Australian Journal of Crop Science AJCS
AJCS 6(5):846-853 (2012) ISSN:1835-2707

Cd^{2+} stress induces two waves of H_2O_2 accumulation associated with ROS-generating system and ROS-scavenging system in cultured tobacco cells

Jin-Fen Wen[1,2], Ming-hua Deng[3], Ming Gong[1*]

[1]College of Life Science, Engineering Research Center of Sustainable Development and Utilization of Biomass Energy of Education, Key Laboratory of Biomass Energy and Environmental Biotechnology of Yunnan Province, Yunnan Normal University, Kunming 650092, P.R.China
[2]College of Modern Agricultural Engineering, Kunming University of Science and Technology, Kunming 650224, P.R.China
[3]College of Horticulture and Landscape, Yunnan Agricultural University, Kunming 650201, P.R. China

***Corresponding author: gongming63@163.com, jinfenwen2000@yahoo.com.cn**

Abstract

Cadmium (Cd) stress at 1.0 mmol/L for 64 h induced two waves of hydrogen peroxide (H_2O_2) accumulation in cultured tobacco cells, which peaked at 30 min and about 48–56 h, respectively. Investigation of the changes of reactive oxygen species (ROS)-generating and ROS-scavenging enzyme activities indicated that tobacco cells actively produced the first wave of H_2O_2 accumulation, possibly by rapid activation of ROS-generating enzymes, including plasma membrane NADPH oxidase, by ironically and covalently-bound cell wall peroxidase (POD), cell wall polyamine oxidases and superoxide dismutase. However, prolonged Cd^{2+} treatment induced the second wave of H_2O_2 accumulation, accompanied by rapid increases in cell death rate and malondialdehyde content. The second H_2O_2 wave may have been produced passively due to decreased activities of the soluble POD, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reductase, and lowered content of reduced glutathione, caused by prolonged Cd^{2+} stress. Our results showed that H_2O_2 played dual roles in tobacco cells, acting as a signal molecule at relatively low concentration and triggering adaptive responses in the early phase of Cd^{2+} stress, and as a highly toxic molecule at high concentration inducing severe oxidative stress and leading to cell death.

Keywords: cadmium; H_2O_2; antioxidative system; oxidative stress; cultured tobacco cells.
Abbreviations: APX, ascorbate peroxidase; CAT, catalase; Cd^{2+}, cadmium; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2DCF-DA, 2,7,-dichlorodihydrofluorescein; MDA, malondialdehyde; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); PAO, polyamine oxidases; PM, plasma membrane; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

Introduction

Heavy metals are important environmental pollutants and their toxicity is a problem of increasing significance for ecological, evolutionary, nutritional and environmental reasons (Benavides et al., 2005). Cadmium (Cd) accumulates not only in the natural environment, but also the aquatic environment, through industrial activities such as mining, plating and metal refining (Choi et al., 2008). Cd has no known biological function and is extremely toxic, even at low concentration (Benavides et al., 2005). When taken up by plants, Cd directly or indirectly inhibits physiological processes such as light harvesting center, photosystem II (Krupa, 1988), chlorophyll metabolism (Hsu and Kao, 2004), plant–water relationships (Perfus-Barbeoch et al, 2002), nutrient levels (Sandalio et al, 2001), potassium uptake and cytokinin content (Veselov et al, 2003), resulting in poor growth and low biomass (Sanitá di Toppi and Gabbrielli, 1999; Benavides et al., 2005). At cellular level, Cd enhances lipid peroxidation, hydrogen peroxide (H_2O_2) generation, and alters the level of antioxidant enzymes and ascorbate–glutathione cycle metabolism (Anjum et al, 2011).
In plant, reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl radical (HO·) are normal by-products of various metabolic pathways and are also produced under stress conditions in various cellular compartments, including chloroplasts, mitochondria, peroxisomes, the endoplasmic reticulum and plasma membranes (PM) (Karuppanapandian et al, 2011). They are highly reactive and toxic molecules causing oxidative damage to DNA, proteins and lipids (Apel and Hirt, 2004). However, in recent years, a wide range of plant responses have been found to be triggered by ROS, such as the cell cycle, programmed cell death, stomatal closure, biotic and abiotic stress responses and development (Gill and Tuteja, 2010). These studies have revealed a dual role for H_2O_2 in plants acting as either toxic by-products of aerobic metabolism, or signal molecules to regulate growth, development and defense pathways, or both. However, there is little direct supporting evidence. Cd exposure can promote the generation of H_2O_2 in mung bean (Anjum, et al., 2011), potato (Stroiński and Zielezińska, 1997), scots pine (Schützendübel et al., 2001), rice (Kuo and Kao, 2004; Hsu and Kao, 2007), pea (Romero-Puertas et al., 2004), *Brassica juncea* L (Verma et al., 2008)and tobacco cells (Olmos et al., 2003; Garnier et al.,

2006). Some previous studies showed that under Cd^{2+} stress, H_2O_2 was a signaling molecule in plants (Kuo and Kao, 2004), and others suggested that it was toxic （Anjum, et al., 2011; Hsu and Kao, 2007）. The present study was designed to investigate the change pattern and possible pathways of H_2O_2 accumulation in tobacco suspension cells during a short-term Cd^{2+} exposure as well as a long-term Cd^{2+} stress to better understand physiological significance of H_2O_2 under Cd^{2+} stress.

