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Hydrogen peroxide is required for abscisic acid-induced NH_4^+ accumulation in rice leaves

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Summary

The role of H_2O_2 in abscisic acid (ABA)-induced NH_4^+ accumulation in rice leaves was investigated. ABA treatment resulted in an accumulation of NH_4^+ in rice leaves, which was preceded by a decrease in the activity of glutamine synthetase (GS) and an increase in the specific activities of protease and phenylalanine ammonia-lyase (PAL). GS, PAL, and protease seem to be the enzymes responsible for the accumulation of NH_4^+ in ABA-treated rice leaves. Dimethylthiourea (DMTU), a chemical trap for H_2O_2, was observed to be effective in inhibiting ABA-induced accumulation of NH_4^+ in rice leaves. Inhibitors of NADPH oxidase, diphenyleneiodonium chloride (DPI) and imidazole (IMD), and nitric oxide donor (*N-tert*-butyl-α-phenylnitrone, PBN), which have previously been shown to prevent ABA-induced increase in H_2O_2 contents in rice leaves, inhibited ABA-induced increase in the content of NH_4^+. Similarly, the changes of enzymes responsible for NH_4^+ accumulation induced by ABA were observed to be inhibited by DMTU, DPI, IMD, and PBN. Exogenous application of H_2O_2 was found to increase NH_4^+ content, decrease GS activity, and increase protease and PAL-specific activities in rice leaves. Our results suggest that H_2O_2 is involved in ABA-induced NH_4^+ accumulation in rice leaves.

Introduction

NH_4^+ is a central intermediate of nitrogen metabolism (Miflin and Lea, 1976). Glutamine synthetase (GS) is the key enzyme in NH_4^+ assimilation and catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine (Miflin and Lea, 1976). Phenylalanine ammonia-lyase (PAL) catalyzes the elimination of NH_4^+ from phenylalanine and produces *trans*-cinnamate (Hahlbrock and

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Abbreviations: ABA, abscisic acid; AOS, active oxygen species; c-PTIO, 2-(4-carboxy-2-phenyl)-4, 4, 5, 5-tetramethylimidazoline-1-oxyl-3-oxide; DMTU, dimethylthiourea; DPI, diphenyleneiodonium chloride; FW, fresh weight; GS, glutamine synthetase; IMD, imidazole; NO, nitric oxide; PAL, phenylalanine ammonia-lyase; PBN, *N-tert*-butyl-α-phenylnitrone

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Grisebach, 1979). NH_4^+, released from PAL reaction, is known to be trapped in the glutamine molecule by the action of GS (Razel et al., 1996; van Heerden et al., 1996). Sakurai et al. (2001) provided evidence to show that GS is partly coupled to the reaction of PAL in developing rice leaves. Cd-induced NH_4^+ accumulation in rice leaves has been proved to be associated with the decrease in GS activity and the increase in PAL-specific activity (Hsu and Kao, 2004).

It is generally believed that GS activity in plants is regulated at the transcriptional level (Hirel et al., 1987; Forde et al., 1989; Edwards et al., 1990; Roche et al., 1993; Sukanya et al., 1994). Beside transcriptional regulation, GS activity in plants might also be regulated at the level of turnover. Oxidative modification of GS has been implicated as the first step in the turnover of GS in bacteria (Levine, 1983; Rivett and Levine, 1990). Stieger and Feller (1997) have shown that GS degradation in illuminated chloroplasts requires the function of the photosynthetic electron transport chain. Chloroplastic GS of wheat seedlings has been reported to be particularly prone to degradation under oxidative stress conditions (Palatnik et al., 1999). By incubating soybean root extracts enriched in GS in a metal-catalyzed oxidation system to produce the hydroxyl radical, Ortega et al. (1999) have shown that GS is oxidized and that the oxidized GS is inactive and more susceptible to proteolysis than nonoxidized GS. It is clear that GS degradation requires the production of active oxygen species (AOS). We also demonstrated that paraquat, which is known to produce AOS, decreased GS activity and increased NH_4^+ content in rice leaves in the light (Chien et al., 2002). It has been shown that the specific activity of protease (or proteolysis) increased under photooxidative environmental conditions and treatment with a hydroxyl radical-generating system or H_2O_2 (Casano and Trippi, 1992; Casano et al., 1990, 1994). Kumar and Knowles (2003) demonstrated that the specific activity of PAL induced by wounding in potato tuber is related to the ability to produce superoxide radicals.

