The participation of hydrogen peroxide in methyl jasmonate-induced NH_4^+ accumulation in rice leaves

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Summary

Ammonium is a central intermediate in the nitrogen metabolism of plants. We have previously shown that methyl jasmonate (MJ) not only increases the content of H_2O_2 , but also causes NH_4^+ accumulation in rice leaves. More recently, H_2O_2 is thought to constitute a general signal molecule participating in the recognition of and the response to stress factors. In this study, we examined the role of H_2O_2 as a link between MJ and subsequent NH_4^+ accumulation in detached rice leaves. MJ treatment resulted in an accumulation of NH_4^+ in detached 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 appear to be the enzymes responsible for the accumulation of NH_4^+ in MJ-treated detached rice leaves. Dimethylthiourea (DMTU), a chemical trap for H_2O_2 , was observed to be effective in inhibiting MJ-induced NH_4^+ accumulation in detached rice leaves. Scavengers of free radicals (sodium benzoate, SB, and glutathione, GSH), nitric oxide donor (*N*-tert-butyl- α -phenylnitron, PBN), the inhibitors of NADPH oxidase (diphenyleneiodonium chloride, DPI, and imidazole, IMD), and inhibitors of phosphatidylinositol 3-kinase (wortmannin, WM, and LY 294002, LY), which have previously been shown to prevent MJ-induced H_2O_2 production in detached rice leaves, inhibited MJ-induced NH_4^+ accumulation. Similarly, changes in enzymes responsible for NH_4^+ accumulation induced by MJ were observed to be inhibited by DMTU, SB, GSH, PBN, DPI, IMD, WM, or LY. Seedlings of rice cultivar Taichung Native 1 (TN1) are jasmonic acid (JA)-sensitive and those of cultivar Tainung 67 (TNG67) are JA-insensitive. On treatment with JA, H_2O_2 accumulated in the leaves of TN1 seedlings but not in the leaves of TNG67. Ethylene action inhibitor, silver thiosulfate, was observed to inhibit MJ- and abscisic

Abbreviations: ABA, abscisic acid; DMTU, dimethylthiourea; DPI, diphenyleneiodonium chloride; FW, initial fresh weight; GS, glutamine synthetase; IMD, imidazole; JA, jasmonic acid; LY, LY 294002; MJ, methyl jasmonate; PAL, phenylalanine ammonia-lyase; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; ROS, reactive oxygen species; STS, silver thiosulfate; TN1, Taichung Native 1; TNG67, Tainung 67; WM, wortmannin

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acid-induced accumulation of NH_4^+ and changes in enzymes responsible for NH_4^+ accumulation in detached rice leaves, suggesting that the action of MJ and ABA is ethylene dependent.

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Introduction

Ammonium is a central intermediate in the nitrogen metabolism of plants. Glutamine synthetase (GS) is a key enzyme in NH_4^+ assimilation and catalyzes the ATP-dependent condensation of NH_4^+ with glutamate to produce glutamine (Mifflin and Lea, 1976). Phenylalanine ammonia-lyase (PAL) catalyzes the elimination of NH_4^+ from phenylalanine producing *trans*-cinnamic acid (Hahlbrock and Grisebach, 1979). NH_4^+ , released from the PAL reaction, is known to be incorporated into glutamine 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 is associated with decreases in GS activity and increases in PAL specific activity (Hsu and Kao, 2004).

GS activity in plants is known to be regulated at the levels of transcription and 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 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 participation of reactive oxygen species (ROS). We also demonstrated that paraquat, which is known to produce ROS, decreased GS activity and increased NH_4^+ content in rice leaves in the light (Chien et al., 2002). It has been shown that protease specific activity (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 PAL specific activity induced by wounding in potato tubers is related to the ability to produce superoxide radicals.

Recently, researchers have focused on the functional aspects of H_2O_2 . H_2O_2 is a constituent of oxidative metabolism and is itself a ROS. Because H_2O_2 is a small, diffusible, and ubiquitous molecule that can be synthesized, as a stimulus, it fulfills the important criteria for an intracellular messenger (Neill et al., 2002; Foyer and Noctor, 2005). Thermoprotection obtained by spraying salicylic acid or by heat acclimation was suggested to be achieved by a common signal transduction pathway involving very early increases in H_2O_2 content (Dat et al., 1998). In tomato plants, H_2O_2 has been shown to act as a second messenger for induction of defense genes in response to wounding and systemin (Orozco-Cárdenas et al., 2001). It has been demonstrated that H_2O_2 is required for the induction of cytosolic ascorbate peroxidase mRNA by oxidative stress (Morita et al., 1999). H_2O_2 has now also been shown to be a critical component of abscisic acid (ABA)-induced stomatal closure (Pei et al., 2000; Zhang et al., 2001; Kwak et al., 2003) and ABA-induced rice leaf senescence (Hung and Kao, 2004b), ABA-induced activities of ascorbate peroxidase and glutathione reductase in rice roots (Tsai and Kao, 2004), and gibberellic acid-induced programmed cell death in barley aleurone cells (Fath et al., 2001).

