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Involvement of Protein Phosphorylation and Reactive Oxygen Species in Jasmonate-elicited Accumulation of Defense/stress-related Proteins in Rice Seedlings

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Abstract: Calcium chelators/antagonist effectively blocked the jasmonic acid (JA) induced accumulation of a basic 28 kDa Bowman-Birk proteinase inhibitor and an acidic 17 kDa pathogenesis-related class 1 protein in rice seedling leaf sheaths. In gel kinase assays revealed rapid changes in the phosphorylation activities of a 46 and 56 kDa Myelin Basic Protein Kinase (MBPK) and a 48 kDa putative Ca^{2+}-dependent protein kinase (CDPK) by JA and leaf sheath cutting and these changes were determined to be cytosolic in nature. ***In vitro*** protein phosphorylation of crude and cytosolic protein extracts, followed by two-dimensional polyacrylamide gel electrophoresis showed considerable enhancement in the Ca^{2+}-dependent phosphorylation of certain proteins as early as 15 min after JA treatment, particularly a 56 kDa phosphoprotein. Hydrogen peroxide (a potent reactive oxygen species, ROS) and oxyfluorfen (a lipid peroxidizing agent) caused a significant increase in the levels of these two proteins over the cut control. Whereas, when JA was co-applied with 3-(3`, 4`-dichlorophenyl)-1,1-dimethylurea, a photosystem II electron transport inhibitor and n-propyl gallate, a free radical scavenger, the accumulation of these induced proteins were considerably reduced. These results suggest an involvement of kinase-signaling cascade(s) and the ROS in JA elicited accumulation of these defense/stress-related proteins in rice.

Key words: *Oryza sativa* L., calcium, jasmonic acid, protein phosphorylation, reactive oxygen species, ribulose-1, 5-bisphosphate carboxylase/oxygenase

Introduction

Jasmonic Acid (JA) and its methyl ester, methyl jasmonate, commonly referred to as jasmonates, are two of the most potent plant global signaling molecules and are involved in the regulation of many different processes in plants (Lee ***et al.***, 2001; Reymond and Farmer, 1998). Using proteome analysis, recently it has been observed that JA potently induce the accumulation of defense/stress-related proteins, including a novel basic 28 kDa Bowman-Birk proteinase inhibitor (BBPIN) and an acidic 17 kDa pathogenesis-related class 1 (PR1a) protein, in rice seedling tissues (Rakwal and Komatsu, 2000). A complex network of signal transduction is involved in plant defense responses and many of the downstream signaling events remain to be elucidated.

Protein phosphorylation and dephosphorylation have been shown to play an important role in the response to various plant hormones (Knetsch ***et al.***, 1996; Novikova ***et al.***, 2000; Seo ***et al.***, 1999; Suzuki and Shinshi, 1995; Yang and Komatsu, 2000; Zhang and Klessig, 1997). Phosphorylation of proteins is a transient process and increased phosphorylation of certain proteins involved in signal transduction can be brought about by inhibition of corresponding protein phosphatase using protein phosphatase inhibitors (Millward and Zolnierowicz, 1999). An important second messenger involved in mediating plant responses to stress is Ca^{2+} and therefore, we examined whether chemical agents that affect Ca^{2+} metabolism could influence the induction of BBPIN and PR1a proteins by JA. In related experiments, the effects of JA on downstream kinase-signaling cascade(s) were also investigated using in-gel kinase assays [for myelin basic protein kinase (MBPK) and Ca^{2+} dependent protein kinase (CDPK) activities] and ***in vitro*** protein phosphorylation (Yang and Komatsu, 2000) which revealed the activation of these signaling components by JA and leaf sheath cutting.