Recently, many investigators have focused on the functional aspects of H_2O_2. H_2O_2 is a constituent of oxidative metabolism and is itself an AOS. Because H_2O_2 is relatively stable and diffusible through membrane, it is generally thought to serve as a signal molecule under various abiotic stresses (Neill et al., 2002), in acclimation to photooxidative stress (Karpinski et al., 1999), in plant–pathogen interactions (Levine et al., 1994), and in abscisic acid (ABA)-induced stomatal closure (Zhang et al., 2001).

We have previously shown that NH_4^+ accumulation is associated with ABA-promoted senescence of rice leaves (Chen et al., 1997). Evidence was also presented to show that ABA-induced NH_4^+ in rice leaves is attributed to a decrease in GS activity (Chen et al., 1997). In recent studies, we found that ABA increases the content of H_2O_2 in rice leaves (Hung and Kao, 2003). Here we have examined the possible involvement of H_2O_2 in ABA-induced NH_4^+ accumulation in rice leaves.

Materials and methods

Plant materials

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. These seeds were then germinated in Petri dishes with wetted filter paper at 37 °C under dark conditions. After 48 h in 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 at 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 10 segments was floated in a Petri dish containing 10 mL of test solution. Incubation was carried out at 27 °C in the dark.

Determination of NH_4^+

NH_4^+ was extracted by homogenizing leaf segments with a pestle and mortar using 0.3 mmol L^{-1} sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000g_n. The supernatant was used to determine NH_4^+ content by the method of Weatherburn (1967). NH_4^+ content was calculated using an extinction coefficient of 3.9982 $\mu mol^{-1} cm^{-1}$ and expressed as $\mu mol\, g^{-1}$ fresh weight (FW).

Enzyme assays

For extraction of GS, leaf samples were homogenized with 10 mmol L^{-1} Tris–HCl buffer (pH 7.6, containing 1 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} EDTA and 1 mmol L^{-1} 2-mercaptoethanol) using a chilled mortar and pestle. The homogenate was centrifuged at 15,000g_n 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 with the 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 min. PAL was extracted and determined according to Hyodo and Fujinami (1989). The calculation was based on the extinction coefficient [9500 $(\text{mmol L}^{-1})^{-1}\,\text{cm}^{-1}$] for *trans*-cinnamic acid. One unit of activity for PAL was defined as the amount of enzyme which caused the formation of 1 nmol *trans*-cinnamic acid h^{-1}. For protease extraction, leaf samples were homogenized in prechilled mortar and pestle with 10 mmol L^{-1} Tris–HCl buffer (pH 7.4) containing 10 mmol L^{-1} 2-mercaptoethanol at 4 °C. The homogenate was centrifuged at 15,000g_n 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 $A_{280}\,h^{-1}$. The method of Bradford (1976) was used to determine protein content in enzyme extracts.

Statistical analysis

Statistical differences between measurements ($n = 4$) on different treatments or on different times were analyzed following the Duncan's multiple range test or Student's *t*-test.

Results and discussion

NH_4^+ content in the control leaves remained unchanged during the first 24 h of incubation in the dark and increased subsequently (Fig. 1). It is clear that ABA-treated rice leaves had higher NH_4^+ content than the control leaves at 48 and 72 h after treatment (Fig. 1).