Results

Two waves of H_2O_2 accumulation during Cd^{2+} stress

Cd^{2+} stress led to a transient increase of H_2O_2 concentration in cultured tobacco cells. When the cells were challenged with 0.5, 1.0 or 3.0 mM Cd^{2+}, a significant increase of H_2O_2 concentration was detected with 5 min, and a H_2O_2 peak occurred at 30, 30 and 60 min for 3.0, 1.0 and 0.5 mM Cd^{2+} stress, respectively (Fig. 1A).Furthermore, prolonged 1.0 mM Cd^{2+} treatment for 64 h led to a second peak of H_2O_2 accumulation after 32 h in cultured tobacco cells, which reached a maximum at about 48–56 h (Fig. 1B). The results showed that Cd^{2+} stress induced two waves of H_2O_2 accumulation, with the second wave higher and longer lasting than the first. To investigate the physiological importance of the two Cd^{2+}-induced waves of H_2O_2 accumulation, we examined the change in death rate of cultured tobacco cells under 1.0 mM Cd^{2+} stress. Cd^{2+} stress for 32 h led to a slight increase in cell death rate; thereafter the death rate rose rapidly and reached about 90% (Fig. 2). This sharp increase in cell death rate was consistent with the second wave of dramatic H_2O_2 accumulation (Fig. 1B).

Possible enzymatic pathways of Cd^{2+}-induced H_2O_2 production

NADPH oxidase transfers electrons from NADPH to O_2 to form $O_2^{\cdot-}$, followed by dismutation of $O_2^{\cdot-}$ to H_2O_2, and has been suggested to be the main producer of ROS in plants challenged with all kinds of environmental stress (Hao et al., 2008). The present results showed that 1.0 mM Cd^{2+} treatment induced a transient increase in NADPH oxidase activity, detectable as early as 10–30 min after Cd^{2+} treatment, and lasted progressively up to 12 h, then declined gradually (Fig. 3). Cell wall polyamine oxidase (PAO) oxidizes polyamines and directly releases H_2O_2. The Cd^{2+} stress led to a sharp increase of cell wall PAO activity within 10–90 min after Cd^{2+} treatment, then the PAO activity dropped rapidly, and even lowered below the control after 16 h (Fig. 4). Cell wall peroxidases (POD) are another extracellular way of producing H_2O_2 in plants, which generates H_2O_2 through oxidation of NADH (Ranieri et al., 2001). We detected the ironically and covalently-bound cell wall fractions of POD activity. The activity of ironically-bound cell wall POD was immediately stimulated by 1.0 mM Cd^{2+} treatment and reached a peak at about 30 min, then declined sharply (Fig. 5A). The Cd^{2+} stress also caused a rapid and relatively long-lasting increase of covalently-bound cell wall POD activity, which peaked at about 16–24 h, and then decreased gradually (Fig. 5B).

Cd^{2+} stress-induced oxidative stress and changes in the intracellular antioxidative system

Since Cd^{2+} treatment induced a significant accumulation of H_2O_2 (Fig. 1), which would lead to oxidative stress, we determined the change in MDA level, an indicator of

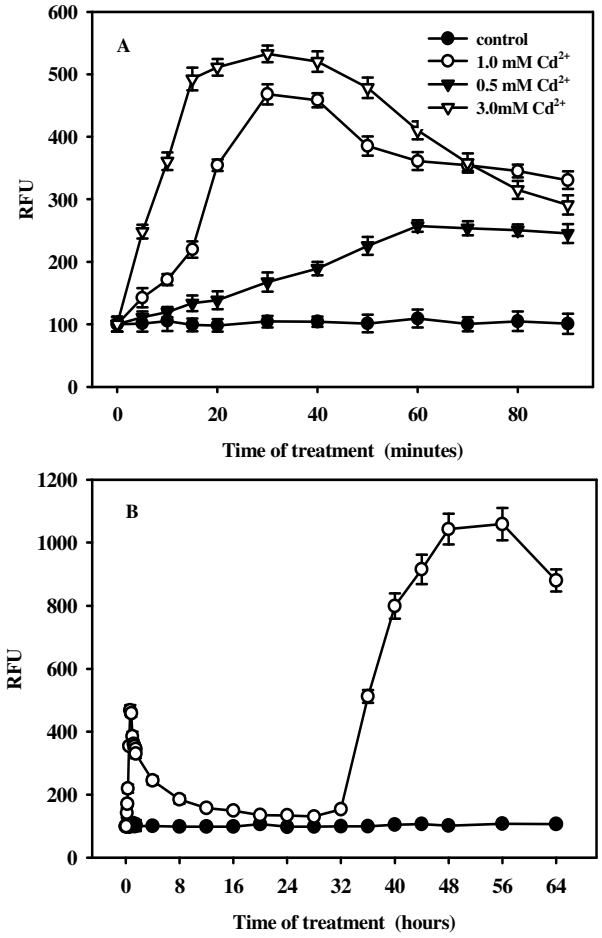

Fig 1. Cd^{2+} stress-induced two waves of H_2O_2 accumulation in cultured tobacco cells. A: transient change of H_2O_2 accumulation in cultured tobacco cells challenged with either 3.0, 1.0 or 0.5 mM Cd^{2+} with 90 min, and same volume of DDS water was added as the control; B: Prolonged change of H_2O_2 concentration in cultured tobacco cells during 1.0 mM Cd^{2+} stress for 64 h. Each point represents Means ± SD at last of three independent experiments. Indicate what does RFU correspond to (Y axis).

