Antioxidant Enzyme Activity and Gene Expression in Response to Lead Stress in Perennial Ryegrass

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ABSTRACT. Lead pollution is an important issue in the world. Perennial ryegrass (*Lolium perenne*), as one of the widely used turfgrass and forage species, has a potential for bioremediation. The objective of this study was to investigate how antioxidant enzymes and their gene transcripts respond to Pb stress in perennial ryegrass. Ryegrass seedlings were subjected to 0, 0.5, and 3.2 mM of Pb(NO₃)₂ for 7 days in a hydroponic system maintained in a greenhouse. Both root and shoot growths were inhibited by Pb compared with the control. However, contents of chlorophyll (Chl) *a* and total Chl were unaffected by Pb treatment. Results from this study showed a substantial increase of malondialdehyde (MDA) content in leaf tissues when perennial ryegrass was exposed to Pb at 3.2 mM. The MDA content from plants in the 0.5 mM Pb treatment was lower than the control, indicating that an effective defense mechanism existed. Circumstantial evidence came also from the content of soluble protein in 0.5 mM Pb treatment, which was not different from the control. Furthermore, the activity of catalase (CAT) increased at 0.5 mM Pb compared with the control, indicating that CAT might play an important role in scavenging reactive oxygen species (ROS). The expression profiles of eight genes encoding antioxidative enzymes were upregulated within 24 hours of Pb treatment. In conclusion, antioxidant enzymes responded to Pb at an early stage of exposure and their gene expression profiles provided more details in time courses of the activation of those systems.

Heavy metal pollution is a worldwide ecological problem because of its impact on plants and animals and ultimately on the health of human beings via the food chain. Lead is one of the most abundant and widely distributed heavy metals because of its various human activities, such as mining and smelting of lead ores, paint manufacturing, gasoline production, lead linings, and so on. As a result, Pb is readily enriched into ecosystems. As a nonessential element for plants, Pb impedes plant growth by affecting physiological process and metabolic pathways such as photosynthesis and nutrient acquisition (Godbold and Kettner, 1991; Kastori et al., 1992; Rashid and Popovic, 1990; Verma and Dubey, 2003).

Like many other toxic metals, Pb induces production and accumulation of free radicals and ROS in plant tissues and causes oxidative stress in plants. Excessive ROS can result in irreversible oxidation of lipids, proteins, chloroplastic pigments, and nucleic acids (Foyer et al., 1994; Malecka et al., 2001; Reddy et al., 2005; Schutzendubel and Polle, 2002; Verma and Dubey, 2003). Plants have developed both enzymatic and nonenzymatic defense systems to scavenge and detoxify ROS (Mittler, 2002).

For example, enhanced activities of superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR), and other antioxidative enzymes were detected in both horse gram (*Macrotyloma uniflorum*) and bengal gram (*Cicer arietinum*) subjected to Pb, and the levels of enzymes were dependent on the concentration of Pb (Reddy et al., 2005). Increased levels of POD and GR, in response to Pb, were also reported in *Arabidopsis thaliana* and maize (*Zea mays*) calli (Verma and Dubey, 2003; Zacchini et al., 2003). Elevated levels of antioxidant enzymes including SOD, glutathione peroxidase (GPX), ascorbate peroxidase (APX), GR, and CAT were found in coontail (*Ceratophyllum demersum*) when exposed to Pb (Mishra et al., 2006). On the contrary, there were also reports indicating decreased or unchanged activities of SOD and POD in plants subjected to Pb or other heavy metals (Islam et al., 2008; Liu et al., 2008; Sun et al., 2009). The discrepancies regarding the responses of SOD and POD to Pb stress may attribute to the different metal concentrations and/or plant developmental stages at which the investigations were conducted.

