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The involvement of hydrogen peroxide in abscisic acid-induced activities of ascorbate peroxidase and glutathione reductase in rice roots

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Abstract

We have monitored the changes in antioxidant enzyme activities and H_2O_2 concentrations in roots of rice (*Oryza sativa* L., cv. Taichung Native 1) seedlings treated with exogenous abscisic acid(ABA). Decrease in superoxide dismutase (SOD) and catalase (CAT) activities was observed in rice roots in the presence of ABA. However, ascorbate peroxide (APX) and glutathione reductase (GR) activities were increased after the ABA treatment. ABA treatment resulted in an increase in H_2O_2 concentrations in rice roots. Pretreatment with dimethylthiourea, a chemical trap for H_2O_2, and diphenyleneiodonium chloride (DPI), a well known inhibitor of NADPH oxidase, inhibited ABA-induced accumulation of H_2O_2 and ABA-induced activities of APX and GR. ABA-induced accumulation of H_2O_2 was found to be prior to ABA-induced activities of APX and GR. Our results suggest that H_2O_2 is involved in ABA-induced APX and GR activities in rice roots.

Abbreviations: ABA – abscisic acid; APX – ascorbate peroxidase; CAT – catalase; DMTU – dimethylthiourea; DPI – diphenyleneiodonium chloride; DW – dry weight; GR – glutathione reductase; SOD – superoxide dismutase

Introduction

The plant hormone abscisic acid (ABA) is a sesquiterpenoid synthesized from xanthophylls (Creelman 1989; Seo and Koshiba 2002) and appears to influence several physiological and developmental events (Creelman 1989; Kende and Zeevaart 1997). It has been shown that ABA can increase the generation of active oxygen species (AOS) such as O_2^- and H_2O_2 (Guan et al. 2000; Pei et al. 2000; Jiang and Zhang 2002, 2003; Hung and Kao 2003) and enhance the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase, ascorbate peroxidase (APX), and glutathione reductase (GR) in plant tissues (Anderson et al.1994; Bueno et al. 1998; Gong et al. 1998; Jiang and Zhang 2001, 2002; Hung and Kao 2003).

Recently, many researchers have focused on functional aspects of H_2O_2 generation. H_2O_2 is a constituent of oxidative metabolism and is itself an AOS. Because H_2O_2 is a relatively stable and diffusible through membrane, H_2O_2 is thought to constitute a general signal indicating cellular stress (Foyer et al. 1997; Neill et al. 2002). Earlier work demonstrated that ABA-induced expression of CAT gene in maize was mediated by increased H_2O_2 (Guan et al. 2000). H_2O_2 was

recently identified as a component of ABA signaling in guard cells (Zhang et al. 2001). It has also been shown that the ABA-induced antioxidant enzyme activities in maize leaves require the participation of H_2O_2 (Jiang and Zhang 2002, 2003).

We have previously shown that the reduction of root growth of rice seedlings by ABA is correlated with an increase in H_2O_2 concentration (Lin and Kao 2001). In this study, an effort was made to discover a possible link between H_2O_2 and antioxidant enzyme activities in roots of rice seedlings exposed to ABA. First of all, the change in the concentration of H_2O_2 and the activities of antioxidant enzymes such as SOD, CAT, APX and GR in roots of rice seedlings were monitored after the treatment of ABA. H_2O_2-manipulators, such as diphenyleneiodonium chloride (DPI), a well known inhibitor of NADPH oxidase (O_2^- synthase) (Levine et al. 1994; Bolwell et al. 1998; Papadakis and Roubelakis-Angelakis 1999; Pei et al. 2000; Orozco-Cardenas et al. 2001; Jiang and Zhang 2003), and dimethylthiourea (DMTU), a trap of H_2O_2 (Levine et al. 1994; Rao et al. 1997; Casano et al. 2001), were then used. The manipulation of H_2O_2 concentrations in the ABA-treated roots may help to assess the possible link between H_2O_2 and ABA-enhanced antioxidant enzyme activities.