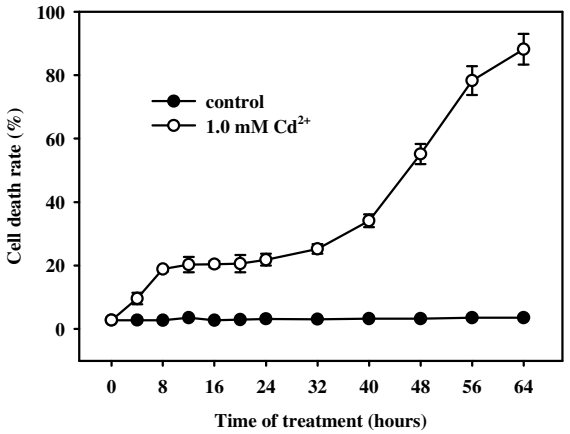

Fig 2. Change of death rate of cultured tobacco cells under Cd^{2+} stress at 1.0 mM.

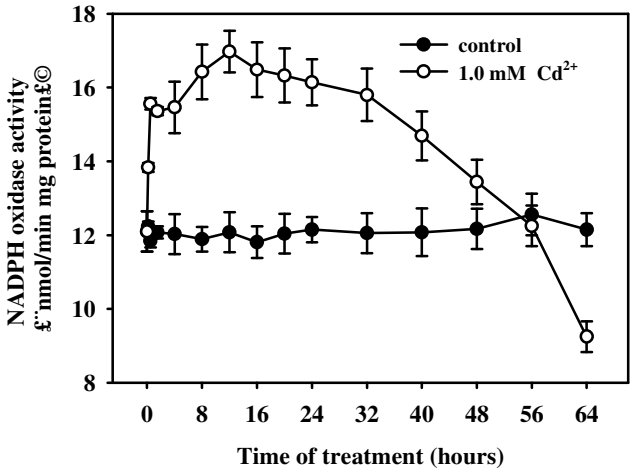

Fig 3. Change of Plasma membrane NADPH oxidase activity in cultured tobacco cells during Cd^{2+} stress at 1.0 mM.

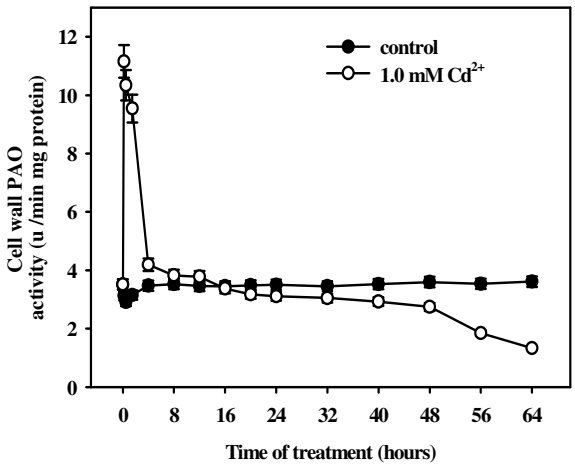

Fig 4. Change of cell wall polyamine oxidases (PAO) activity in cultured tobacco cells during Cd^{2+} stress at 1.0 mM.

membrane lipid peroxidation (Hodges et al., 1999). The 1.0 mM Cd^{2+} treatment firstly led to a gentle increase of MDA content in cultured tobacco cells; and this rise became rapid after 40 h (Fig. 6), consistent with the second wave of H_2O_2 accumulation (Fig. 1B). Plants possess very efficient antioxidative systems to scavenge ROS and protect them from destructive oxidative stress. The antioxidative system in plants comprises a variety of antioxidases and antioxidants (Posmyk et al., 2009). To investigate the relationship between the occurrence of the two H_2O_2 waves and changes in the antioxidative system in cultured tobacco cells under Cd^{2+} stress, we assayed activities of several main antioxidases and content of an antioxidant, reduced glutathione (GSH). The changes in activity of different antioxidases differentially responded to Cd^{2+} stress (Fig. 7, 8A–E). Of the main sources of H_2O_2 production, dismutation of O_2^- into H_2O_2 by superoxide dismutase (SOD), plays an important role in H_2O_2 production (Apel and Hirt, 2004). The 1.0 mM Cd^{2+} treatment led to a significant increase of SOD activity as early as 10–30 min, and this rise lasted up to 32 h, and then SOD activity declined rapidly (Fig. 7). Soluble POD activity in cultured tobacco cells