Other critical downstream components of the defense response pathways are the ROS and accumulating evidence indicates that hydrogen peroxide (H_2O_2) functions as a signaling molecule in plants; H_2O_2 is one of the earliest cellular responses to potential pathogens and elicitor molecules (Lamb and Dixon, 1997). The ROS also serve to activate phytoalexin biosynthesis and induction of defense-related genes/proteins (Levine ***et al.***, 1994 and Low and Merida, 1996) and H_2O_2 has been proposed to be a key factor mediating programmed cell death in response to pathogens, elicitors and hormones (Bethke and Jones, 2001; Desikan ***et al.***, 2001 and Tenhaken ***et al.***, 1995). Therefore, we used H_2O_2 and ROS generating chemicals, including a photosystem II electron transport chain inhibitor and a free radical scavenger to examine the involvement of ROS in mediating the JA induced accumulation of the two defense/stress related proteins. Taken together, the present study provides evidence for early changes in kinase activities and the potential involvement of the ROS in potentiating JA-induced accumulation of these two defense/stress-related proteins, the 28-kD BBPIN and the 17-kD PR1a in rice seedling leaf sheaths.

Materials and Methods

Plant material

Two-week-old rice (***Oryza sativa*** L. cv. Nipponbare) seedlings were grown under white fluorescent light (wavelength 390-500 nm, 150 μmol $m^{-2}s^{-1}$, 12 h light period/day) at 25°C and 70% relative humidity, in a growth chamber, as described previously (Rakwal and Komatsu, 2000; Rakwal and Komatsu, 2001a and Rakwal ***et al.***, 2001b). For treatment with JA, 3 cm long leaf sheath segments were floated on a 10 ml solution of 100 μM JA in plastic Petri dishes under continuous white fluorescent light (150 μmol m^{-2} s^{-1}), either in the presence or absence of the inhibitors described in the figure legends and incubated as above for various time periods as indicated. The leaf sheath segments floated on Milli Q (MQ) water served as the cut control (labeled as CONTROL or CON in the Fig.). For sampling, leaf sheath segments were blotted dry on a Kimwipe tissue, weighed and immediately frozen at -80°C until proteins were extracted for analysis.

Chemicals

JA (raecemic mixture), methyl viologen (MV), n-propyl gallate (nPG), an antioxidant, ethylene glycol-bis (â-aminoethyl ether)-***N,N,N`,N`***-tetraacetic acid (EGTA), â-glycerophosphate (disodium salt), sodium orthovanadate (Na_3VO_4), sodium fluoride (NaF), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), myelin basic protein (MBP) and histone III-S were purchased from Sigma (St. Louis, MO, USA). Calcium chloride ($CaCl_2$), ethylenedinitrilotetraacetic acid (EDTA), sodium pyrophosphate decahydrate, trichloroacetic acid (TCA), zinc chloride ($ZnCl_2$) and H_2O_2 were obtained from Wako Pure Chemicals (Tokyo, Japan). The photosystem II electron transport chain inhibitor 3-(3`, 4`-dichlorophenyl)-1,1-dimethylurea (DCMU) was from Nacalai Tesque (Kyoto, Japan) and the low molecular weight (LMW) markers were from Amersham Pharmacia Biotech (Uppsala, Sweden). Oxyfluorfen (OXY) was a kind gift from Dr. Peter Boger (University of Konstanz, Konstanz, Germany).

Preparation of crude protein extract and subcellular fractions

All procedures were carried out at 4°C. For preparation of crude protein extract, 100 mg of leaf sheath segments were cut into small pieces with a clean scissor and immediately homogenized in 400 µl of cold homogenization buffer (0.2 M Tris-HCl, pH 7.8; containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 10% v/v glycerol for SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting); (0.2 M Tris-HCl, pH 7.8; containing 5 mM EDTA, 14 mM 2-mercaptoethanol, 10% w/v glycerol, plus 1 mM PMSF, 1 mM Na_3VO_4 and 50 mM β-glycerophosphate for in-gel kinase assay) in a chilled glass mortar and pestle. The homogenates were centrifuged at 20,600 × g, at 4°C, first for 5 min and then for 10 min and the final supernatant was used as the crude protein extract. For separation of proteins into the cytosolic and membrane fractions, 50 mg leaf sheaths were homogenized in 200 µl cold in-gel kinase assay homogenization buffer, as above and centrifuged for 600×g for 5 min. The resulting supernatant was ultracentrifuged at 100,000 rpm (Beckman Ultracentrifuge, rotor, TLA 100.2) for 15 min and the cytosolic fraction was obtained by collecting the supernatant. The pellet was resuspended in 100 µl of cold in-gel kinase homogenization buffer and washed by centrifugation at 100,000 rpm for 15 min. The pellet was resuspended in 100 µl cold membrane solubilizing buffer (the same in-gel kinase assay homogenization buffer containing 1% Triton X-100) and solubilized for 30 min on ice. The membrane fraction was obtained from the supernatant after centrifugation at 100,000 rpm for 7 min. Total protein concentrations were determined by a dye-binding assay with BSA as a standard (Bradford, 1976).

