Effect of cadmium on peroxidase isozyme activity in roots of two *Oryza sativa* cultivars

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ABSTRACT. Cadmium (Cd) decreased the growth of rice expressed as fresh weight. Root growth was inhibited more severely in Taichung Native 1 rice than in Tainung 67. The differences in peroxidase (POX) activity and lignin content between Cd-tolerant and Cd-sensitive rice varieties were compared. In our study, POX activity in Tainung 67 increased significantly in Cd-treated tissues. However, enhanced POX activity in Cd-treated tissues was accompanied by a H_2O_2 decrease. The H_2O_2 accumulation in the Cd-treated tissues of Taichung Native 1 rice may be due to the lower amount of POX enhancement induced by Cd. The increased activity in cationic (pI 8.6) and anionic (pI 4.5) POXs correlated with the increase in POX transcripts, and therefore was mostly due to the *de novo* synthesis of the cationic (pI 8.6) and anionic (pI 4.5) POXs in Cd-treated roots of Tainung 67. For promoter analysis, Tainung 67 (Japonica type) owned ten conserved cores of CURECORECR sequence (5′-GTAC-3′), a copper-response element (CuRE), involved in heavy metals response. Taichung Native 1 (Indica type) had only eight CURE. This implies that a Cd-tolerant cultivar, Tainung 67 (Japonica type), may receive more signals with Cd treatment, enhancing more POX synthesis, which leads to the production of more lignin to deal with Cd stress.

Keywords: Cadmium; Lignin; *Oryza sativa*; Oxidative stress; Peroxidase; Promoter.

Abbreviations: Cd, cadmium; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **POX,** peroxidase; **ROS,** reactive oxidative species; **pI,** isoelectric point; **CuRE,** copper-response element.

INTRODUCTION

Cadmium is a non-essential element in plants that negatively affects their growth and development. In most environments, Cd first enters plant roots, where initial damage is most likely to take place (Sanità di Toppi and Gabbrielli, 1999). Cd easily penetrates the root through the cortical tissue and is translocated to the aboveground tissues (Williams et al., 2000). Cadmium is reported to affect stomatal openings, transpiration, and photosynthesis. Chlorosis, leaf rolls and stunting are the main and easily visible symptoms of cadmium toxicity in plants. Several studies have suggested that an oxidative stress could be involved in Cd toxicity, by either inducing oxygen free radical production, or by decreasing enzymatic and non-enzymatic antioxidants (Balestrasse et al., 2001; Benavides et al., 2005; Cho and Seo, 2005; Fornazier et al., 2002).

Several studies reported that Cd treatment increased the activities of those antioxidative enzymes (Dixit et al., 2001). Cho and Seo (2005) reported that Cd-induced oxidative stress in *Arabidopsis* is due to H_2O_2 accumulation. Romero-Puertas et al. (2004) studied the involvement of H_2O_2 and O_2^- in the signalling events that lead to CAT, GR and CuZn-SOD transcript level variations in pea plants under Cd stress. H_2O_2 can induce many reactions involving peroxidase activation, secondary metabolism stimulation, and structural changes such as lignin deposition.

In the presence of H_2O_2, secreted class III plant POX oxidizes a vast array of compounds (hydrogen donors). Plant POXs are heme-containing glycoproteins and are usually classified as acidic, neutral, or basic, according to their isoelectric points. Most of the higher plants possess a large number of POX isoenzymes, which are encoded by multigene families (Hiraga et al., 2001). The Arabidopsis genome contains 73 POX genes (Tognolli et al.,

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2002; Welinder et al., 2002). In rice, 138 POX genes are distributed among the 12 rice chromosomes (Passardi et al., 2004). Several physiological functions for POXs in plants have been reported, such as H_2O_2 removal, toxic reductants oxidation, cell wall lignin biosynthesis and degradation (Lagrimini, 1991), auxin catabolism (Gazaryan and Lagrimini, 1996), defensive responses to wounding, response to anoxia (Lee and Lin, 1995) and defense against pathogen or insect attack (Passardi et al., 2005). The diversity of processes catalyzed by POXs as well as the large number of their genes suggests a possible functional specialization for each isoform. In addition, POXs promoter sequences are very divergent and have variable regions (Cosio and Dunand, 2009). The great diversity of the promoter and intronic sequences partially explains why all kinds of internal or external stimuli regulate the gene expression (Cosio and Dunand, 2009; Mathé et al., 2010).

POX is involved in lignin synthesis (Li et al., 2001; Liu and Ger, 1997; Quiroga et al., 2000; Yang et al., 2007; Cho et al., 2011). POX has been proposed as a candidate enzyme for catalyzing the final step in the polymerization of monolignols like p-coumaroyl alcohol, coniferyl alcohol, 5-hydroxyconiferyl alcohol and sinapyl alcohol (Hatfield and Vermerris, 2001).

Rice (*Oryza sativa* L.) belongs to the *Oryza* genus of the Graminceae family, and is divided into three subspecies: *Indica*, *Japonica* and *Javanica*. Rice (*O. sativa* L.) has played a central role in human nutrition and culture for the past 10,000 years, is the most important food crop in the world, and feeds over half of the global population. It has important syntenic relationships with the other cereal species, and is a model plant for the grasses. To sequence the rice genome with high accuracy and reliability, a clone-by-clone sequencing strategy was adopted by the International Rice Genome Sequencing Project (IRGSP) (Sasaki and Burr, 2000). In December 2004, the IRGSP completed the sequencing of the rice (*Oryza sativa* L. ssp. *Japonica* cv. Nipponbare) genome. The high quality map-based sequence of the entire genome is now available in public databases. An Indica type cultivar and a Japonica cultivar were used in this study. The Indica type cultivar (*Oryza sativa* L. cv. Taichung Native 1) is known to be sensitive to Cd, and the Japonica cultivar (*Oryza sativa* L. cv. Tainung 67) is known to show significant tolerance to Cd (Hsu and Kao, 2003). If a mechanism related to oxidative stress is involved in Cd toxicity, this mechanism should then be differently expressed in plants tolerant and sensitive to Cd.

The purpose of this study is to understand the effects of cadmium on two rice (*Oryza sativa* L.) cultivars by examining changes in peroxidase (POX) activities and in hydrogen peroxide and lignin amounts, to gain more insight into the role of POX in lignin synthesis and in the cadmium tolerance levels of two rice cultivars. To understand the regulation of POX genes by cadmium, the potential regulatory regions within the promoter in both Tainung 67 Japonica type) and Taichung Native 1 (Indica type) were also investigated.

MATERIALS AND METHODS

Plant materials

Two rice cultivars, an *Indica* type cultivar (*Oryza sativa* L. cv. Taichung Native 1) and a *Japonica* cultivar (*Oryza sativa* L. cv. Tainung 67), were germinated with water in the dark for two days, then transferred to a container containing Murashinge-Skoog medium (half-strength). The seedlings were grown at $30 \pm 0.5°C$ under a 16 hrs/ 8 hrs light/ dark period and illuminated with white fluorescent light at an irradiance of 200 μmol $m^{-2}s^{-1}$. Six-day-old rice seedlings were cultured in varying concentrations of $CdCl_2$ (50, 100, 150, 300 μM) and Murashinge-Skoog medium (control) for various times. The fresh weights of rice roots were measured after culturing. Ten to thirty roots were collected and kept frozen at -80°C for peroxidase, H_2O_2, and lignin assays. Data were analyzed using Dunnett's multiple comparison test.

H_2O_2 determination

Rice roots (100 mg) were homogenized with 0.6 mL of 50mM phosphate buffer (pH 6.5) with 10 mM 3-amino-1, 2, 4-triazole. The homogenates were centrifuged at 6,000 *g* for 25 min 100 μL of supernatant was added to 900 μL of reaction mixture that contained 100 μM Xylenol orange, 250 μM $FeSO_4$, 100 mM sorbitol, and 25 mM H_2SO_4. The reaction mixture was incubated for 45 min at room temperature, 10 μL catalase added, vortexed vigorously, centrifuged at 12,000 *g* for 2 min, and supernatant absorption was measured at 560 nm to determine the level of H_2O_2, using the H_2O_2 standard curve (0.5 μM - 2.5 μM) (Jiang et al., 1990).