Materials and methods

Plant material

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. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pre-treated at 37 °C for 1-day in darkness. Uniformly germinated seeds were selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No.1 filter paper moistened with 10 ml of distilled water for 2 days. Roots of 2-day-old seedlings were then treated with distilled water, ABA (9 μM) or H_2O_2 (10 mM). Root growth of rice seedlings grown in distilled water is similar to that grown in medium containing inorganic salts, thus seedlings grown in distilled water were used as the controls. For the experiments examining the role of H_2O_2 in regulating ABA-induced APX and GR activities in roots, DPI (0.1 μM) and DMTU (5 mM) were used. Each Petri dish contained 20 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27 °C in darkness.

H_2O_2 determination

The H_2O_2 concentration was colorimetrically measured as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenising roots with 3 ml of phosphate buffer (50 mM, pH 6.8) including 1 mM hydroxylamine. The homogenate was centrifuged at $6000 \times g$ for 25 min. To determine H_2O_2 concentrations, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride (Aldrich) in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at $6000 \times g$ for 15 min. The intensity of yellow colour of supernatant was measured at 410 nm. H_2O_2 concentration was calculated using the extinction coefficient 0.25 μmol^{-1} cm^{-1}. H_2O_2 concentration was expressed on the basis of tissue dry weight (DW).

Enzyme extraction and assays

For extraction of enzymes, root tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. For analysis of APX activity, 2 mM AsA was added to the extraction buffer. The homogenate was centrifuged at $12{,}000 \times g$ for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extraction procedure was carried out at 4 °C. CAT activity was assayed by measuring the initial rate of disappearance of H_2O_2(Kato and Shimizu 1987). The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) for H_2O_2. One unit of CAT was defined as the amount of enzyme, which degrades 1 nmol H_2O_2 per min. SOD was determined according to Paoletti et al. (1986). One unit of SOD was defined as the amount of enzyme that inhibits by 50% the rate of NADH oxidation observed in blank. APX

was determined according to Nakano and Asada (1981). The decrease in ascorbate concentration was followed as a decline in optical density at 290 nm and activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1} at 290 nm) for ascorbate. One unit of APX was defined as the amount of enzyme that degrades 1 μmol of ascorbate per min. GR was determined by the method of Foster and Hess (1980). One unit of GR was defined as the amount of enzyme that decreases 1 A_{340} per min. Activities of all enzymes were expressed on the basis of DW.

Statistical analysis

The results presented were the mean of four replicates. Means were compared by either Student's *t*-test or Duncan's multiple range test.

Results

The changes in antioxidant enzyme activities (SOD, CAT, APX and GR) in roots of rice seedlings after treatment of 9 μM ABA are presented in Figure 1. The ABA-treated roots had higher activities of APX and GR than the control (Figure 1b, c). On the contrary, the ABA-treated roots had lower activities of SOD and CAT than the control (Figure 1a, d).

ABA treatment caused an increase in H_2O_2 concentration in rice roots (Figure 2). The increase in H_2O_2 was evident 8 h after treatment of ABA. These results suggest that H_2O_2 may play an important role in regulating the increase of APX and GR activities in rice roots treated with ABA.

To test whether H_2O_2 is involved in ABA-induced APX and GR activities in roots of rice seedlings, DMTU, a chemical trap for H_2O_2 (Levine et al. 1994; Rao et al. 1997; Casano et al. 2001), was used. Roots of rice seedlings were pre-treated with or without DMTU for 12 h and then treated with 9 μM ABA for 24 h. As indicated in Figure 3a, when rice roots were pre-treated with DMTU, ABA-induced accumulation of H_2O_2 in rice roots was significantly reduced. DMTU pre-treatment was also observed to be effective in inhibiting the increase in the activities of