Immunoblot analysis

The anti 28 kDa BBPIN and 17 kDa PR1a antibodies were prepared in a previous study (Rakwal and Komatsu, 2000). SDS-PAGE was performed as above, except for loading of equal volume (2.5 µl) of the samples for each treatment and control, thereby having a representative level of the protein after each treatment with respect to the control(s). Immediately after completion of the run, the separated proteins were electroblotted onto a polyvinylidene difluoride membrane (Fluorotrans, Pall BioSupport Division, Port Washington, NY, USA) using a semi-dry

transfer blotter (Nippon Eido, Tokyo, Japan) and detected by peroxidase enzyme immunoassay (Rakwal and Komatsu, 2000).

In-gel kinase assay

Crude protein extracts prepared from two-week-old rice seedling leaf sheaths treated with 100 μM JA or the cut control were subjected to in-gel kinase assay, with either MBP or histone III-S as the substrate. For determining MBP phosphorylation activity, protein extracts (approximately10 μg) were separated by 15% SDS-PAGE containing 0.25 mg ml^{-1} MBP as substrate in the separating gel, following the previously published procedure of Romeis (Romeis ***et al.***, 1999). Briefly, after electrophoresis, SDS was removed and the proteins denatured by washing the gel with buffer A (25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 0.5 mg ml^{-1} BSA, 0.1% Triton X-100 and 5 mM NaF) for 1 h. The separated proteins were then renatured in buffer B (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF) for another 1 h at room temperature with constant shaking, before being kept overnight at 4°C. The gels were then equilibrated in 25 mM Tris-HCl (pH 7.5), 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT and 0.1 mM Na_3VO_4 for 30 min at room temperature. The reaction was initiated by adding 5 μM [γ-^{32}P]ATP (110 TBq $mmol^{-1}$, Amersham Pharmacia Biotech, Buckinghamshire, UK) and incubated at 30°C for 30 min. The reaction was stopped by extensive gel washing with 5% (w/v) TCA containing 1% (w/v) sodium pyrophosphate until background radioactivity decreased. The gels were stained with Coomassie brilliant blue (CBB) R-250, destained, dried and exposed to an X-ray film (Kodak, Rochester, NY, USA) at -80°C for 3 days. For demonstrating CDPK activity using histone III-S as a substrate, the same samples that were used for determining MBP phosphorylation activity, were subjected to in-gel kinase assay, following a previously published procedure (Yang and Komatsu, 2000). CBB staining also confirmed an almost equal loading.

***In vitro* protein phosphorylation**

Five μl protein extracts (approximately 5 μg) were incubated in a 25 μl reaction mixture that consisted of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1.25 mM $CaCl_2$ and 39 μM [γ-^{32}P]ATP (110 TBq $mmol^{-1}$). The reaction mixture was incubated for 10 min at 30°C and terminated by addition of an equal volume (25 μl) of lysis buffer (O'Farrel, 1975) to the reaction mixture. The samples were then subjected to two-dimensional-PAGE (2D-PAGE) according to the procedure of O`Farrel (1975). Proteins were separated in the first dimension by isoelectric focusing and in the second dimension by 15% SDS-PAGE. The separated proteins on gels were stained with CBB, destained, dried and exposed to an X-ray film at -80°C for 3 days.