Peroxidase extraction and activity assay

Rice roots (100 mg) were homogenized in 0.1 mL of 10 mM phosphate buffer (pH 7.0). The homogenates were centrifuged at 14,000 *g* for 20 min and the supernatant was saved for analysis. POX activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm after 20 min incubation at room temperature (Liu and Ger, 1997). The reaction mixtures contained 25 μL of 50 mM H_2O_2, 5 μL of 250 mM guaiacol, 195 μL of 12.5 mM 3, 3-dimethylglutaric acid (pH 6.0) and 25 μL of enzyme extract. One unit of POX isoenzymes was defined as the amount of enzyme that caused the formation of 1 nmol tetraguaiacol per min (extinction coefficient is 26.6 $mM^{-1}cm^{-1}$ at 470 nm) (Lin and Kao, 2001).

Peroxidase isoelectric focousing gel electrophoresis and peroxidase activity staining

POX extracts were subjected to analytical flat bed isoelectric focusing on polyacrylamide gels containing ampholines in the 4-6.5 and 5.5-8.5 pH range (GE Healthcare Bio-Sciences AB, Sweden). The samples were subjected to electrophoresis for 2.5 hrs at 25W at 12°C. After focusing, the gels were soaked in 500 mL of PBS (10 mM sodium phosphate buffer (pH 6.0), 150 mM NaCl) for 30 min with

shaking to remove ampholines and equalize the pH. The POX isoenzymes were detected by soaking the gel for 10 min in 200 mL of the phosphate buffer containing 0.6 mg mL^{-1} 4-chloro-1-naphthol and 0.16% H_2O_2 (Ye et al., 1990).

RNA Isolation and Reverse Transcription of RNA

Total RNA was prepared from frozen roots (-80°C) using the Plant Total RNA Miniprep System (Viogene, Sunnyvale, CA, USA) and quantified with UV absorption at 260 nm. Four micrograms of total RNA were incubated at 70°C for 10 min with 500 ng of Oligo-p(dT)15 (Life Technologies, Gaithersburg, MD, USA), then quickly chilled on ice. The mixture was added to obtain a 20 µL reaction volume containing 20 mM Tris-HCl (pH 8.3), 55 mM MgCl$_2$, 10 mM DTT, 0.5 mM each of dNTP and 10 U of SUPERSCRIPT^{TM} II RNase H-Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA). The contents were then incubated at 42°C for 50 min. Heating at 70°C for 15 min stopped the reaction. The solution was then stored at -20°C.

PCR primers and PCR products quantification

We designed one set of POX primer according to the POX gene documented in GeneBank. The sequences POX 8.6 F5´-ATGCAGTTACTCTGTCCCGACC-3´ and R5´-CTACTTCGACCTCATCGCCAAG-3´ (AF019743) were derived from the pI 8.6 POX gene. The pI 8.6 POX PCR products were 455 bps in length. The sequences of POX 4.5 F5´-ACGTT TTCGA CAACC GCTAC-3´ and R5´-TGGAT CACAT CTTTG TTGAC G-3´ (D14481), were derived from the pI 4.5 POX gene. The pI 4.5 POX PCR products were 410 bps in length. The sequences of POX 5.1 F5´-TCGAGCACTTCTCTCTCTTCC-3´ and R5´-GAGAGCACCACCATGTCCTC-3´ (AK120411), were derived from the pI 5.1 POX gene. The pI 5.1 PCR products were 734 bps in length. Based on the rice actin (ACT) gene, we designed one pair of actin primer: F5´-GGAACTGGTATGGTCAAGGCT-3´ and R5´-AGTCTCATGGATAACCGCAG-3´, as a control.

To a sterile 0.2-mL tube were added 5 µL of 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl and 15 mM MgCl$_2$), 4 µL of 2.5 mM dNTP mixture, 1 µL of the sense and antisense primers (20 µM), 25 ng to 100 ng of the cDNA template and an appropriate amount of water to make a total volume of 50 µL. After adding 0.25 µL of *Taq* polymerase (5 units/µL) (TaKaRa Shuzo, Japan), the tube was heated for 3 min at 94°C in a thermocycler (Gene CyclerTM, Bio-Rad, Hercules, CA, USA). The PCR cycles for POXs were 30 s at 95°C, 30 s at 50°C and 1 min at 72°C (pI 8.6 POX); 30 s at 95°C, 30 s at 58°C and 1 min at 72°C (pI 4.5 POX); 30 s at 95°C, 30 s at 58 °C and 1 min at 72°C (pI 5.1 POX); 30 s at 95°C, 30 s at 56°C and 1 min at 72°C (ACT). The PCR product was electrophoresed in 1.5 % agarose gel and stained with ethidium bromide. The approximate length of the PCR product was obtained by comparing the markers (100 bps DNA Ladder, Life Technologies). We recorded the electrophoresis using a CCD camera (Eastman Kodak company, Rochester, NY, USA) and compared the band concentrations using LabImage 2.7.2. (Kapelan GmbH Bio-Imaging Solutions, Halle, Germany).

Lignin determination

Lignin was extracted and measured using the method of Bruce and West (1989). Rice roots (1 g) were homogenized in 6 mL of 99.5% ethanol and the extract was centrifuged at 10,000 g for 15 min. The pellet was transferred to several glass Petri-dishes and air-dried overnight. Ten mg of dried residue was placed in a screw-cap tube, and 1 mL of 2 NHCl and 0.1 mL of thioglycolic acid were added. The sealed tube was heated to 100°C for 12 hrs. After cooling, the contents were centrifuged at 14,000 g for 30 min at 4°C.The pellet was washed once with 1 mL of water and centrifuged at 4°C, then resuspended in 1 mL of 2.5 N NaOH. The solution was agitated gently at 25rs for 18 h. After centrifugation at 14,000 g for 30 min, the supernatant was transferred to a test tube. One mL of concentrated HCl was added to the test tube and the lignin thioglycolate was allowed to precipitate at 4°C for 6 h. After centrifugation at 14,000 g for 30 min, the pellets were dissolved in 1 mL of 0.5 N NaOH. The absorbance was measured against a NaOH blank at 280 nm. The amount of lignin was calculated from a linear calibration curve (10-030 µg) with commercial alkali lignin (Sigma-Aldrich, Steinheim, Germany). Data were analyzed using Dunnett's multiple comparison test.

DNA isolation and restriction digestion

Genomic DNA from rice roots was harvested and isolated using the Plant Genomic DNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA). For complete digestion, 2.5 µg of genomic DNA was restricted with 80 U of *DraI*, *EcoRV*, *PvuII*, and *StuI* ,separately, at 37°C for 18 h. The digested DNA was ethanol precipitated after phenol:chloroform (1:1) extraction. The DNA pellet was resuspended in TE buffer and 0.5 µg of completely-digested DNA was used to ligate with the "Genome Walker Adaptor" (Clontech, USA) according to the manufacturer's protocol.

Primers: The following gene-specific and adaptor-specific primers were used. Rice (Indica type) pI 4.5 POX gene (CT831866) specific primers: GSP1-1: (5´-GGCATCTCGCACCCCGAGTATTAAGGC-3´); GSP1-2: (5´-TCACAGAGCTCTTGATTTCTATTCCAA- 3´); GSP2-1: (5´-CATCTCTCACAGAAGTTACTGCCCT GCT-3´); GSP2-2: (5´-AGAACACATGAGCTGATTAC AATTTAG-3´). Adaptor-specific primers: AP1: (5´-GTAATACGACTCACTATAGGGC-3´); AP2: (5´-ACTATAGGGCACGCGTGGT-3´).