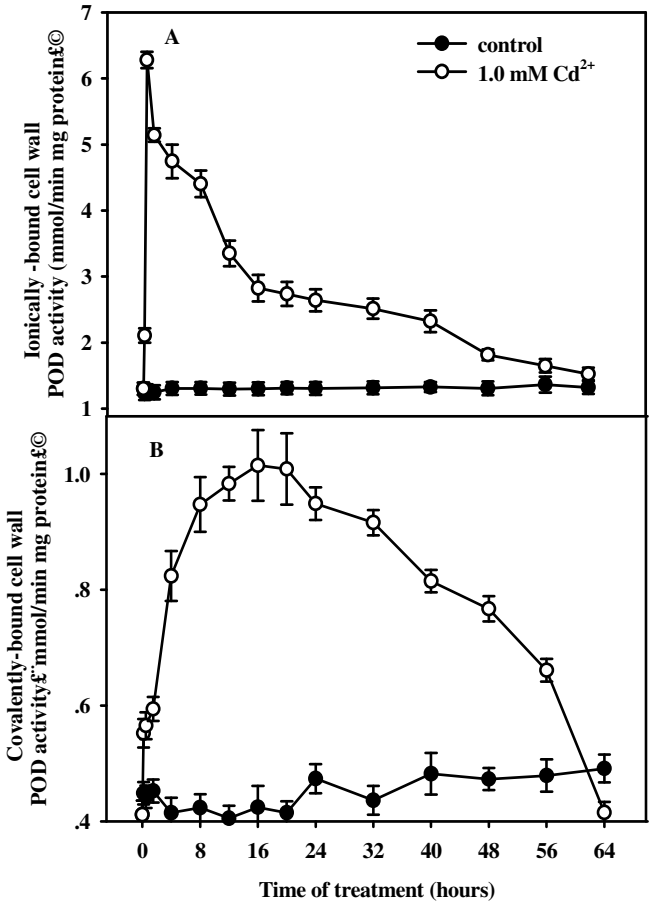

Fig 5. Change of cell wall peroxidase (POD) activity in cultured tobacco cells during Cd^{2+}stress at 1.0 mM, (A) ionically-bound cell wall POD, (B) covalently-bound cell wall POD.

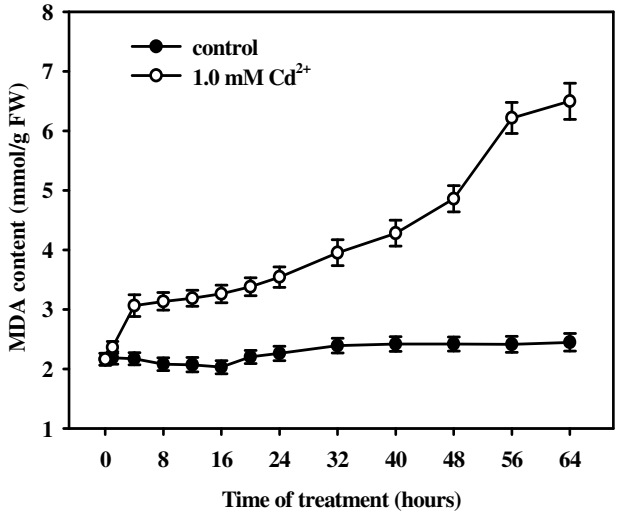

Fig 6. Change of malondialdehyde (MDA) content in cultured tobacco cells during Cd^{2+} stress at 1.0 mM.

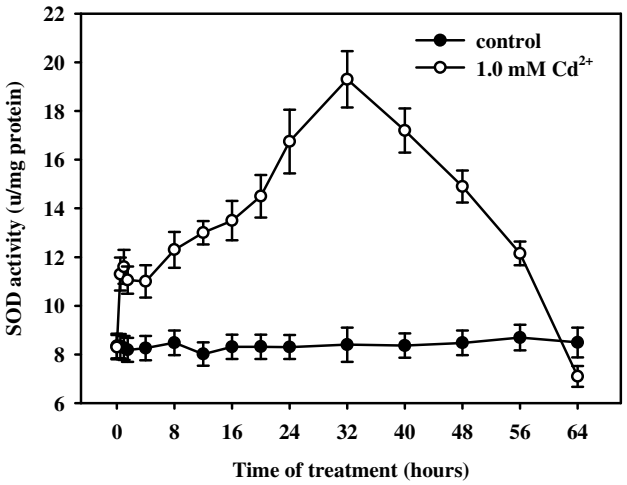

Fig 7. Change of superoxide dismutase (SOD) activity in cultured tobacco cells during Cd^{2+} stress at 1.0 mM.

rapidly increased in response to Cd^{2+} treatment, and reached 1.2-fold higher than controls at 4 h, and then declined sharply (Fig. 8A). Catalase (CAT) activity rose significantly and peaked at 20 h under Cd^{2+} stress, then dropped substantially with longer treatments of Cd^{2+} (Fig. 8B). Similar to the change in CAT activity, glutathione peroxidase (GPX) activity exhibited a gradual rise and reached a peak at 20 h under Cd^{2+} stress, and then declined (Fig. 8C). In addition, ascorbate peroxidase (APX) activity was immediately stimulated at 4 h under Cd^{2+} stress, then declined slightly until 20 h, increased sharply up to 32 h and was followed by a drastic drop (Fig. 8D). APX activity in Cd^{2+}-treated cultured tobacco cells at 32 h was about 10.5 times higher than in controls. Glutathione reductase (GR) activity responded rapidly to Cd^{2+} stress and increased up to 2.3 times higher than in controls at 16 h, and then declined gradually (Fig. 8E). The general trend for the abovementioned antioxidase activities was a significant increase in the early phase and gradual decrease in the late stage under Cd^{2+} stress at 1.0 mM (Figs. 7-8). GSH is the major low molecular weight thiol compound in most plants and acts as a disulfide reductant to protect thiol groups on enzymes, regenerate ascorbate and react with $^1O_2$ and HO· (Nagalakshmi and Prasad, 2001). Cd^{2+} stress firstly led to a significant increase of GSH content in the early phase, then a sharp decrease in the late phase (Fig. 9A). However, oxidized glutathione (GSSG) content exhibited only a slight rise in the early stage of Cd^{2+} stress, the increased rapidly in the late phase of Cd^{2+} stress (Fig. 9B). The GSH/GSSG ratio represents intracellular redox state (Foyer and Noctor, 2005), and showed a similar change to GSH content: firstly reaching a peak at 24 h, and then declining sharply with prolonged Cd^{2+} stress (Fig. 9C).