Methyl jasmonate (MJ) was first considered to be secondary metabolite with a possible application in the perfume industry (Demole et al., 1962). It is now evident that jasmonates are a class of plant hormones, which mediate various aspects of developmental and stress responses (Creelman and Mullet, 1997). MJ has been shown to cause H_2O_2 production in parsley suspension-cultured cells (Kauss et al., 1994) and to act as a signal molecule for the induction of defense genes in tomato plants (Orozco-Cárdenas et al., 2001). We have previously shown that MJ not only increases the content of H_2O_2 (Hung and Kao, 2004a), but also causes NH_4^+ accumulation (Chen and Kao, 1998) in rice leaves. In this paper, we have examined the possible involvement of H_2O_2 in MJ-induced NH_4^+ accumulation in rice leaves.

Materials and methods

Plant materials and treatments

Rice (*Oryza sativa* L., cv. Taichung Native 1, TN1, or Tainung 67, TNG 67) 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 of incubation, uniformly germinated seeds were selected and cultivated in a 500 ml beaker containing half-strength Kimura B solution as described previously (Hsu and Kao, 2005). The hydroponically cultivated seedlings were grown for 12 days in a phytotron (College of Agriculture, National Taiwan University, Taipei Taiwan) with natural sunlight at 30 ± 1 °C (day)/25 ± 1 °C (night) and 90% relative humidity. The apical 3 cm of the third leaf of TN1 was used in 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. In experiments with intact leaves of TN1 and TNG67 seedlings, jasmonic acid (JA) was added directly to half-strength Kimura B solution at the time when the third leaf was fully expanded.

Determination of NH₄⁺ and H₂O₂

NH₄⁺ was extracted by homogenizing leaf segments with a pestle and mortar using 0.3 mM sulfuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 39,000g_n. The supernatant was used to determine NH₄⁺ content by the method of Weatherburn (1967). NH₄⁺ content was calculated using an extinction coefficient of 3.9 μmol⁻¹ cm⁻¹ and expressed as μmol g⁻¹ initial fresh weight (FW). H₂O₂ content was measured colorimetrically as described by Jana and Choudhuri (1982). H₂O₂ was extracted by homogenizing leaf samples with phosphate buffer (50 mmol L⁻¹, pH 6.5) containing 1 mmol L⁻¹ hydroxylamine. The homogenate was centrifuged at 6,000g_n for 24 min. To determine H₂O₂ content, the extracted solution was mixed with 0.1% titanium chloride in 20% (v/v) H₂SO₄. The mixture was then centrifuged at 6000g_n for 25 min. The absorbance was measured at 410 nm. Using this method, we obtained that absorbance increased linearly with the amount of H₂O₂ and addition of H₂O₂ to extracts resulted in the predicted increase of absorbance, i.e. added H₂O₂ was fully recovered (data not shown). The H₂O₂ content in leaf extracts was calculated using an extinction coefficient of 0.28 μmol⁻¹ cm⁻¹.

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 MgCl₂, 1 mmol L⁻¹ EDTA and 1 mmol L⁻¹ 2-mercaptoethanol) using a chilled pestle and mortar. 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 Oaks 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 MgSO₄, and 16 μmol NH₂OH; 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) FeCl₃ 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 (mmol L⁻¹)⁻¹ 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 μmol *trans*-cinnamic acid per h. 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 A₂₈₀ per h. The method of Bradford (1976) was used to determine protein content in enzyme extracts.

Preparation of silver thiosulfate (STS)

A stock of STS was prepared by mixing equal volumes of 0.01 mol L⁻¹ AgNO₃ and 0.04 mol⁻¹ Na₂S₂O₃ (Liu et al., 1990).