PCR amplification

The PCR reactions were carried in a total volume of 20 µL containing the following to a final concentration: 10X

Advantage® 2 PCR Buffer, 0.2 mM of each (dATP, dTTP, dCTP, dGTP), 0.2 μM of each primer (adaptor-specific and gene-specific); 0.4 μl of ligated DNA as template (either total digestion or partially digested and size selected), and 0.4 μL of 50X Advantage® 2 Polymerase Mix (Clontech, USA). The first PCR conditions were 94°C, 25 s; 72°C, 3 min for 7 cycles; 94°C, 25 s; 68°C, 3 min for 32 cycles, and an additional cycle at 68°C for 7 min using thermocycler (Gene Cycler™, Bio-Rad, Hercules, CA, USA).

Gene-specific primer

[GSP1-1 (5´-GGCATCTCGCACCCCGAGTATTAA GGC-3´) and GSP1-2 (5´-TCACAGAGCTCTTGATTT CTATTCCAA -3´)] and an adaptor-specific primer [AP1(5´-GTAATACGACTCACTATAGGGC-3´)] were used for the rice (Indica) pI 4.5 POX gene. PCR products from the first run were diluted 50 fold and 0.4 μL of diluted PCR products were used as a template for the second PCR. The second PCR reactions were carried in a total volume of 20 μL containing the following to a final concentration: 10X Advantage® 2 PCR Buffer,
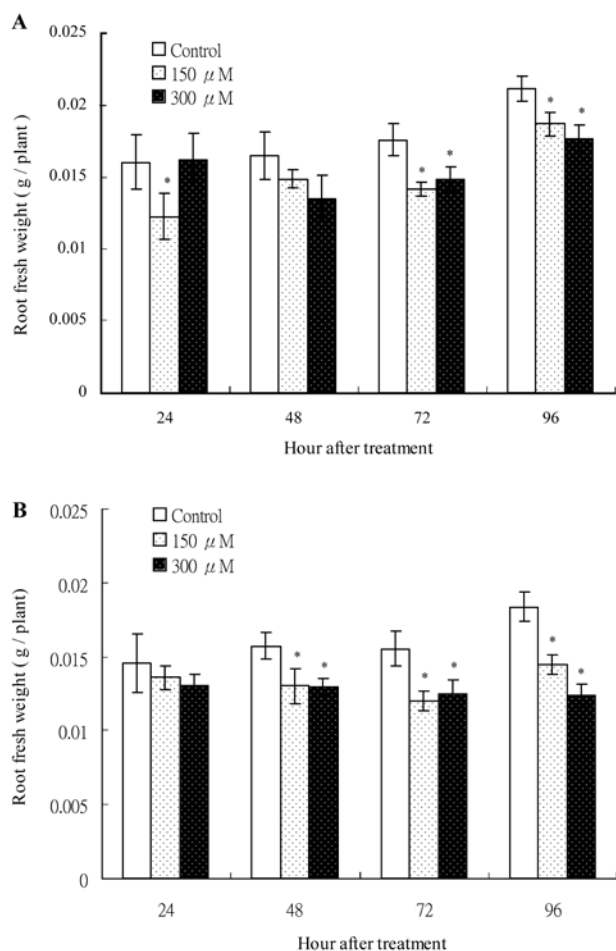
Figure 1. Effect of Cd on rice root growth. Values are the means ± SE of six replicates. Means significantly lower that the experiment control (Dunnett's multiple comparison test, P≦0.05) are marked *. (A) for Tainung 67 and (B) for Taichung Native 1.
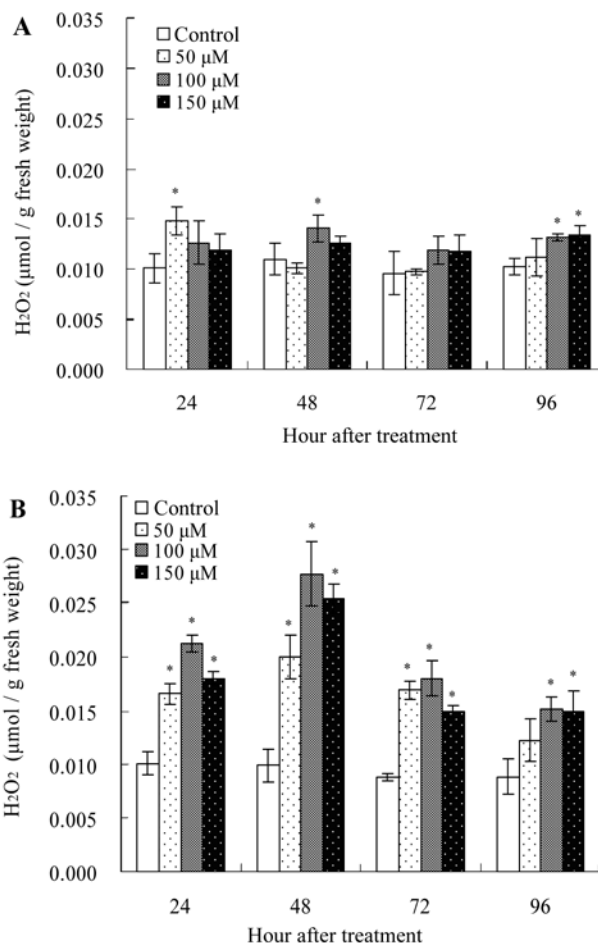
Figure 2. Effect of Cd on H_2O_2 accumulation in rice roots. Values are the means ± SE of three replicates. Means significantly higher that the experiment control (Dunnett's multiple comparison test, P≦0.05) are marked *. (A) for Tainung 67 and (B) for Taichung Native 1.

0.2 mM of each (dATP, dTTP, dCTP, dGTP), 0.2 μM of each primer (adaptor-specific and gene-specific); 0.4 μL of ligated DNA as template (either total digestion or partially digested and size selected), and 0.4 μL of 50X Advantage® 2 Polymerase Mix. The second PCR conditions were: 94°C, 25 s; 72°C, 3 min for 5 cycles; 94°C, 25 s; 68°C, 3 min for 25 cycles, and an additional cycle at 68°C for 7 min. Gene-specific primer [GSP2-1 (5´-CATCTCTCACAGAAGTTACTGCCCTGCT-3´) and GSP2-2 (5´-AGAACACATGAGCTGATTACAATTTAG-3´)] and an adaptor specific primer [AP2 (5´-ACTATAGGGCACGCGTGGT-3´)] were used for the rice (Indica) pI 4.5 POX gene. The amplified products from the second PCR reaction were resolved using 1% (w/v) agarose and 0.5X TBE buffer.

PCR products cloning and sequencing

The PCR-amplified products for both rice type pI 4.5 POX genes were resolved with low melting agarose 1% (w/v), and the obtained fragments (~5 kb and 200 bp)

Figure 3. Effect of Cd on peroxidase activity in rice roots. One unit of peroxidase activity was defined as the amount of enzyme that causes the formation of 1 nmol tetraguaiacol per min. (Extinction coefficient is 26.6 mM^{-1}cm^{-1} at OD. 470.) Values are the means ± SE of three replicates. Means significantly higher or lower that the experiment control (Dunnett's multiple comparison test, P≦0.05) are marked *. (A) for Tainung 67 and (B) for Taichung Native 1.
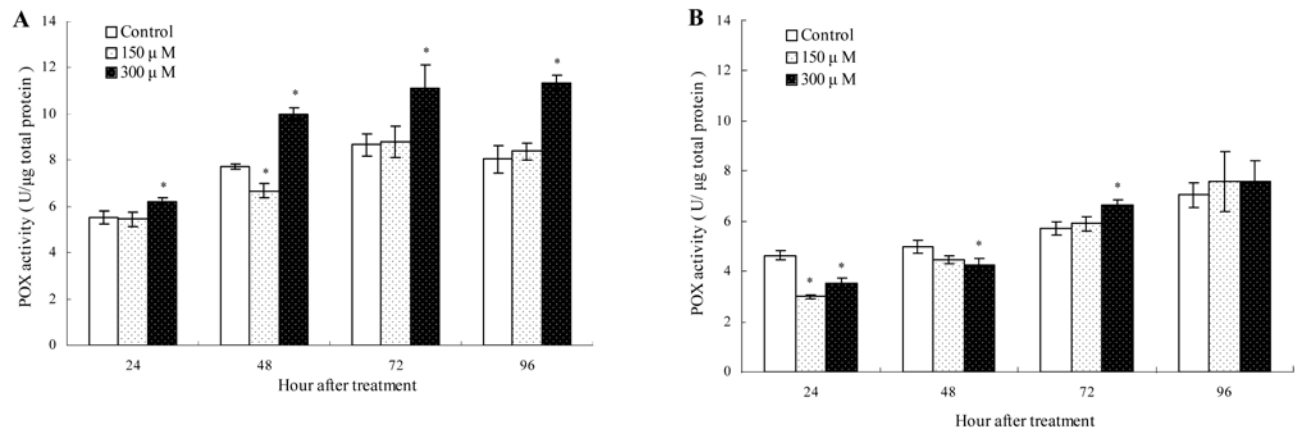
were excised, purified using the Gel-M^{TM} Gel Extraction System (Viogene, Sunnyvale, CA, USA), and ligated into the pGEM T-Easy cloning vector (Promega, USA). The ligation reactions transformed into *E. coli* DH5α competent cells. To confirm the presence of the rice pI 4.5 POX gene, the bacterial colonies carrying the inserts were identified by blue/white selection, and clones were sequenced.

