Phosphatidylinositol 3-phosphate is required for abscisic acid-induced hydrogen peroxide production in rice leaves

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Abstract

Rice leaves produce H_2O_2 in response to abscisic acid (ABA), which results in induction of senescence and accumulation of NH_4^+ . The upstream steps of the ABA-induced H_2O_2 production pathway in rice leaves remain largely unclear. In animal cells, H_2O_2 production in neutrophils is activated by phosphatidylinositol 3-phosphate (PI3P), a product of phosphatidylinositol 3-kinase (PI3K). In the present study, we examined whether PI3P plays a role in H_2O_2 production in rice leaves exposed to ABA. We found that PI3K inhibitors LY 294002 (LY) or wortmannin (WM) inhibited ABA-induced H_2O_2 production, senescence and NH_4^+ accumulation. Hydrogen peroxide almost completely rescued the inhibitory effect of LY or WM. It appears that PI3P plays a role in ABA-induced H_2O_2 production, senescence, and NH_4^+ accumulation in rice leaves.

Abbreviations: ABA – abscisic acid; AOS – active oxygen species; DMSO – dimethyl sulfoxide; FW – fresh weight; GS – glutamine synthetase; LY – LY 294002; MDA – malondialdehyde; PAL – phenylalanine ammonia-lyase; PI3K – phosphatidylinositol 3-kinase; PI3P – phosphatidylinositol 3-phosphate; WM – wortmannin

Introduction

Recently, many researchers have focused on the functional aspect of H_2O_2 . H_2O_2 is a constituent of oxidative metabolism and is itself an active oxygen species (AOS). Because H_2O_2 is a small, diffusible, and ubiquitous molecule that can be synthesized, as well as destroyed, rapidly in response to external stimuli, it fulfills the important criteria for an intracellular messenger.

AOS, originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O_2 to form $O_2^{\cdot-}$, followed by dismutation of $O_2^{\cdot-}$ to H_2O_2 , has been a recent

focus in AOS signaling. The neutrophil NADPH oxidase consists of two plasma-membrane proteins, gp91^{phox} and p22^{phox}, which together form heterodimeric flavocytochrome b₅₅₈, and three cytosolic regulatory proteins, p40^{phox}, p47^{phox}, and p67^{phox}, which translocate to the plasma-membrane after stimulation to form the active complex (Segal and Abo 1993; Henderson and Chappell 1996). Plant homologues of the animal NADPH oxidase protein subunits have been identified, and some of their genes have been sequenced (Xing et al. 1997; Keller et al. 1998; Torres et al. 1998; Sagi and Fluhr 2001). These observations have demonstrated the presence of a plant NADPH oxidase resembling

the neutrophil NADPH oxidase. In fact, in several plants systems investigated, the oxidative burst and the accumulation of H_2O_2 are mediated by the activation of plasma-membrane NADPH oxidase (Ogawa et al. 1997; del Río et al. 1998; Potikha et al. 1999; Pei et al. 2000; Orozco-Cárdenas et al. 2001; Jiang and Zhang 2002; Hung and Kao 2004).

Since the discovery in the early to mid-1980s, the enzyme responsible for D-3 phosphorylation of phosphoinositides, that is the phosphatidylinositol 3-kinase (PI3K), has been the subject of intensive study (Charpenter and Cantley 1996). There are three types of PI3K in animal cells (Toker and Cantley 1997). In plants, only type III PI3K has been reported (Hong and Verma 1994; Welters et al. 1994; Bunney et al. 2000). It has been shown that NADPH oxidase is activated by binding phosphatidylinositol 3-phosphate (PI3P), a product of PI3K, to the PX domain of $p40^{phox}$ (Ellson et al. 2001). Jung et al. (2002) demonstrated that guard cells overexpressing PI3P-binding protein resulted in decreased stomatal closure in response to ABA. They also showed the similar effects in guard cells treated with PI3K inhibitors wortmannin (WM) or LY 294002 (LY) and concluded that PI3P is required for ABA-induced stomatal closure (Jung et al. 2002). This conclusion is supported further by the recent observation that H_2O_2 partially reversed the effect of WM or LY on ABA-induced stomatal closure (Park et al. 2003).