Statistical analysis

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

Results and discussion

MJ increases NH₄⁺ and H₂O₂ contents

NH₄⁺ content in the control leaves remained unchanged during 48 h of incubation (Fig. 1A). It is clear that MJ-treated rice leaves had higher NH₄⁺ contents than the control leaves 36 and 48 h after treatment (Fig. 1A). MJ treatment caused an increase in H₂O₂ content (Fig. 1B). Wounding is known to induce H₂O₂ production (Orozco-Cárdenas et al., 2001). When detached rice leaves are used, 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, H₂O₂ production of detached rice leaves in the presence or absence of MJ is unlikely to be complicated by the wounding effect. The increase in H₂O₂ in detached rice leaves was evident 12 h after MJ treatment (Fig. 1B), and preceded the increase in NH₄⁺. These results suggest

Figure 1. Changes in NH_4^+ (A) and H_2O_2 (B) contents in detached rice leaves treated with either water or $45 \mu\text{mol L}^{-1}$ MJ in the dark. Vertical bars represent standard errors ($n = 4$). Asterisks indicate values that are significantly different between H_2O and MJ treatments at $P < 0.05$ by Student's *t*-test.

that H_2O_2 may play a role in regulating NH_4^+ accumulation in detached rice leaves induced by MJ.

Effect of MJ on the enzymes related to NH_4^+ accumulation

GS is the primary enzyme responsible for NH_4^+ assimilation in plants (Mifflin and Lea, 1976). It is clear that the decrease in GS activity (expressed on the basis of g FW), rather than GS specific activity (expressed on the basis of mg protein), induced by MJ is closely associated with NH_4^+ accumulation (Fig. 2A and D). Since NH_4^+ is known to be released through the action of PAL, the first enzyme in the pathway of phenylpropanoid biosynthesis (Hahlbrock and Grisebach, 1979), it is likely that MJ-induced NH_4^+ accumulation is associated with an

increase in the activity or the specific activity of PAL in rice leaves. NH_4^+ accumulation caused by MJ was 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 MJ-treated rice leaves is most likely related to the increase in the activity or the specific activity of protease. As shown in Fig. 2C and F, MJ was observed to be effective in increasing the specific activity, rather than activity of protease. MJ-induced decrease in the activity of GS and increases in the specific activities of PAL and protease in rice leaves (which occurred 24 h after treatment) occur prior to MJ-induced accumulation of NH_4^+ (which occurred 36 h after treatment) and after the onset of H_2O_2 production (which occurred 12 h after treatment) (Figs. 1 and 2). It appears that GS, PAL, and protease are the enzymes responsible for MJ-induced NH_4^+ accumulation. The fact that MJ treatment resulted in an increase in the specific activities of both protease and PAL (Fig. 2E and F), suggests that PAL is resistant to proteolysis in detached rice leaves.

There is a dramatic increase in NH_4^+ content between 36 and 48 h for MJ treatment (Fig. 1A), which does not correspond to the kinetic of GS activity and PAL specific activity (Fig. 2A and E), suggesting that other factors may also contribute to the increase in NH_4^+ content. NH_4^+ is known to be produced during nitrate assimilation and photorespiration (Mifflin and Lea, 1976). Previously, we have shown that NH_4^+ accumulation in MJ-treated detached rice leaves is attributable to an increase in reduction of nitrate (Chen and Kao, 1998). Since our experiments were conducted in the dark, NH_4^+ accumulation induced by MJ is unlikely to have been produced from photorespiration.

Effect of sodium benzoate (SB), reduce glutathione (GSH), and nitric oxide (NO) donor

Previously, we have shown that free radical scavengers, such as SB and GSH, partially inhibited the increase in H_2O_2 content in rice leaves caused by MJ (Hung and Kao, 2004a). NO is a bioactive free radical implicated in a number of physiological processes in plants (Lamattina et al., 2003). We have shown that MJ-induced H_2O_2 production in rice leaves can be reduced by the NO donor *N*-tert-butyl- α -phenylnitrone (PBN) (Hung and Kao, 2004a). Here, we show that SB, GSH, and PBN are effective in reducing MJ-induced accumulation

Figure 2. Changes in the activities and the specific activities of GS (A, D), PAL (B, E), and protease (C, F) in detached rice leaves treated with either water or 45 $\mu\text{mol L}^{-1}$ MJ in the dark. Vertical bars represent standard errors ($n = 4$). Asterisks indicate values that are significantly different between H₂O and MJ treatments at $P < 0.05$ by Student's t -test.

of NH₄⁺ (Fig. 3D), decreasing the activity of GS (Fig. 3A), and increasing the specific activities of protease and PAL (Fig. 3B and C) in rice leaves. Meanwhile, the 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. 3), suggesting that the PBN effects are attributable to NO release.

