Journal of Plant Physiology **161** (2004) 1347–1357

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JOURNAL OF PLANT PHYSIOLOGY

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Hydrogen peroxide is necessary for abscisic acid-induced senescence of rice leaves

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Received 16 March 2004; accepted 12 May 2004

KEYWORDS:
Abscisic acid;
H_2O_2;
Leaf senescence;
Lipid peroxidation;
Oryza sativa

Summary

The role of H_2O_2 in abscisic acid (ABA)-induced rice leaf senescence is investigated. ABA treatment resulted in H_2O_2 production in rice leaves, which preceded the occurrence of leaf senescence. Dimethylthiourea, a chemical trap for H_2O_2, was observed to be effective in inhibiting ABA-induced senescence, ABA-increased malondialdehyde (MDA) content, ABA-increased antioxidative enzyme activities (superoxide dismutase, ascorbate peroxidase, glutathione reductase and catalase), and ABA-decreased antioxidant contents (ascorbic acid and reduced glutathione) in rice leaves. Diphenyleneiodonium chloride (DPI) and imidazole (IMD), inhibitors of NADPH oxidase, and KCN and NaN_3, inhibitors of peroxidase, prevented ABA-induced H_2O_2 production, suggesting NADPH oxidase and peroxidase are H_2O_2-generating enzymes in ABA-treated rice leaves. DPI, IMD, KCN, and NaN_3 also inhibited ABA-promoted senescence, ABA-increased MDA contents, ABA-increased antioxidative enzyme activities, and ABA-decreased antioxidants in rice leaves. These results suggest that H_2O_2 is involved in ABA-induced senescence of rice leaves.
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Introduction

The plant hormone abscisic acid (ABA) is a sesquiterpenoid synthesized from xanthophylls (Creelman, 1989; Taylor et al., 2000; Seo and Koshiba, 2002) and appears to influence several physiological and developmental events (Creelman, 1989; Kende and Zeevaart, 1997). It has been suggested that ABA is one of the most effective plant hormones in terms of promoting leaf senescence (Nooden, 1988). Applied ABA has been found to promote leaf senescence in a wide range of plant

Abbreviations: AOS, Active oxygen species; APOD, Ascorbate peroxidase; AsA, Ascorbic acid; CAT, Catalase; DMTU, Dimethylthiourea; DPI, Diphenyleneiodonium chloride; FW, Fresh weight; GR, Glutathione reductase; GSH, Reduced glutathione; MDA, Malondialdehyde; SOD, Superoxide dismutase

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doi:10.1016/j.jplph.2004.05.011

species (Nooden, 1988; Creelman, 1989). An increase in endogenous ABA has been shown to coincide with senescence of leaves (Gepstein and Thimann, 1980; Yang et al., 2002).

Lipid peroxidation is considered to be an important mechanism of leaf senescence (Thompson et al., 1987). Active oxygen species (AOS) can initiate lipid peroxidation (Kellogg and Fridovich, 1975). It has been shown that ABA causes generation of AOS including H_2O_2 (Guan et al., 2000; Pei et al., 2000; Jiang and Zhang, 2001; Hung and Kao, 2003) and lipid peroxidation expressed as malondialdehyde (MDA) production in plant cells (Bueno et al., 1998). Thus, ABA leads to oxidative stress in plant cells.

Recently, many researchers have focused on the functional aspects of H_2O_2. H_2O_2 is a constituent of oxidative metabolism and is itself an AOS. It has been shown that H_2O_2 promotes leaf senescence (Parida et al., 1978; Mondal and Choudhuri, 1981; Begam and Choudhuri, 1992; Lin and Kao, 1998) and induction of senescence is accompanied by an increase in endogenous H_2O_2 content (Mondal and Choudhuri, 1981; Hung and Kao, 2003). 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 (Chamnongpol et al., 1998; Neill et al., 2002), in acclimation to photooxidative stress (Karpinski et al., 1999), in plant–pathogen interactions (Levine et al., 1994), and in ABA-induced stomatal closure (Zhang et al., 2001).

We have previously shown that ABA not only increases the content of H_2O_2 and the activities of superoxide dismutase (SOD), ascorbate peroxidase (APOD), glutathione reductase (GR), and catalase (CAT), but also causes a decrease in ascorbic acid (AsA) and glutathione (GSH) contents in rice leaves (Hung and Kao, 2003). Meanwhile, protein loss (senescence) and lipid peroxidation were observed in ABA-treated rice leaves (Hung and Kao, 2003). All these results suggest that ABA causes oxidative stress and ABA-promoted senescence of rice leaves is mediated through oxidative stress. Here, we have examined the role of H_2O_2 as a connection between ABA and subsequent antioxidant defense and senescence in rice leaves.

