Arch Environ Contam Toxicol (2009) 56:723–731
DOI 10.1007/s00244-008-9226-2

Relationship Between Proline and Hg^{2+}-Induced Oxidative Stress in a Tolerant Rice Mutant

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Received: 29 February 2008 / Accepted: 18 August 2008 / Published online: 12 September 2008

Abstract There has been little agreement regarding the mechanism by which proline reduces heavy metal stress. The present work examines the relationship between Hg^{2+}-induced oxidative stress and proline accumulation in rice and explores the possible mechanisms through which proline protects against Hg^{2+} stress. The effect of proline on alleviation of Hg^{2+} toxicity was studied by spectrophotography and enzymatic methods. Hg^{2+} induced oxidative stress in rice by increasing lipid peroxidation. Pretreatment of the rice with 2 mM proline for 12 h profoundly alleviated Hg^{2+}-induced lipid peroxidation and minimized H_2O_2 accumulation. Proline pretreatment significantly reduced ($p < 0.01$) the Hg^{2+} content in rice leaves. A comparison of the effects of proline pretreatment on H_2O_2 accumulation by Hg^{2+} and aminotrazole suggested that proline protected cells from Hg^{2+}-induced oxidative stress by scavenging reactive oxygen species. The present work demonstrates a protective effect of proline on Hg^{2+} toxicity through detoxifying reactive oxygen species, rather than chelating metal ions or maintaining the water balance under Hg^{2+} stress.

Abbreviations

AT	Aminotriazole
GSH	Glutathione
GSSG	Oxidative glutathione
MDA	Malonaldehyde
ROS	Reactive oxygen species
TBA	2-Thiobarbituric acid

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Mercury, being one of the major heavy metal pollutants, has highly poisonous effects on living organisms. Untreated industrial wastes, gold and silver mining, and industrial process have been recognized as the major sources of mercury (Hg) in agricultural lands (Cabrera et al. 1999; Lin et al. 2006). The main forms of Hg in plant-soil systems include electrochemically uncharged or volatile Hg, the mercuric ion that predominates in many mercury-contaminated soils. Like other heavy metals, such as cadmium, copper, lead, and zinc, Hg^{2+}, which is highly water soluble and reactive, accumulates readily in plants including rice, with significant potential to endanger human health and ecological integrity (Patra and Sharma 2000; Rubén et al. 2006).

Plants exposed to toxic concentrations of heavy metals show visible injuries such as chlorosis, necrosis, and root growth inhibition (Carrier et al. 2003; Sandalio et al. 2001). At the molecular level, underlying mechanisms such as metals bind to sulfydryl groups of proteins, leading to inhibition of activity or disruption of structure, or replace an essential element, resulting in deficiency effects (Van Assche and Clijsters 1990). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species (ROS), resulting in oxidative stress (Dietz et al. 1999). Although Hg as a transition metal is well known to encourage oxidative stress via Fenton-type reaction, the molecular mechanisms of Hg-induced oxidative damage and tolerance in plants have not been fully understood.

Proline accumulation often occurs in a variety of plants in the presence of elevated levels of heavy metals, but a consensus has not emerged on its role in combating metal stress (Schat et al. 1997; Shah and Dubey 1998; Mehta and Gaur 1999; Zengin and Munzuroglu 2005). Some researchers assume that proline accumulation is a symptom of injury which does not confer protection against metal stress (Lutts et al. 1996). On the contrary, it has been suggested that proline might protect plants from heavy metal toxicity (Mehta and Gaur 1999; Siripornadulsil et al. 2002). There appears to be a relationship between lipid peroxidation and proline accumulation in plants subjected to diverse kinds of stress (Molinari et al. 2007). If such a relationship exists, proline accumulation might play an important role in inhibiting heavy metal-induced lipid peroxidation.

Rice is an important crop worldwide. It is also considered to be a model plant for monocots because of its relatively small genome size. To contribute to our understanding of the mechanisms by which proline offers protection against heavy metal-induced oxidative stress, in the present work we investigated the role of proline in facilitating Hg^{2+} detoxification in rice by comparing the response of the wild type with a mutant of rice, as the free proline level is higher in the mutant than in the wild type. We demonstrated that the higher free proline level provided effective protection against Hg^{2+}-induced stress, by reducing free radical damage in the cells.