Results and Discussion

Co-application of EGTA, EDTA and $ZnCl_2$ with JA block the induction of BBPIN and PR1a proteins

As calcium serves as an essential second messenger during a variety of stress responses in plants, the effect of chemical agents that affect calcium metabolism, such as EGTA, an extracellular Ca^{2+} chelator, EDTA, a divalent cation chelator and $ZnCl_2$, a Ca^{2+} antagonist was examined on the induction of these two defense/stress related proteins in leaf sheaths. These

chemicals were applied simultaneously with JA and the induction profiles were examined by immunoblot analysis (Fig. 1). It was found that these chemical agents almost completely blocked the induced accumulation of the BBPIN and PR1a proteins, strongly suggesting an additional involvement of Ca^{2+} in mediating JA action in rice. Although, to best of our knowledge, there are no reports about these chemicals blocking PR protein accumulation(s) in rice (and other plants), these same chemicals have been recently shown to prevent victorin (a host-specific toxin from *Puccinia coronata* f. sp. *avenae* causing crown rust disease of oats) induced DNA-ladder formation (a sign of apoptosis) in oat leaves, suggesting that it was a Ca^{2+}-required active process (Tada *et al*., 2001).

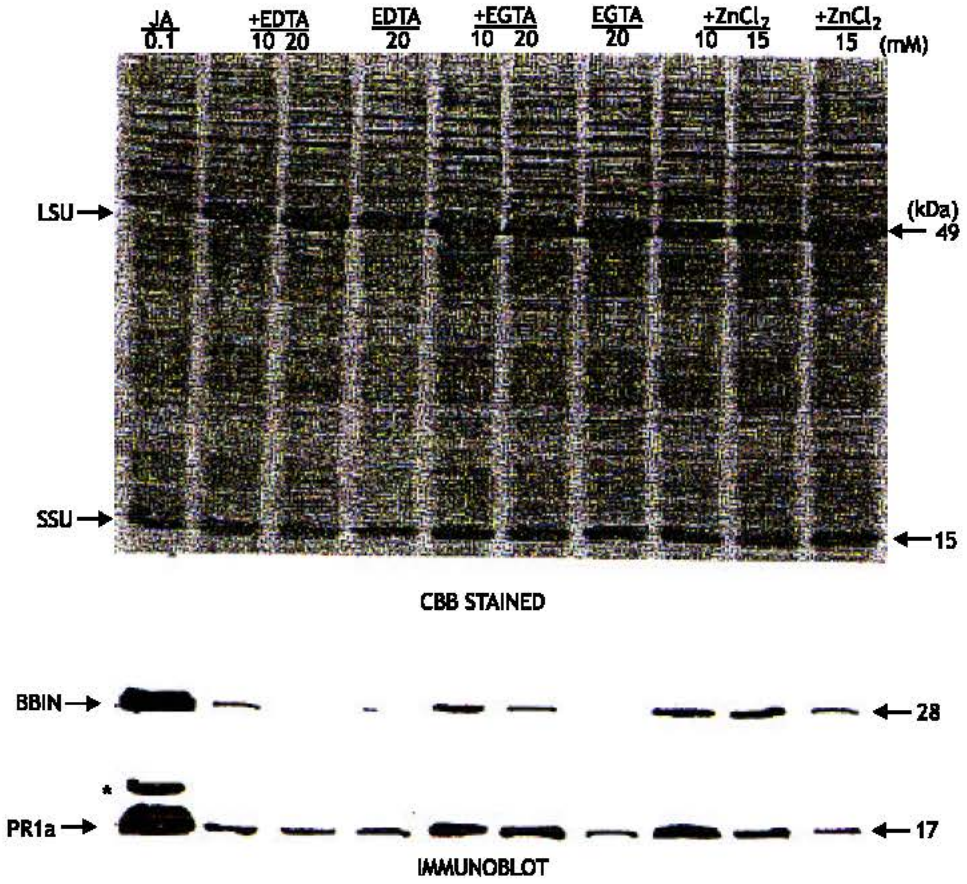

Fig. 1: Effect of EDTA, EGTA and $ZnCl_2$ on the JA-induced leaf sheath proteins. EDTA, EGTA (10 and 20 mM) and $ZnCl_2$ (10 and 15 mM) were co-applied with 100 μM JA. Leaf sheaths treated with 20 mM EDTA, EGTA and 15 mM $ZnCl_2$ alone served as a control. Upper panel shows the CBB stained polypeptides separated on a 15% polyacrylamide gels. Lower panel shows the immunostained 28 kDa BBPIN and the 17 kDa PR1a. The asterisk shows low molecular weight polypeptides that cross-react with the anti 28 kDa BBPIN antibody. Protein markers (LMW) are indicated at the left in kDa.