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 light 30 °C day (12 h)/25 °C night (12 h) 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.

Determinations of protein, H_2O_2, lipid peroxidation, GSH, and AsA

The senescence of detached rice leaves was followed by measuring the decrease of protein content. For protein extraction, leaf segments were homogenized in 50 mmol l^{-1} sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600g_n for 20 min, and the supernatants were used for determination of protein by the method of Bradford (1976) and enzyme activities. 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 mmol l^{-1}, pH 6.5) containing 1 mmol l^{-1} hydroxylamine. The homogenate was centrifuged at 6000g_n 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 6000g_n for 25 min. The absorbance was measured at 410 nm. The H_2O_2 content was calculated using the extinction coefficient 0.28 μmol^{-1} 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). GSH in 3% sulfosalicylic acid extract and AsA in 5% (w/v) trichloroacetic acid extract were determined as described by Smith (1985) and Laws et al. (1983), respectively.

Enzyme assays

The enzyme assays in detail have been described previously (Hurng and Kao, 1994). 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 absorbance at 240 nm, and activity was calculated using the extinction coefficient

[40 (mmol l^{-1})$^{-1}$ cm^{-1} at 240 nm] for H_2O_2 (Kato and Shimizu, 1987). SOD was determined according to Paoletti et al. (1986). APOD was determined according to Nakano and Asada (1981). The decrease in AsA concentration was followed as the decline in optical density at 290 nm and activity was calculated using the extinction coefficient [2.8 (mmol l^{-1})$^{-1}$ cm^{-1} at 290 nm] for AsA. GR was determined by the method of Foster and Hess (1980). One unit of activity for CAT, SOD, APOD, and GR was defined as the amount of enzyme which degraded 1 μmol H_2O_2 per min, inhibited 50% the rate of NADH oxidation observed in control, degraded 1 μmol of AsA per min, and decreased 1 A_{340} per min, respectively.

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 at 5% level of significance.

Results

Yellowing is an obvious expression of leaf senescence and chlorophyll loss is often viewed as the principal criterion of senescence. The protein degradation during leaf senescence has been realized from earliest studies. We have shown that protein degradation 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. MDA is routinely used as an indicator of lipid peroxidation. The changes in protein and MDA contents in detached rice leaves treated with 45 μmol l^{-1} ABA in the dark are shown in Figs. 1A and B. The decrease in protein and increase in MDA was evident at 36 h after ABA treatment. Clearly, ABA is effective in promoting senescence of rice leaves. ABA treatment resulted in an increase in MDA, indicating that ABA brings about lipid peroxidation. Lipid peroxidation is caused by AOS (Kellogg and Fridovich, 1975; Thompson et al., 1987). ABA treatment also caused an increase in H_2O_2 content (Fig. 1C). The increase in H_2O_2 was evident at 24 h after treatment of ABA, which preceded the decrease in protein and increase in MDA. These results suggest that H_2O_2 may play an important role in regulating the senescence of rice leaves induced by ABA.

To demonstrate the involvement of H_2O_2 in the effects induced by ABA in rice leaves, namely the decrease in protein content and the increase in MDA content, dimethylthiourea (DMTU), a chemical trap for H_2O_2 (de Agazio and Zacchini, 2001), was used. Detached rice leaves were incubated in a solution containing 45 μmol l^{-1} ABA with or without 5 mmol l^{-1} DMTU. As indicated in Figs. 2A and B, the decrease in protein and the increase in MDA in rice leaves caused by ABA were reduced by DMTU. Previously, we have shown that ABA increased the