Discussion

H_2O_2 appears to be a central signal component of plant adaptation to both biotic and abiotic stresses (Gill and Tuteja, 2010). Previous studies have given several lines of evidence indicating that Cd exposure increased the production of H_2O_2 in tobacco, rice and *B. juncea* (Garnier et al., 2006; Hsu and Kao, 2007; Verma et al., 2008). Cd^{2+} was found to affect tobacco cells by a series of three waves of H_2O_2 (Garnier et al., 2006). Firstly, transient NADPH oxidase-dependent accumulation of H_2O_2 followed by accumulation of $O_2^{\cdot-}$ in mitochondria. A third wave of H_2O_2 consisted of fatty acid hydroperoxide accumulation. $CdCl_2$ treatment resulted in H_2O_2 production in detached rice leaves, and NADPH oxidase was found to be a H_2O_2-generating enzyme in $CdCl_2$-treated detached rice leaves （Hsu and Kao et al., 2007). An increased level of H_2O_2 in roots of Cd-treated seedlings of *B. juncea* was directly correlated with increased activity of ironically-bound cell wall POD (Verma et al., 2008). The present study indicated that H_2O_2 content under Cd^{2+} stress showed no single change, and that tobacco cells responded to Cd^{2+} treatment with two waves of H_2O_2 accumulation. The first wave peaked at 30 min, and the second at 48–56 h at a much higher level (Fig. 1). Thus an interesting question arises: what is the physiological importance of the two waves of H_2O_2 accumulation under Cd^{2+} stress? H_2O_2 production under stress conditions is often attributed to the complex up-regulation of several H_2O_2-generating enzymes (Hao et al., 2008).

Our studies showed that Cd^{2+} could immediately enhance the activities of NADPH oxidase, cell wall PAO, and ironically-bound and covalently-bound cell wall POD after a short period of Cd^{2+} treatment (Figs. 3–5). The results suggested that Cd^{2+} treatment could lead to the first wave of H_2O_2 accumulation by up-regulating the activities of the four enzymes involved in generating H_2O_2. The tobacco cells could actively produce the first wave of H_2O_2 accumulation, which could act as signal molecules to induce adaptive responses of the tobacco cells to Cd^{2+}stress, including up-regulation of the antioxidase activities and increasing antioxidant GSH content, as observed in the early phase of Cd^{2+} treatment (Figs. 7-9). ROS levels in plant tissues are elaborately controlled by ROS-scavenging enzymes as well as by non-enzymatic antioxidants (Gill and Tuteja, 2010, Čabala et al., 2010). With increased activities of antioxidases and enhanced content of the antioxidant GSH after short-term stress, the H_2O_2 level in tobacco cells declined gradually to control levels, which may be attributed to the enhanced activity of the antioxidative system induced by the short-term Cd^{2+} treatment and the consequent first wave of H_2O_2 accumulation (Fig. 1). Cd^{2+} is a highly toxic ion, and sustained Cd^{2+} exposure will disturb physiological and biochemical events and cause cell damage in plants (Benavides et al., 2005). In the present experiment, with prolonged Cd^{2+} stress for more than 28–32 h, activities of the antioxidases SOD, CAT, GPX, APX and GR declined rapidly (Figs. 7-8), and GSH content and GSH/GSSG ratio dropped significantly (Fig. 9). The second wave of H_2O_2 accumulation appeared after Cd^{2+} stress for 32 h (Fig. 1B) and was accompanied by a sharp increase in content of the lipid peroxidation product, MDA (Fig. 6), and a high death rate of tobacco cells (Fig. 2) – which could be caused by severe oxidative stress due to excess H_2O_2 accumulation in the second wave. In conclusion, the present results suggested that tobacco cells under Cd^{2+} stress could actively produce the first wave of H_2O_2 accumulation in the early phase by activation of enzymes involved in ROS generation. The first H_2O_2 wave was transitory and could play a signal role to activate adaptive response to Cd^{2+} stress. However, prolonged Cd^{2+} treatment led to decreased activity of the antioxidative system and disturbed equilibrium of intracellular redox state. The second wave of H_2O_2 accumulation was passively produced and followed by a high level of cell death. H_2O_2 obviously played dual roles in tobacco cells, acting as a signal molecule at relatively low concentration and triggering adaptive responses in the early phase of Cd^{2+} stress; and as a highly toxic molecule at high concentration and inducing severe oxidative stress and leading to cell death.