GS is the primary enzyme responsible for NH_4^+ assimilation in plants (Miflin and Lea, 1976). We observed that GS activity in the control leaves remained unchanged during 48 h of incubation and the decrease in GS activity in ABA-treated rice leaves was evident 36 h after treatment (Fig. 2A). However, ABA had no effect on the specific activity of GS in rice leaves (Fig. 2D). Since NH_4^+ is known to be released through the action of PAL, the first enzyme in the phenylpropanoids (Hahlbrock and Grisebach, 1979), it is possible that ABA-induced NH_4^+ accumulation is associated with the increase in the activity or the specific activity of PAL in rice

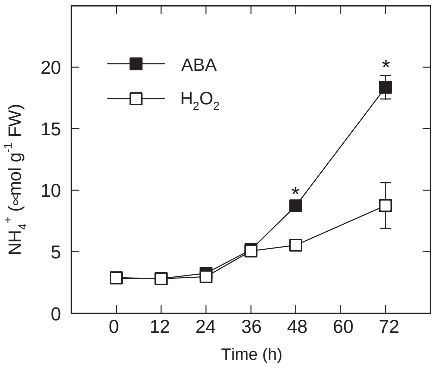

Figure 1. Changes in NH_4^+ contents in rice leaves treated with either water or 45 μmol L^{-1} ABA in the dark. Vertical bars represent standard errors ($n = 4$). Asterisk represents value that is significant at $P < 0.05$ level by Student's *t*-test when compared to water control.

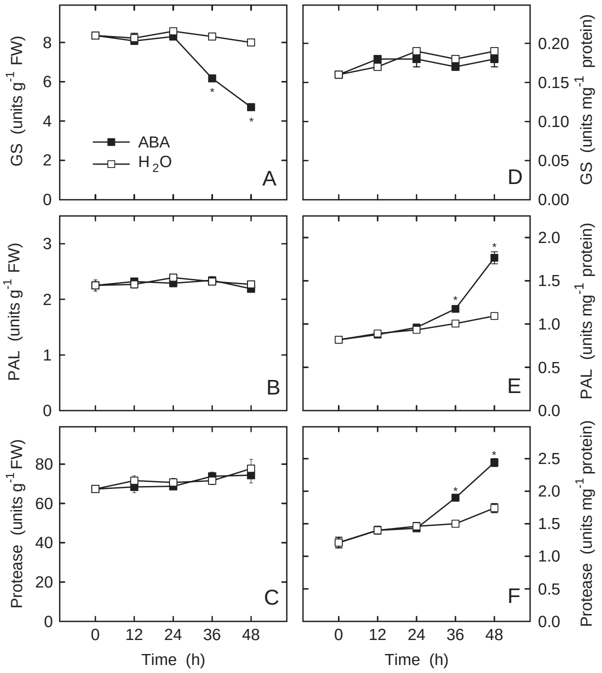

Figure 2. Changes in the activities and the specific activities of GS (A,D), PAL (B,E) and protease (C,F) in rice leaves treated with either water or 45 μmol L^{-1} ABA in the dark. Vertical bars represent standard errors ($n = 4$). Asterisk represents values that are significant at $P < 0.05$ level by Student's *t*-test when compared to water control.

leaves. It was found that NH_4^+ accumulation caused by ABA is associated with the specific activity of PAL (Fig. 2E), but not the activity of PAL (Fig. 2B). GS activity in plants might be regulated at the level of turnover (Stieger and Feller, 1997; Ortega et al., 1999; Palatnik et al., 1999). The decrease in GS activity in ABA-treated rice leaves is most likely related to the increase in the activity or the specific activity of protease. As shown in Figs. 2C and F, ABA was observed to be effective in increasing the specific activity, rather than activity of protease. ABA-induced decrease in the activity of GS and increase in the specific activities of PAL and protease in rice leaves (which occurred 36 h after treatment) are prior to ABA-induced accumulation of NH_4^+ (which was observed 48 h after treatment) (Figs. 1 and 2). It appears that GS, PAL, and protease are the enzymes responsible for ABA-induced NH_4^+ accumulation.

NH_4^+ is a central intermediate in the metabolism of nitrogen in plants. NH_4^+ can also be produced during nitrate assimilation and photorespiration (Miflin and Lea, 1976). Since our experiments were conducted in the dark, NH_4^+ accumulation induced by ABA is unlikely to have been produced from photorespiration. Previously, we have shown that NH_4^+ accumulation in rice leaves by methyl jasmonate is attributable to an increase in reduction of nitrate (Chen and Kao, 1998). ABA is biologically similar to methyl jasmonate (Weidhase et al., 1987). It appears that an increase in nitrate reduction induced by ABA may also contribute NH_4^+ accumulation in ABA-treated rice leaves. Further experiments are required to clarify this possibility.