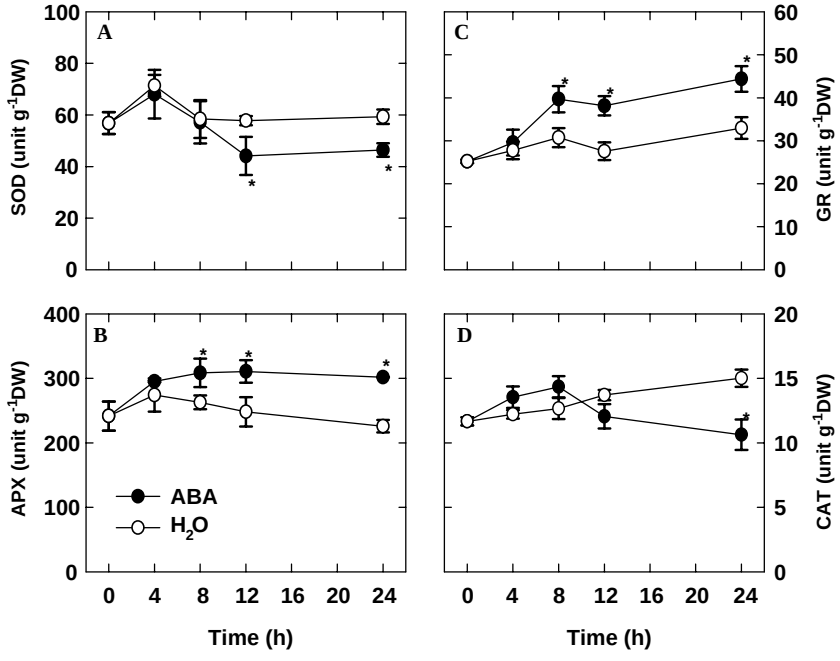

Figure 1. Changes in the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) in roots of rice seedlings in the presence or absence of ABA. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and 9 μM ABA, respectively. Asterisks indicate values that are significant at $p < 0.05$ by Student's *t*-test.

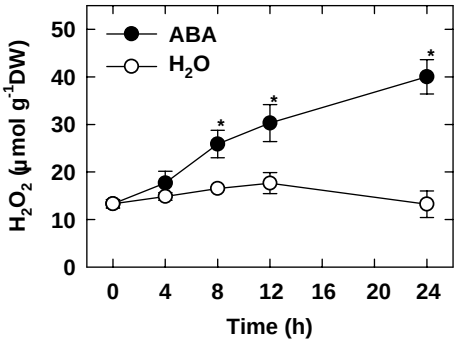

Figure 2. Changes in H_2O_2 concentrations in roots of rice seedlings in the presence or absence of ABA. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and 9 μM ABA, respectively. Asterisks indicate values that are significant at $p < 0.05$ by Student's *t*-test.

APX and GR in rice roots caused by ABA (Figure 3b, c).

AOS originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O_2 to form O_2^-, followed by dismutation of O_2^- to H_2O_2, has been a recent focus in AOS signaling. In several model systems investigated in plants, the oxidative burst and the accumulation of H_2O_2 appear to be mediated by the activation of plasma-membrane NADPH oxidase complex (Pei et al. 2000; Orozco-Cardenas et al. 2001; Jiang and Zhang 2002, 2003). DPI, a chemical inhibitor of the NADPH oxidase complex found in mammalian neutrophils, inhibits the pathogen-, elicitor-, wound-, and ABA-induced accumulation of H_2O_2 in plants (Levine et al. 1994; Bolwell et al. 1998; Papadakis and Roubelakis-Angelakis 1999; Pei et al. 2000; Orozco-Cardenas et al. 2001; Jiang and Zhang 2003). As shown in Figure 3a, when rice roots were pre-treated with 0.1 μM DPI, ABA-induced accumulation of H_2O_2 in rice roots was completely inhibited. DPI also inhibited ABA-enhanced APX and GR activities in rice roots (Figure 3b, c).

To study the effect of exogenous H_2O_2, 10 mM H_2O_2 was added to the root medium. The effect of exogenous H_2O_2 on the changes in the concentrations of endogenous H_2O_2 and the activities of APX and GR in rice roots is shown in Figure 4. The results indicated that exogenous H_2O_2 increased the concentrations of endogenous H_2O_2 and the activities of APX and GR in rice roots.

