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Research Article

Differential responses of peroxidases in sweetpotato suspension-cultured cells to cadmium treatment

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Abstract As cultured plant cells can grow in high oxidative stress conditions, they form an excellent system to study antioxidant mechanisms and the mass production of antioxidants. Oxidative stress is a major cause of damage in plants exposed to various types of environmental stress, including heavy metals, such as cadmium (Cd). Heavy metal accumulation can interfere with many cell functions and plant growth. To evaluate the contribution of oxidative stress to Cd-induced toxicity, cultured sweetpotato (*Ipomoea batatas*) cells were treated with increasing concentrations of Cd (0, 10, 25, and 50 μM) and cultured further. Cell growth was significantly inhibited by 25 and 50 μM of Cd, and the total protein content increased with 50 μM of Cd. Additionally, the activity of peroxidase (POD) and ascorbate peroxidase (APX), antioxidant enzymes that remove hydrogen peroxide (a reactive oxygen species), increased in the cells after treatment with 50 μM of Cd. The expression analysis of *POD*, *APX*, and peroxiredoxin (*PRX*) isolated from sweetpotato cultured cells in a previous study revealed the differential expression of *POD* in response to Cd. In this study, the expression levels of several acidic *POD* (*swpa2, swpa3*, and *swpa4*) and basal POD (*swpb1, swpb2*, and *swpb3*) genes were increased in Cd-treated cultured cells. These results indicate that Cd-mediated oxidative stress is closely linked to improved POD-mediated antioxidant defense capacity in sweetpotato suspension-cultured cells.

Keywords cadmium, oxidative stress, peroxidase, suspension cultured cells, sweetpotato

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Introduction

Cadmium (Cd) pollution is rapidly increasing due to global urbanization and industrialization, and the consequent increase in contaminated farmland and agricultural water supply has seriously impacted safe agricultural crop production (Farooq et al. 2018; Jan et al. 2015; Khan et al. 2015). Cd is highly mobile in soil, and crops grown in soils with low Cd concentrations easily contaminate agricultural products through uptake and transport. Cd accumulation can lethally damage plants and humans by biochemically weakening the affinity of sulfhydryl groups (-SH) in proteins and interfering with the activity of metabolically important enzymes (Jung et al. 2016; 2018; Meuwly and Rauser 1992). In addition to inhibiting normal plant growth, Cd can substitute cofactors for essential metal ions in important proteins via the Fenton reaction (Jung et al. 2016; 2018; Loix et al. 2017; Peroza et al. 2009; Singh et al. 2016). In this process, reactive oxygen species (ROS), such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$), are generated. Oxidative stress due to excess ROS accumulation not only destroys cell membranes through intracellular lipid peroxidation, but also reduces the biosynthesis of primary metabolites necessary for plant life, such as nucleic acids, proteins, carbohydrates, and adversely affects photosynthesis, respiration, and ion absorption (Asgari Lajayer et al. 2017; Jung et al. 2016; Singh et al. 2016). Therefore, plants mitigate or minimize oxidative stress caused by the excessive uptake and accumulation of Cd by using various physiological and biochemical detoxification systems (Jung et al. 2016; 2018; Singh et al. 2016; Srivastava et al. 2014).

Peroxidases (PODs) catalyze the reduction of H_2O_2 by transferring electrons to various donor molecules, such as lignin precursors, phenolic compounds, and secondary metabolites (Passardi et al. 2005). POD has been implicated