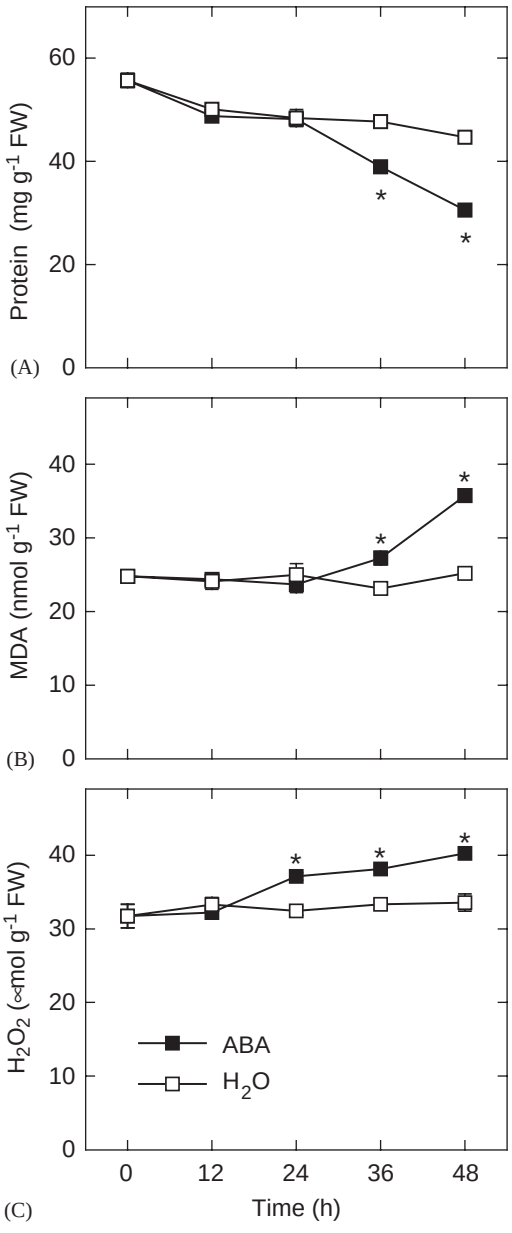

Figure 1. Changes in the contents of protein (A), MDA (B), and H_2O_2 (C) in rice leaves treated with either water or 45 μmol l^{-1} ABA in the dark. Values are means ± SE ($n = 4$). Asterisks represent values that are significant at $P < 0.05$ level by Student's *t*-test when compared to water control.

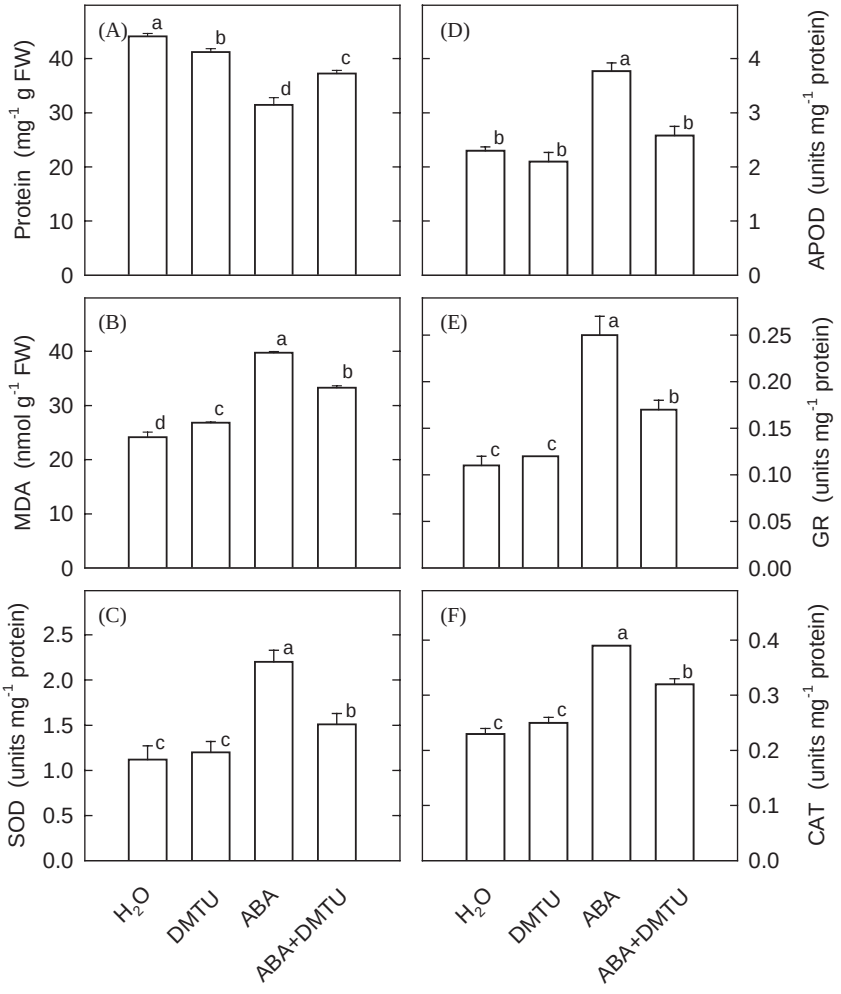

Figure 2. Effect of DMTU on the contents of protein (A), and MDA (B), and the activities of SOD (C), APOD (D), GR (E), and CAT (F) in rice leaves treated with ABA. The concentrations of ABA and DMTU were 45 μmol l^{-1} and 5 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means ± SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

activities of SOD, APOD, GR, and CAT and decreased the contents of AsA and GSH in rice leaves (Hung and Kao, 2003). DMTU was also observed to be effective in inhibiting ABA-increased activities of SOD (Fig. 2C), APOD (Fig. 2D), GR (Fig. 2E), and CAT (Fig. 2F) and ABA-decreased contents of AsA (Fig. 3A) and GSH (Fig. 3B) in rice leaves.

