Real-time Quantitative PCR Monitoring of Antioxidant Enzyme Gene Expression in Wheat Radicles Treated With Cu^{2+} and Cd^{2+}

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Abstract: Real-time quantitative PCR was used to study the differential expression of three antioxidant enzyme genes copper/zinc-superoxide dismutase (Cu/Zn-SOD)、peroxidase (POD) and Glutathione S-transferase (GST) – in winter wheat *(Triticum aestivum L.)* radicles following treatment with two heavy metals (Cu and Cd). The effects of varying the concentration of the heavy metals and the duration of exposure were investigated. It was found that Cd stress has a more profound effect than Cu on antioxidant gene expression for all tested mass concentrations and that heavy metal exposure induces GST expression more strongly than that of POD or Cu/Zn-SOD, with POD being expressed more strongly than Cu/Zn-SOD.

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Key words: antioxidant enzyme genes; heavy metals stress; real-time quantitative PCR.

Abbreviations:

AOS	active oxygen species
Cu/Zn-SOD	copper/zinc-superoxide dismutase
GSH	glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
·OH	hydroxyl radicals
POD	peroxidase
ROS	reactive oxygen species

1.Introduction

During growth and development, a plant has to cope with a range of different internal and external stresses, so its ability to adapt to metabolic and environmental changes is essential for survival. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and the more toxic hydroxyl radicals ($OH\cdot$) and singlet oxygen ($^1O_2$), are produced continuously during plant growth and development, but their abundance increases when plants are exposed to various biotic and abiotic stresses (Elstner, 1982; Asada, 1994; Dat et al., 2000). These toxic ROS oxidize proteins, unsaturated fatty acids and DNA, causing cellular damage and cell death.

Plants have a number of different defense mechanisms by which they respond to oxidative stress. These include producing non-enzymatic antioxidants such as ascorbate and glutathione, and enzymatic antioxidants such as catalase, superoxide dismutase and ascorbate peroxidase. If these defenses fail to protect the plant from the ROS, cell death will result. Several heavy metals have become widely distributed in the environment due to human activities such as mining and the disposal of garbage and sewage sludge in field sites. In terms of their environmental impact, the most important of these heavy metals are cadmium, zinc, copper, and lead. However, the accumulation of large amounts of any heavy metal in the organism as a whole or in specific organs can cause significant damage (Clemens and Krämer 2003).

Heavy metal stress is one of the major abiotic stresses affecting germination, crop growth and productivity. In nature, plants encounter a number of biotic and abiotic stress factors simultaneously, including drought, heat, shock and heavy metals. Copper is an essential trace mineral that is present in almost all living organisms; it is a cofactor that is required to maintain the structural and catalytic properties of various enzymes. Cadmium has a wide range of toxic effects, which are exacerbated by its long biological half-life and low excretion rate (Tully et al. 2000). Its acute toxicity in mammals has been attributed in part to its ability to induce oxidative stress. In plants, Cd affects photosynthesis, respiration and nitrogen metabolism, and causes oxidative damage similar to that observed in mammals (Dixit et al. 2001).

Under optimal conditions, cellular homeostasis is achieved by the coordinated action of several biochemical pathways. Stress factors can

have different effects on these pathways and their coordination; in plants, this can change the flow of metabolites through the various homeostatic pathways. Metabolic activity results in the continuous formation of reactive oxygen species (ROS), and their abundance increases under stress conditions. This increase is accompanied by the activation of defense genes whose products have a variety of functions that may include ROS scavenging. ROS such as the superoxide radical (O_2^-) cause oxidative damage to various cellular components such as membrane lipids and oxidation-sensitive enzymes, affect vital processes such as the synthesis and denaturation of proteins, and can induce mutations in DNA. Cells therefore produce a number of protective enzymes that are activated in response to oxidative stress. These include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), and Glutathione S-transferase (GST). Glutathione-dependent enzymes and metabolites are useful target species for biomonitoring of oxidative damage and the effects of ROS because glutathione is involved in phytochelatin biosynthesis (Rauser 1990) and glutathione S-transferases (GST, Habig et al. 1974; Schröder and Berkau 1993) are potent detoxification enzymes, catalyzing the nucleophilic attack of reduced glutathione on electrophilic pollutant molecules and products of oxidative stress.

