Journal of Plant Physiology 169 (2012) 478–486

Contents lists available at SciVerse ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Involvement of hydrogen peroxide in heat shock- and cadmium-induced expression of ascorbate peroxidase and glutathione reductase in leaves of rice seedlings

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ARTICLE INFO

Article history:
Received 9 September 2011
Received in revised form
28 November 2011
Accepted 30 November 2011

Keywords:
Ascorbate peroxidase
Cadmium
Glutathione reductase
Heat shock
Hydrogen peroxide
Oryza sativa

ABSTRACT

Hydrogen peroxide (H_2O_2) is considered a signal molecule inducing cellular stress. Both heat shock (HS) and Cd can increase H_2O_2 content. We investigated the involvement of H_2O_2 in HS- and Cd-mediated changes in the expression of ascorbate peroxidase (APX) and glutathione reductase (GR) in leaves of rice seedlings. HS treatment increased the content of H_2O_2 before it increased activities of APX and GR in rice leaves. Moreover, HS-induced H_2O_2 production and APX and GR activities could be counteracted by the NADPH oxidase inhibitors dipehenylene iodonium (DPI) and imidazole (IMD). HS-induced *OsAPX2* gene expression was associated with HS-induced APX activity but was not regulated by H_2O_2. Cd-increased H_2O_2 content and APX and GR activities were lower with than without HS. Cd did not increase the expression of *OsAPX* and *OsGR* without HS treatment. Cd increased H_2O_2 content by Cd before it increased APX and GR activities without HS. Treatment with DPI and IMD effectively inhibited Cd-induced H_2O_2 production and APX and GR activities. Moreover, the effects of DPI and IMD could be rescued with H_2O_2 treatment. H_2O_2 may be involved in the regulation of HS- and Cd-increased APX and GR activities in leaves of rice seedlings.

Introduction

Cadmium (Cd) is a non-essential heavy metal. The uptake and accumulation of Cd in plants represent the main entry pathway into humans and mammals. In plants, Cd causes severe physiological and morphological effects such as chlorosis and growth reduction. Cd is a non-redox metal unable to participate in Fenton-type reactions, but it causes oxidative stress by generating reactive oxygen species (ROS) (Garnier et al., 2006). ROS react with lipids, proteins, nucleic acids, and pigments and cause lipid peroxidation, membrane damage and inactivation of enzymes, which results in toxic effects. To protect against the toxic effects of these damaging ROS, plants have evolved highly regulated enzymatic and non-enzymatic mechanisms to keep a balance between ROS production and destruction for maintaining cellular redox homeostasis. Plants use antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) as well as antioxidants such as ascorbate (AsA) and glutathione (GSH) to scavenge ROS (Noctor and Foyer, 1998; Amudha and Balasubramani, 2011; Foyer and Noctor, 2011).

APX (EC 1.11.1.11) belongs to the class I heme-containing peroxidases found in higher plants (Takeda et al., 1998) and catalyzes the conversion of H_2O_2 to H_2O and O_2 with AsA used as the specific electron donor (Asada, 1999). GR (EC 1.6.4.2) is a flavoenzyme and has been found in all organisms examined. It catalyzes the reduction of oxidized glutathione (GSSG) to GSH with accompanying oxidation of NADPH. APX and GR, the first and last enzymes, respectively, in the AsA–GSH cycle, are responsible for ROS detoxification (Noctor and Foyer, 1998; Foyer and Noctor, 2011).

APX and GR cDNAs from various plant species have been cloned and their sequences can be found in GenBank. In plants, APX and GR are located in different cellular compartments. Eight types of APX have been described for *Oryza sativa*: 2 cytosolic (*OsAPX1* and *OsAPX2*), 2 putative peroxisomal (*OsAPX3* and *OsAPX4*), and 4 chloroplastic isoforms (*OsAPX5*, *OsAPX6*, *OsAPX7*, and *OsAPX8*) (Teixeira et al., 2004). Three genes encoding GR have also been described for *O. sativa*: a cytosolic isoform (*OsGR2*) (Kaminaka et al., 1998) and 2 chloroplastic isoforms (*OsGR1* and *OsGR3*) (Bashir et al., 2007).