Promoter sequence analysis

The 1,269-bp upstream of the transcription rice pI 4.5 POX gene start site was analyzed using PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) software (http://www.dna.affrc.go.jp/ PLACE/ and http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RESULTS

Effect of cadmium on the root growth in rice

Cd treatment turned roots brown. As the incubation time was prolonged to 96 hrs, in Tainung 67, the root reduction was about 16% under 300 μM of Cd treatment compared to the control (Figure 1A). In Taichung Native 1, the growth reduction was 32% in roots under 300 μM of Cd treatment compared to the control (Figure 1B). Root growth was more severely inhibited in Taichung Native 1 than in Tainung 67.

Effect of cadmium on H$_2$O$_2$ content and peroxidase activity

H$_2$O$_2$ content in Tainung 67, was not significantly altered by Cd treatment (Figure 2A), but accumulated remarkably in Cd-treated Taichung Native 1 (Figure 2B). Rice seedlings were cultured in MS-medium with Cd or without (control) for 24, 48, 72 and 96 h. In, the POX activity was slightly enhanced after 24 h treatment. As the incubation time was prolonged to 96 hrs, the activity of POX in Cd-treated Tainung 67 increased 1.4 fold
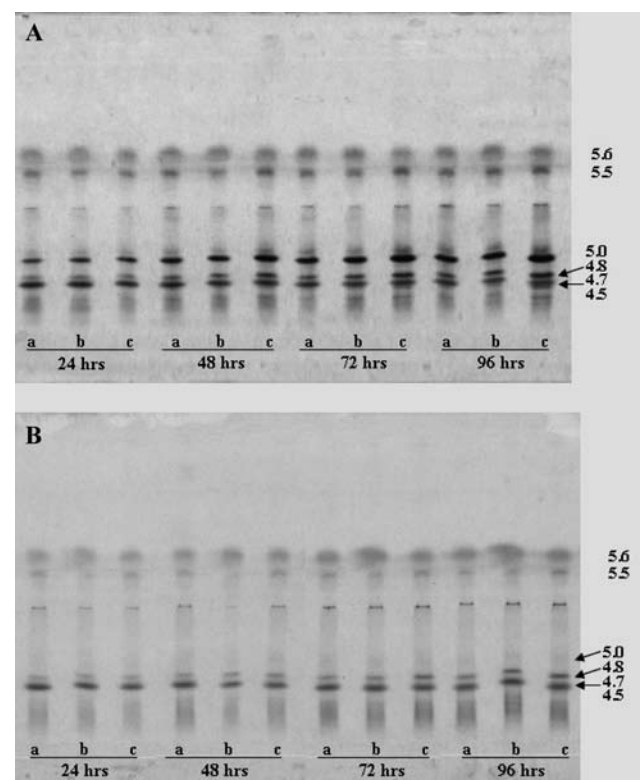
Figure 4. Effect of Cd on anionic peroxidase isozyme activity in rice roots. Lane a (control), lane b (150 μM CdCl$_2$ treatment), lane c (300 μM CdCl$_2$ treatment). Isoelectric points of peroxidases are marked on the right. (A) for Tainung 67 and (B) for Taichung Native 1.

compared to the control (Figure 3A). Cd treatment slightly enhanced the POX activity in Taichung Native 1. As the incubation time was prolonged to 96 hrs, the activity of POX in Cd-treated Tainung 67 was about 1.1 fold compared to the control (Figure 3B). The POX activity in the control did not significantly change during the course of incubation. The changes in cationic and anionic POXs is
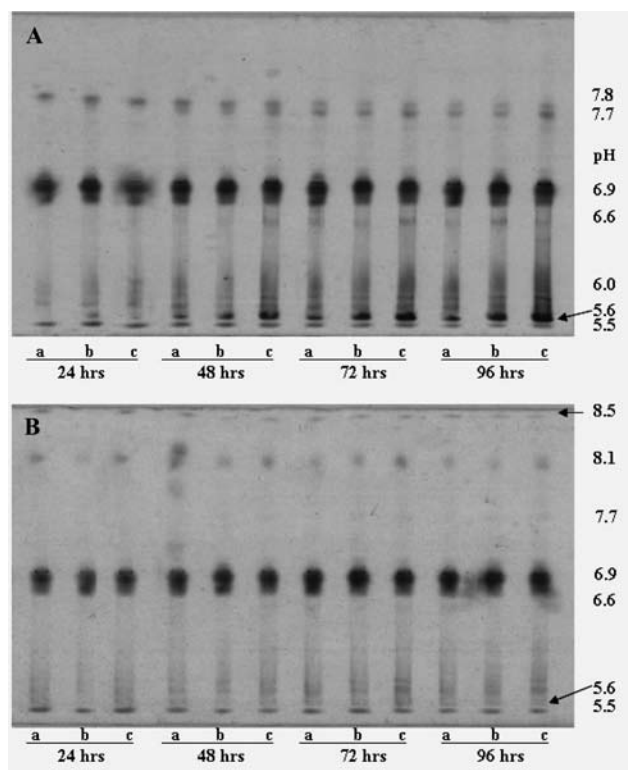
Figure 5. Effect of Cd on cationic peroxidase isozyme activity in rice roots. Lane a (control), lane b (150 μM CdCl$_2$ treatment), lane c (300 μM CdCl$_2$ treatment). Isoelectric points of peroxidases are marked on the right. (A) for Tainung 67 and (B) for Taichung Native 1.

shown in Figures 4 and 5. Rice roots were homogenized, extracted and subjected to polyacrylamide gels for isoelectric-focusing electrophoresis. In Tainung 67, there were more than fifteen POX isozymes identified by isoelectric points in the extracts of Cd-treated roots. In the first 24 hrs of Cd treatment, the cationic and anionic POX isozyme activities did not significantly change in Tainung 67 roots. When the incubation time was prolonged to 96 hrs, the POX activity (pI 4.5, 4.8, 5, 5.5, 5.6, 6.0, 6.6, 7.7, 7.8) in Cd-treated roots increased significantly compared to the controls (Figures 4A and 5A). In Taichung Native 1, there were more than fourteen POX isozymes identified by isoelectric points in the extracts of Cd-treated roots (Figure 4B and 5B). Only anionic pI 4.8 POX and pI 5.6 POX activity were significantly enhanced compared to the control in roots (Figure 4B), otherwise, some POX isozymes only appeared in one of two cultivars. For example, cationic (pI 7.8) POX only showed their activity in Tainung 67. Cationic (pI 8.5) POX, however, only appeared in Taichung Native 1 (Figures 4 and 5).

