance and reduced translocation of Cd may be related to the increase of sulfhydryl-containing molecules.

As an important detoxification enzyme in plants, GST catalyzes the conjugation of glutathione with various compounds and facilitating their transport to vacuole or apoplast for detoxification (Adamis et al, 2004). There is a significant positive dose-response relationship between Cd stress and GST activity (Zhang and Ge, 2008). It was reported that GSH content and GST activity in Phragmites australis with 50 μmol/L Cd$^{2+}$ was higher than those in control (Iannelli et al, 2002). In our work, under the Cd exposure, GST activity in roots of the two cultivars were enhanced, and that of N07-63 increased more than N07-6 ($P<0.05$). Apart from this, the effect was more obvious with H$_2$O$_2$&Cd treatment (Table 5) owing to the increase of NPT, GSH, PCs and the accumulation of Cd. H$_2$O$_2$ functions as a trigger of programmed death in challenged cells and as a diffusible signal for the induction in adjacent cells of genes encoding cellular protectants such as glutathione S-transferase (Levine et al, 1994). Thus, it might be hypothesized that more GST isozymes in roots were activated by Cd exposure for detoxification, especially in N07-63.

Taken together, evidence from this study showed that H$_2$O$_2$ increased the levels of sulfhydryl-containing compounds and enhanced the activity of GST in rice roots subjected to Cd stress, thus suppressing Cd-induced oxidative damage and reducing Cd translocation. In addition, this study revealed differences between cultivars in terms of underlying mechanism of enhancing Cd tolerance and lowering Cd translocation.

**ACKNOWLEDGEMENTS**

We thank the financial support from the Natural Science Foundation of China (Grant No. 30700479) and the Ph.D. Programs Foundation of Ministry of Education of China (Grant No. 20090097110035).

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Bai Xiao-juan, et al. Effect of H$_2$O$_2$ Pretreatment on Cd Tolerance of Different Rice Cultivars