Materials and Methods

Plant Material and Treatment

Immature embryos of *japonica* rice (*Oryza sativa* cv. "Zhonghua 11") were used to initiate embryogenic calli. After calli were cocultured with *Agrobacterium tumefaciens* strain EHA105 harboring binary vector pSMR-J18R, the Hg^{2+}-tolerant rice mutant was obtained form the T-DNA (Ac/Ds) mutant population, and our present results showed that the Hg^{2+}-tolerant rice mutant was a somaclonal mutant. After exposure to 0.2 mM Hg^{2+} for 10 days, remarkable phenotypic differences were observed between wild-type and mutant plants:, chlorosis appeared in the seedlings of the wild type, while the mutant seedlings remained green after an exposure of 5 days. Wild-type seedlings appeared to be dead, while mutant seedlings were growing well on day 10.

Rice seeds (offered by China National Rice Research Institute) were surface-sterilized with sodium hypochlorite (0.5%, w/v) for 25 min, rinsed extensively with distilled water, and soaked in deionized water overnight. The seeds were dark-incubated in petri dishes covered with moist filter paper for 1 day at 30°C. After germination, the seeds were transferred to a plastic screen floating on distilled water at 28°C for 7 days, then seedlings of uniform size were cultivated in black polyethylene barrels (16 seedlings per pot) containing 6 L normal rice nutrient solution (IRRI, pH 5.1). The nutrient solution was renewed every 7 days. A 14-h photoperiod at 65% relative humidity, a photon flux density of 170 μmol quanta m^{-2} s^{-1}, and day/night temperatures of 32/27°C were used to cultivate the rice seedlings. At the six-leaf stage, 0.2 mM Hg was added to the nutrient solution. Seedlings were uprooted at 12-h intervals up to 48 h. Samples were used with the second fully expanded upper leaves (physiological characteristics of stability numerate representative), which were immediately frozen in liquid nitrogen and stored at −80°C for biochemical analysis or dried at 80°C for elemental analysis. In order to clarify the relationship between proline and Hg^{2+}-induced oxidative stress in this experiment, we used three treatments: control, exposure to Hg^{2+}, and proline pretreatment. All experiments were performed in triplicate.

Proline Determination

Proline was determined according to the method of Bates et al. (1975). Plant leaf tissues (0.5 g) were extracted with 5 mL 3% sulfosalicylic acid and centrifuged at 5,000 rpm min^{-1} for 20 min. A 2-mL sample of the supernatant was reacted with 2 mL of ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene and mixed vigorously with a test tube stirrer for 15–20 s. The chromophore containing toluene was extracted from the aqueous phase, then warmed to room temperature, and the absorbance was read at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% (w/v) sulfosalicylic acid and covering the concentration range 1–10 μg mL^{-1}.

Determination of Leaf Water Potential

Leaf total water potential was measured with water potential instrument (Model PSYPRO, Wescor Corp., USA) on the second fully expanded upper leaf (three leaflets per plot) at 12 h interval.

Determination of ROS Production

Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA) with 2-thiobarbituric acid (TBA) according to Zhao et al. (1994). Fresh leaf samples (1.0 g) were ground with 5 mL 0.6% TBA in 10% trichloroacetic acid (TCA), using a mortar and pestle. The

mixture was heated at 100°C for 15 min. After cooling in ice, the mixture was centrifuged at 5,000 rpm min^{-1} for 10 min. The absorbances of the supernatant at 450, 532 and 600 nm were measured. The MDA content was calculated on a fresh weight basis as follows: (μmol MDA $g^{-1}FW$) = 6.45 (OD_{532}-OD_{600}) −0.56 OD_{450}. H_2O_2 content was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 0.5 g leaf tissues with 5 mL acetone. The homogenate was centrifuged at 6,000 rpm min^{-1} for 25 min. To determine H_2O_2 content, 3 mL extracted solution was mixed with 1 mL 0.1% titanium chloride in 20% (v/v) H_2SO_4 and the mixture centrifuged at 6,000 rpm min^{-1} for 15 min. The absorbance of the supernatant at 410 nm was used to calculated H_2O_2 content using the extinction coefficient 0.28 μmol cm^{-1}. $O_2^{-\bullet}$ -generation rate was measured by the spectrophotometric method of Wang and Luo (1990).

Glutathione (GSH) Determination

The content of reduced and oxidized glutathione was determined with an enzyme-recycling assay spectrophotometrically at 412 nm according to Li et al.(2003). The assay was based on sequential oxidation of glutathione by DTNB and reduction by NADPH in the presence of a known amount of GR. To quantify GSSG content, 2-vinylpyridine was added to the extract. Standard curves were generated with reduced and oxidized glutathione.

Chlorophyll Determination

Chlorophyll content was determined according to Arnon (1949) with UV-2550 UV–Visible spectrophotometer.