Effect of cadmium on peroxidase gene transcription

The transcription levels of genes encoding the anionic pI 4.5 POX and the cationic pI 8.6 POX in roots of the two cultivars were examined using reverse transcriptase polymerase chain reaction (RT-PCR) (Figure 6A and 6B). We perfomed a linear calibration in the templates and the PCR products of pI 4.5, pI 8.6 and actin. In Tainung 67,
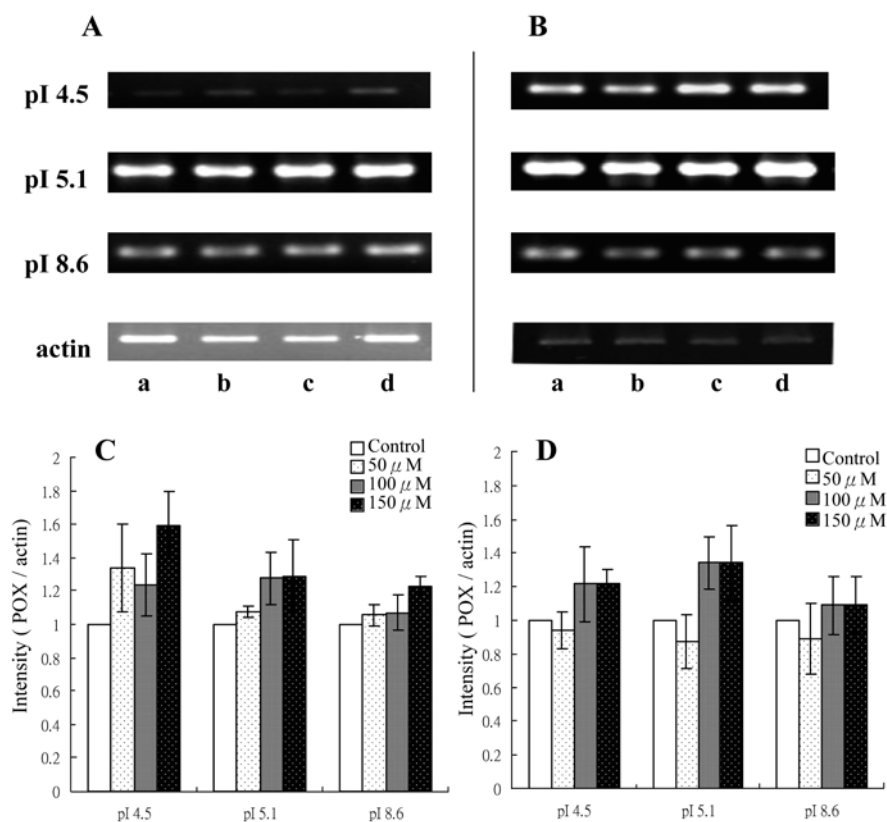
Figure 6. Effect of Cd (72 hr treatment) on the transcript accumulation of the peroxidase isozymes in the rice roots. Lane a (control), lane b (50 μM CdCl$_2$ treatment), lane c (100 μM CdCl$_2$ treatment) and lane d (150 μM CdCl$_2$ treatment). Actin is used as an internal control. (A) for Tainung 67 and (B) for Taichung Native 1. Quantitative data of peroxidase transcription level were converted from (A) to get (C) and from (B) to get (D).
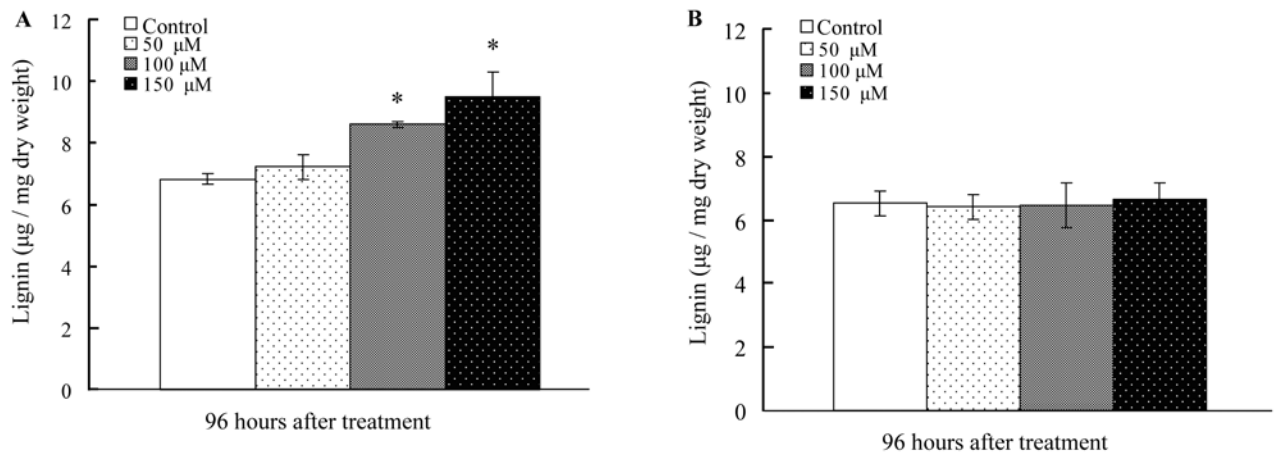
Figure 7. Effect of Cd on lignin contents 96 h after treatment in rice roots. Means significantly higher that the experiment control (Dunnett's multiple comparison test, $P \leqq 0.05$) are marked *. Values are the means ± SE of three replicates. (A) for Tainung 67 and (B) for Taichung Native 1.

Table 1. Main cis-acting elements present in 1,225 bps upstream rice (Japonica type) pI 4.5 POX gene transcription start site.

Motif name	Sequence	Function	Position
AGCBOXNPGLB/ GCC box	AGCCGCC	Elicitation, wounding and pathogen	-100(+)
ARFAT	TGTCTC	Auxin response factor (ARF); AuxRE	-516(-)
Box 4	ATTAAT	Part of a conserved DNA module involved in light responsiveness	-1221(+), -562(+), -903(+)
CAREOSREP1	CAACTC	GARE; gibberellin	-812(-), -512(-)
CAT-box	GCCACT	Cis-acting regulatory element related to meristem expression	-420(+)
CCAAT-box	CAACGG	MYBHv1 binding site	-425(-)
CCAATBOX1	CCAAT	Heat shock element (HSE)	-496(+), -528(-)
CURECORECR	GTAC	Copper-response element (CuRE)	-1151(+/-), -778(+/-), -768(+/-), -754(+/-), -648(+/-),
GARE20SREP1	TAACGTA	GA-responsive element (GARE)	-328(-)
G-Box/ G-box	CACGTT	Cis-acting element involved in light responsiveness	-1050(+), -979(-)
GT1-motif	GGTTAA	Light responsive element	-38(-)
I-BOX	GATAAG	Part of light responsive element	-962(-), -388(-), -383(-)
I-box	GATAAGATA	Part of light responsive element	-386(-)
LTRECOREATCOR15	CCGAC	Cis-acting element involved in low-temperature responsiveness	-83(+)
MBS	CGGTCA TAACTG	MYB Binding site, Involved in drought-inducibility	-716(+), -411(+)
Sp 1	GGGCGG	Involved in light-responsiveness	-98(-)
MYCATRD22	CACATG	ABA-induction	-955(+), -1170(-)
TC-rich repeats	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness	-17(-)
TCA-element	CAGAAAAGGA TCAGAAGAGG CCATCTTTTT	Involved in salicylic acid responsiveness	-746(+), -481(-), -643(-), -270(-)