This paper describes an investigation into the activity of antioxidant enzymes under various conditions and the expression of the corresponding genes in the wheat radicle. The results obtained provide increased understanding of plant responses to Cu^{2+} and Cd^{2+} stresses and to stressful environmental conditions in general, and will be useful in future crop engineering programs aimed at adapting crops to challenging environments and increasing their agronomic value.

2.Materials and Methods

Sowing and heavy metal treatments

The crop examined in this work was the "Aikang 58" variety of winter wheat (*Triticum aestivum).* Seeds of uniform size were selected, washed with distilled water and treated with 0.1% mercuric chloride (w/v) for 5 min. The seeds were then thoroughly washed with deionized water and sown in petri dishes (100 seeds per dish) lined with filter paper and precultured for 72h. Sets of Cu and Cd solutions containing 5 mg/L, 30 mg/L, and 60 mg/L of the metal salt were prepared by dissolving copper sulfate ($CuSO_4 \cdot H_2O$) or $CdCl_2$ in tap water; control experiments were conducted using tap water alone. The precultured seeds were then soaked in tap water or the appropriate metal ion solution and left to grow for 24h, 48h, 72h, or 96h before subsequent experimentation.

Total RNA extraction and cDNA synthesis

0.1g of wheat radicles were ground in liquid nitrogen. Total RNA was extracted using the RNAiso Plus kit (TaKaRa/Invitrogen, Japan) according to the manufacturer's instructions. All preparation and handling steps involving RNA were performed in a laminar flow hood, under RNase-free conditions. RNA was used to generate single-stranded cDNA by reverse transcription using an oligo-dT primer and the ABI PCR System (Applied Biosystems, Foster City, CA, USA) in conjunction with the PrimeScript™ RT-PCR Kit (TaKaRa, Japan). Reverse transcription was performed according to the kit manufacturer's instructions.

Design and identification of qRT-PCR primers

Real-time PCR primers for the amplification of Cu/Zn superoxide dismutase (SOD), peroxidase (POD) and Glutathione S-transferase (GST) were designed based on the wheat gene sequences in GenBank using the Primer Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), along with a primer for the 18S rRNA as a control. The primers used to quantify the mRNA levels of the genes of interests were given in Table 1. The mRNA sequences of *Cu/Zn-SOD*, *POD*, *GST* and *18S rRNA* were obtained from the following GenBank accession numbers: TAU69536, X56011, AJ441055 and AJ272181, respectively. The extracted RNA samples were subjected to DNA-free treatment to avoid genomic DNA contamination, and amplified PCR products of all four genes were sequenced and blasted to ensure that the correct mRNA sequences were quantified.

Quantitative real-time RT–PCR (qRT-PCR)

QRT–PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in conjunction with the SYBR Premix Ex Taq™ kit (TaKaRa, Japan), using the procedure specified by the kit manufacturer. Each sample was split into three separate sub-samples and three reactions were performed in parallel. A standard two-step amplification procedure was carried out as follows: 95°C for 30s, then 95°C for 5s and 60°C for 34s go-around 35 cycles. Following amplification, the samples were subjected to the dissociation protocol.. Each sample was done in triplicate.

Statistical analysis

The expression data were analyzed using the SPSS 11.5 statistical package for Windows (SPSS Inc., USA). Analyses of variation were computed for statistically significant differences determined using the appropriate F-tests. Results are presented as the means ± SD of at least three independent replicates. Mean differences were compared utilizing Tukey's

test at P<0.05.