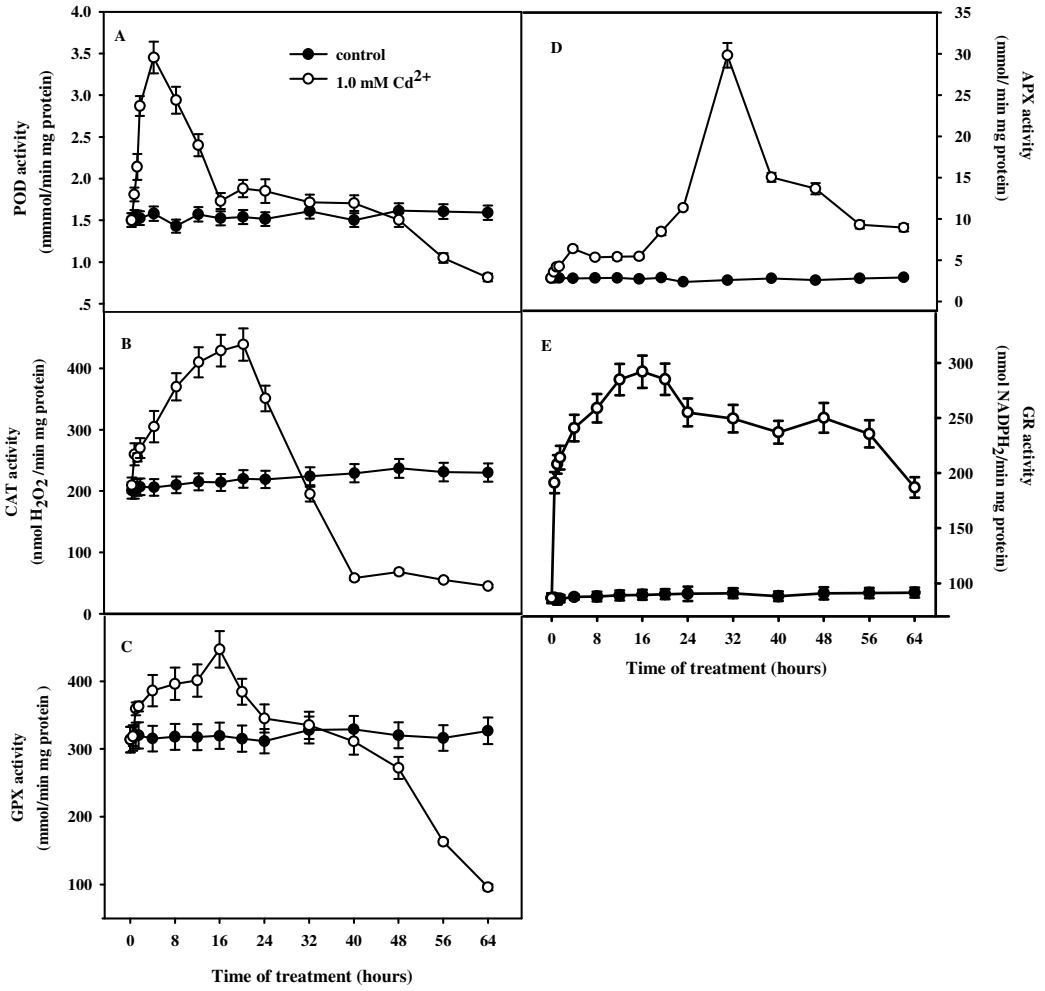

Fig 8. Change of **a**ntioxidase activities in cultured tobacco cells during Cd^{2+} stress at 1.0 mM. A: peroxidase (POD); B: catalase (CAT); C: glutathione peroxidase (GPX); D: ascorbate peroxidase (APX); E: glutathione reductase (GR)

Materials and methods

Plant material, culture and Cd^{2+} treatments

Tobacco (*Nicotiana tabacum cv*. Bright Yellow) callus was initiated from stem tissue. Sterile stem pieces were transferred to MS (Murashige and Skoog, 1962) agar medium (pH 5.8, Ca^{2+} concentration of 3 mmol/L) containing 2 μg/mL of glycine, 5 μg/mL of nicotinic acid, 10 μg/mL of pyridoxine, 10 μg/mL of thiamine hydrochloride, 1 mg/mL of casein acid hydrolysate, 3% (w/v) of sucrose, 2 μg/mL of 2,4-dichlorophenoxacetic acid and 0.1 μg/mL of kinetin. This was then incubated in darkness at 25°C for 2–4 weeks. Cultured tobacco cells were initiated from these calli into an identical liquid medium and incubated in a shaker incubator at 120 rpm at 25°C. After 7 d, 5.0 mL of the culture medium was taken and fresh medium was added to make up a volume of 50 mL. Cells were kept for 4 d under these conditions and were then processed. After the second sub-culturing, 4-d-old cultured tobacco cells were filtered through a mesh (pore size 40.0 μm) and 1.0 g (fresh weight) of the cells was resuspended in 25.0 mL of MS solution (pH 6.0) containing 200 mM sucrose. After 4 h, the cultured tobacco cells were submitted to assays on the effect of $CdCl_2$ (0–3.0 mM), and 1.0 mM $CdCl_2$ was chosen for the main experiments. Cells harvested at distinct periods were suction-dried and weighed for packed cell volume and stored at –80°C for further analyses.

H_2O_2 measurement

The generation of H_2O_2 was monitored by the method of Hao et al. (2008) with some modification. Briefly, 1.5 mL of the cultured tobacco cells treated with Cd^{2+} for distinct periods was incubated at a final concentration of 10 μM H_2DCF-DA for 15 min with shaking in darkness. The fluorescence of the reaction mixture was monitored immediately on a RF-5301 fluorescence spectrophotometer (Shimadzu, Japan) with 488 nm excitation wavelength and 525 nm emission wavelength.