AOS, originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O_2 to form $O_2^{-\bullet}$, followed by dismutation of $O_2^{-\bullet}$ 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 (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., 1977; Alvarez et al., 1998; Orozco-Cárdenas and Ryan, 1999; Jiang and Zhang, 2002). As shown in Fig. 4, when detached rice leaves were treated with a solution of DPI (25 μmol l^{-1}) and IMD (0.1 mmol l^{-1}), ABA-induced accumulation of H_2O_2 in rice leaves was reduced (Figs. 4C and F). DPI and IMD also inhibited ABA-promoted leaf senescence (Figs. 4A and D), ABA-increased contents of MDA (Figs. 4B and E) and activities of SOD (Figs. 5A and 6A), APOD (Figs. 5B and 6B), GR (Figs. 5C and 6C), and CAT (Figs. 5D and 6D), and ABA-decreased contents of AsA (Fig. 3A) and GSH (Fig. 3B).

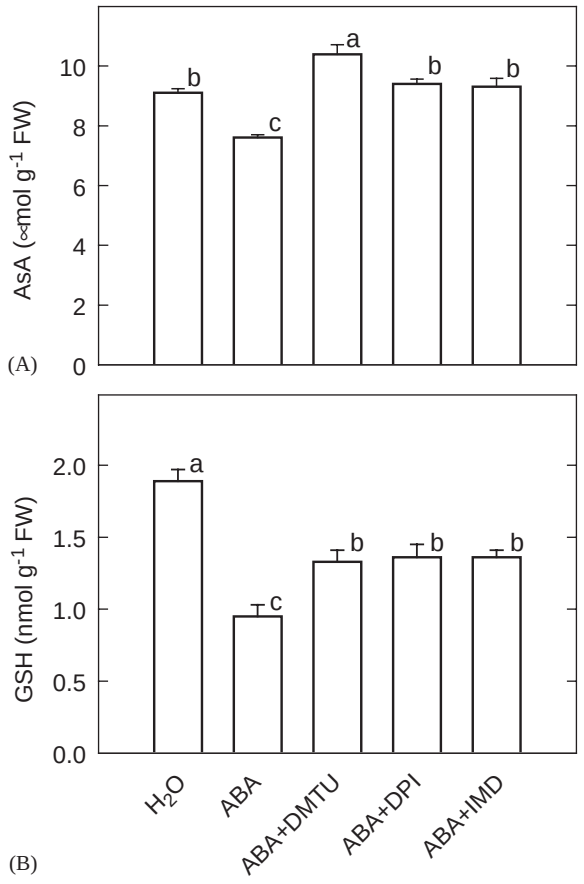

Figure 3. Effect of DMTU, DPI, and IMD on the contents of AsA (A) and GSH (B) in rice leaves treated with ABA. The concentrations of ABA, DMTU, DPI, and IMD were 45 μmol l^{-1}, 5 mmol l^{-1}, 25 μmol l^{-1}, and 0.1 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means ± SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

Another potential enzymatic source of H_2O_2 production is cell wall peroxidase (Papadakis and Roubelakis-Angelakis, 1999; Blee et al., 2001). To test the possible involvement of cell wall peroxidase, peroxidase inhibitors such as KCN and NaN_3 were used. As is well known, KCN and NaN_3 are also inhibitors of mitochondria respiration. To distinguish the effect of KCN and NaN_3 on respiration and peroxidase activity, the concentrations of KCN and NaN_3 of 10 μmol l^{-1} and 1 mmol l^{-1}, respectively, were used, since these concentrations did not inhibit the respiration rate of detached rice leaves (unpublished). Figure 7C shows that the addition of KCN and NaN_3 to detached rice leaves restored the ABA-induced H_2O_2 production to control levels. KCN and NaN_3 also inhibited ABA-promoted senescence (Fig. 7A), ABA-increased content of MDA (Fig. 7B) and activities of SOD (Fig. 8A), APOD (Fig. 8B), GR (Fig. 8C), and CAT (Fig. 8D), and ABA-decreased contents of AsA (Fig. 8E) and GSH (Fig. 8F).