We have previously shown that ABA-induced H_2O_2 production in rice leaves is evident 24 h after treatment (Hung and Kao, 2004). In several plant systems, H_2O_2 has been shown to function as a signal molecule (Levine et al., 1994; Rao et al., 1997; Karpinski et al., 1999; Casano et al., 2001; Zhang et al., 2001; Neill et al., 2002). It seems that the accumulation of H_2O_2 in rice leaves induced by ABA may play an important role in regulating the increase in NH_4^+ content in rice leaves. To test this hypothesis, dimethylthiourea (DMTU), a chemical trap for H_2O_2 (Levine et al., 1994; Rao et al., 1997; Casano et al., 2001), was used. As indicated in Fig. 3D, ABA-induced NH_4^+ accumulation was significantly reduced by DMTU. DMTU treatment was also observed to be effective in inhibiting the decrease in the activity of GS and the increase in the specific activities of protease and PAL in rice leaves caused by ABA [Figs. 3(A)–(C)].

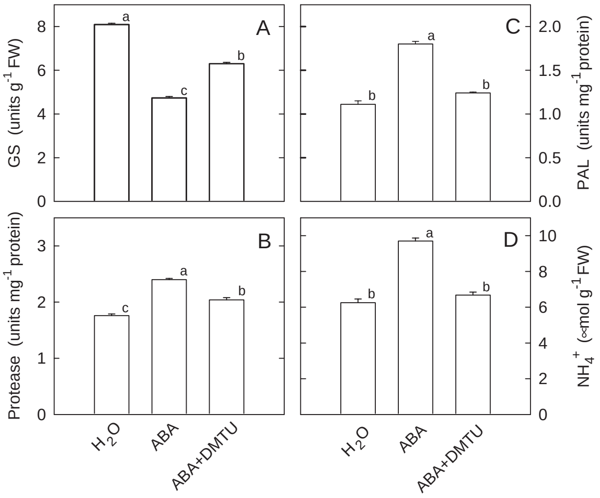

Figure 3. Effect of DMTU on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH_4^+ (D) in rice leaves treated with ABA. The concentrations of ABA and DMTU were 45 μmol L^{-1} and 5 mmol L^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P<0.05$ level, according to Duncan's multiple range test.

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. There are reports indicating that oxidative burst and the accumulation of H_2O_2 are mediated by the activation of plasma-membrane NADPH oxidase complex (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). Some chemical inhibitors of the NADPH oxidase complex found in mammalian neutrophils, such as diphenyleneiodonium chloride (DPI) and imidazole (IMD), inhibit the pathogen-, elicitor-, wound-, and ABA-induced accumulation of H_2O_2 in plants (Levine et al., 1994; Auh and Murphy, 1995; Bestwick et al., 1997; Alvarez et al., 1998; Orozco-Cárdenas and Ryan, 1999; Jiang and Zhang, 2002). Previously, we also demonstrated that ABA-induced H_2O_2 accumulation in rice leaves can be inhibited by low-concentration (25 μmol L^{-1}) DPI and 0.1 mmol L^{-1} IMD, indicating that ABA-dependent H_2O_2 generation originated, at least in part, from plasma-membrane NADPH oxidase (Hung and Kao, 2005). As shown in Fig. 4D, when rice leaves were treated with DPI and IMD, ABA-induced accumulation of NH_4^+ in rice leaves was reduced. DPI and IMD also inhibited ABA-induced changes in the activities or specific activities of the enzymes responsible for NH_4^+ accumulation [Figs. 4(A)–(C)].