Cell death assay

An aliquot of cultured tobacco cells was incubated with

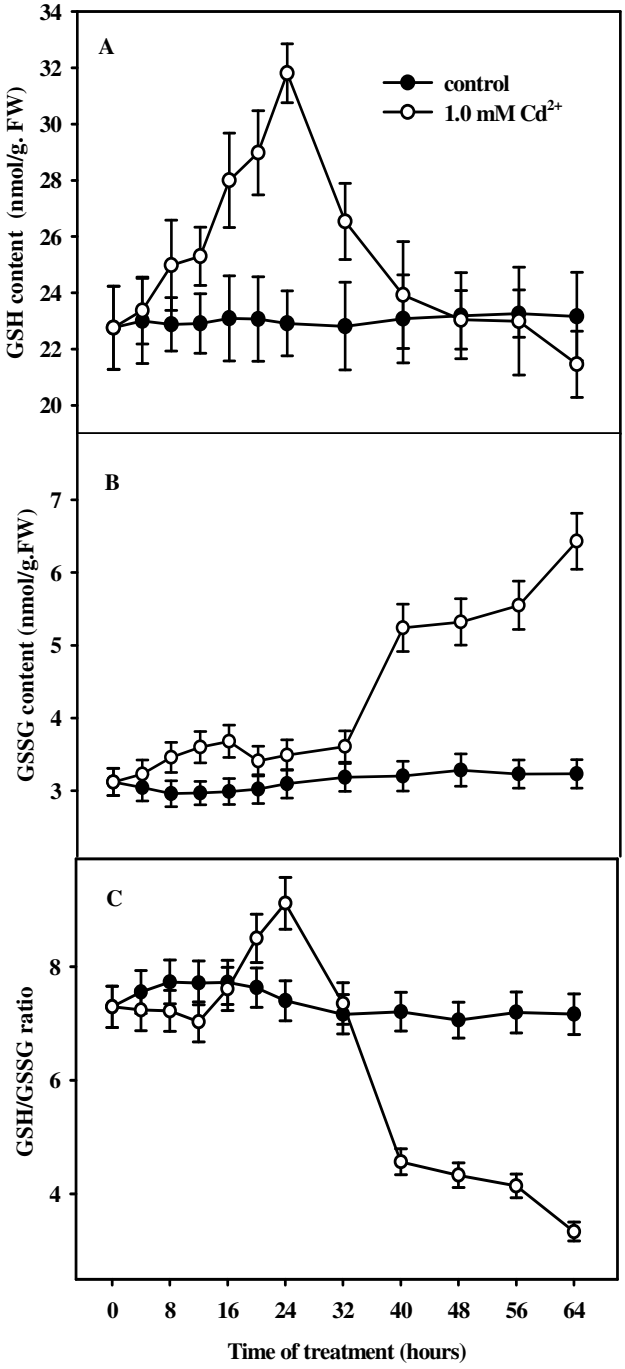

Fig 9. Change of reduced glutathione (GSH A), Oxidized glutathione (GSSG B), and GSH/GSSG ratio (C) in cultured tobacco cells during Cd^{2+} stress at 1.0 mM.

0.025% (w/v) Evans Blue at room temperature for 10 min and subsequently washed with 100 mM $CaCl_2$ (pH 5.6) solution. The stained dead cells were observed by light microscopy (Yano et al., 1999).

MDA assay

Lipid peroxidation in cultured tobacco cells was determined by estimating the MDA content according to the method of Hodges et al. (1999).

Determination of H_2O_2-generating enzymes

Determination of (PM) NADPH oxidase activity

PM was isolated using the two-phase aqueous polymer partition system (Hao et al., 2008). NADPH oxidase activity of the plasma membrane vesicles was assayed by measuring SOD inhibition and the NADPH-dependent reduction of sodium , 3'-[1-[phenylamino-carbony]-3,4-tetrazolium]-bis(4-methoxy-6 -nitro) benzenesulfonic acid hydrate by O_2^- according to the method of Hao et al. (2008).

Determination of cell wall PAO activity

The cell wall PAO activities were determined as described by Kaur-sawheny et al. (1981).

Determination of ionically and covalently-bound cell wall POD activity

The covalently and ionically-bound cell wall POD activities were determined as described by Ranieri et al. (2001).

Extraction and assay of antioxidative enzymes

Extraction of antioxidative enzymes

The cultured tobacco cells (0.1 g) were homogenized with 0.02 g of hydrated polyvinylpyrrolidone in 1 mL of 0.1 M phosphate buffer (pH 7.8) supplemented with 2 mM dithiothreitol, 0.1 mM ethylenediamine-tetraacetic acid and 1.25 mM PEG-4000 and centrifuged (16,000g for 15 min). The supernatant was then stored in separate aliquots at –86°C.

Assay of antioxidative enzymes

SOD (EC 1.15.1.1) activity was measured according to Giannopolitis and Ries (1977). One unit of SOD was defined as the enzyme activity that inhibited the photoreduction of nitroblue tetrazolium (NBT) to blue formazan by 50%, and SOD activity of the extracts was expressed as NBT units mg protein^{-1} min^{-1}. CAT (EC 1.11.1.6) activity was measured at 25°C according to Aebi (1984). CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm and expressed as nmol of H_2O_2 decomposed mg protein^{-1} min^{-1}. POD (EC 1.11.1.7) was assayed by the method of Chance and Maehly (1955) and expressed as mmol of guaiacol oxidized mg protein^{-1} min^{-1}. APX (EC 1.11.1.11) activity was measured at 25°C according to Nakano and Asada (1981). APX activity was determined by following the decrease in absorbance of ascorbate at 290 nm, and expressed as mmol of ascorbate oxidized mg protein^{-1} min^{-1}. GPX (EC 1.11.1.9) activity was determined at 30°C according to the method described by Nagalakshmi and Prasad (2001), and expressed as mmol of NADPH oxidized mg protein^{-1} min^{-1}. GR (EC 1.6.4.2) activity was determined at 25°C according to Halliwell and Foyer (1978), and expressed as nmol of NADPH2 oxidized mg protein^{-1} min^{-1}.

Assay of GSH contents

GSH contents were determined according to Nagalakshmi and Prasad (2001).

Determination of protein contents

Protein contents were measured as described by Bradford (1976) using bovine serum albumin as a standard.