Discussion

Pei et al. (2000) were the first to demonstrate the generation of H_2O_2 and its effects in guard cells caused by ABA. In subsequent work, ABA-induced increase in H_2O_2 has been reported for maize seedlings, rice roots, and rice leaves (Jiang and Zhang, 2001; Lin and Kao, 2001; Hung and Kao, 2003, Fig. 1C). On the other hand, ABA decreased the release of H_2O_2 from germinating radish seeds (Schopfer et al., 2001). It seems that H_2O_2 generation is not a common response to ABA and this response is not confined to guard cells.

In guard cells, ABA-induced H_2O_2 generation is regulated by plasma-membrane NADPH oxidase (Pei et al., 2000; Zhang et al., 2001). Recently, Jiang and Zhang (2002) also reported that plasma-membrane NADPH oxidase is involved in ABA- and water stress-induced antioxidant defense in leaves of maize seedlings. Here, we show that DPI and IMD, inhibitors of NADPH oxidase (Levine et al., 1994; Auh and Murphy, 1995; Bestwick et al., 1977; Alvarez et al., 1998; Orozco-Cárdenas and Ryan, 1999; Pei et al., 2000; Orozco-Cárdenas et al., 2001; Jiang and Zhang, 2002), reduced ABA-induced H_2O_2 production (Figs. 4C and F) and lipid peroxidation (Figs. 4B and E), ABA-promoted senescence (Figs. 4A and D), ABA-increased antioxidative enzyme activities (Figs. 5 and 6), and ABA-decreased antioxidants (Fig. 3) in rice leaves. Similar results were obtained by using DMTU, a chemical trap for H_2O_2 (Figs. 2 and 3). Furthermore, the increase in H_2O_2 content by ABA was observed to be preceded the occurrence of leaf senescence and the increase in MDA content (Figs. 1A and C). It appears that H_2O_2 is involved in ABA-induced senescence of rice leaves and that NADPH oxidase in rice leaf cells is involved in ABA-induced H_2O_2 production.

It has been shown that a high concentration of DPI can affect other enzymes potentially involved in the generation of AOS, including cell wall peroxidase and nitric oxide synthase (Bolwell et al., 1998; Orozco-Cárdenas et al., 2001; Schopfer et al., 2001). The fact that ABA-induced H_2O_2 accumulation in rice leaves can be inhibited by low concentration (25 μmol l^{-1}) DPI, and can be inhibited by both DPI and IMD strongly suggest that ABA-dependent H_2O_2 generation originated, at least in part, from plasma membrane NADPH oxidase.