The data have been obtained with PlantCare and PLACE. Indicated positions are relative to the Transcriptional start site. Strands are indicated as: +, forward; -, reverse.
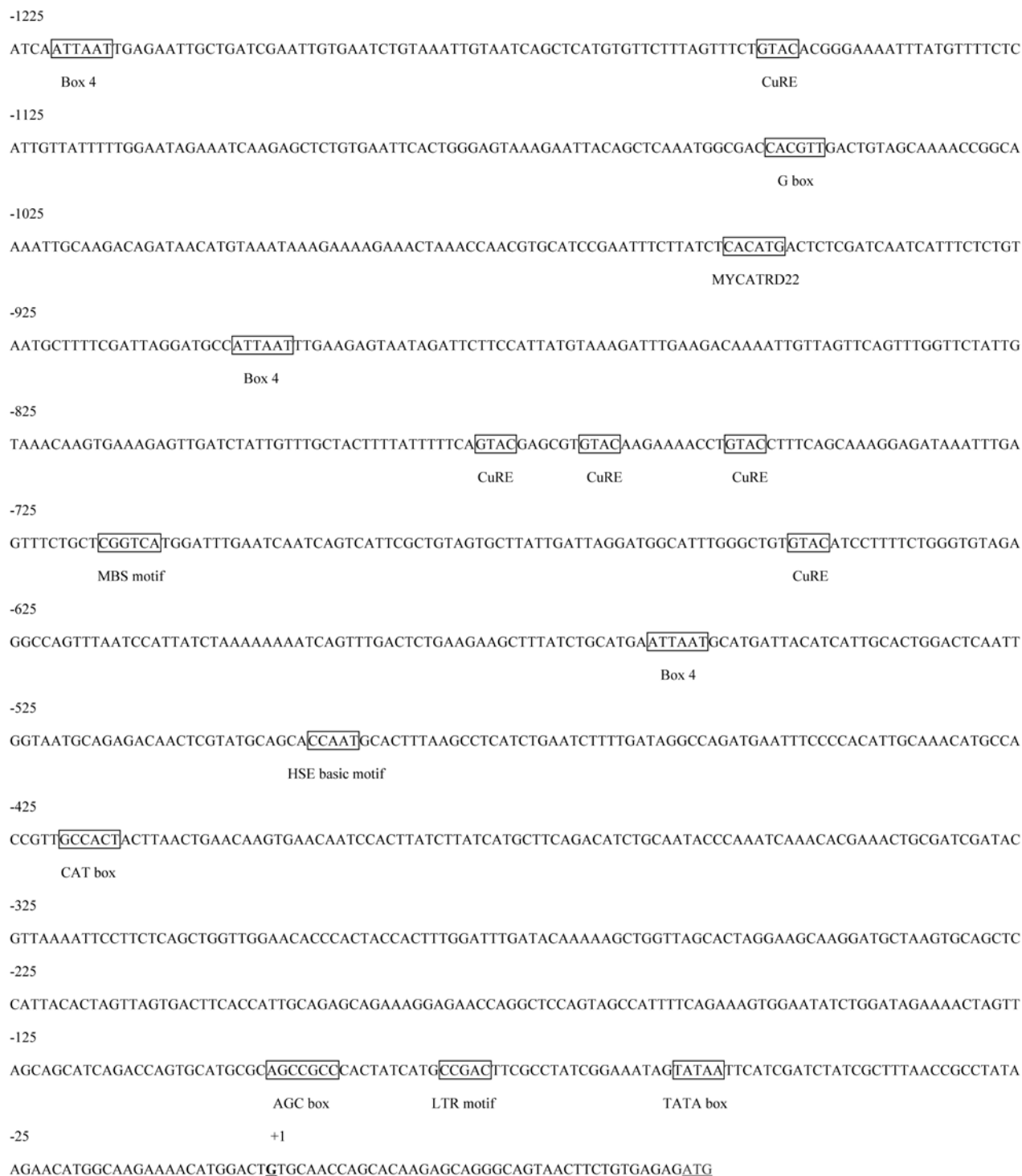
transcript levels in 150 μM of Cd-treated tissues were about 1.8-fold for pI 4.5 POX and about 1.2-fold for pI 8.6 POX compared with the control (Figure 6C and 6D). In Taichung Native 1, the transcription levels of pI 4.5 and pI 8.6 did not significantly change in Cd-treated tissues compared to the control. The transcription level of pI 5.1 POX did not significantly change in either Cd-treated cultivar

tissues compared to the control. All PCR products were sequenced and the POX sequences of Tainung 67 and Taichung Native 1 were almost the same (similarity > 97%).

Effect of cadmium on lignin synthesis

As shown in Figure 7A, the lignin contents in Tainung 67 significantly increased, to about 140%, in 150 μM of

-1225
ATCA ATTAAT TGAGAATTGCTGATCGAATTGTGAATCTGTAAATTGTAATCAGCTCATGTGTTCTTTAGTTTCT GTAC ACGGGAAAATTTATGTTTTCTC
          Box 4   CuRE

-1125
ATTGTTATTTTTGGAATAGAAATCAAGAGCTCTGTGAATTCACTGGGAGTAAAGAATTACAGCTCAAATGGCGAC CACGTT GACTGTAGCAAAACCGGCA
                                                                                                                   G box

-1025
AAATTGCAAGACAGATAACATGTAAATAAAGAAAAGAAACTAAACCAACGTGCATCCGAATTTCTTATCT CACATG ACTCTCGATCAATCATTTCTCTGT
                                                                                                        MYCATRD22

-925
AATGCTTTTCGATTAGGATGCC ATTAAT TTGAAGAGTAATAGATTCTTCCATTATGTAAAGATTTGAAGACAAAATTGTTAGTTCAGTTTGGTTCTATTG
                                       Box 4

-825
TAAACAAGTGAAAGAGTTGATCTATTGTTTGCTACTTTTATTTTTCA GTAC GAGCGT GTAC AAGAAAACCT GTAC CTTTCAGCAAAGGAGATAAATTTGA
                                                                                   CuRE   CuRE   CuRE

-725
GTTTCTGCT CGGTCA TGGATTTGAATCAATCAGTCATTCGCTGTAGTGCTTATTGATTAGGATGGCATTTGGGCTGT GTAC ATCCTTTTCTGGGTGTAGA
                  MBS motif   CuRE

-625
GGCCAGTTTAATCCATTATCTAAAAAAAATCAGTTTGACTCTGAAGAAGCTTTATCTGCATGA ATTAAT GCATGATTACATCATTGCACTGGACTCAATT
                                                                                            Box 4

-525
GGTAATGCAGAGACAACTCGTATGCAGCA CCAAT GCACTTTAAGCCTCATCTGAATCTTTTGATAGGCCAGATGAATTTCCCCACATTGCAAACATGCCA
                                       HSE basic motif

-425
CCGTT GCCACT ACTTAACTGAACAAGTGAACAATCCACTTATCTTATCATGCTTCAGACATCTGCAATACCCAAATCAAACACGAAACTGCGATCGATAC
          CAT box

-325
GTTAAAATTCCTTCTCAGCTGGTTGGAACACCCACTACCACTTTGGATTTGATACAAAAAGCTGGTTAGCACTAGGAAGCAAGGATGCTAAGTGCAGCTC

-225
CATTACACTAGTTAGTGACTTCACCATTGCAGAGCAGAAAGGAGAACCAGGCTCCAGTAGCCATTTTCAGAAAGTGGAATATCTGGATAGAAAACTAGTT

-125
AGCAGCATCAGACCAGTGCATGCGC AGCCGCC CACTATCATG CCGAC TTCGCCTATCGGAAATAG TATAA TTCATCGATCTATCGCTTTAACCGCCTATA
                                       AGC box   LTR motif   TATA box

-25   +1
AGAACATGGCAAGAAAACATGGACT**G**TGCAACCAGCACAAGAGCAGGGCAGTAACTTCTGTGAGAG_ATG_

Figure 8. Nucleotide sequence of rice pI 4.5 POX gene (Japonica type) promoter region (-1225/+44). The bold nucleotide represents the transcription start residue and is numbered as +1. The putative TATA-box is underlined and labeled. Nucleotides are numbered from the transcription start site (TSS) residue. Significant putative cis-acting elements are underlined and labeled.

Cd-treated tissues compared to the control. The lignin contents in Cd-treated tissues of Taichung Native 1did not significantly increase (Figure 7B).