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in a wide range of physiological processes in plants, including suberization, lignification, crosslinking of cell wall compounds, and defense against various environmental stimulus, such as heavy metals (Cosio and Dunand 2009; Passardi et al. 2005). In the tissues of many plant species, POD activity correlates significantly with Cd accumulation levels (Schutzendubel and Polle 2002). In the presence of Cd, the increased POD production is believed to be caused by phytotoxic metal fractions or free metals that are not bound to cell walls or accumulate in vacuoles. Thus, increased POD activity in response to Cd stress may play an important role in cellular defense mechanisms against toxicity (Van Assche and Clijsters 1990). Additionally, POD activity is closely related to changes in gas exchange, transpiration, photosynthesis, and respiration, all of which potentially serve as indicators of impaired metabolic activity in Cd accumulation (MacFarlane and Burchett 2001). Ascorbate peroxidases (APXs) uses ascorbic acid (AsA) as a specific electron donor to reduce H_2O_2 to water. APX and the AsA-glutathione (GSH) cycle play an important role in scavenging ROS in various organelles, including chloroplasts (Asada 1992; Mittler et al. 2004; Noctor and Foyer 1998). ROS scavenging enzymes in plants have been extensively studied and it has been demonstrated that APX activity generally increases along with the activity of other enzymes, such as CAT, SOD, and GSH reductases, in response to various stress conditions, including Cd accumulation (Shigeoka et al. 2002).

Cultured plant cells are a good system for studying antioxidant enzymes because they can be grown in high oxidative stress conditions (Kim et al. 2004). Plant cell suspension culture is of great importance in the field of plant biotechnology for the development of transgenic study, biosynthesis of useful pharmaceutical proteins, and mass propagation. Previously we investigated the levels of various antioxidant proteins such as CAT, POD, and SOD, as well as low molecular-weight metabolites (AsA and GSH) in 100 cell lines derived from different plant species. Cultured cells of plant have significantly higher levels of antioxidant activities, such as enzymes of SOD and POD, than plant tissue (Kim et al. 1994, 1999; You et al. 1996). In previous studies, the POD and APX genes were isolated from cultured cells, and their expression levels were characterized to understand their physiological functions in response to environmental conditions such as abiotic stress and pathogen infection (Huh et al. 1997; Jang et al. 2004; Kim et al. 1999; 2007; Park et al. 2003). In addition, changes in POD activity and gene expression were investigated in sweetpotato roots under various heavy metal treatment conditions, such as Cd, Cu, and Zn (Kim et al. 2010). However, the responses of POD and APX in sweetpotato cultured cells to Cd treatment, a typical heavy metal stressor, have not yet been clarified in detail. Therefore, in this study, changes in POD and APX activity and gene expression were analyzed in Cd-treated sweetpotato suspension-cultured cells.

Materials and Methods

Plant material and cell culture conditions and Cd treatment

Sweetpotato suspension cultures (*Ipomoea batatas* (L) *Lam.* cv. White Star) was used (Kim et al. 1994). Subcultured cells (1 g fresh weight) were subcultured every 14 days in 50 ml of MS basal medium (Murashige and Skoog 1962) supplemented with 1 mg/L 2,4-dichlorophenoxy-acetic acid and 30 g/L sucrose and cultured at 25°C in the dark. For Cd metal treatment, 14-days cultured cells containing various concentrations of $CdSO_4$ (0, 10, 25 and 50 μM) were cultured for 14 days. All treated material was immediately frozen in liquid nitrogen and stored at -70°C until further analysis.

POD and APX activity assays

Total soluble protein was extracted from sweetpotato suspension culture cells using extraction buffer and concentration was determined using the Bio-Rad Protein Assay (Bradford 1976). Extracted proteins were used for measurement of POD and APX activities. POD activity was investigated by method of Kwak et al. (1995) using pyrogallol as a substrate. One unit of POD activity was defined as the amount of enzyme required to form 1 mg of purpurogalin from pyrogallol in 20 seconds, as measured by absorbance at 420 nm. APX activity was measured according to the method of Nakano and Asada (1981) using AsA as a substrate. AsA oxidation was initiated by H_2O_2 and the decrease in absorbance at 290 nm was monitored for 1 minute 30 seconds. One unit of APX was defined as the amount of enzyme that oxidized 1 mol of AsA per minute.