To determine the concentration of absorbed Hg^{2+} in leaves, fresh samples were thoroughly rinsed with distilled water to remove excess surface bound Hg^{2+} and then dried in oven at 80°C for 1 day. Dried leaf samples were ground to a fine powder by pulverator, then transported to a digestion mixture containing H_2SO_4–HNO_3 at 1:1 ratio. Digestion was performed on a hotplate at 80°C until the solution became colourless. The residue was dissolved in 2% (v/v) HNO_3 and the final volume was adjusted to 5 mL. The digested samples were analyzed for metal content with an atomic fluorescence spectrophotometer (AFS-930).

Statistical Analysis

Variances among different treatments were analyzed using the multi-way ANOVA. All the data reported in this paper are means of three replicates. Significant difference among means were determined by Tukey, at $p = 0.05$ and $p = 0.01$. DPS software (Tang and Feng 2002) was used in all analyses.

Results

Analysis of the Hg^{2+}-Tolerant Rice Mutant

The PCR amplification result indicated that the mutation was not caused by T-DNA insertion, but most probably caused by tissue culture since the mutant was derived from a transgenic rice line.

Effect of Hg^{2+} and Proline Pretreatment on Proline Accumulation in Leaves

Although the constitutive content of proline was almost same in both the wild type and the mutant, proline content in the mutant was 4-fold higher than that of the control after exposed to Hg^{2+} for 12 h. After reaching the peak at 12 h, proline content in the mutant decreased rapidly in the next 12 h. After then the proline content in the mutant kept increased until the end of the experiment. A increase in the proline content was observed in the wild type at 12 and 36 h after the exposure to Hg^{2+}. The proline content in the wild type was consistently lower than that in the mutant.

Pretreatment of wild type and mutant with proline significantly enhanced the level of endogenous proline (Fig. 1). As the exposure to 0.2 mM Hg^{2+} enlonged, there was a concomitant decrease in endogenous proline content.

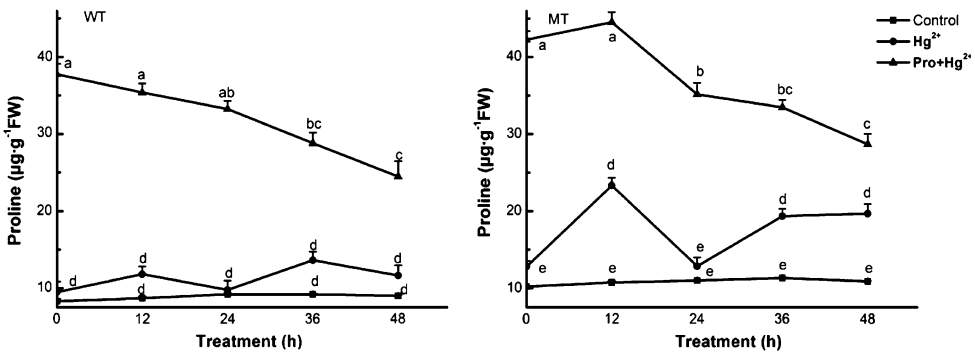

Fig. 1 Effect of proline pretreatment (2 mM, 12 h) on endogenous proline content in leaves of the wide-type and the mutant. Seedlings were exposed to a nutrient solution containing 0.2 mM Hg^{2+} at the time intervals shown in the figure

In 0.2 mM Hg^{2+}-enriched media, proline accumulation was significantly higher in the mutant than in the wild type. Through the multi-way ANOVA, we found that different cultivators ($F_{1,29} = 78.89$, $p < 0.01$), treatments ($F_{2,29} = 927.09$, $p < 0.01$) and exposing time ($F_{4,29} = 11.76$, $p < 0.01$) would all have a significantly different effect on the proline content, and the interact of different cultivators and treatments ($F_{2,29} = 6.77$, $p < 0.05$), and the interact of treatments and exposing time ($F_{8,29} = 17.479$, $p < 0.01$) would both affect the proline content significantly. From the different exposure time, we found that the wild type has a significant difference only during the pretreatment of proline. But for the mutant, the two treatments both induced significant difference in different time. all the results present here indicate that the mercury-tolerant mutant has a much more effective proline synthesis.

Effect of Hg^{2+} on Leaf Water Potential

The present work demonstrated that water status in mutant seedlings did not undergo a significant decrease ($p < 0.05$) within 48 h exposure to 0.2 mM Hg^{2+}. Difference in leaf water potential between Hg^{2+}-treated wild type and control was significant only after 48 h. About 12, 24, and 36 h-exposure of mutant seedlings to the metal did not result in any significant reduction in their leaf water potential when compared to control (Table 1). Through the multi-way ANOVA, we can found that Hg^{2+} different exposed time ($F_{4,59} = 66.22$, $p < 0.01$), united factors between different types and treatments ($F_{1,59} = 24.67$, $p < 0.01$), different types and exposed time ($F_{4,59} = 22.99$, $p < 0.01$), and the three factors ($F_{4,59} = 8.44$, $p < 0.01$) would induce a significant difference.