Plant responses to Pb have been studied at gene levels using DNA microarray technique, which overcame some of the disadvantages related to enzyme analysis (Magrini et al., 2008). Upregulated expressions were observed for type-2 metallothionein, aminocyclopropane carboxylic acid synthase/oxidase, and other genes induced by abiotic stress in rattlebox (*Sesbania drummondii*) subjected to Pb treatment (Srivastava et al., 2007). Using quantitative real-time polymerase chain reaction (Q-PCR)

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technique, Brunet et al. (2009) detected the increased expression of GR, APX, and glutathione S-transferase gene in the roots of grass pea (*Lathyrus sativus*) exposed to Pb for 96 h in a hydroponic system. Kovalchuk et al. (2005) analyzed the global genome expression of transgenic *A. thaliana* plants chronically exposed to Pb using DNA microarray technique and revealed 19 upregulated genes and 76 downregulated genes. Liu et al. (2009) reported that more than 1310 genes were affected in the expression profiles of *A. thaliana* in response to different concentrations of Pb (1, 10, and 100 μM) during the early stage of treatment, and most of the upregulated genes were also found under other stress. The genetic techniques provided effective tools to investigate plant responses to heavy metals and can be supplementary to those used at enzyme levels.

Perennial ryegrass is one of the most widely used species for turf and forage in temperate regions (Bidar et al., 2007; Wilkins and Humphreys, 2003). Perennial ryegrass is capable of accumulating heavy metals (Pichtel and Salt, 1998) and was defined as a facultative metallophyte by Smith and Bradshaw (1979). Arienzo et al. (2004) reported that a healthy vegetation of ryegrass could be established on metal-polluted soils. Carlson and Rolfe (1979) found that the growth of perennial ryegrass did not begin to decrease until Pb treatment reached 1000 $\mu\text{g}\cdot\text{g}^{-1}$. Little is known about the mechanisms driving the responses in perennial ryegrass to Pb. Understanding of the physiological responses and transcription profiles of the genes coding for antioxidant enzymes in perennial ryegrass under Pb stress can provide valuable information and assessment tools to breeders.

The objectives of this study were to investigate the physiological responses of perennial ryegrass seedlings to a wide range of Pb concentrations concerning both antioxidant enzymes and transcription profiles of the genes coding for those enzymes.

Material and Methods

PLANT MATERIALS. Perennial ryegrass ‘Quickstart II’ was seeded to plastic pots (7.5 cm diameter and 9.0 cm deep) with sand medium in July 2010. The pots were watered twice daily and fertilized twice weekly with half-strength Hoagland’s solution (Hoagland and Arnon, 1950) until germination. Thereafter, the plants were watered daily to pot capacity and clipped at 8 cm above the soil surface. The plants were maintained in a greenhouse with a 14-h light period from natural light and temperatures of 20/16 °C (day/night).

TREATMENTS. At about seven-leaf stage, 40 d after germination, the plants were transplanted into 300-mL conical flasks containing 290 mL half-strength Hoagland’s solutions, after the roots were washed free of sand using distilled water. The Hoagland nutrition contained also 0.1 μM of CaO_2 for providing plants additional oxygen. The flasks were wrapped with aluminum foil to block light. Ten plants were maintained in each flask. After 1 week of adaptation in the hydroponics system, the plants were transferred to new flasks and subjected to Pb treatments by adding 0, 0.5, and 3.2 $\text{mmol}\cdot\text{L}^{-1}$ $\text{Pb}(\text{NO}_3)_2$ to the half-strength Hoagland’s solution containing CaO_2 . During the period of experiment, the nutrient solution was supplemented every 2 d. The experiment was arranged in a randomized complete block design with five replicates and each flask as one treatment unit. Two identical experiments were established for the measurements of enzymes and gene expression analysis, respectively.

GROWTH AND ENZYME. Shoot and root length were measured at 7 d after Pb treatment. Immediately following the measurement of plant growth, leaves were harvested, weighed, and frozen in liquid nitrogen before storing under -70°C for further measurement of Chl content, SOD, CAT, POD, MDA, and soluble protein using the following procedures.

Chlorophyll content in leaves was measured by the method of Hiscox and Israelstam (1979). In summary, 0.1 g of fresh leaves was cut into small pieces and placed into 15-mL tubes containing 10 mL of dimethylsulfoxide (DMSO). The tubes were kept under 25 °C in darkness for 48 h and shaken 15 s every 12 h for thorough mixing of the extraction. Thereafter, 1 mL extraction was transferred into a new tube and 2 mL of DMSO was added for dilution. The diluted samples were mixed and immediately measured for light absorption at 645 and 663 nm. The content of Chl *a* and Chl *b* (milligram per gram fresh weight) was calculated based on the equations developed by Hiscox and Israelstam (1979).