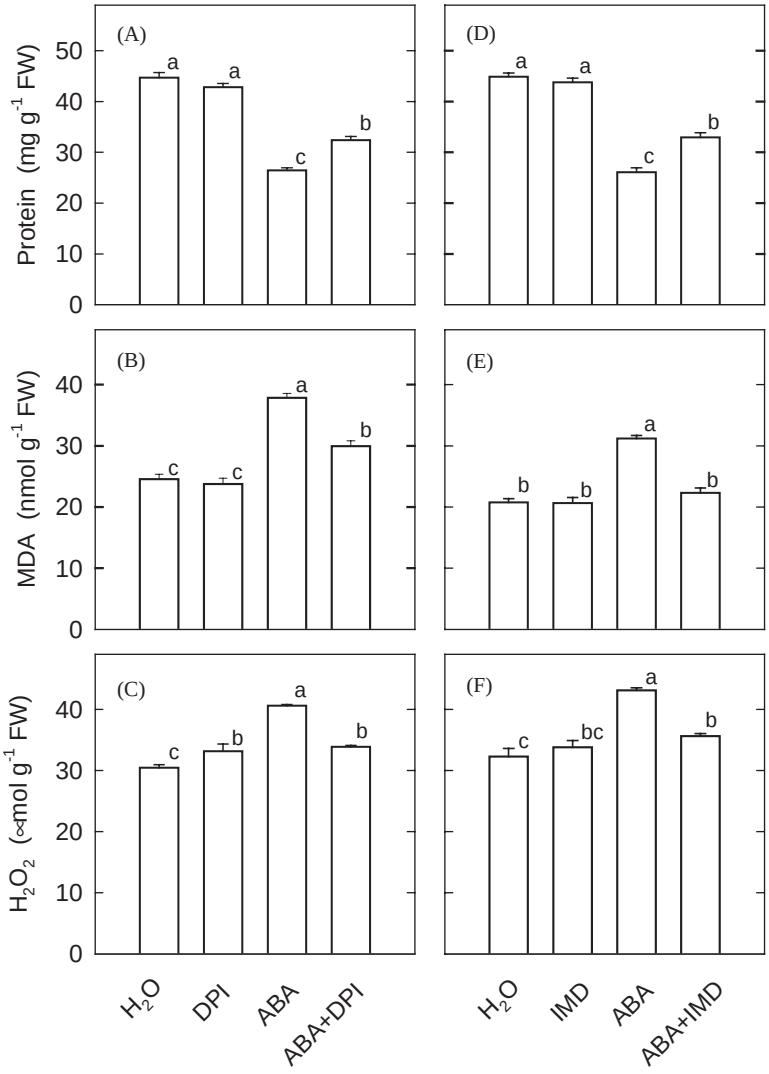

Figure 4. Effect of DPI and IMD on the contents of protein (A, D), and MDA (B, E), and H_2O_2 (C, F) in rice leaves treated with ABA. The concentrations of ABA, DPI, and IMD were 45, 25 μmol l^{-1}, and 0.1 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means ± SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

Recently, a cell wall peroxidase has been identified in French bean (Bolwell et al., 1998; Blee et al., 2001), and a potentially peroxidase-mediated H_2O_2 production has been demonstrated in *Arabidopsis* cultures challenged with a fungal elicitor (Bolwell et al., 2002). *Arabidopsis* plants transformed with an antisense bean peroxidase construct are hypersensitive with a fungal elicitor (Bolwell et al., 2002). Here, we show that KCN and NaN_3, inhibitors of peroxidase, prevented ABA-induced H_2O_2 production (Fig. 7C) and lipid peroxidation (Fig. 7B), ABA-promoted senescence (Fig. 7A), ABA-increased antioxidative enzyme activities (Figs. 8(A)–(D)), and ABA-decreased antioxidants in rice leaves (Figs. 8E and F). It appears that peroxidase is another H_2O_2-generating enzyme in ABA-treated rice leaves. However, the endogenous rice peroxidase has yet to be identified.

Plasma-membrane NADPH oxidase transfers electrons from cytoplasmic NADPH to O_2 to form $O_2^{-\bullet}$, which is then dismutated to H_2O_2 by the action of apoplastic SOD. It has been shown that NaN_3 inhibits apoplastic Cu-Zn SOD (Ogawa et al., 1997). Furthermore, KCN has the ability to scavenge H_2O_2 (Baker et al., 1998). It appears that KCN and NaN_3 are not fully specific to peroxidase, which would explain the results that the addition of KCN and NaN_3 to detached rice leaves restored the ABA-induced H_2O_2 production to control levels (Fig. 7C).

In plants, polyamines are thought to play an important role in growth, development and stress

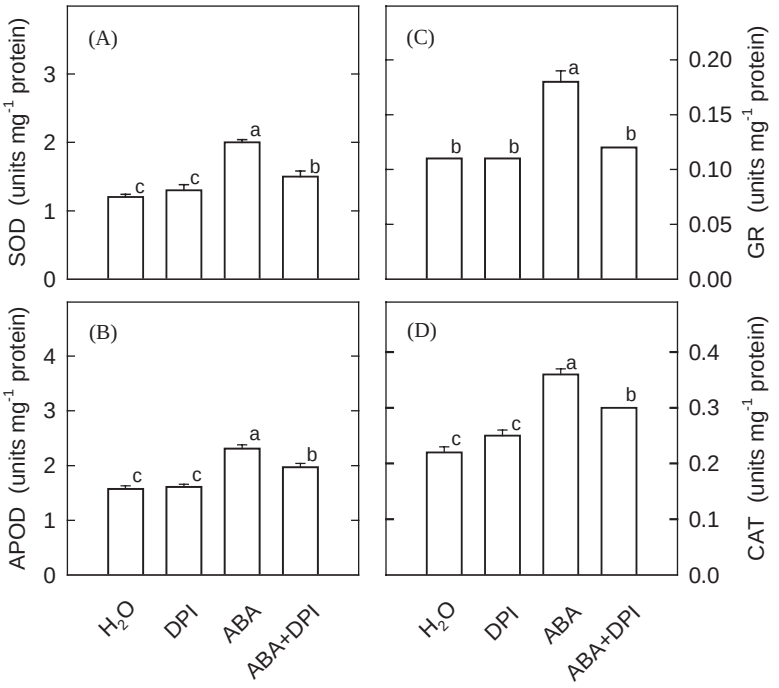

Figure 5. Effect of DPI on the activies of SOD (A), APOD (B), GR (C), and CAT (D) in rice leaves treated with ABA. The concentrations of ABA and DPI were 45 and 25 μmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means $\pm$ SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