Effect of Proline on Alleviating Hg^{2+}-Induced Oxidative Damage

Figure 2 showed that a time-dependent increasement of MDA content was generated during the exposure of the seedlings to Hg^{2+}. However, the increased MDA level was lower in the mutant than the wild type. A lower MDA level was also observed in the pretreatment of proline than in the Hg treatments. Among the three factors, expect the united factors of cultivars and treatments ($F_{2,29} = 1.02$, $p > 0.05$), most of them induced a significant difference. Also we can found some interesting things in the two cultivars of different exposure time in detail: the MDA content of mutant only have significant difference with Hg^{2+} treatment, but almost none with the pretreatment of proline; the MDA content of wild type is similar to mutant. These data indicates that high concentration of proline synthesized endogenously in mutant provides a means to reducing the level of free radicals generated during heavy metal stress.

The effect of proline on suppressing $O_2^{-\bullet}$ - generation in rice seedlings is shown in Fig. 3. Pretreated with proline can mitigate the $O_2^{-\bullet}$ - production of both the wild type and the mutant. Evidently, a higher level of $O_2^{-\bullet}$ - production was observe only in the seedlings treated with Hg^{2+}. Fig. 4 also indicated that H_2O_2 level increased significantly in Hg^{2+}-treated rice leaves. However, H_2O_2 level in the seedlings pretreated with proline was greatly diminished throughout the entire duration of the incubation. It is similar to the alternation of $O_2^{-\bullet}$ - production. The difference between them is: the single factors and the union effect all can induced a significant difference to $O_2^{-\bullet}$ - production in wild type; in the mutant, the union effect of cultivars and treatments ($F_{2,29} = 4.37$, $p > 0.05$), union effect of cultivators and exposure time ($F_{4,29} = 2.53$, $p > 0.05$) can not induce a significant difference to the H_2O_2 level. These observations indicate that the supply of exogenous proline significantly reduced damage from oxidative stress generated by Hg^{2+}.

Effect of Hg^{2+} Stress on GSH and GSSG Content

As shown in Table 2, the GSH content in leaves of the wild type dropped down synchronously in time-dependent manner. On the other hand, there was a nearly 3-fold increase in GSSG level in the wild type after treated for

Table 1 Effect of Hg^{2+} on water potential in leaves of the wild type and the mutant

Exposing time/h	Water potential/Mp			
	WT(CK)	WT(+Hg^{2+})	MT(CK)	MT(+Hg^{2+})
0	−1.72 ± 0.14a	−1.72 ± 0.14a	−2.20 ± 0.23abcd	−2.20 ± 0.22abcd
12	−3.14 ± 0.07fgh	−3.30 ± 0.08fgh	−2.45 ± 0.09bcde	−2.44 ± 0.10cde
24	−1.77 ± 0.07ab	−2.06 ± 0.07abc	−2.28 ± 0.07def	−2.72 ± 0.11abcde
36	−3.42 ± 0.10gh	−3.33 ± 0.07fgh	−2.93 ± 0.10fgh	3.18 ± 0.082efgh
48	−2.39 ± 0.10bcde	−3.53 ± 0.06 h	−2.23 ± 0.07defg	−2.84 ± 0.24abcd

Seedlings were exposed to a nutrient solution containing 0.2 mM Hg^{2+} at the time intervals shown in this table

Note: Values are means of three replicates ± SE. Different letters in the same column indicate a significant difference at $p < 0.05$

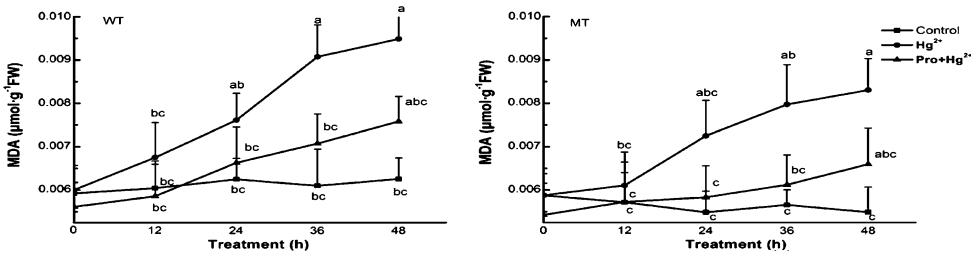

Fig. 2 Effect of proline pretreatment (2 mM, 12 h) on lipid peroxidation (measured as MDA content) in leaves of rice exposed to 0.2 mM Hg^{2+} at the time intervals shown in the figure