Supplied Cd increases the activities of APX and GR in plants (Chaoui et al., 1997; Kuo and Kao, 2004; Smeets et al., 2005, 2008; Liu et al., 2007; Ekmekci et al., 2008; Nahakapam and Shah, 2011)

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; FW, fresh weight; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HS, heat shock; hsp, heat shock protein; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.

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doi:10.1016/j.jplph.2011.11.012

and the expression of *APX* and *GR* genes (Smeets et al., 2008; Tamas et al., 2008). However, a high concentration of Cd (50 μM) had no effect on the activities of APX and GR in pea plants (Romero-Puertas et al., 2007).

Exposure of plants to temperatures 5–15 °C above the normal growing conditions for approximately 15 min to a few hours is usually considered as heat shock (HS) treatment. HS pretreatment can increase the tolerance of plants to subsequent Cd stress (Neumann et al., 1994; Orzech and Burke, 1988; Kochhar and Kochhar, 2005; Hsu and Kao, 2007a). HS was involved in increased APX activity and expression of cytosolic *APX* (Mittler and Zilinskas, 1992; Karpinski et al., 1997; Sato et al., 2001), and increased GR activity in leaves of rice seedlings (Hsu and Kao, 2007a); however, whether HS treatment induces the expression of *GR* gene in rice or other plants is unknown.

Both HS and Cd increase H_2O_2 content (Gong et al., 2001; Romero-Puertas et al., 2004; Hsu and Kao, 2007a,b; Rodriguez-Serrano et al., 2009). Experiments with inhibitors suggested that the main source of H_2O_2 induced by HS and Cd could be an NADPH oxidase (Romero-Puertas et al., 2004; Rodriguez-Serrano et al., 2006; Volkov et al., 2006; Hsu and Kao, 2007a,b). Recently, researchers have focused on functional aspects of H_2O_2 generation. H_2O_2 is a constituent of oxidative metabolism. Because H_2O_2 is relatively stable and diffusible through the membrane, it is thought to constitute a general signal molecule inducing cellular stress (Nell et al., 2002). H_2O_2 increased APX and GR activities in maize leaves (Pastori and Trippi, 1992, 1993) and rice roots (Tsai et al., 2005) and the expression of gene encoding APX in germinating rice embryos (Morita et al., 1999) and *Arabidopsis* leaves (Karpinski et al., 1999). We also found that H_2O_2 treatment enhanced *OsAPX8*, *OsGR2* and *OsGR3* expression in rice roots (Hong et al., 2007, 2009). However, the failure of induction of *APX* and *GR* by H_2O_2 has also been reported (Babiychuk et al., 1995; Vansuyt et al., 1997; Xiang and Oliver, 1998).

In the present study, we examined the effect of HS and Cd on the activities of APX and GR and the expression of *OsAPX* and *OsGR* genes in leaves of rice seedlings. We aimed to determine whether HS- and Cd-mediated changes in APX and GR activities are associated with the expression of *OsAPX* and *OsGR* genes. HS and Cd induce H_2O_2 accumulation in leaves of rice seedlings (Kuo and Kao, 2004; Hsu and Kao, 2007a,b). H_2O_2 could be a candidate for signal transduction of oxidative stress. Thus, we examined the role of H_2O_2 in HS- and Cd-mediated changes in APX and GR activities and *OsAPX* and *OsGR* expression in rice seedlings.