We have previously shown that ABA not only increases the content of H_2O_2 , but also promotes senescence (protein loss) and induces lipid peroxidation, as judged by the content of malondialdehyde (MDA), in rice leaves (Hung and Kao 2003). Recently, we also found that NADPH oxidase is an enzyme responsible for H_2O_2 generation in ABA-treated rice leaves and H_2O_2 is involved in ABA-induced senescence of rice leaves (Hung and Kao 2004). In this paper, we investigated the possibility that PI3P, as found in animal cells and guard cells, activates H_2O_2 generation in ABA-treated rice leaves by using PI3K inhibitors WM and LY.

Materials and methods

Plant material and chemicals

Rice (*Oryza sativa* L., cv. Taichung Native 1) was sterilized with 2.5% sodium hypochlorite for

15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes with wetted filter paper at 37 °C under dark conditions. After 48 h incubation, uniformly germinated seeds were selected and cultivated in a 500 ml beaker containing half-strength Kimura B solution as described previously (Chu and Lee 1989). The hydroponically cultivated seedlings were grown for 12 days in a Phytotron with natural sunlight 30 °C day/25 °C night and 90% relative humidity. The apical 3 cm of the third leaf was used in all experiments. A group of ten segments was floated in a Petri dish containing 10 ml of test solution. Incubation was carried out at 27 °C in the dark.

Test solutions included ABA (45 μ M), H_2O_2 (1 mM), and PI3K inhibitors, LY (100 μ M) and WM (1 μ M). All chemicals were purchased from Sigma Co. (St. Louis, MO, USA). LY and WM stock solutions were prepared in 100% dimethyl sulfoxide (DMSO).

Determinations of protein, H_2O_2 , lipid peroxidation, and NH_4^+

For protein extraction, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at $17,600 \times g$ for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976). The H_2O_2 content was measured colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing leaf tissue with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at $6000 \times g$ for 25 min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium sulphate in 20% (v/v) H_2SO_4 . The mixture was then centrifuged at $6000 \times g$ for 25 min. The absorbance was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient $0.28 \mu\text{mol}^{-1} \text{cm}^{-1}$. MDA, routinely used as an indicator of lipid peroxidation, was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968). NH_4^+ was extracted and its concentration determined as described previously (Chien and Kao 2000).

Enzyme assays

For extraction of glutamine synthetase (GS), leaf samples were homogenized with 10 mM Tris-HCl buffer (pH 7.6, containing 1 mM MgCl_2 , 1 mM EDTA and 1 mM 2-mercaptoethanol) using a chilled pestle and mortar. The homogenate was centrifuged at $15,000 \times g$ for 30 min and the resulting supernatant was used for determination of GS activity. The whole extraction procedure was carried out at 4 °C. GS was assayed by the method of Oak et al. (1980). The reaction mixture contained in a final volume of 1 ml was 80 μmol Tris-HCl buffer, 40 μmol L-glutamic acid, 8 μmol ATP, 24 μmol MgSO_4 , and 16 μmol NH_2OH , the final pH was 8.0. The reaction was started by addition of the enzyme extract and, after incubation for 30 min at 30 °C, was stopped by adding 2 ml 2.5% (w/v) FeCl_3 and 5% (w/v) trichloroacetic acid in 1.5 N HCl. After centrifugation the absorbance of the supernatant was read at 540 nm. One unit of GS activity is defined as 1 μmol L-glutamate γ -monohydroxamate formed per minute. Phenylalanine ammonia-lyase (PAL) was extracted and determined according to Hyodo and Fujinami (1989). The calculation was based on the extinction coefficient ($9500 \text{ M}^{-1} \text{ cm}^{-1}$) for *trans*-cinnamic acid. One unit of activity for PAL was defined as the amounts of enzyme which caused the formation of 1 nmol *trans*-cinnamic acid per hour. For protease extraction, leaf samples were homogenized in prechilled pestle and mortar with 10 mM Tris-HCl buffer (pH 7.4) containing 10 mM 2-mercaptoethanol at 4 °C. The homogenate was centrifuged at $15,000 \times g$ for 30 min and the resulting supernatant was used for protease assay. Protease was assayed according to the method described by Sheoran and Garg (1978). One unit of protease activity was defined as the amount of enzyme which increased 0.01 A_{280} per hour.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or on different times were analyzed following Duncan's multiple range test.

Results

The most obvious character of leaf senescence is yellowing. Chlorophyll loss has long been considered the principal criterion of senescence. The protein breakdown that occurs during leaf senescence has been recognized since the earliest studies performed. We have shown that protein breakdown precedes chlorophyll loss during rice leaf senescence (Kao 1980). Thus, senescence of rice leaves in the present investigation was followed by measuring the decrease of protein.