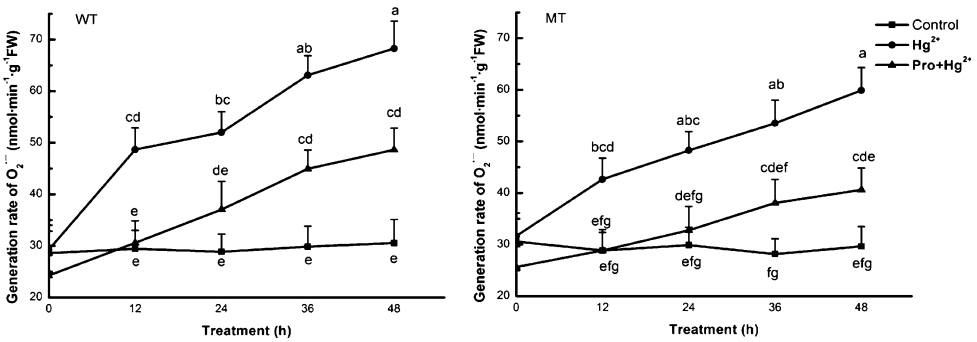

Fig. 3 Effect of proline pretreatment (2 mM, 12 h) on $O_2^{-\bullet}$ - generation rate in leaves of rice exposed to 0.2 mM Hg^{2+} at the time intervals shown in the figure

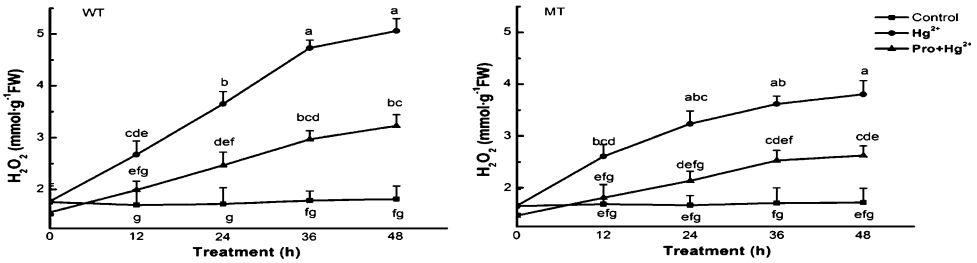

Fig. 4 Effect of proline pretreatment (2 mM, 12 h) on H_2O_2 content in leaves of rice exposed to 0.2 mM Hg^{2+} at the time intervals shown in the figure

48 h. By contrast, there was no significant ($p > 0.05$) increase in GSSG level in the mutant during the experiment period. When the reduced and oxidized forms of GSH were expressed as a molar ratio (GSH/GSSG) (Table 2), it was shown that the wild type seedlings had a 4-fold reduction in their GSH/GSSG ratio than the mutant seedlings exposed to Hg^{2+}. These results suggested that in the presence of Hg^{2+}, the redox state of the cytoplasm of the mutant cells remain more reducing than that of the wild type cells as a higher content of endogenous proline existed in the mutant.

Effect of Proline Pretreatment on Hg^{2+} Concentration and Chlorophyll Content in Leaves of Rice

Hg^{2+} concentration in leaves of rice seedlings exposed to 0.2 mM Hg^{2+} for 48 h was shown in Table 3. The mutant accumulated more Hg^{2+} than the wild type. After pretreatment of proline, the Hg^{2+} concentration in leaves decreased in both the rice seedlings of the wild type and the mutant. Hg^{2+} concentration in leaves decreased by 50% and by 30% in the wild type and in the mutant, respectively.

Results in Table 4 indicated that the leaf chlorophyll content decreased during exposed to 0.2 mM Hg^{2+} in both the wild type and the mutant, and the decreased extent of the chlorophyll content without pretreatment with proline is larger than that of pretreatment with proline, showing that proline pretreatment significantly ameliorated reduction of chlorophyll by Hg^{2+}. This amelioration was more pronounced in the case of the mutant than in the wild type. we also can found it with the multi-way ANOVA: the united effect of the types and treatments ($F_{2,89} = 3.59$, $p < 0.05$) induced a difference.