Promoter sequence analysis

We performed genome walking using PCR and analy-

sis of the upstream region of pI 4.5 POX genes in rice (Japonica and Indica type). In this study, we cloned the upstream genomic DNA region of rice pI 4.5 POX gene from rice (Japonica and Indica) total genomic DNA. We sequenced about 1.3 kb of the POX promoter regions. The putative TSS (transcription start site) mapped to the A resi-

-1225

ATCA ATTAAT TGAGAATTGTTGATCGAATTGTGAACCTGTAAATTGTAATCAGCTCATGTGTTCTTTAGTTTCT GTAC ACGGGAAAATTTATGTTTTCTC
         Box 4   CuRE

-1125

ATTGTTATTTTTGGAATAGAAATCAAGAGCTCTGTGAATTCACTGGGAGTAAAGAATTACAGCTCAAATGGCGAC CACGTT GACTGTAGCAAAACCGGCA
                                                               G box

-1025

AAATTGCAAGACAGATAACGTGTAAATAAAGAAAAGAAACTAAACCAACGTGCATCCGAATTTCTTATCT CACATG ACTCTCGATCAATCATTTCTCTGT
                                                           MYCATRD22

-925

AATGCTTTTCGATTAGGATGCC ATTAAT TTGAAGAGTAATAGATTCTTCCATTATGTAAAGATTTGAAGACAAAATTGTTAGTTCAGTTTGGTTCTATTG
                        Box 4

-825

TAAACAAGTGAAAGAG TTGACC TATTGTTTGCTACTTTTATTTTTCA GTAC GAGCGT GTAC AAGAAAACCT GTAC CTTTCAGCAAAGGAGATAAATTTGA
                 Box-W1/ W box   CuRE   CuRE   CuRE

-725

GTTTCTGCT CGGTCA TGGATTTGAATCAATCAGTCATTCGCTGTAGTGCTTATTGATTAGGATGGCATTTGGGCTGTGAACATCCTTTTTTGGGTGTAGA
            MBS motif

-625

GGCCAGTTTAATCCATTATCTAAAAAAAAATCAGTTTGACTCTGAAGAAGCTTTATCTGCATGA ATTAAT GCATGATTACATCATTGCACTGGACTCAATT
                                                         Box 4

-525

GGTAATGCAGAGACAACTCGTATGCAGCA CCAAT GCACTTTAAGCCTCATCTGAATCTTTTGATAGGCCAGATGAATTTCCCCACATTGCAAACATGCCA
                          HSE basic motif

-425

CCGTT GCCACT ACTTAACTGAACAAGTGAACAATCCACTTATCTTATCATGCTTCAGACATCTGCAATACCCAAATCAAACACGAAACTGCGATCGATAC
            CAT box

-325

GTTAAAATTCCTTCTCAGCTGGTTGGAACACCCACTACCACTTTGGATTTGATACAAAAAGCTGGTTAGCACTAGGAAGCAAGCATGCTAAGTGCAGCTC

-225

CATTACACTAGTTAGTGACTTCACCATTGCAGAGCAGAAAGGAGAACCAGGCTCCAGTAGCCATTTTCAGAAAGTGGAATATCTGGATAGAAAACTAGT

-125

AGCAGCATCAGACCAGTGCATGCGC AGCCGCC CACTATCATG CCGAC TTCGCCTATCGGAAATAG TATAA TTCATCGATCTATCGCTTTAACCGCCTATA
                         AGC box   LTR motif   TATA box

-25   +1

AGAACATGGCAAGAAAACATGGACT**G**TGCAACCAGCACAAGAGCAGGGCAGTAACTTCTGTGAGAGATG

Figure 9. Nucleotide sequence of rice pI 4.5 POX gene (Indica type) promoter region (-1225/+44). The bold nucleotide represents the transcription start residue and is numbered as +1. The putative TATA-box is underlined and labeled. Nucleotides are numbered from the transcription start site (TSS) residue. Significant putative cis-acting elements are underlined and labeled.

due location at position -41, with respect to the translation start codon (ATG). About 1.3 kb upstream genomic DNA region of both two rice pI 4.5 POX genes cDNA corresponding to the ATG start codon were cloned from the rice (Indica and Japonica) genomic DNA. Thus, the identified 1.3 kb upstream genomic DNA regions contain a 1,269-bp putative pI 4.5 POX gene promoter sequence. The promoter sequence of the Indica type rice pI 4.5 POX gene was almost the same as that of the Japonica type. They differed by only six nucleotides (Figures 8 and 9). Analysis of both rice pI 4.5 POX gene promoters using the PLACE (Higo et al., 1999) and PlantCARE (Lescot et al., 2002) software (http://www.dna.affrc.go.jp/PLACE/ and http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) showed that a number of potential cis-acting elements responded to environmental signals (Tables 1 and 2).

The promoter regions of both cultured rice varieties contained three Box 4 (-1221/1216, -903/-898 and -562/-557), two putative G Boxes/G boxes were present in-1050 /-1046 and -979/-974 (in reverse orientation, -), one GT1-motif was located in -38/-33 (in reverse orientation, -), three putative I BOX (-962/-957, -388/-383 and -383/-378, all in reverse orientation), one putative I-box (-386/-378, in reverse orientation) and one SP1 (-98/-93, in reverse orientation). These motifs and elements were thought to be involved in light response. The ARFAT (5´-TGTCTC-3´) for auxin response factor (ARF) was present in (-516/-511, in reverse orientation). Two CAREOSREP1 (5´-CAACTC-3´) and one GARE20SREP1 (5´-TAACGTA-3´) of GA response element (GARE) were present at position (-812/-807 and -512/-507, both in reverse orientation), and (-328/-322, in reverse orientation), respectively. Two abscisic acid response elements (ABA-induction) MYCATRD22 (5´-CACATG-3´) were found at position (-955/-950 and -1170/-1175 (in reverse orientation)). Two putative basic motifs of (5´-CCAAT-3´) of heat shock ele-

Table 2. Main cis-acting elements present in 1,225 bps upstream rice (Indica type) pI 4.5 POX gene transcription start site.

Motif name	Sequence	Function	Position
AGCBOXNPGLB/ GCC box	AGCCGCC	Elicitation, wounding and pathogen	-100(+)
ARFAT	TGTCTC	Auxin response factor (ARF); AuxRE	-516(-)
Box 4	ATTAAT	Part of module involved in light responsiveness	-1221(+), -562(+), -903(+)
CAREOSREP1	CAACTC	GARE; gibberellin	-812(-), -512(-)
Box-W1/W box	TTGACC	Fungal elicitor responsive element; wounding	-809(+)
CAT-box	GCCACT	Cis-acting regulatory element related to meristem expression	-420(+)
CCAAT-box	CAACGG	MYBHv1 binding site	-425(-)
CCAATBOX1	CCAAT	Heat shock element (HSE)	-496(+), -528(-)
CURECORECR	GTAC	Copper-response element (CuRE)	-1151(+/-),-778(+/-), -768(+/-), -754(+/-),
GARE20SREP1	TAACGTA	GA-responsive element (GARE)	-328(-)
G-Box/ G-box	CACGTT	Cis-acting element involved in light responsiveness	-1050(+), -979(-), -1009(-)
GT1-motif	GGTTAA	Light responsive element	-38(-)
I-BOX	GATAAG	Part of light responsive element	-962(-), -388(-), -383(-)
I-box	GATAAGATA	Part of light responsive element	-386(-)
LTRECOREATCOR15	CCGAC	Cis-acting element involved in low-temperature responsiveness	-83(+)
MBS	CGGTCA TAACTG	MYB Binding site, Involved in drought-inducibility	-716(+), -411(+)
Sp1	GGGCGG	Involved in light-responsiveness	-98(-)
MYCATRD22	CACATG	ABA-induction	-955(+), -1170(-)
TC-rich repeats	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness	-17(-)
TCA-element	CAGAAAAGGA TCAGAAGAGG CCATCTTTTT	Involved in saliciylic acid responsiveness	-746(+), -481(-), -643(-), -270(-)

The data have been obtained with PlantCare and PLACE. Indicated positions are relative to the Transcriptional start site. Strands are indicated as: +, forward; -, reverse.

ments (HSE) were present in (-496/-492 and -528/-524 (in reverse orientation)). Two MBS motifs (5´-CGGTCA/ TAACTG-3´) which were involved in drought induction to be found at position (-716/-711 and -411/-406). One AGCBOXNPGLB/GCC box (5´-AGCCGCC-3´) was present in -100/-94. One TC-rich repeats motif (5´-GTTTTCTTAC-3´) in -17/-26 (in reverse orientation), and four TCA-elements (5´-CAGAAAAGGA/TCAGAA-GAGG/ CCATCTTTTT-3´), one was present in -746/-737 (in forward orientation), the others in -643, -481 and -270 were all in reverse orientation. These motifs and/or elements thought to be involved in elicitation, wounding, pathogen, defense and stress response were also found. The significant difference in promoter regions between the Japonica and Indica type was that the Japonica type owned ten conserved cores of the CURECORECR sequence (5´-GTAC-3´), a copper-response element (CuRE), involved in heavy metals response. The Indica type had only eight CuRE, their motifs were located at positions: -1151/-1148 (+/-), -778/-775 (+/-), -768/-765 (+/-), -754/-751 (+/-) and -648/-645(+/-) (in forward orientation /+ and reverse orientation /-).