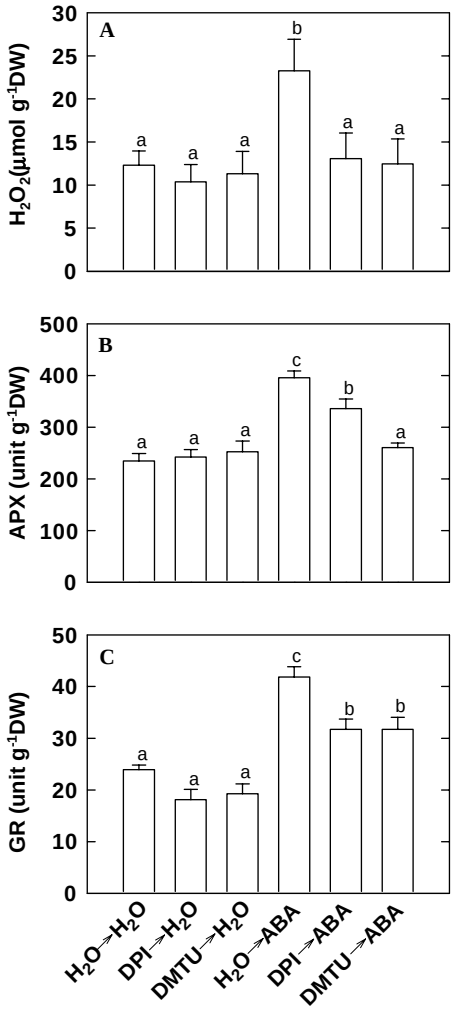

Figure 3. Effect of pre-treatments with dimethylthiourea (DMTU) and diphenyleneiodonium chloride (DPI) on the concentrations of H_2O_2 and the activities of ascorbate peroxidase (APX) and glutathione reductase (GR) in rice roots exposed to ABA. Two-day-old seedlings were pre-treated with 0.1 μM DPI and 5 mM DMTU, respectively, for 12 h and then treated with distilled water or 9 μM ABA for 24 h. Values with the same letter are not significantly different at $p < 0.05$, according to Duncan's multiple range test.

Discussion

The enzyme SOD brings about the dismutation of the radical O_2^- to H_2O_2, and plays an important role in protecting cells against the toxic effect of O_2^-. It has been shown that ABA treatment results in a significant increase in SOD activity in tobacco BY-2 cells (Bueno et al. 1998), maize seedlings (Jiang and Zhang 2001, 2002, 2003), and rice leaves