Effect of Proline Pretreatment on H_2O_2 Content in Rice Exposed to AT Stress

The seedlings treated with 2 mM AT in dark showed a rapid and continuous loss of catalase activities compared with the untreated seedlings (data not shown). After 48-h exposure to AT, a significant increase in the content of hydrogen peroxide was observed in both the wild type and the mutant seedlings. Proline pretreatment caused similar levels of decline (significantly) in accumulation of H_2O_2 by seedlings subjected to Hg^{2+} stress (Table 5). The results indicated that the different cultivators and different treatments could affect the accumulation of H_2O_2 significantly ($F_{2,12} = 124.62$, $p < 0.01$).

Table 2 Effect of Hg^{2+} on GSH and GSSG content in leaves of the wild type and the mutant. Seedlings were exposed to 0.2 mM Hg^{2+} at the time intervals shown in this table

Exposing time/h	GSH/nmol·mg^{-1}protein			
	WT(CK)	MT(CK)	WT(+Hg^{2+})	MT(+Hg^{2+})
0	131.25 ± 0.99fg	146.13 ± 0.39cdef	133.62 ± 1.95efg	150.03 ± 1.36cd
12	130.24 ± 0.99g	150.17 ± 0.88cd	123.54 ± 4.90g	203.64 ± 2.22a
24	127.51 ± 1.70g	149.76 ± 1.26cd	122.72 ± 2.11g	160.96 ± 9.37bc
36	128.21 ± 1.94g	148.89 ± 2.04cd	100.54 ± 1.73h	165.16 ± 1.69b
48	126.39 ± 1.95g	147.75 ± 1.07cde	88.07 ± 1.74h	137.48 ± 1.30defg
Exposing time/h	GSSG/nmol·mg^{-1}protein			
	WT(CK)	MT(CK)	WT(+Hg^{2+})	MT(+Hg^{2+})
0	36.81 ± 0.65fg	37.41 ± 0.86fg	36.62 ± 0.69fg	37.54 ± 0.96fg
12	39.03 ± 0.61efg	35.93 ± 0.95g	48.50 ± 1.14d	40.92 ± 1.40efg
24	40.68 ± 0.90efg	36.88 ± 0.97fg	59.25 ± 1.13c	42.27 ± 1.16ef
36	39.11 ± 1.18efg	39.14 ± 1.12efg	72.08 ± 1.26b	44.39 ± 0.67de
48	40.52 ± 0.78efg	39.49 ± 1.31efg	100.26 ± 1.25a	49.22 ± 1.20d
Exposing time/h	GSH/GSSG			
	WT(CK)	MT(CK)	WT(+Hg^{2+})	MT(+Hg^{2+})
0	4.00 ± 0.09cdef	4.37 ± 0.09bcd	4.09 ± 0.11bcdef	4.47 ± 0.08bc
12	3.74 ± 0.08defg	4.69 ± 0.12hdefg	2.86 ± 0.20hi	5.58 ± 0.15a
24	3.51 ± 0.10fg	4.55 ± 0.10bc	2.32 ± 0.08i	4.27 ± 0.33bcde
36	3.68 ± 0.11efg	4.27 ± 0.11bcde	1.56 ± 0.00j	4.17 ± 0.10bcde
48	3.50 ± 0.07fgh	4.23 ± 0.12bcde	0.98 ± 0.03j	3.12 ± 0.10gh

Note: Values are means of three replicates ± SE. Different letters in the same column indicate a significant difference at $p < 0.05$

Table 3 Effect of proline pretreatment (2 mM, 12 h) on Hg^{2+} concentration in leaves of the wild type and the mutant exposed to 0.2 mM Hg^{2+} for 48 h

Treatment	Hg^{2+} concentration/mg·kg^{-1}DW	
	WT	MT
CK	8.66 ± 0.04d	9.86 ± 0.24d
Hg^{2+}	94.17 ± 1.31b	126.12 ± 0.69a
Proline +Hg^{2+}	47.96 ± 0.95c	93.85 ± 0.93b

Note: Values are means of three replicates ± SE. Different letters in the same column indicate a significant difference at $p < 0.05$

Discussion

In plants, the most general symptom of Hg^{2+} toxicity is chlorosis. In previous work, we have shown that rice seedlings treated with Hg^{2+} show chlorosis and biomass reduction, and rice accumulated large amount of Hg^{2+} when incubated in Hg^{2+}-enriched culture medium (Zeng et al. 2008). Chlorophyll is a major pigment participating in photosynthesis process which is one of the main sites of heavy metal injury in plants (Shakya et al. 2008). Results in this study indicated that Hg^{2+} caused reduction of chlorophyll in both the wild type and the mutant, and proline pretreatment significantly ameliorated reduction of chlorophyll by Hg^{2+}.