3. Results

Quantitative analysis of *Cu/Zn-SOD* mRNA expression following Cu and Cd treatment

To examine the ability of different heavy metals to induce transcription of *Cu/Zn-SOD* gene, wheat radicles were exposed to aqueous solutions of Cu or Cd salts containing 0, 5 mg/L, 30 mg/L, or 60 mg/L of the metal salt. Quantitative RT-PCR analysis indicated that Cd stress induced significantly stronger expression of the Cu/Zn-SOD gene than did Cu stress at all tested mass concentrations, as shown in Figure 1. For both metal salts, the level of Cu/ZN-SOD expression decreased over time following exposure, with the lowest levels being observed after 96h. For all treatments, the level of Cu/Zn-SOD expression was significantly lower than that in the control and was reduced by exposure to higher mass concentrations of the metal salt.

Quantitative analysis of *POD* mRNA expression following Cu and Cd treatment

POD is important because it prevents the accumulation of H_2O_2, which oxidizes membrane lipids to malondialdehyde (MDA). It thus plays a vital role in maintaining the integrity of the cell membrane. As shown in Figure 2, quantitative RT-PCR analysis revealed that Cu stress reduced the expression of the POD gene significantly more than did Cd stress. Treatment with either of the two heavy metals caused a rapid decrease in POD expression compared to the control, with levels beginning to increase towards the end of the treatment. Treatment with low mass concentrations of Cu caused POD gene expression to remain consistently low throughout the experiment whereas treatment with higher mass concentrations caused POD expression to increase towards the end of the treatment; the lowest level of POD expression observed occurred with the 30 mg/L treatment, 72 hours after exposure. Cd stress caused POD expression to decrease initially but in most cases, it then began to increase later in the experiment – typically, after 48 hours. The lowest level of POD expression was observed after treatment with the 5mg/L Cd solution.

Quantitative analysis of *GST* mRNA expression following Cu and Cd treatment

As shown in Figure 3, GST gene expression increased in response to treatment with either Cu or Cd. The abundance of *GST* mRNA in treated wheat radicles increased significantly between 24h and 48h after treatment for all concentrations of Cd (by a factor of 1.5-2 at 24h and 1-3 at 48h). Cu exposure caused rapid increases in the abundance of GST mRNA, especially following treatment with the 60mg/L and 30mg/L solutions. In general, GST gene expression was initially reduced by treatment with the metal salts but began to recover as the experiment proceeded.

4. Discussion

In this work, wheat radicles were treated with solutions of Cu and Cd salts in order to investigate the expression of genes encoding antioxidant enzymes following heavy metal stress. In general, copper salts had more pronounced effects on the expression of the Cu/ZN SOD, POD, and GST genes than did cadmium salts. In addition, GST was expressed more strongly than POD, which in turn was expressed more strongly than Cu/Zn-SOD.

There seem to be species-specific differences in the induction of anti-oxidant gene expression following heavy metal stress: in some plants, primary defense genes such as SOD, catalase, and POD are expressed more strongly whereas in others, the main response involves glutathione-dependent enzymes. In the case of wheat, it seems that some of its detoxification capacity – specifically, that originating from glutathione-based enzymes – is initially employed to deal with heavy metal stress but is quickly reallocated for other purposes. It has previously been reported that glutathione S-transferases generally respond strongly to heavy metal exposure (Noctor et al. 2002).

Table 1. Genes targeted for expression profile analysis and primer sequences for their cDNA

Gene	Acc.no	Primer sequence	Product（bp）
18SrRNA	AJ272181	F-TTAGTTGGTGGAGCGATTT R-TGTTATTGCCTCAAACTTCC	145
Cu/Zn-SOD	TAU69536	F-CGATAGCCAGATTCCTTTG R- AACCAGCGACCTACAACG	176
POD	X56011	F-CAGCGACCTGCCAGGCTTTA R-GTTGGCCCGGAGAGATGTGG	196bp
GST	AJ441055	F-GGAGCACAAGAGCCCCGAGC R-CGGGTTGTAGGTGTGCGCGT	217bp

Note: PCR primers were designed for three antioxidant genes (Cu/Zn-SOD, POD and GST) and one housekeeping genes (18S rRNA).