For enzyme extraction, ≈ 0.3 g of leaf samples were homogenized with 5 mL of 50 mM buffer solution (containing 0.7% of $\text{NaH}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$ and 1.64% $\text{Na}_2\text{HPO}_4\cdot 12\text{H}_2\text{O}$, pH 7.8) subjected to grinding with an ice-cooled mortar and pestle and finally centrifuged at 5000 g_n for 25 min at 4 °C. The supernatant was collected for the determination of soluble protein content, SOD, CAT, and POD activity, and MDA content. CAT and POD activities were determined following the method described by Chance and Maehly (1955). SOD activity was measured according to the method used by Chowdhury and Choudhuri (1985) and Zhang and Kirkham (1994). The content of MDA was determined according to the method of Heath and Packer (1968). The total soluble protein content was estimated using bovine serum albumin as a standard (Bradford, 1976).

GENE EXPRESSION OF ANTIOXIDANT ENZYMES. Leaf samples were collected at 2, 4, 6, 24, and 48 h after the initiation of Pb treatment and flash frozen in liquid nitrogen and then stored at -70°C for gene expression analysis.

Total RNA was isolated from ≈ 0.1 g leaves of each treatment using Trizol reagent (Invitrogen, Carlsbad, CA) and then treated with RNase-free DNase I to remove DNA. The quality of RNA was examined by electrophoresis in 1.5% agarose gel. Concentration of RNA was determined using a spectrophotometer and then diluted to 400 $\text{ng}\cdot\mu\text{L}^{-1}$ using RNase free water; 2 μg RNA was reversely transcribed with oligo (dT) primer using first strand cDNA synthesis kit (Fermentas Canada, Burlington, ON, Canada) following the manufacturer’s instructions. The resultant cDNA was then diluted 10 times and kept at -20°C for Q-PCR.

Primers of different genes were synthesized based on the previous reports for use in Q-PCR (Table 1). The specification of each pair of primers was confirmed by randomly sequencing PCR products and further consolidated by the melting curve analysis using Q-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid. Only primers with amplification efficiency above 95% were used in this study. The Q-PCR was conducted with SYBR Green I (Sigma-Aldrich, St. Louis, MO) as a fluorogenic intercalating dye on a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA). Each 20 μL of reaction solution contained 3.5 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2 , 1 unit PCR buffer, 0.5 $\mu\text{M}\cdot\text{L}^{-1}$ of each primer, 10 $\mu\text{M}\cdot\text{L}^{-1}$ dNTPs, 0.5 unit SYBR Green I, 1 unit Taq polymerase (Fermentas Canada), 0.6 μL DMSO (Sigma-Aldrich), and 1.5 μL of template cDNA. A parallel

control containing no templates was run to determine contaminations and formation of primer dimers. An internal control using *eEF1A* (s) gene also was included. The conditions and parameters for PCR were as follows: an initial denaturing step at 95 °C for 3 min, followed by 40 cycles of 94 °C for 15 s, 50–55 °C for 20 s, and 72 °C for 20 s. Melting curve analysis of the amplification products was performed at the end of each PCR reaction to ensure that a single PCR product was detected. The PCR amplification data were analyzed with Option Monitor version 2.03 (MJ Research).

STATISTICS. One-way analysis of variance was conducted using SPSS (version 17.0; IBM Corp., Armonk, NY). Treatment means were separated using Fisher's least significant difference at 0.05 significant levels.

Results

PLANT GROWTH AND STRESS. Shoot and root length of perennial ryegrass decreased with increasing Pb concentrations (Table 2). The concentration of Chl *a* and total Chl was not affected by Pb treatment (Table 2). At 3.2 mM of Pb, perennial ryegrass showed a higher level of Chl *b* compared with the control and 0.5 mM Pb treatment. MDA content increased substantially in the 3.2 mM Pb treatment compared with the control. On the contrary, MDA content in the 0.5 mM Pb treatment was lower than the control (Table 3). Soluble protein content decreased when the concentration of Pb increased to 3.2 mM (Table 3).