Hg^{2+} can inactivate the cellular antioxidant pool and disrupt the metabolic balance, eventually enhancing the load of ROS, such as $O_2^{-\bullet}$, ·OH, H_2O_2 and $^1O_2$ (Briat 2002). ROS in turn causes damage to the biomolecules such as membrane lipids, proteins, chloroplast pigments, enzymes, nucleic acids, etc. Lipid peroxidation is a biochemical marker for the free radical mediated injuries. Measurement of the level of MDA in the tissues is widely used as an index of lipid peroxidation. Our results showed a time-dependent increase in the level of MDA in seedlings exposed to Hg^{2+}, indicating that Hg^{2+} induces oxidative stress in rice plants. These results are in conformity with the observations of many other authors (Xu et al. 2000; Patra and Sharma 2000). Also in our early studies, we found that Hg^{2+} Stress led to a large number of H_2O_2 accumulation in the wild-type and the mutant, and speeded up the rate of the $O_2^{-\bullet}$, and MDA formation (Zeng et al. 2008).

Most heavy metals cause oxidative stress via generation of ROS (Dietz et al. 1999). It has been proposed that

Table 4 Effect of proline pretreatment (2 mM, 12 h) on the chlorophyll contents in leaves of the wild type and the mutant exposed to 0.2 mM Hg^{2+}

Exposing time/h	Chlorophyll-a + -b/mg·g^{-1}FW					
	WT(CK)	MT(CK)	WT(Hg^{2+})	MT(Hg^{2+})	WT(Pro)	MT(Pro)
0	6.70 ± 0.66abcd	7.55 ± 0.41ab	6.70 ± 0.66abcd	7.55 ± 0.41abcd	7.74 ± 0.03d	6.34 ± 0.11abcd
12	6.71 ± 0.22abcd	5.84 ± 0.17ab	6.70 ± 0.39abcd	6.96 ± 0.53abcd	6.40 ± 0.14cd	6.23 ± 0.72abcd
24	7.11 ± 0.28a	6.45 ± 0.34abcd	6.13 ± 0.40abcd	6.79 ± 0.17abcd	5.72 ± 0.24abcd	7.25 ± 0.16abcd
36	6.17 ± 0.06abcd	5.35 ± 0.41abcd	5.26 ± 0.03bcd	5.89 ± 0.03abc	5.31 ± 0.28bcd	6.22 ± 0.35bcd
48	6.77 ± 0.24abcd	6.64 ± 0.19abcd	5.21 ± 0.38bcd	5.76 ± 0.39cd	5.66 ± 0.33bcd	6.18 ± 0.26abcd

Note: Values are means of three replicates ± SE. Different letters in the same column indicate a significant difference at $p < 0.05$

Table 5 Effect of proline pretreatment (2 mM, 12 h) on H_2O_2 content in the wild type and the mutant exposed to 2 mM AT for 48 h

Treatment	H_2O_2 (mmol g^{-1}FW)	
	WT	MT
CK	1.77 ± 0.44d	1.65 ± 0.06a
AT	3.34 ± 0.08d	3.29 ± 0.10c
Proline +AT	2.24 ± 0.00a	2.86 ± 0.1b

Note: Values are means of three replicates ± SE. Different letters in the same column indicate a significant difference at $p < 0.05$

proline act as a free radical scavenger to protect plants away from damage by oxidative stress (Alia and Matysik 2001). The present study indicated that proline was involved in the Hg^{2+} tolerance of rice. Some researchers showed that proline involved in abating heavy metal stress stems from its enhanced accumulation in the plants exposed to metals (Wu et al. 1998; Backor et al. 2004; Tripathi and Gaur 2004). Taking advantage of mutant that produced high level of endogenous proline, we carried out measurements on MDA and free radical level in wild type and mutant seedlings during Hg^{2+} stress with and without the addition of exogenous proline. This hypothesis has been examined in those study. Although the scavenging reaction of ROS with other amino acids, such as tryptophan, tyrosine, histidine, etc. are more effective compared with proline (Michaeli and Feitelson 1994), proline is of special interest because of its extensive accumulation in plants during environmental stress (Chai et al. 1998). As depicted in Fig. 3, both the test plant species showed enhancement in the content of proline within 12 h of the metal treatment, which was in agreement with reports on other higher plants (Schat et al. 1997; Tripathi and Gaur 2004). Nevertheless, they differed in the content in their tissues and proline accumulation increased drastically in the mutant under longer Hg^{2+} treatment. After proline pretreatments, the proline content also increased within 12 h in the mutant while the wild type showed a time-dependent decrease. These results suggest that the regulation of endogenous proline biosynthesis under Hg^{2+} stress is correlated with the tolerance of mutant rice. There are three possible reasons of the free proline accumulation under stress: first, stimulation of proline synthesis from glutamic acid (Girousse et al. 1996), which has been found to be dependent on the abscisic acid concentration; second, inhibition of proline oxidation to other soluble compounds; and third, inhibition of protein synthesis. In contrast to its metabolism, the physiological significance of proline accumulation has been less studied recently (Sharma and Dietz 2006).