DISCUSSION

Cadmium decreased the growth of rice expressed as fresh weight. Root growth was inhibited more severely in Taichung Native 1 than in Tainung 67 (Figure 1). Our data confirm the results reported by Hsu and Kao (2003) indicating that the Japonica cultivar (*Oryza sativa* L. cv. Tainung 67) is more tolerant to Cd than is the Indica cultivar (*Oryza sativa* L. cv. Taichung Native 1). For the 24-hr Cd treatment, the H_2O_2 accumulation significantly increased in the Taichung Native 1 root (Figure 2B). Plant cells demonstrated an oxidative burst or the rapid production of ROS with a subsequent cascade of plant responses to biotic and abiotic stress (Hippeli et al., 1999; Low and Merida, 1996). Hydrogen peroxide and superoxide release is one of the early events induced by pathogen attack on plant cells (Murphy and Auh, 1996) and similarly by hyposmotic and mechanical stress (Yahraus et al., 1995). Cd can promote the generation of H_2O_2 (Cho and Seo, 2005; Hsu and Kao, 2007). The level of ROS produced from oxidative stress regulates the induction of stress-activated 40-kDa MBP (myelin basic protein) kinase signal transduction pathways by Cd (Yeh et al., 2007). Through the regulatory MAPKs pathways, POXs and other antioxidative enzymes may be activated to remove H_2O_2 or other species of ROS. In our studies, the Tainung 67 POX activity increased significantly in Cd-treated tissues (Figure 3). However, the decrease in H_2O_2 level was accompanied by an enhancement of POX activity in Cd-treated tissues (Figures 2A and 3A). In Taichung Native 1, the accumulation of H_2O_2 in Cd-treated tissues could be due to the lower amount of POX enhancement induced by Cd (Figures 2B and 3B).

In our investigation, the isozyme pattern of POXs in isoelectric focusing gels showed different increases in the POX activity of the two cultivars (Figures 4 and 5). Anion-ic (pI 5.6, pI 5) POXs and cationic (pI 7.7, pI 7.8) POXs were significantly enhanced by Cd in Tainung 67. On the contrary, anionic (pI 4.8) POXs and cationic (pI 8.1, pI 8.5) POXs were specific enhanced by Cd in Taichung Native 1. These different POX isozyme patterns in the two cultivars may be related to their tolerance of Cd. In Tainung 67, the POXs synthesized more lignin than did Taichung Native 1 (Figure 7A). The activation of the lignin synthesis was a typical defense against both biotic and abiotic environmental stresses (Li et al., 2001; Wang and Liu, 1999; Yang et al., 2007). In our studies, the discussion focused on the final phase-the formation of the lignin macromolecule. The last step in lignin biosynthesis involves the oxidation of monolignols and is catalyzed by peroxidases (POXs) and/or laccases. POXs can generate phenoxy radicals from monolignols using hydrogen peroxide as the oxidant. The phenoxy radicals are then coupled to generate lignin polymers (Li et al., 2003). The soluble class III plant POX participate directly or indirectly in a broad range of physiological processes, such as lignin and suberin formation, cross-linking of cell wall components, and synthesis of phytoalexins at the infection site associated with limited pathogen development (Almagro et al., 2009). However, despite the knowledge about the exact location of a POX, it is often difficult to reveal the functions of each single enzyme due to the large number of similar isozymes and their broad substrate specificity (Mika et al., 2004). Both Quiroga et al. (2000) and Chen et al. (2002) indicated that both cationic and anionic POXs were involved in lignin synthesis in tomato and radish plants. Our results suggested that the increase in the cationic (pI 7.8 and pI 7.7) and anionic (pI 5.6, pI 4.8, pI 4.5) POXs in Tainung 67 may be responsible for the late stage lignin polymerization in Cd-treated roots (Figures 3A and 7A). The increased activity in cationic (pI 8.6) and anionic (pI 4.5) POXs correlated with the increase in POX transcripts (Figure 6A), and therefore was mostly due to the *de novo* synthesis of the cationic (pI 8.6) and anionic (pI 4.5) POXs in Cd-treated roots of Tainung 67. In our studies, the value of pI was predictive so that it was possible to estimate different pI from amino acid sequence and the Rf value of isoelectric focusing gels. The decimal fraction of pI may have been incorrect. The transcription level of pI 5.1 POX may be pI 5.0 POX, shown in isoelectric focusing gels, and the same in Tainung 67 and Taichung Native 1. On the other hand, the pI 5.1 POX was significantly active in Tainung 67 but not in Taichung Native 1 as shown in isoelectric focusing gels. Pathirana et al. (2005) reported that removing the N-linked glycan structure from peanut peroxidase can influence protein stability and activity. The difference in pI 5.1 POX activity might not be due to the different transcription level, but from the different translation or glycosylation level. In addition, the POX sequences of Tainung 67 and Taichung Native 1 were almost the same (GenBank accession no. D14481 and CT 831866), indicating that the POX gene of corresponding pI in different rice cultivars didn't have too much divergence.

It was suggested that the different regulation on the pro-

moter region in POX genes might occur in two Cd-treated rice cultivars. For promoter analysis between Japonica type and Indica type, the significant difference is that Japonica owned ten conserved cores of the CURECORE-CR sequence (5´-GTAC-3´), a copper-response element (CuRE), involved in heavy metals response (Quinn and Merchant, 1995; Kropat et al., 2005; Qi et al., 2007). Indica had only eight CuRE. Until now, no cadmium-response element has been found in the data base. Here, we assume that the copper-response element (CuRE), the only heavy metal response element reported so far (Qi et al., 2007), can recognize Cd stimuli. Therefore, the tolerant cultivar Tainung 67 (Japonica type) might receive more signals from Cd treatment, synthesize more POX, then produce more lignin to deal with Cd stress.

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鎘對兩個不同栽培品系水稻根部之過氧化同功異構酶活性的影響

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　　鎘處理之水稻（*Oryza sativa* L. cv. Taichung Native 1 and *O. sativa* L. cv. Tainung 67）幼苗根的生長受到抑制，根組織內過氧化酶活性亦有增加。台農 67 號（Japonica type）之水稻根部鎘處理後可能藉調控啓動子區域，影響過氧化酶基因表現或是藉由將過氧化酶糖基化，引發過氧化酶活性的增加，加強根部組織清除由鎘引起之過氧化氫的能力，同時合成較多木質素。台中在來一號（Indica type）水稻品系則在鎘處理後，過氧化酶增加量少，造成較多的過氧化氫之累積，並且生成的木質素較少，故台中在來一號（Indica type）水稻對於鎘較敏感。因此，台農 67 號（Japonica type）之水稻對鎘耐受性較台中在來一號（Indica type）佳。由過氧化酶基因啓動子區域之分析，台農 67 號（Japonica type）之水稻根部細胞內過氧化酶基因含有十個重金屬反應元素（CURE），而台中在來一號（Indica type）則含八個重金屬反應元素（CURE）。我們推測台農 67 號（Japonica type）之水稻可能較容易接受重金屬鎘之刺激的訊息，進而調控更多的過氧化酶之合成，進而應付鎘之衝擊。

關鍵詞：鎘；過氧化氫；木質素；水稻；過氧化酶；氧化逆境；啟動子。