ACTIVITY OF ANTIOXIDANT ENZYMES. The plants treated with 0.5 mM Pb exhibited elevated CAT activity compared with the control, whereas plants treated with 3.2 mM of Pb showed suppressed CAT activity compared with the control (Table 4). The levels of POD in 0.5 mM Pb-treated plant did not change compared with the untreated plants. However, there was a higher activity of POD in 3.2 mM Pb treatment than both untreated and 0.5 mM treatment (Table 4). The SOD activity was inhibited in Pb-treated plants compared with the control. Higher concentration of Pb resulted in more inhibition. The SOD activity of plants exposed to 3.2 and 0.5 mM Pb was 74% and 55% lower than the control, respectively (Table 4).

GENE EXPRESSION OF ANTIOXIDANT ENZYMES. The expressions of *MnSOD* were upregulated at 4 through 24 h after the application of 3.2 mM Pb compared with the control. At the 0.5 mM level, its expression was upregulated only at 24 h after treatment. The expression of Chl *Cu/Zn-SOD* gene was upregulated in both 0.5 and 3.2 mM Pb treatments at 2, 4, and 6 h and was then below the levels of control at 24 h after the application of Pb, and the

Table 1. Primer sequences used for quantitative polymerase chain reaction analyses of perennial ryegrass subjected to 0.5 and 3.2 mM Pb in a hydroponic system using half-strength Hoagland's solution.

Gene ^z	Primer ^y	Primer sequence (5'–3')	Product size (base pair)	Reference
<i>GPX</i>	F	GCCGAGTATCCGATTTTGA	195	Byrne et al. (2010)
	R	TCGATACTGAGCGGAGAGGT		
<i>GR</i>	F	TGTGCTGTTTTCTGCATTCC	175	Byrne et al. (2010)
	R	AGTCTCAGCATCAACCACCA		
<i>pAPX</i>	F	CCTGAAAGGTCTGGGTTTGA	173	Foito et al. (2009)
	R	TCCTTGGCATAAAGGTCCAC		
<i>Cyt Cu/ZnSOD</i>	F	GACACMACAAATGGHTGCAT	221	Bian and Jiang (2009)
	R	TCATCBGGATCGGCATGGACAAC		
<i>Chl Cu/ZnSOD</i>	F	ATGGGTGCATATCDAYAG	271	Bian and Jiang (2009)
	R	GCCAGTCTTCCACCAGCAT		
<i>FeSOD</i>	F	TGCACTTGGTGATATTCCACTC	297	Kim et al. (2007)
	R	CGAATCTCAGCATCAGGTATCA		
<i>MnSOD</i>	F	CAGRGBGCCATCAAGTTCAACG	338	Bian and Jiang (2009)
	R	TACTGCAGGTAGTACGCATG		
<i>POD</i>	F	AGGCCAGTGCTHCAMCTTC	220	Bian and Jiang (2009)
	R	TTGGTGTAGTAGGCGTTGTC		
<i>eEF1A</i> (s)	F	CCGTTTTGTCTGAGTTTGGT	113	Lee et al. (2010)
	R	AGCAACTGTAACCGAACATAGC		

^zGPX = glutathione peroxidase, GR = glutathione reductase, pAPX = peroxisomal type ascorbate peroxidase, Cyt Cu/ZnSOD = cytosolic Cu/ZnSOD, Chl Cu/ZnSOD = chloroplastic Cu/ZnSOD, POD = peroxidase.

^yF and R represent forward and reverse.

Table 2. Shoot and root growth of perennial ryegrass subjected to 0.5 and 3.2 mM Pb in a hydroponic system using half-strength Hoagland's solution for 7 d.