Many researchers suggested that proline plays a pivotal role in imparting plants tolerance to stress that lower the water potential of ambient environment (Colmer et al. 1996; Schat et al. 1997). Sharma et al. (1998) maintained that water deficit is the primary reason of proline accumulation because such accumulation is not induced in plants exposed to elevated concentrations of metals at high humidity levels. But in the present study, we found that a significant difference in water stress is simultaneous with a lower proline content of wild type at 48 h exposed to Hg^{2+} which is not existing in mutant, the possible reason is the high endogenous proline content in the mutant. On the other hand, the levels of proline in plants have been observed to be enhanced even by UV light (Tripathi and Gaur 2004) which does not lead to osmotic stress or create low water potential.

It has been suggested that free proline chelates with heavy metal ions in cells, therefore decreasing their availability and toxicity to sensitive cellular sites (Farago and Mullen 1979). However, the present work demonstrated that proline helps rice to tolerate metal stress primarily by scavenging free radicals rather than by chelating with heavy metal ions. This point is upheld by the fact that reduction of AT-induced lipid peroxidation was as much as that of the Hg^{2+}-induced lipid peroxidation resulting from proline pretreatment. There is at least one similarity in the mode of action of heavy metals and herbicide: both of them induce oxidative stress (Vangronsveld and Clijsters 1994). AT is an herbicide which irreversibly inactivates catalase, which cannot chelate with proline. In

present study, 2 mM AT treatment resulted in approximately 2-fold increase in H_2O_2 content both in the wild type and the mutant seedlings. Proline pretreatment caused similar level of decline in accumulation of H_2O_2 by seedlings subjected to AT and Hg^{2+} stress. Proline pretreatment provided protection by reducing Hg^{2+}-induced lipid peroxidation. This suggests a protective role of proline against Hg^{2+}-induced oxidative damage. Furthermore, proline pretreatment resulted in less Hg^{2+} accumulation and reduction of chlorophyll in leaves of the wild type and the mutant, which also endorsed the role of proline as an antioxidant rather than Hg^{2+} chelator (Matysik et al. 2002). It has been reported that heavy metal can affect the photosynthetic apparatus (Clemens 2006), also the generated ROS such as ·OH radicals are known to damage biological membranes (Halliwell and Gutteridge 1989), and proline has been found to protect cell membranes of onion against salt injury (Mansour 1998), so we presume that proline pretreatment mitigated the membrane damage (Shakya et al. 2008; Tatar and Gevrek 2008).

GSH is a scavenger of many reactive oxygen radicals (May et al. 1998). During oxidative stress, GSH is utilized by the cells, thereby leading to its depletion. Furthermore, GSH is the precursor of phytochelatins (PCs) which are synthesized enzymatically (Grill et al. 1989). PC, believed to be the major heavy metal chelator in plants, could serve to shuttling poisonous ions from the cytosol to the vacuole (Sanita and Gabbrielli 1999; Cobbett 2000). Proline may physically quench singlet oxygen or react directly with hydrogen peroxide. These reactions result in reducing free radical damage and a more reducing cellular environment (higher GSH/GSSG value). The high GSH levels in turn facilitate PC synthesis and sequestration of Hg^{2+}-PC compound into the vacuole.

According to our experimental results, a positive relationship between Hg^{2+} toxicity and proline accumulation suggests a protective role of this amino acid against heavy metal toxicity. The present work clearly shows the protective role of proline against metal toxicity, although, it is difficult to elaborate the manner in which this role was executed. It might be that proline reduces the production of harmful ROS, or sequestrates them.

In conclusion, the mutant rice can be well adapted to the stress of Hg^{2+}, which was due to increased level of proline that minimized the damage caused by toxic oxygen species and protected the cell membranes by stabilizing plasmalemma permeability, so that less Hg^{2+} is taken up by the cells. Furthermore, the results confirmed the conclusion drawn in previous publication (Siripornadulsil et al. 2002): proline reduces heavy metal stress by detoxification of free radicals produced as a result of heavy metal poisoning.

Acknowledgments This work was supported by the National Key Technologies R&D Program of China during the 11th Five-Year Plan Period (NO. 2006BAK02A18), the Natural Science Foundation of Zhejiang Province (NO. Z306300), and the National Key Basic Research and Development Program (NO. 2002CB410804).

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