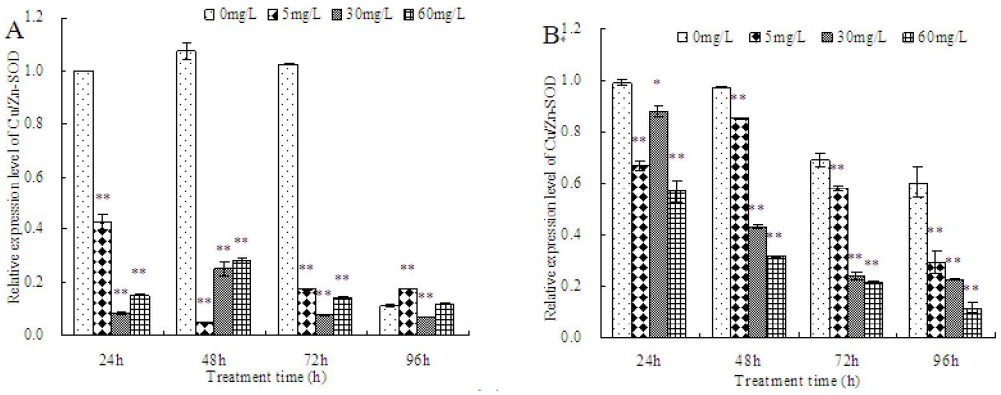

Figure 1. Quantitative real-time PCR data on the number of mRNA transcripts of the *Cu/Zn-SOD* gene in wheat radicles treated with 0 (control), 5, 30, or 60mg/L of Cu (A) or Cd (B) for 24h, 48h, 72h, 96h. The values reported are means ±SD (n=3). Single asterisks indicate significant deviation from the control and double asterisks indicate extremely significant deviance form the control (P<0.05) as determined by ANOVA followed by a multiple range test (LSD) with respect to duration of exposure.

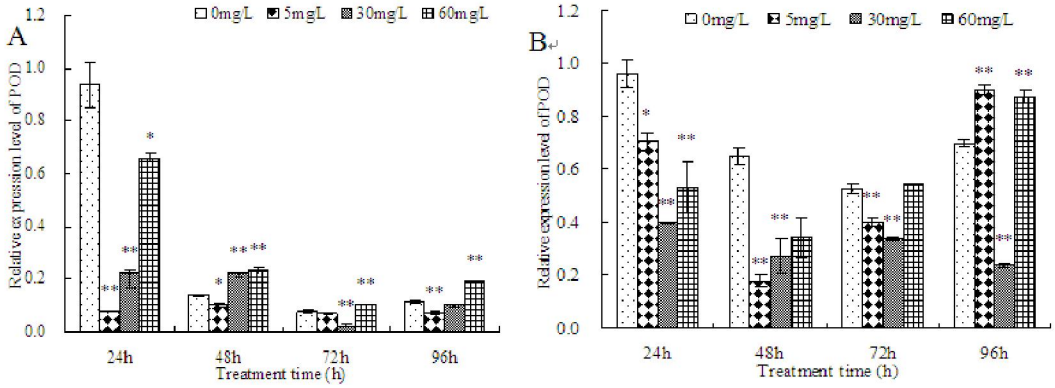

Figure 2. Quantitative real-time PCR data on the number of mRNA transcripts of the POD gene in wheat radicles treated with 0 (control), 5, 30, or 60mg/L of Cu (A) or Cd (B) for 24h, 48h, 72h, 96h. The values reported are means ±SD (n=3). Single asterisks indicate significant deviation from the control and double asterisks indicate extremely significant deviance form the control (P<0.05) as determined by ANOVA followed by a multiple range test (LSD) with respect to duration of exposure.