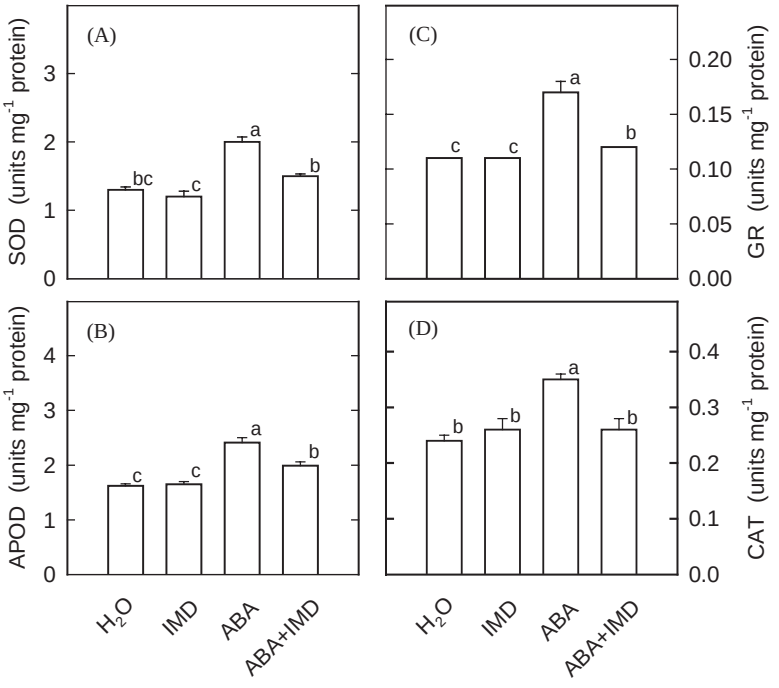

Figure 6. Effect of IMD on the activies of SOD (A), APOD (B), GR (C), and CAT (D) in rice leaves treated with ABA. The concentrations of ABA and IMD were 45 μmol l^{-1} and 0.1 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means $\pm$ SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

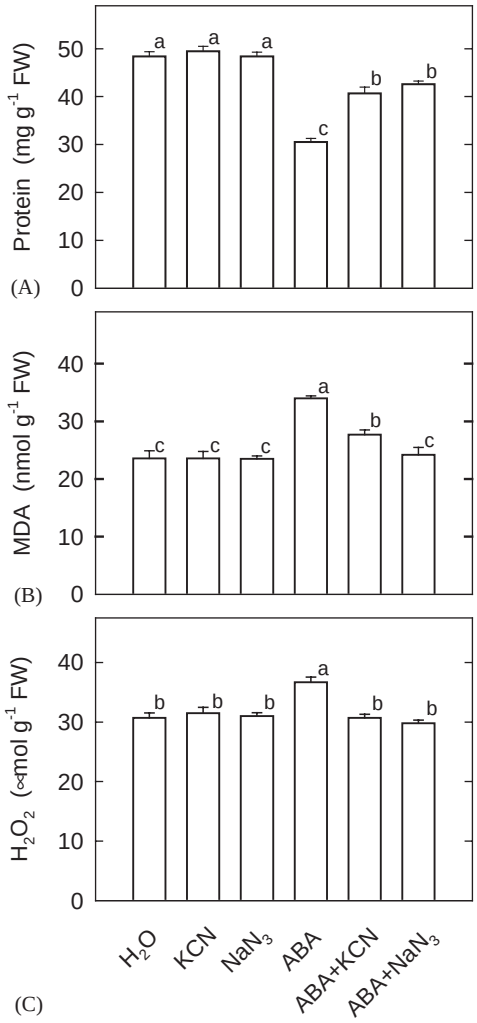

Figure 7. Effect of KCN and NaN_3 on the contents of protein (A), and MDA (B), and H_2O_2 (C) in rice leaves treated with ABA. The concentrations of ABA, KCN, and NaN_3 were 45, 10 μmol l^{-1}, and 1 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means ± SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

response (Walden et al., 1997). It has been shown that H_2O_2 produced by diamine or polyamine oxidase induced hypersensitive cell death in plants (Yoda et al., 2003). Previously, we have shown that ABA treatment had no effect on polyamine content in rice leaves (Chen and Kao, 1991). Thus, diamine or polyamine oxidase is unlikely to be affected by ABA in rice leaves. An alternative source for H_2O_2 includes oxalate oxidase, an enzyme that degrades oxalate to CO_2 and H_2O_2 (Dumas et al., 1995). Oxalate oxidase gene expression is induced by salt stress, salicylate, and methyl jasmonate (Hurkman and Tanaka, 1996). It is not known whether ABA will activate oxalate oxidase in rice leaves. Further work is necessary to clarify this possibility.