Effect of dimethylthiourea (DMTU), a chemical trap for H₂O₂ and NADPH oxidase inhibitors

To demonstrate the involvement of H₂O₂ on the effects induced by MJ in detached rice leaves, namely the increase in NH₄⁺ content and the changes in enzymes responsible for NH₄⁺ accumulation, DMTU, a chemical trap for H₂O₂ (de Agazio and Zacchini, 2001), was used. Detached rice leaves were incubated in a solution containing 45 $\mu\text{mol L}^{-1}$ MJ with or without 5 mmol L⁻¹ DMTU. The increase in the content of NH₄⁺, the decrease in

the activity of GS, and the increase in the specific activities of PAL and protease caused by MJ was observed to be reduced by DMTU (Fig. 4).

ROS, originating from the plasma-membrane NADPH oxidase, which transfers electrons from cytoplasmic NADPH to O₂ to form O₂^{-•}, followed by dismutation of O₂^{-•} to H₂O₂, has been a recent focus in ROS signaling research (Neill et al., 2002). Diphenyleneiodonium chloride (DPI) and imidazole (IMD) are known to be inhibitors of NADPH oxidase (Orozco-Cárdenas et al., 2001). Recently, we also demonstrated that MJ-induced H₂O₂ accumulation in detached rice leaves can be inhibited by low concentrations of DPI (1 $\mu\text{mol L}^{-1}$) and IMD (100 $\mu\text{mol L}^{-1}$) (Hung et al., 2006), indicating that MJ-dependent H₂O₂ generation originates, in part, from plasma-membrane NADPH oxidase. When detached rice leaves were treated with DPI and IMD, MJ-induced NH₄⁺ accumulation in rice leaves was reduced (Fig. 4D). DPI and IMD also inhibited the decrease in the activity of GS and the increase in the specific activities of PAL and protease caused by MJ (Fig. 4A, B and C).

Figure 3. Effect of SB, GSH, and PBN on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH₄⁺ (D) in MJ-treated detached rice leaves in the presence or absence of c-PTIO. The concentrations of MJ, SB, GSH, PBN, and c-PTIO were 45 μmol L⁻¹, 10 mmol L⁻¹, 30 mmol L⁻¹, 100 μmol L⁻¹, 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, according to Duncan's multiple range test.

Figure 4. Effect of DMTU, DPI, and IMD on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH₄⁺ (D) in detached rice leaves treated with MJ. The concentrations of MJ, DMTU, DPI, and IMD were 45 μmol L⁻¹, 5 mmol L⁻¹, 1 μmol L⁻¹, 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, according to Duncan's multiple range test.

Collectively, these results appear to suggest that the changes in enzyme activities or specific activities related to NH₄⁺ accumulation, and the increase in NH₄⁺ content in detached rice leaves are a consequence of H₂O₂ production caused by MJ. This suggestion is supported further by the observations that exogenous application of H₂O₂ is able to increase NH₄⁺ content, decrease GS activity, and increase protease and PAL specific activities in detached rice leaves (Hung and Kao, 2005).

Effect of phosphatidylinositol 3-kinase (PI3K) inhibitors

The neutrophil NADPH oxidase consists of two plasma-membrane proteins, gp91^{phox} and p22^{phox}, which together form heterodimeric flavocytochrome *b*₅₅₈, 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 (Sagel and Abo, 1993; Henderson and Chappell, 1996). In neutrophils, 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). It has been shown that PI3P is important in

NADPH oxidase-mediated H₂O₂ production during ABA-induced stomatal closure (Jung et al., 2002; Park et al., 2003), ABA-promoted leaf senescence (Hung and Kao, 2004b), MJ-promoted leaf senescence (Hung et al., 2006), and during auxin-induced root gravitropic responses (Joo et al., 2005). LY 294002 (LY) and wortmannin (WM) are inhibitors of PI3K, a product of which is PI3P. Recently, we also demonstrated that LY (100 μmol L⁻¹) or WM (1 μmol L⁻¹) inhibited MJ-induced H₂O₂ production in detached rice leaves (Hung et al., 2006). When detached rice leaves were treated with a solution of LY or WM, MJ-induced accumulation of NH₄⁺ in detached rice leaves was reduced (Fig. 5D). LY or WM also inhibited the decrease in the activity of GS (Fig. 5A) and the increase in the specific activities of PAL and protease (Fig. 5B and C) in rice leaves caused by MJ. Exogenous H₂O₂ (1 mmol L⁻¹) was observed to reverse the inhibitory effect of LY or WM on MJ-induced accumulation of NH₄⁺ and changes of enzymes responsible for the accumulation of NH₄⁺ (Fig. 5).