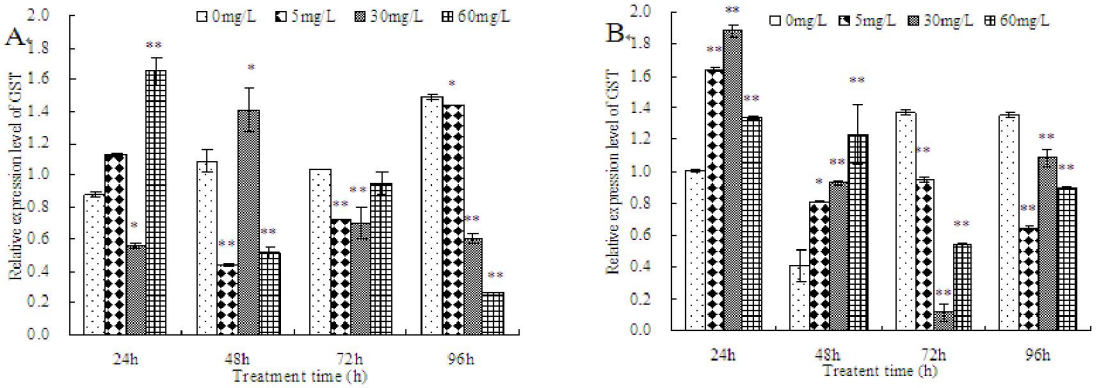

Figure 3. Quantitative real-time PCR data on the number of mRNA transcripts of the GST gene in wheat radicles treated with 0 (control), 5, 30, or 60mg/L of Cu (A) or Cd(B) for 24h, 48h, 72h, 96h. The values reported are means ±SD (n=3). Single asterisks indicate significant deviation from the control and double asterisks indicate extremely significant deviance form the control (P<0.05) as determined by ANOVA followed by a multiple range test (LSD) with respect to duration of exposure.

Long-term exposure to heavy metals can affect plant physiological processes and reduce cellular control over the formation and destruction of ROS (Srivalli et al., 2003). Plants have well-organized antioxidative defense systems comprising enzymatic and non-enzymatic antioxidants to scavenge ROS. The cooperative functioning of antioxidants such as SOD, POD and GST plays an important role in scavenging ROS and maintaining the organism's physiological redox balance (Wise, 1995; Foyer and Nector, 2000; Cho and Seo, 2005). Under normal growing conditions, oxidative damage to cellular components is minimized by efficient elimination of ROS. However, if ROS production exceeds the capacity of the cell's antioxidant systems, damage will start to accumulate.

The superoxide dismutases (SODs) are a family of metalloenzymes with a range of different isoforms, including Cu-Zn-SOD, Mn-SOD and Fe-SOD. They are present in almost all cells that are exposed to oxygen and are the primary scavengers of O_2^- radicals (Alscher et al., 2002). To determine how SOD expression is affected by heavy metal stress, we monitored the abundance of Cu/Zn-SOD mRNA in wheat radicles that had been treated with Cu or Cd. Our results indicate that there were significant differences in Cu/Zn-SOD expression between groups and that heavy metal exposure reduced the abundance of Cu/Zn-SOD mRNA relative to the control group (Figure 1). This demonstrates that heavy metals can affect SOD levels in wheat plants at both the molecular and the cellular levels.

Peroxidases (PODs) are widely distributed in the plant kingdom and play a major role in eliminating active oxygen species (AOS). They catalyze the oxidation of a sacrificial substrate by H_2O_2. A previous study investigated changes in the expression profiles of 10 POD genes in sweet pea following exposure to air pollutants and UV radiation (Kim et al., 2007). It was found that certain POD genes played specific roles in defending against oxidative stress. In another study, it was demonstrated that treating plants with three heavy metals – Cd, Cu and Zn – affected POD expression and that the magnitude of the effect varied depending on the duration of exposure (Kim et al., 2010). The results obtained in the current study suggest that POD enzymes are important in protecting the wheat radical from oxidative stress induced by heavy metal treatment (Fig. 2). Significant changes in POD expression were observed irrespective of the type of metal applied; while the response to Cu stress was more pronounced than that induced by Cd, both metals resulted in substantial changes compared to the control. POD destroys H_2O_2 by oxidizing various hydrogen donor molecules and thus protects plant tissues that have been exposed to direct oxidants or heavy metals (Wang and Yang, 2005; Song et al., 2007; Xue et al., 2008). The abundance of POD mRNA may therefore be a useful indicator of damage to plant tissues caused by heavy metal exposure.