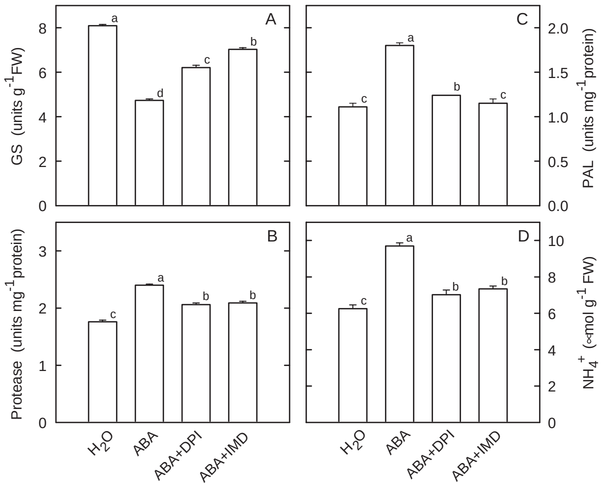

Figure 4. Effect of DPI and IMD on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH_4^+ (D) in rice leaves treated with ABA. The concentrations of ABA, DPI, and IMD were 45 and 25 μmol L^{-1}, and 0.1 mmol L^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

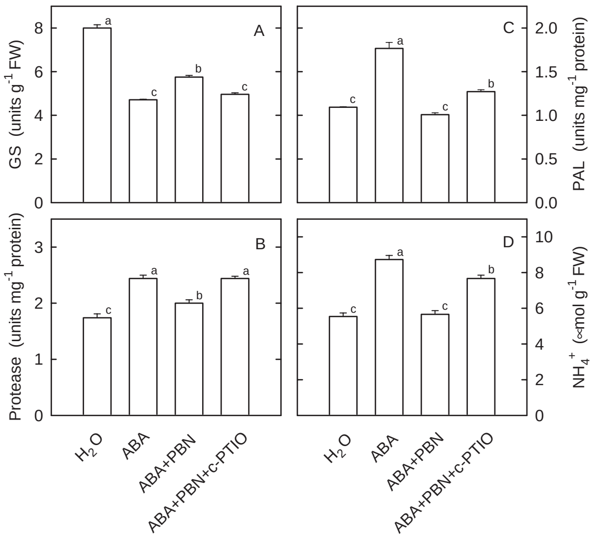

Figure 5. Effect of PBN on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH_4^+ (D) in ABA-treated rice leaves in the presence or absence of c-PTIO. The concentrations of ABA, PBN, and c-PTIO were 45 and 100, and 100 μmol L^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

The mechanism of AOS production and the molecules involved have been well investigated in animal cells, particularly in neutrophils. The NADPH oxidase complex, which consists of many components, is responsible for AOS production in neutrophil cells, and is activated by the binding of phosphatidylinositol 3-phosphate to one of the components (Ellson et al., 2001). Phosphatidylinositol 3-phosphate is a product of phosphatidylinositol 3-kinase. Recently, Jung et al. (2002) and Park et al. (2003) demonstrated that wortmannin or LY 294002, inhibitors of phosphatidylinositol 3-kinase, inhibited ABA-induced H_2O_2 production and stomatal closing and H_2O_2 partially reversed the effects of wortmannin or LY 294002 on ABA-induced stomatal closing. They suggested that phosphatidylinositol 3-phosphate is important in NADPH oxidase-mediated H_2O_2 production during ABA-induced stomatal closing. We have preliminary data indicating that wortmannin or LY 294002 prevented ABA-induced H_2O_2 production and ABA-induced NH_4^+ accumulation in rice leaves (Hung and Kao, unpublished observations). Work in this direction is presently under further investigation.

Nitric oxide (NO) is a bioactive free radical implicated in a number of physiological processes in plants, including growth, development, and defense responses (Lamattina et al., 2003). It has been shown that NO is able to counteract the toxicity of paraquat and diquat, which is known to generate superoxide radicals, in potato and rice leaves (Beligni and Lamattina, 1999; Hung et al., 2002). More recently, we have shown that ABA-induced H_2O_2 production in rice leaves can be reduced by NO donor *N-tert*-butyl-α-phenylnitrone (PBN) (Hung and Kao, 2003). Here, we show that PBN is effective in reducing ABA-induced accumulation of NH_4^+ (Fig. 5D), decrease in the activity of GS (Fig. 5A), and increase in the specific activities of protease and PAL (Figs. 5B and C) in rice leaves. Meanwhile, these PBN effects can be reversed by 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), a NO-specific scavenger (Fig. 5), suggesting that the PBN effects are attributable to NO released.