Native poly-acrylamide gel electrophoresis (PAGE)

Native gel electrophoresis of POD and APX was performed on 7.5% gels at 4°C, 120 V (Beauchamp and Fridovich 1971). POD gel analysis was performed by Kim et al. (1994) with modifications. After electrophoresis, the gel was

stained with 1.5% H_2O_2 and 1% benzidine. APX isozyme was prepared by equilibrating the gel in a solution consisting of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM AsA for 30 minutes, followed by equilibration of the gel in a solution consisting of 50 mM sodium phosphate buffer (pH 7.0) contains 4mM AsA and 2mM H_2O_2. It was detected by equilibrating the gel in solution for 20 minutes. After washing with 50 mM sodium phosphate buffer (pH 7.0) for 1 minute, the gel was immersed in 50 mM sodium phosphate buffer (pH 7.8) containing 1.25 mM NBT and 28 mM TEMED for 10 minutes at room temperature (Mittler and Zilinskas 1994).

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from sweetpotato suspension cultured cells using Trizol reagent (Invitrogen, USA). First-strand cDNA was synthesized from total RNA (1 μg) using the Improm-II reverse transcription kit (Promega, USA) according to the manufacturer's instructions. As an internal control, expression of 18S internal standard (Ambion, USA) was also analyzed. Gene-specific primers are designed in the 3'-UTR or region near the stop codon of each gene. Sequences of primers used for amplification of the POD gene are described (Kim et al. 2007; Park et al. 2003).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Subsequent multiple comparison of means was examined based on the least significant difference (LSD) test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 12; IBM, Armonk, NY, USA) and statistical significance was set at $P < 0.05$.

Results and Discussion

Effects of Cd treatment in suspension-cultured cells

To confirm the effect of different Cd concentrations on sweetpotato suspension-cultured cells, fresh cell weight and total protein concentration were measured after treatment with 0, 10, 25, and 50 uM Cd (Fig. 1). Sweetpotato suspension-cultured cells were subcultured every 14 days and cultured for 30 days, and the fresh cell weight and total protein concentration were measured on the 14th day after subculture after treatment with Cd at different concentrations. There was no significant change in fresh cell weight with the low-concentration 10 uM treatment, and it decreased with the 25 uM treatment compared with control conditions (Fig. 1A). After treatment with 50 uM Cd, approximately 85% of the fresh cell weight was reduced compared with the untreated control condition. There was no significant change in total protein content with the 10 uM and 25 uM treatment from the control group; however, there was a 1.77-fold increase in total protein content after treatment with 50 uM Cd compared with the untreated control conditions in the suspension-cultured cells of sweetpotato (Fig. 1B).

The effects of Cd treatments have been confirmed in cultured cells of several plant species. Changes in secondary metabolites were confirmed after Cd treatment in grape cultured cells, and the growth of the cultured cells decreased after treatment with 1 and 1.5 mM Cd (Cetin et al. 2014). On the other hand, the total phenol and tocopherol content increased on the second day after treatment, which is the sampling period at the beginning of treatment. In addition, the degree of cell damage in tobacco BY2 cultured cells according to Cd concentration (100, 150, 250, and 500

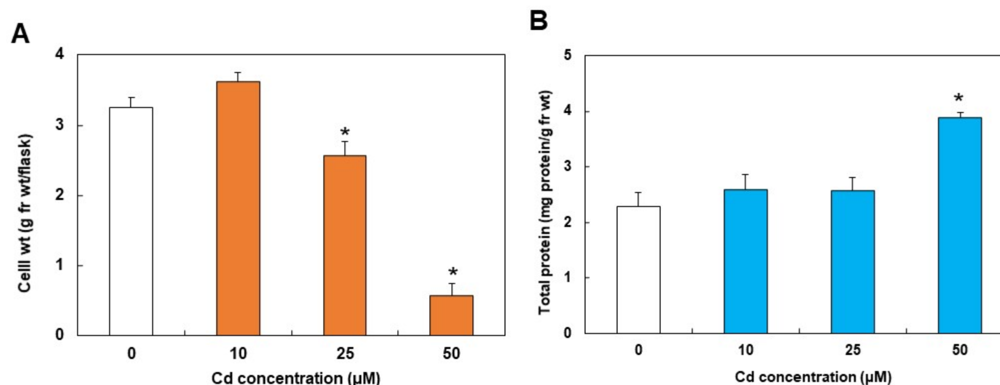

Fig. 1 Effect of cell growth and total protein contents in the sweetpotato suspension-cultured cells under Cd treatment conditions. (A) Cell fresh weight under 0, 10, 25, and 50 μM of Cd. (B) Total protein amounts under 0, 10, 25, and 50 μM of Cd. Data are presented as the average of three replicates. Asterisks mark statistically significant differences (*$P < 0.05$) between the control and treatment conditions, determined using a one way ANOVA with the LSD post hoc test