The glutathione S-transferases (GSTs) are dimeric and multifunctional enzymes that are ubiquitous in aerobic organisms (Edwards et al., 2000). GSTs play a crucial role in detoxifying xenobiotic compounds in cells by catalyzing the nucleophilic attack of the thiol group (SH) of reduced glutathione (GSH) on diverse electrophilic molecules, including herbicides, insecticides, carcinogens and other xenobiotics (Pascal et al., 1998; Edwards et al., 2000; Yin et al., 2008). They play a central role in the antioxidant defense mechanisms of both eukaryotes and prokaryotes. Exposure to environmental stress (acidic pH) has been shown to induce GST expression in shrimp . Moreover, it has been reported that cadmium exposure affects the expression of a number of GST isoforms in the river puffer fish *T. obscurus* (Kim et al., 2010). Our results suggest that GST is one of the main antioxidant components in the responses to Cu and Cd exposure in wheat, since its expression increased significantly following treatment. For all metal treatments examined in this work, the level of GST expression was substantially higher than in the control in the 48 hours following the initiation of the treatment and then began to gradually decrease. This suggests that GST plays an important role in detoxifying heavy metals in wheat radicles.

The results obtained in this work demonstrate that antioxidant gene expression following exposure to Cu and Cd varies over time and depends on the concentration of the metal salt. The origins of the differences between the two metals in terms of gene expression are unclear and there are several factors that should be considered when searching for explanations. First, we observed differences in the responses of antioxidant genes to different superoxide-generating compounds, although these were relatively minor; in general, the responses to the two metals were similar for all three genes considered. Second, antioxidant gene expression is controlled by a wide range of different promoters and control elements. The number, order, and types of protein binding sequences present in promoters play a major role in determining gene expression patterns . Third, cadmium is very toxic towards a wide variety of species, affecting the behavior, growth, and physiology of many plants (Liu et al., 2007). Cd has multiple effects on cells and can interfere with cell cycle progression, proliferation, differentiation, DNA replication and repair, and apoptotic pathways (Bertin et al., 2006). It also induces oxidative stress

by increasing the concentration of the superoxide anion and hydrogen peroxide in cells (Szuster-Ciesielska et al., 2000). Cadmium ions (Cd^{2+}) can bind to free thiol (SH) groups in proteins, cysteine, and glutathione, and inhibit the functions of these biomolecules. It seems that Cd can both induce and inhibit GST activity, depending on its concentration (Yano et al., 2005). Moreover, our results indicate that the duration of Cd exposure has a significant effect on the expression of three antioxidant genes (Cu/Zn-SOD, POD, and GST). The expression of all three genes considered in this work increased following Cd exposure.

Copper is an essential trace mineral that is present in almost all organisms. It functions as a cofactor and is required for the structural and catalytic properties of various enzymes. Cells must balance their need for small quantities of copper against its toxic effects when present in excessive quantities. Cu deficiency decreases the activities of enzymes involved in oxidation defense systems and also alters the cellular abundance of ROS scavengers, such as metallothioneins and glutathione (Uriu-Adams et al., 2005). Excessive Cu levels cause ROS formation by promoting hydroxyl radical formation. These are the most strongly oxidizing ROS and are highly toxic (Gaetke et al., 2003). Our results indicate that Cu/Zn-SOD and POD expression are less strongly induced by Cu exposure than is that of GST, which suggests that ROS generated by Cu exposure are primarily detoxified by glutathione peroxidase.

The findings obtained in this work constitute an important contribution to our understanding of plants' responses to stress factors and provide some baseline information on the cascades or networks of events that are triggered by heavy metal stress. In the long run, unraveling the stress response mechanisms of plants will be extremely useful because it will expand our understanding of gene regulation in all eukaryotes and may eventually allow us to design or adjust mechanisms that regulate gene expression to create crops that are better adapted to challenging environments and have increased agronomic value.

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