The fact that PI3K inhibitors, which reduced the MJ-induced H₂O₂ production (Hung et al., 2006), were able to prevent the MJ-induced NH₄⁺ accumulation and changes in enzymes related to NH₄⁺

Figure 5. Effect of LY and WM on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH₄⁺ (D) in MJ-treated detached rice leaves in the presence or absence of H₂O₂. The concentrations of LY, WM, MJ, and H₂O₂ were 100 μmol L⁻¹, 1 μmol L⁻¹, 45 μmol L⁻¹ and 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, according to Duncan's multiple range test.

accumulation in detached rice leaves (Fig. 5), strengthen further our conclusion that H_2O_2 is necessary for MJ-induced NH_4^+ accumulation and changes in enzymes related to NH_4^+ accumulation.

JA induces H_2O_2 and NH_4^+ accumulation in the leaves of cultivar TN1 seedlings but not in cultivar TNG67

Fig. 6 shows the effect of JA concentrations, in the range $5\text{--}40\ \mu\text{mol L}^{-1}$, applied over a period of 3 days, on the contents of NH_4^+ and H_2O_2 in the second leaves of rice seedlings. It is clear that increasing JA concentration progressively increases NH_4^+ content in leaves of TN1 seedlings but not in leaves of TNG67 (Fig. 6B and D). It appears that, in terms of NH_4^+ accumulation, TNG67 is a JA-insensitive cultivar and TN1 is a JA-sensitive. If H_2O_2 is important in regulating NH_4^+ accumulation, then H_2O_2 content is expected to be increased in JA-treated seedlings of TN1 but not in TNG67. As indicated in Fig. 6A and C, this is the case.

An increase in ethylene sensitivity is associated with MJ- and ABA-induced NH_4^+ accumulation in detached rice leaves

In our recent work, we reported that H_2O_2 is necessary for ABA-induced NH_4^+ accumulation in detached rice leaves (Hung and Kao, 2005). Since endogenous ABA content decreased in MJ-treated rice leaves (Wang and Kao, 1999), it is unlikely that the effect of MJ on NH_4^+ accumulation in detached rice leaves is mediated through ABA. Previously, we demonstrated that MJ- and ABA-induced H_2O_2 production in detached rice leaves was inhibited by STS, an inhibitor of ethylene action, and concluded that one of the earliest events following MJ or ABA treatment is modulating ethylene sensitivity, which then causes the production of H_2O_2 (Hung et al., 2006). Here, we also observed that treatment of rice leaves with STS inhibited the accumulation of NH_4^+ , the decrease in GS activity, and the increase in protease and PAL specific activities caused by MJ and ABA (Fig. 7). Results suggest that ABA- or MJ-induced NH_4^+ accumulation is ethylene dependent.

Figure 6. Effect of JA on the contents of H_2O_2 (A, C), and NH_4^+ (B, D) in the second leaves of rice seedlings. Rice seedlings were cultivated in Kimura B solution in a Phytotron with natural sunlight at 30°C (day)/ 25°C (night) at 90% relative humidity. JA was added to the Kimura B solution when the third leaves were fully expanded. H_2O_2 and NH_4^+ contents were determined 3 days after adding JA. Vertical bars represent standard errors ($n = 4$). Values with the same letter are not significantly different at $P < 0.05$, according to Duncan's multiple range test.

Figure 7. Effect of STS on the activities of GS (A), the specific activities of protease (B) and PAL (C), and the content of NH₄⁺ (D) in detached rice leaves in the presence or absence of ABA and MJ. The concentrations of STS, ABA and MJ were 200, 45, and 45 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, according to Duncan's multiple range test.

Conclusion

Our results indicated that H₂O₂ production preceded the changes in enzyme activity associated with NH₄⁺ accumulation in MJ-treated detached rice leaves. In terms of NH₄⁺, it was observed that rice seedlings of TN1 are JA-sensitive and those of TNG67 are JA-insensitive. On treatment with JA, H₂O₂ accumulated in the leaves of TN1 seedlings but not in the leaves of TNG67. This work establishes the links between MJ (or JA) treatment, H₂O₂, enzymes responsible for NH₄⁺ accumulation, and NH₄⁺ accumulation.

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