uM) was measured through ion conductance analysis (Shi et al. 2020). Cd treatment affected the growth of plant cultured cells, increased ROS, and caused a change in antioxidant substances. In this study, a significant decrease in cell growth and an increase in total protein content occurred after treatment with 50 uM Cd (Fig. 1).

Changes in POD and APX activity in suspension-cultured cells to Cd treatment

As Cd treatment induces oxidative stress through the induction of ROS, changes in the activities of the representative antioxidant enzymes POD and APX, which remove H_2O_2 (a representative ROS), were investigated in Cd-treated sweetpotato suspension-cultured cells (Fig. 2). Compared with the untreated control conditions, there was no change in POD activity with 10 uM Cd, and POD activity decreased with 25 uM Cd (Fig. 2A). On the other hand, treatment with 50 uM Cd caused a weak 1.15-fold increase in POD activity. Treatment with 10 uM Cd resulted in a 1.6-fold increase in APX activity compared with the control group, and 50 uM Cd caused a 3.16-fold increase in APX activity (Fig. 2B). In addition, native gel analysis showed that POD and APX isoenzyme patterns were similar to their activity results.

In a previous study, treatment of sweetpotato roots with 0, 0.1, 0.5, and 1 mM Cd resulted in higher ROS accumulation in the roots than in the leaves (Kim et al. 2010). Treatment with 1 mM Cd, the highest treatment condition, caused the highest increase in POD activity in leaves and roots. In this study, an increase in POD activity in sweetpotato cultured cells was also confirmed with the highest Cd concentration. In addition, it has been confirmed that SOD and APX activities increase with increases in H_2O_2 and lipid peroxidation in Cd-treated rice plants (Chiao et al. 2020). Therefore, it is thought that the increase in ROS-induced oxidative stress after Cd treatment affects the activities of the H_2O_2-scavenging antioxidant enzymes POD and APX in sweetpotato cultured cells.

Differential responses of POD and APX genes in suspension-cultured cells to Cd treatment

Previous studies have isolated genes of various antioxidant enzymes derived from sweetpotato cultured cells and characterized their expression patterns (Huh et al. 1997;