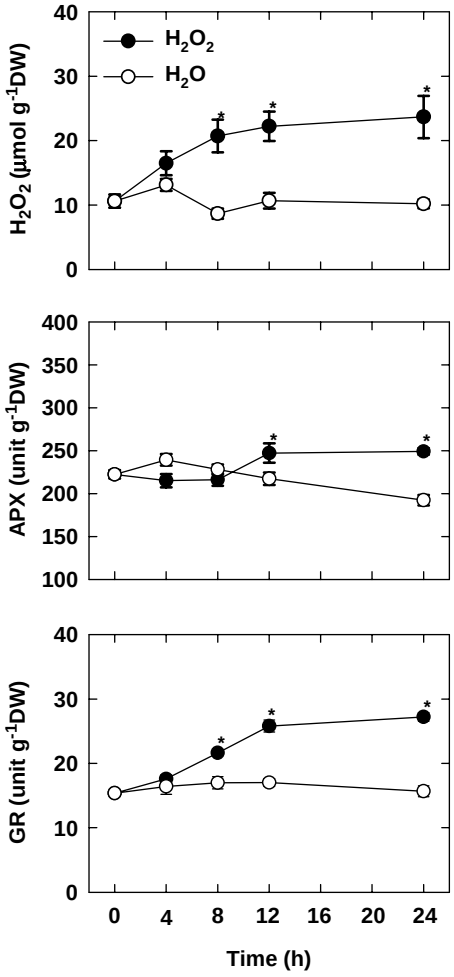

Figure 4. Change in the concentrations of H_2O_2 and the activities of ascorate peroxidase (APX) and glutathione reductase (GR) in roots of rice seedlings in the presence or absence of external H_2O_2. Rice seeds were germinated in distilled water for 2 days and then were transferred to distilled water and 10 mM H_2O_2, respectively. Asterisks indicate values that are significant at $p < 0.05$ by Student's *t*-test.

(Hung and Kao 2003). However, we observed that treatment with 9 μM ABA caused a decrease in SOD activity in rice roots (Figure 1a). CAT is known to dismutate H_2O_2 into H_2O and O_2 in plants. CAT activity appears to be increased by ABA in maize seedlings (Jiang and Zhang 2001, 2002, 2003). However, Bueno et al. (1998) demonstrated that CAT activity in tobacco BY-2 cells remained constant after the ABA treatment. In this study we observed that CAT was reduced in rice roots after 24 h of ABA treatment (Figure 1d).

The role of APX and GR in the H_2O_2 scavenging in plant cells has been well established in ascorbate–glutathione cycle (Foyer et al. 1997). APX activity in rice roots was increased by 9 μM ABA (Figure 1b). Similar results have been reported in maize seedlings (Jiang and Zhang 2001, 2002, 2003) and tobacco BY-2 cells (Bueno et al. 1998) subjected to ABA treatment. GR activity was increased in maize seedlings (Jiang and Zhang 2001, 2002, 2003) but was reduced in tobacco BY-2 cells after the ABA treatment (Bueno et al. 1998). In this study, we show that GR activity is enhanced by ABA in rice roots (Figure 1c).

ABA-induced H_2O_2 generation was first observed in guard cells (Pei et al. 2000; Zhang et al. 2001). In subsequent work, ABA-induced increases in H_2O_2 have been reported for maize seedlings (Jiang and Zhang 2002, 2003), rice leaves (Hung and Kao 2003) and rice roots (Lin and Kao 2001) (Figure 2). On the other hand, ABA decreased the release of H_2O_2 from germinating radish seeds (Schopfer et al. 2001). It appears that the increase in H_2O_2 generation is not a common response to ABA and this response is not confined to guard cells.

It has been shown that high concentration of DPI can affect other enzymes potentially involved in the generation of AOS, including extracellular peroxidase and nitric oxide synthase (Bolwell et al. 1998; Orozco-Cardenas et al. 2001; Schopfer et al. 2001). The fact that ABA-induced H_2O_2 accumulation can be inhibited by low concentration (0.1 μM) of DPI (Figure 3a) strongly suggests that ABA-dependent H_2O_2 generation originated, at least in part, from plasma membrane NADPH oxidase. Since inhibition of CAT was inhibited 24 h after the ABA treatment (Figure 1d), the possibility that CAT may contribute to H_2O_2 generation at a later stage of the ABA treatment cannot be excluded, but seems unlikely.

The present study indicated that H_2O_2 participated in the regulation of ABA-induced APX and GR activities in rice roots. This conclusion was based on observations that (a) ABA treatment induced generation of H_2O_2 in rice roots, (b) pre-treatment with DMTU inhibited ABA-induced generation of H_2O_2 and ABA-increased activities of APX and GR in rice roots, (c) pre-treatment with DPI reduced ABA-induced generation of H_2O_2 and ABA-increased activities of APX and GR in rice roots, and (d) exogenous application of H_2O_2 increased the concentrations of endogenous H_2O_2 and the activities in APX and GR in rice

roots. Since pre-treatment with DMTU and DPI completely reduced ABA-induced H_2O_2 generation but only partially inhibited ABA-increased APX and/or GR in rice roots, suggesting that other signal molecules may also participate in regulating ABA-increased APX and GR activities in rice roots.

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