LY and WM have been shown to be inhibitors for PI3K in animal cells (Vlahos et al. 1994; Ui et al. 1995). These inhibitors also affected production of PI3P in *Arabidopsis* protoplasts (Kim et al. 2001). Figure 1 shows that the effect of LY or WM concentration on protein content of rice leaves in the presence or absence of 45 μM ABA. ABA alone decreased protein content in rice leaves, indicating that ABA is effective in promoting senescence of rice leaves. LY or WM alone had no effect on protein content. When applied together with ABA, LY concentrations between 10 and 100 μM produced a clear protection against protein loss. The optimal concentration of LY in inhibiting ABA-promoted senescence of rice leaves was observed to be 100 μM . WM at 1 μM was observed to be equally effective as 10 μM in inhibiting ABA-promoted protein loss in rice leaves. Visual observation indicated that appearance of rice leaves treated with ABA + LY or ABA + WM is basically similar to that of control leaves, indicating that the effect of LY or WM on ABA-induced senescence is biologically significant.

MDA is routinely used as an indicator of lipid peroxidation. It is known that ABA is effective in causing lipid peroxidation in rice leaves (Hung and Kao 2003). Recently, we demonstrated that H_2O_2 is involved in ABA-induced lipid peroxidation of rice leaves (Hung and Kao 2004). Thus, we investigated the effect of LY or WM on ABA-induced MDA and H_2O_2 production in rice leaves. Detached rice leaves were treated with 45 μM ABA, 100 μM LY, 1 μM WM or 0.2% (w/v) DMSO (solvent control) and the contents of MDA and H_2O_2 were determined 2 days after treatment. LY or WM alone had no effect on MDA and H_2O_2 contents in rice leaves (Figures 2a, b). However, LY or WM significantly reduced the

Figure 1. Effect of LY or WM concentration on protein content in rice leaves treated with or without ABA. Protein content was determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $p < 0.05$ level, according to Duncan's multiple range test.

ABA-induced MDA and H_2O_2 production in rice leaves (Figures 2a, b).

NH_4^+ is a central intermediate of nitrogen metabolism (Mifflin and Lea 1976). GS is the key enzyme in NH_4^+ assimilation and catalyzes that ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine (Mifflin and Lea 1976). GS in plants has been reported to be particularly prone to proteolysis under oxidation stress conditions (Ortega et al. 1999; Palatnik et al. 1999; Ishida et al. 2002). PAL catalyzes the elimination of NH_4^+ from phenylalanine and the production of *trans*-cinnamate (Hahlbrock and Grisebach 1979). NH_4^+ released from PAL reaction is known to be trapped in glutamine molecule by the action of GS (Sakurai et al. 2001). Our recent work indicated that ABA-induced NH_4^+ accumulation is attributable to the ABA-induced increase in the specific activity of PAL and protease and decrease in the activity of GS in rice leaves, and meanwhile, H_2O_2 has been shown to be required for the ABA-induced NH_4^+ accumulation in rice leaves (Hung and Kao 2005). It seems that in rice leaves one of the earliest events following ABA treatment is the generation of H_2O_2 , which then causes changes in PAL, GS and protease and subsequent accumulation of NH_4^+ (Figure 3). If PI3P indeed plays a role in the ABA-induced H_2O_2 generation in rice leaves, then NH_4^+ accumulation caused by ABA are expected to be inhibited by LY or WM. As shown in Figure 4d, it is indeed the case. LY or WM treatment was also observed to be effective in inhibiting the decrease in the activity of GS (Figure 4a) and the increase in the specific activities of protease (Figure 4b) and PAL (Figure 4c) in rice leaves caused by ABA.

Figure 2. Effect of LY or WM on the contents of MDA (a) and H_2O_2 (b) in ABA-treated rice leaves in the presence or absence of H_2O_2 . The concentrations of ABA, LY, WM, and H_2O_2 were 45 μ M, 100 μ M, 1 μ M, and 1 mM, respectively. All measurements were determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $p < 0.05$ level, according to Duncan's multiple range test.