Results observed in the present study suggest that NADPH oxidase, which shows sensitivity to DPI and IMD, and peroxidase, which is sensitive to KCN and NaN_3, are operating in ABA-treated rice leaves. These two H_2O_2-generating enzymes were also observed in tobacco protoplasts (Papadakis and Roubelakis-Angelakis, 1999). It appears that when rice leaves are treated with ABA, H_2O_2 is generated in the apoplast. Because apoplast has only a small proportion of the cell's antioxidant capacity, H_2O_2 will rapidly move into the cell to exert its effect on senescence. It has been suggested that peroxiporins or water channels (aquaporins) may serve as conduits for trans-membrane H_2O_2 transport (Neill et al., 2002). Thus, H_2O_2 can function as a mobile signal in ABA-treated rice leaves, but whether H_2O_2 is the sole signal remains to be determined.

When detached rice leaves are used to study senescence, wounding is always a problem. However, in the present study, each long and narrow rice leaf was cut transversely, thus the area of wounding was very small. Therefore, H_2O_2 generation and senescence of detached leaves induced by ABA are unlikely to be complicated by the wounding effect. Since ABA is known to inhibit ethylene production in detached rice leaves (Kao and Yang, 1983), ABA-induced H_2O_2 production and senescence do not seem to be mediated through ethylene production.

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 posphatidylinositol 3-phosphate to one of the components (Ellson et al., 2001). Phosphatidylinositol 3-phosphate is a product of phosphatidylinositol 3-kinase, which phosphorylates the D-3 position of phosphoinositides. 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 and LY 294002 prevented ABA-induced H_2O_2 production and ABA-promoted senescence in rice leaves (Hung and Kao, unpublished observation). Work in this direction is presently under further investigation.

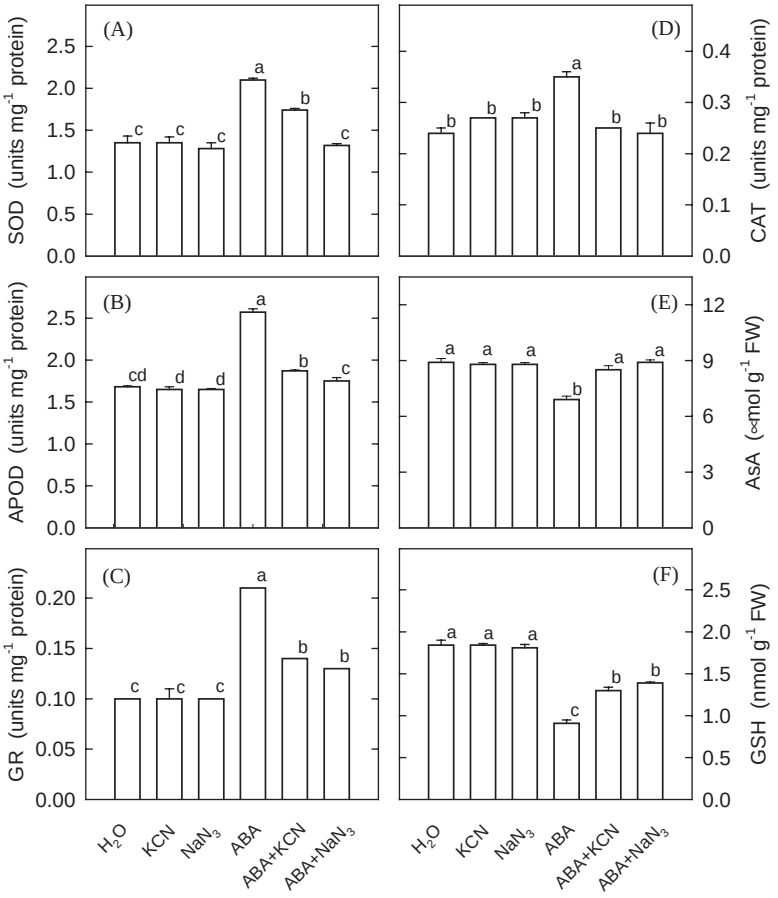

Figure 8. Effect of KCN and NaN_3 on the activies of SOD (A), APOD (B), GR (C), and CAT (D) and the contents of AsA (E) and GSH (F) in rice leaves treated with ABA. The concentrations of ABA, KCN, and NaN_3 were 45, 10 μmol l^{-1}, and 1 mmol l^{-1}, respectively. All measurements were determined 2 days after treatment in the dark. Values are means $\pm$ SE ($n = 4$). Value with the same letter are not significantly different at $P < 0.05$ level, according to Duncan's multiple range test.

Acknowledgements

This work was supported by grant NSC 90-2313-B-002-267 from the National Science Council of the Republic of China.

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