Pb treatment (mM)	Shoot length (cm)	Root length (cm)	Chl <i>a</i> (mg·g ⁻¹ FW)	Chl <i>b</i> (mg·g ⁻¹ FW)	Chl (<i>a+b</i>) (mg·g ⁻¹ FW)
0	12.1 a ^z	9.0 a	2.20 a	0.46 b	2.65 a
0.5	10.1 b	6.5 b	2.44 a	0.50 b	2.94 a
3.2	4.6 c	4.4 c	2.32 a	0.60 a	2.92 a

^zMeans within a column followed by the same letters were not significantly different at the *P* = 0.05 level based on Fisher's least significant difference test.

FW = fresh weight.

Table 3. Soluble protein and malondialdehyde (MDA) content in perennial ryegrass subjected to 0.5 and 3.2 mM Pb in a hydroponic system using half-strength Hoagland's solution for 7 d.

Pb treatment (mM)	MDA (nM·g ⁻¹)	Soluble protein (mg·g ⁻¹)
0	7.4 b ^z	5.0 a
0.5	6.1 c	5.6 a
3.2	13.4 a	3.5 b

^zMeans within a column followed by the same letters were not significantly different at the *P* = 0.05 level based on Fisher's least significant difference test.

peak value of transcript existed at 4 and 2 h after the application for the 0.5 and 3.2 mM Pb treatments, respectively (Table 5). The transcript of *Cyt Cu/Zn-SOD* mRNA increased at 6 and 4 h after treatment with 3.2 and 0.5 mM Pb, respectively (Table 5). The expression of *FeSOD* gene in perennial ryegrass showed upregulation at 4 and 24 h after the application of 3.2 mM Pb, whereas at 4 and 6 h after the treatment of 0.5 mM Pb (Table 5).

Elevated expression of *APX* gene in perennial ryegrass was only detected 4 h after the application of Pb treatments, with the

Table 4. Antioxidant enzyme activities of perennial ryegrass subjected to 0.5 and 3.2 mM Pb in a hydroponic system using half-strength Hoagland's solution for 7 d.

Pb treatment (mM)	Catalase	Peroxidase	Superoxide dismutase
	(Unit min ⁻¹ .mg ⁻¹ protein)		
0	52.9 b ^z	27.7 b	1.9 a
0.5	60.5 a	20.3 b	1.1 b
3.2	30.7 c	40.8 a	0.5 c

^zMeans within a column followed by the same letters were not significantly different at the *P* = 0.05 level based on Fisher's least significant difference test.

Table 5. Temporal transcription changes of different types of superoxide dismutase (SOD) gene in perennial ryegrass subjected to 0.5 and 3.2 mmol·L⁻¹ Pb in a hydroponic system using half-strength Hoagland's solution at different times after the treatment. Values are presented as a ratio to the untreated control.

Pb treatment (mM)	Sampling time after Pb treatment (h)				
	2	4	6	24	48
	----- <i>MnSOD</i> -----				
0.0	1.00 a ^z	1.00 b	1.00 b	1.00 c	1.00 a
0.5	0.53 ab	0.55 c	0.82 b	3.55 a	1.49 a
3.2	0.24 b	2.77 a	5.73 a	1.24 b	0.11 b
	----- <i>ChlCu/ZnSOD</i> ^y -----				
0.0	1.00 c	1.00 c	1.00 c	1.00 a	1.00 a
0.5	2.94 b	8.37 a	5.82 a	0.17 b	0.09 b
3.2	8.05 a	2.79 b	3.98 b	0.84 a	0.15 b
	----- <i>CytCu/ZnSOD</i> ^y -----				
0.0	1.00 a	1.00 bc	1.00 b	1.00 a	1.00 a
0.5	0.25 b	2.65 a	0.33 c	0.36 b	0.11 b
3.2	1.60 a	1.21 b	2.31 a	1.03 ab	0.44 b
	----- <i>FeSOD</i> -----				
0.0	1.00 a	1.00 c	1.00 b	1.00 b	1.00 a
0.5	0.73 a	1.35 b	1.83 a	0.60 c	0.29 b
3.2	0.85 a	3.18 a	0.52 c	1.58 a	0.41 b

^zMeans within a column followed by the same letters were not significantly different at the *P* = 0.05 level based on Fisher's least significant difference test.