If H_2O_2 generation is the primary function of PI3P in the ABA-induced senescence and NH_4^+ accumulation, then H_2O_2 should be able to bypass

Figure 3. Regulation of NH₄⁺ accumulation in ABA-treated rice leaves. $\rightarrow$ Stimulation of the reaction; $\Rightarrow$ inhibition of the reaction.

a deficiency of PI3K activity and induce senescence and NH₄⁺ accumulation. Indeed, H₂O₂ (1 mM) almost completely reverses the effect of LY or WM on the ABA-induced decrease in protein content (data not shown) and increase in NH₄⁺ content (Figure 4d). We also observed that H₂O₂ reversed the effect of LY or WM on ABA-induced increase in MDA content (Figure 2a), decrease in the activity of GS (Figure 4a), and increase in the specific activities of protease (Figure 4b) and PAL (Figure 4c) in rice leaves.

Discussion

In recent years there has been a growing interest in the functional aspects of H₂O₂. H₂O₂ is a constituent of oxidative metabolism and is itself an AOS. In addition, in view of characteristics of H₂O₂, which can be generated in normal cells, and is relatively stable and diffusible through cell membranes (Foyer et al. 1997; del Río et al. 1998), it is suitable to act as a signal molecule in cell function. It has been shown that H₂O₂ per se promotes senescence and NH₄⁺ accumulation in rice leaves (Lin and Kao 1998; Hung and Kao 2005). There are reports indicating that H₂O₂ is required for ABA-induced senescence and NH₄⁺ accumulation in rice leaves (Hung and Kao 2004, 2005). Induction of senescence and NH₄⁺ accumulation caused by ABA was found to be preceded by an increase in endogenous H₂O₂ content in rice leaves (Hung and Kao 2003, 2005). Recently, we were able to show that NADPH oxidase in rice leaves is involved

Figure 4. Effect of LY or WM on the activities of GS (a), the specific activities of protease (b) and PAL (c), and the content of NH₄⁺ (d) in ABA-treated rice leaves in the presence or absence of H₂O₂. The concentrations of ABA, LY, WM, and H₂O₂ were 45 μ M, 100 μ M, 1 μ M, and 1 mM, respectively. All measurements were determined 2 days after treatment in the dark. Values with the same letter are not significantly different at $p < 0.05$ level, according to Duncan's multiple range test.

ABA-induced H_2O_2 production (Hung and Kao 2004). Inhibitors of NADPH oxidase such as diphenyleneiodonium chloride (DPI) and imidazole (IMD) prevented ABA-induced H_2O_2 production and inhibited ABA-promoted senescence, ABA-induced accumulation of NH_4^+ , and ABA-caused changes in enzymes (PAL, protease, and GS) responsible for NH_4^+ accumulation in rice leaves (Hung and Kao 2004; 2005).

In the present study, we investigated the role of PI3P in ABA-induced H_2O_2 production in rice leaves using PI3K inhibitors. It appears that PI3P is important in ABA-induced H_2O_2 production based on two lines of evidence. First, two PI3K inhibitors, LY or WM, commonly inhibited the ABA-induced H_2O_2 production (Figure 2b). Second, H_2O_2 reversed the inhibitory effect of the PI3K inhibitors on the ABA-induced senescence (data not shown), lipid peroxidation (Figure 2a), NH_4^+ accumulation (Figure 4d), increase in the specific activities of protease (Figure 4b) and PAL (Figure 4c), and decrease in the activity of GS (Figure 4a). PI3P has also been shown to be important in the ABA-induced H_2O_2 generation in guard cells (Jung et al. 2002; Park et al. 2003). It appears that a role of PI3P in the ABA-induced H_2O_2 generation is not confined to guard cells.

NADPH oxidase is known to be H_2O_2 -generating enzyme in the ABA-treated rice leaves (Hung and Kao 2004). In neutrophils, PI3P regulates H_2O_2 production by binding to the noncatalytic component p40^{phox} of the NADPH oxidase (Ellson et al. 2001). However, a rice homolog of p40^{phox} has not been reported. Therefore, the detailed mechanism of the action of PI3P during H_2O_2 production in rice leaves await further investigation.

The fact that PI3K inhibitors, which reduced the ABA-induced H_2O_2 production (Figure 2b), were able to prevent the ABA-induced senescence (Figure 1) and NH_4^+ accumulation (Figure 4d) of rice leaves strengthens further our previous conclusion that H_2O_2 is necessary for ABA-induced senescence and NH_4^+ accumulation of rice leaves (Hung and Kao 2004, 2005).

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