If H_2O_2 indeed plays an important role in ABA-induced accumulation of NH_4^+ in rice leaves, exogenous H_2O_2 is expected to increase the content of NH_4^+ and influence the activities or specific activities of enzymes related to NH_4^+ accumulation in rice leaves. As shown in Fig. 6, it is indeed the case. Meanwhile, PBN counteracted the effect of H_2O_2 and the protective effect of PBN was reversed by c-PTIO (Fig. 6).

We have previously shown that paraquat, an AOS-generating chemical, decreases GS activity and

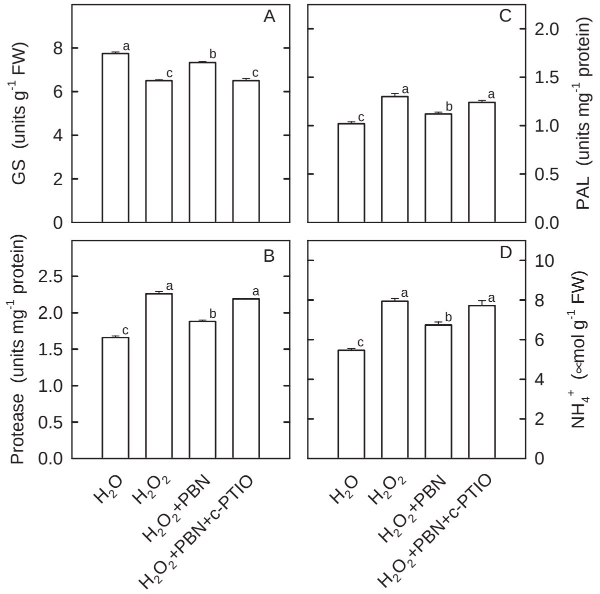

Figure 6. Effect of PBN on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH_4^+ (D) in H_2O_2-treated rice leaves in the presence or absence of c-PTIO. The concentrations of H_2O_2, PBN, and c-PTIO were $10\,\text{mmol}\,L^{-1}$ and 100 and $100\,\mu\text{mol}\,L^{-1}$, respectively. All measurements were determined 2 days after treatment in the dark. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

increases NH_4^+ accumulation in rice leaves in the light (Chien et al., 2002). It has been demonstrated that GS in plants is particularly prone to proteolysis under oxidative stress conditions (Ortega et al., 1999; Palatnik et al., 1999; Chien et al., 2002; Ishida et al., 2002), and the specific protease activity increases under photooxidative environmental conditions and treatment with hydroxyl radical-generating system or H_2O_2 (Casano and Trippi, 1992; Casano et al., 1990, 1994). It appears that the decrease in GS activity in ABA-treated rice leaves (Fig. 2A) may be, at least in part, attributable to the increase in specific protease activity (Fig. 2F).

Kumar and Knowles (2003) demonstrated that specific activity of PAL induced by wounding in potato tuber was related to the ability to produce superoxide radicals. Here, we provided evidence to show that H_2O_2 is involved in ABA-induced specific PAL activity in rice leaves. It is not known whether PAL in plants is prone to proteolysis under oxidative stress conditions. The fact that ABA treatment resulted in an increase in specific activities of both PAL and protease (Fig. 2E and F), suggests that PAL is resistant to proteolysis in rice leaves.

Our results indicated that H_2O_2 production precedes the changes of enzymes associated with NH_4^+ accumulation, and NH_4^+ accumulation in ABA-treated rice leaves. Clearly, the links between ABA treatment, H_2O_2 production, enzymes responsible for NH_4^+ accumulation, and NH_4^+ accumulation are well established. The results reported here also suggest that the changes in enzyme activities or specific activities related to NH_4^+ accumulation, and the increase in NH_4^+ content demonstrated in rice leaves are a consequence of H_2O_2 production caused by ABA.

Acknowledgements

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