^y*Chl Cu/ZnSOD* = chloroplastic Cu/ZnSOD gene, *Cyt Cu/ZnSOD* = cytosolic Cu/ZnSOD gene.

3.2 mM Pb treatment had a higher expression than the 0.5 mM Pb treatment (Table 6). The expression of *GPX* gene showed a similar trend to *APX* gene except the increase was found at 2 h after the application of 0.5 mM Pb. The expression of *GR* showed sustained increase from 4 to 24 h after the application of Pb in both 3.2 and 0.5 mM treatments. The *POD* gene expression was upregulated by 0.5 mM Pb at 4 and 6 h after the treatment. However, increased *POD* mRNA levels were detected for the 3.2 mM Pb treatment at all but the 24 h sampling times after the application of Pb (Table 6).

Discussion

The growth of perennial ryegrass was inhibited when exposed to 0.5 mM Pb, which was in agreement with the findings

Table 6. Temporal transcription changes of peroxisomal-type ascorbate peroxidase (*pAPX*), glutathione peroxidase (*GPX*), glutathione reductase (*GR*), and peroxidase (*POD*) genes in perennial ryegrass subjected to 0.5 and 3.2 mmol·L⁻¹ Pb in a hydroponic system using half-strength Hoagland's solution at different times after the treatment. Values are presented as a ratio to the untreated control.

Pb treatment (mM)	Sampling time after Pb treatment (h)				
	2	4	6	24	48
	----- <i>pAPX</i> -----				
0.0	1.00 a ^z	1.00 c	1.00 b	1.00 a	1.00 a
0.5	0.60 b	1.91 b	0.54 a	0.57 a	0.57 ab
3.2	0.49 b	5.52 a	1.08 ab	0.99 a	0.52 b
	----- <i>GPX</i> -----				
0.0	1.00 b	1.00 b	1.00 a	1.00 a	1.00 a
0.5	2.48 a	2.60 a	1.93 a	0.85 a	0.89 b
3.2	1.11 b	2.85 a	1.26 a	0.50 a	0.48 c
	----- <i>GR</i> -----				
0.0	1.00 a	1.00 b	1.00 b	1.00 c	1.00 a
0.5	0.50 b	2.64 a	1.78 a	6.69 a	1.32 a
3.2	1.00 ab	1.91 ab	2.19 a	2.99 b	0.79 a
	----- <i>POD</i> -----				
0.0	1.00 bc	1.00 b	1.00 b	1.00 a	1.00 b
0.5	1.23 b	6.25 a	4.46 a	1.01 a	0.88 b
3.2	4.48 a	4.15 a	2.35 a	0.45 b	3.96 a

^zMeans followed by the same letters within a column for each gene are not significantly different based on Fisher's least significant difference test at *P* < 0.05.

by Carlson and Rolfe (1979). The concentration of Chl *a* and total Chl was not affected by Pb treatment, whereas the content of Chl *b* was increased by high level of Pb. Since higher concentration of Chl *b* existed in stunted perennial ryegrass, it might be caused by faster decrease of cell number and cell size or by impaired ability of water uptaking resulted by metal toxicity. Therefore, plant biomass may be a better indicator of perennial ryegrass tolerance to Pb than Chl content.

Lipid peroxidation is a sensitive marker of oxidative stress (Polle et al., 1997), which can be reflected by its final decomposition product, MDA. Results from this study showed a substantial increase of MDA content in leaf tissues when perennial ryegrass was exposed to 3.2 mM Pb. However, the MDA content was lower than the control at the 0.5 mM Pb treatment, indicating that an effective defense mechanism was in action. Circumstantial evidence also came from the content of soluble protein in 0.5 mM Pb treatment, which was not different from that of the control. Furthermore, the activity of CAT increased at 0.5 mM Pb compared with the control, indicating that CAT played an important role in scavenging ROS, as was reported in other organisms under low dose of Pb (Cho and Seo, 2005). The present study showed that POD activity in 0.5 mM Pb treatment remained at the same level as that in the control. This may be explained by the fact that POD catalyzes conversion of H₂O₂ to generate phenoxy compounds in the downstream of ROS scavenging (Greppin et al., 1986). This result was also supported by other research findings where POD activity increased in response to Pb in *A. thaliana*, rice (*Oryza sativa*), maize, horse gram, and bengal gram

(Lummerzheim et al., 1995; Reddy et al., 2005; Verma and Dubey, 2003; Zacchini et al., 2003).