Statistical analyses

Data were analyzed with Sigma Plot 8.0. The data reported in this paper are the mean ± standard deviation (SD) of three independent experiments.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (30460016).

References

Aebi H (1984) Catalase *in vitro*. Methods in Enzymology 105:121-126

Anjum NA, Vmar S, Iqbal M, Khan NA (2011) Cadmium causes oxidative stress in Mung Bean by affecting the antioxidant enzyme system and ascorbate-glutathione cycle metabolism. Russ J Plant Physl+ 58:92-99

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373-99

Benavides MP, Gallego SM. Tomaro ML, (2005) Cadmium toxicity in plants. Braz J Plant Physiol 17: 21-34

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248-254

Chance B, Maehly AC (1955) Assay of catalase and peroxidase. Methods in enzymology 2:764-775

Choi YK, Jo PG, Choi CY (2008) Cadmium affects the expression of heat shock protein 90 and metallothionein mRNAin the Pacific oyster, *Crassstrea gigas*. Comp Biochem Physiol C 147:286-292

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248-254

Čabala R, Slováková L, Zohri ME, Frank H (2010) Accumulation and translocation of Cd metal and the Cd-induced production of glutathione and phytochelatins in *Vicia faba* L. Acta Physiol Planta 33:1239-1248

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell 17:1866-1875

Garnier L, Simon-plas F, Thuleau P, Agnel J, Blein J, Ranjeva R, Montillet J (2006) Cadmium affects tobacco cells by a series of three waves of reactive oxygen species that contribute to cytotoxicity. Plant Cell Environ 29:1956-1969

Giannopolitis CN, Ries SK (1977) Superoxide dismutases. I. Occurrence in higher plants. Plant Physiol 54:309-314

Gill SS, Tuteja N (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909-930

Halliwell B, Foyer CH (1978) Properties and physiological function of a glutathione reductase purified from spinach leaves by affinity chromatography. Planta 139:9-17

Hao FS, Zhang JG, Yu ZL, Chen J (2008) Involvement of NADPH oxidase NtrbohD in the rapid production of H_2O_2 induced by ABA in cultured tobacco cell line BY-2. Prog Natl Sci 18:267-271

Hodges MD, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604-611

Hsu YT, Kao CH (2004) Cadmium toxicity is reduced by nitric oxide in rice leaves. Plant Growth Regul 42: 227-238

Hsu YT, Kao CH (2007) Toxicity in leaves of rice exposed to cadmium is due to hydrogen peroxide accumulation. Plant Soil 298:231-241

Karuppanapandian T, Moon J-C, Kim C, Manoharan K, Kim W (2011) Reactive oxygen species in plants: their generation, signal transduction, and scavenging mechanisms. Aust J Crop Sci 5:709-725

Kaur-sawheny R, Flores HE, Galston AW (1981) Polyamine oxidase in oat leaves a cell wall-localized enzyme. Plant Physiol 68:494-498

Krupa，Z (1988) Cadmium-induced changes in the composition and structure of the light-harvesting chlorophyll alb protein complex-II in radish cotyledons. Physiol Plantarum 73:518-524

Kuo MC, Kao CH (2004) Antioxidant enzyme activities are upregulated in response to cadmium in sensitive, but not in tolerant rice (*Oryza sativa* L.) seedlings. Bot Bull Acad Sin 45:291-299

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plantarum 15:473-479

Nagalakshmi N, Prasad MNV (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci 160:291-299

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol 22:867-880

Olmos EO, Martínez-Solano JR, Piqueras A, Hellín E (2003) Early steps in the oxidative burst induced by cadmium in cultured tobacco cells (BY-2 line). J Exp Bot 54:291-301

Pefus-Barbeoch, L, Leonhardt, N, Vavasseur, A, Forestier, C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J 32: 539-548

Posmyk MM, Kontek R, Janas KM (2009) Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotox Environ Safe 72:596-602

Ranier A, Castagna A, Baldan B, Soldatini GF (2001) Iron deficiency differently affects peroxidase isoforms in sunflower. J Exp Bot 354:25-35

Romero-Puertas MC, Rodriguez-Serrano M, Corpas FJ, Gómez M, del Río LA, Sandalio LM (2004) Cadmium induced subcellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in pea leaves. Plant Cell Environ 27:1122-1134

Sandalio LM, Dalurzo HC, Gómez M, Romero-Puertas MC, del Río LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 52:2115-2126

Sanitá di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41:105-130

Schützendübel A, Schwang P, Teichmann T, Gross K, Langenfeld-Heyer R, Godbold DL, Polle A (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in scots pine roots. Plant Physiol 127:887-898

Stroiński A, Zielezińska M (1997) Cadmium effect on hydrogen peroxide, gluthatione and phytochelatins levels in potato tuber. Acta Physiol Planta 19:2127-135

Verma K, Shekhawat GS, Sharma A, Mehta SK, Sharma V (2008) Cadmium induced oxidative stress and changes in soluble and ionically bound cell wall peroxidase activities in roots of seedling and 3 - 4 leaf stage plants of *Brassica juncea* (L.) czern. Plant Cell Rep 27:1261-1269

Veselov D, Kudoyarova G, Symonyan M, Veselov St (2003) Effect of cadmium on ion uptake, transpiration and cytokinin content in wheat seedlings. Bulg J Plant Physiol (S) 353-359

Yano A, Suzuki K, Shinshi HA (1999) Signaling pathway, independent of the oxidative burst, that leads to hypersensitive cell death in cultured tobacco cells includes a serine protease. Plant J 18:105-109