Materials and methods

Plant material and treatments

Seeds of rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water, then germinated in a Petri dish on wet filter papers at 37 °C in the dark. After 48-h incubation, uniformly germinated seeds were cultivated in a beaker containing half-strength Kimura B solution (182.3 μM $(NH_4)_2SO_4$, 91.6 μM KNO_3, 273.9 μM $MgSO_4{\cdot}7H_2O$, 91.1 μM KH_2PO_4, 182.5 μM $Ca(NO_3)_2$, 30.6 μM Fe-citrate, 0.25 μM H_3BO_3, 0.2 μM $MnSO_4{\cdot}H_2O$, 0.2 μM $ZnSO_4{\cdot}7H_2O$, 0.05 μM $CuSO_4{\cdot}5H_2O$, and 0.07 μM H_2MoO_4; Kimura, 1931). Kimura B nutrient solution contains the desired nutrients for growing rice seedlings. Because we used young rice seedlings, the nutrient solution contained no silicon, although silicon is essential for growth of sturdy rice plants in the field.

Hydroponically cultivated rice seedlings were grown in a Phytotron (Agricultural Experimental Station, National Taiwan University, Taipei, Taiwan) with natural sunlight at 30/25 °C day/night and 90% relative humidity. Four beakers were used for each treatment, with 20 seedlings in each beaker. Nutrient solutions (pH 4.7) were replaced every 3 days. When the third leaves of rice seedlings were fully grown (12 days after sowing), the seedlings were treated with HS and 5 μM $CdCl_2$.

HS pretreatment and Cd stress treatment

Twelve-d-old rice seedlings with 3 leaves were exposed to 30 °C (non-HS) and 45 °C (HS) for 3 h in the dark, then grown in half-strength Kimura B solution with or without 5 μM $CdCl_2$ at 30/25 °C day/light. Cd toxicity was first shown in the second leaves of rice seedlings, so the second leaves were used for all chemical measurements and enzyme assays.

Determination of H_2O_2, chlorophyll, and malondialdehyde (MDA) content

H_2O_2 was extracted with sodium phosphate buffer (50 mM, pH 6.8) containing 1 mM hydroxylamine, a catalase inhibitor. H_2O_2 content was measured after reaction with $TiCl_4$ (Tsai et al., 2004). The blank reaction consisted of 50 mM phosphate buffer in the absence of leaf extracts. The absorbance was measured at 410 nm. H_2O_2 content was calculated by use of a standard curve prepared with known concentrations of H_2O_2. The chlorophyll content after extraction in 96% (v/v) ethanol was determined as described (Wintermans and Demots, 1965). Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined by the thiobarbituric acid reaction as described (Heath and Packer, 1968). The contents of H_2O_2, chlorophyll, and MDA were expressed on a basis of fresh weight (FW) basis of leaves before HS pretreatment or Cd treatment.

Extraction and APX and GR assays

Leaf samples were excised and immediately used for enzyme extraction. All operations were carried out at 4 °C. For enzyme extraction, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) with use of a chilled pestle and mortar. For analysis of APX activity, 2 mM AsA was added to the extraction buffer. The homogenate was centrifuged at 12,000 × *g*. APX activity was determined as described (Nakano and Asada, 1981). The decrease in AsA concentration was followed by a decrease in absorbance at 290 nm, and activity was calculated by the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for AsA. One unit of APX activity was defined as the amount of enzyme that degraded 1 μmol of AsA per min. GR activity was determined as described (Foster and Hess, 1980). One unit of GR was defined as the amount of enzyme that decreased 1 optical density min^{-1} at 340 nm. Enzyme activities were expressed on a milligram protein basis. The enzyme extracts were used for determination of protein by the Bradford method (Bradford, 1976).

Semi-quantitative RT-PCR analysis of OsAPX and OsGR genes

Total RNA was isolated from leaves by the TRIzol method (Invitrogen, CA, USA). To prevent DNA contamination, RNA was treated with Turbo DNase I (Ambion, TX, USA) for 30 min at 37 °C before RT-PCR. Control PCR amplifications involved use of RNA as a template after DNase I treatment to verify the elimination of contaminated DNA. Reverse-transcription reactions involved 200 ng total RNA and the SuperScript III first-strand synthesis RT-PCR system (Invitrogen, CA, USA).

Gene-specific primers were designed with the 3′UTR of the rice *OsAPX*, *OsGR*, and *Oshsp17.3* genes. The sequences used and the