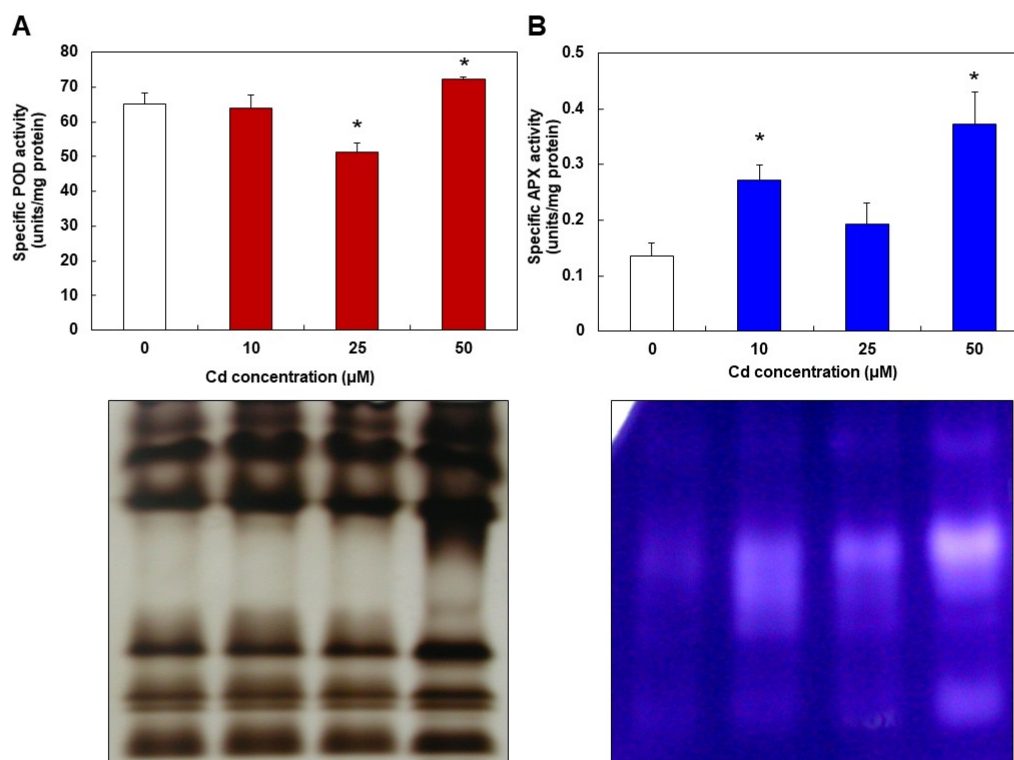

Fig. 2 POD and APX activity levels in the sweetpotato suspension-cultured cells under Cd treatment conditions. (A) POD activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively. (B) APX activity and isoenzyme patterns as determined by spectroscopy and native gel analysis, respectively. Data are presented as the average of three replicates. Asterisks mark statistically significant differences (*P < 0.05) between the control and treatment conditions, determined using a one way ANOVA with the LSD post hoc test

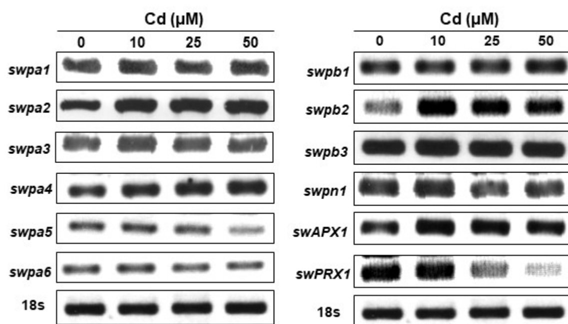

Fig. 3 RT-PCR analysis of the expression of 10 *POD*, 1 *APX*, and 1 *PRX* genes in sweetpotato suspension-cultured cells exposed to Cd treatment. *18S RNA* was used as a control for equal loading

Kim et al. 1999; Park et al. 2003). Therefore, in this study, expression changes of POD and APX genes were investigated in Cd-treated sweetpotato suspension-cultured cells (Fig. 3). Of the 10 POD genes, the expression of the acidic PODs, such as *swpa1*, *swpa2*, *swpa3*, and *swpa4*, was increased by Cd treatment, and the expression of *swpa5* and *swpa6* was decreased. Of the basic PODs, *swpb2* expression was strongly increased by Cd treatment. The expression of *swAPX1*, an APX derived from cultured cells, was increased by Cd treatment, whereas the expression of *swPRX1*, a peroxiredoxin (PRX) gene derived from cultured cells, was decreased by Cd treatment.

In previous studies, the expression of POD genes was increased by Cd treatment in sweetpotato plants (Kim et al. 2010). In particular, the expression of the acidic POD genes *swpa1*, *swpa2*, *swpa3*, and *swpa4* increased strongly. Additionally, the expression of all basic POD genes increased, with *swpb3* increasing strongly. Therefore, the expression of various POD genes is increased in cultured cells and plants by Cd treatment. It is thought that POD genes derived from cultured cells form an antioxidant defense mechanism against Cd-induced oxidative stress in sweetpotato cultured cells and plants. In previous studies, POD gene expression was confirmed under various abiotic and biotic stress conditions (Huh et al. 1997; Jang et al. 2004; Kim et al. 1999; 2007; 2010; Park et al. 2003). These results suggest that POD gene expression is related to the oxidative stress resistance mechanism induced by various environmental stress conditions. In future, detailed functional studies using acid and basic POD genes are needed. Overall, the present study has provided important data that allows us to better understand the mechanism of resistance to oxidative stress in plants.

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