Superoxide dismutase is one of the most important members of the enzymatic antioxidant defense system against ROS (Alscher et al., 2002). At the end of 7 d exposure to Pb, SOD activity was less in the 3.2 mM treatment than in the 0.5 mM treatment. Dose-dependent SOD inhibition by Pb also was reported by Zhang et al. (2007).

Three types of SODs, FeSOD, MnSOD, and Cu/ZnSOD, are currently recognized (Tsang et al., 1991). In this study, Q-PCR demonstrated variations of these genes in terms of expression levels and time course. Similarly, Liu et al. (2009) reported that the *SOD* gene expression was substantially increased within 3 h of Pb treatment and then gradually declined to a stable level. Four genes encoding for SOD enzymes were all upregulated in response to Pb treatment in *A. thaliana* seedlings (Liu et al., 2009). Evidence of involvement of SOD enzymes in Pb tolerance also came from transgenic *A. thaliana* line (Kovalchuk et al., 2005), which showed overexpression of *MnSOD* and increased tolerance to Pb, salinity, drought, and freezing (McKersie et al., 1993; Tanaka et al., 1999; Wang et al., 2004).

Both APX and GR are indispensable components of ascorbate–glutathione (GSH) pathway generated mainly in chloroplasts and other cell organelles. As one of the key enzymes of antioxidative defense system, APX uses the reducing power of ascorbic acid to scavenge harmful H_2O_2 and regulates the cellular concentration of O_2^- and H_2O_2 (Asada, 1992). Glutathione reductase is involved in the formation of reduced GSH molecules, which regulate redox in cells and play a critical role in protecting plants against oxidative stress (Grant et al., 1996; Sanchez-Fernandez et al., 1997). In this study, the Pb-induced expression of *GR* and *APX* suggested that ROS detoxification was at least partly implemented through the ascorbate–GSH cycle in perennial ryegrass. Brunet et al. (2009) reported that there was an overaccumulation of transcripts coding for GR in grass pea, along with slight increase of mRNA of various APX in response to Pb stress. Accumulation of mRNA and increased activity of APX was also found in other plant species (Mishra et al., 2006; Verma and Dubey, 2003).

Results from this study suggested that GPX probably served as an intrinsic defense against Pb-induced oxidative damage in perennial ryegrass. It was consistent with the elevated GPX activity in rice exposed to Pb (Verma and Dubey, 2003). Also, both the 0.5 and 3.2 mM levels of Pb induced significant increase of *POD* mRNA accumulation in perennial ryegrass. This result was in agreement with other study where 20 genes encoding POD enzymes were upregulated in the seedlings of *A. thaliana* in response to Pb treatment (Liu et al., 2009).

In present study, the genes encoding antioxidant enzymes were upregulated within a few hours after the exposure to Pb. However, their expression levels were the same as or below that in untreated controls after 24 or 48 h. Note also, the SOD activity was below the level of control in plants after 7 d of Pb treatment. Therefore, antioxidant enzymes might be responsible for the early stage defense to Pb-induced oxidative stress in ryegrass. Only *POD* gene retained at upregulated levels at 48 h after treatment with 3.2 mM Pb, coincidental with the elevated enzyme activity of POD 7 d after the subjection to Pb at 3.2 mM. The results indicated that the time course of plant defense system in response to Pb was better defined at gene levels than at the enzyme levels, although the results of gene expression conformed to the enzyme activity.

In conclusion, the effects of Pb on perennial ryegrass were reflected at the growth, physiological, enzyme, and gene levels. Although antioxidant enzymes may be used as indicators of the responses in perennial ryegrass to Pb at an early stage of exposure, gene expression profiles provided more accurate time course of the activation of those systems.

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