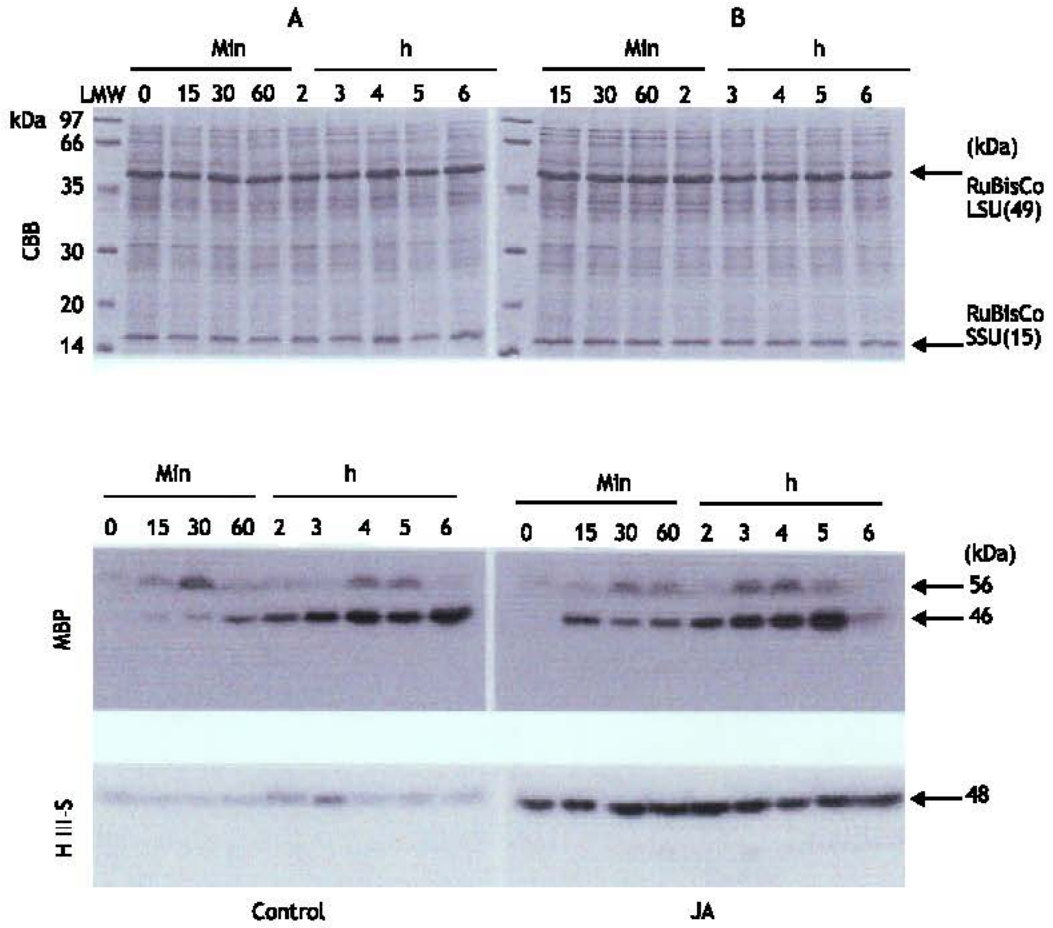

Fig. 2: Jasmonic acid (JA) rapidly activates 46/56 kDa MBPK and 48 kDa putative CDPK activities. Leaf sheaths were treated with 100 μM JA (JA) for the respective time periods as indicated on the top of each lane (min, minutes; h, hours), under continuous light (B). The leaf sheath segments floated on MQ water served as a cut control (A). Proteins were separated on 15% polyacrylamide gels. CBB stained protein profiles of the separated leaf sheath crude protein extracts are shown at the top. The position of low molecular weight (LMW) markers is indicated at the left in kDa. The RuBisCO large chain (LSU, 49 kDa) and small chain (SSU, 15 kDa) are also marked for reference on the right hand side. In-gel kinase assay, with MBP and histone III-S as a substrate, are shown in the middle and lower panels, respectively. These are the representatives of three independent experiments. The 46, 48 and 56 kDa kinase activities are marked on the right

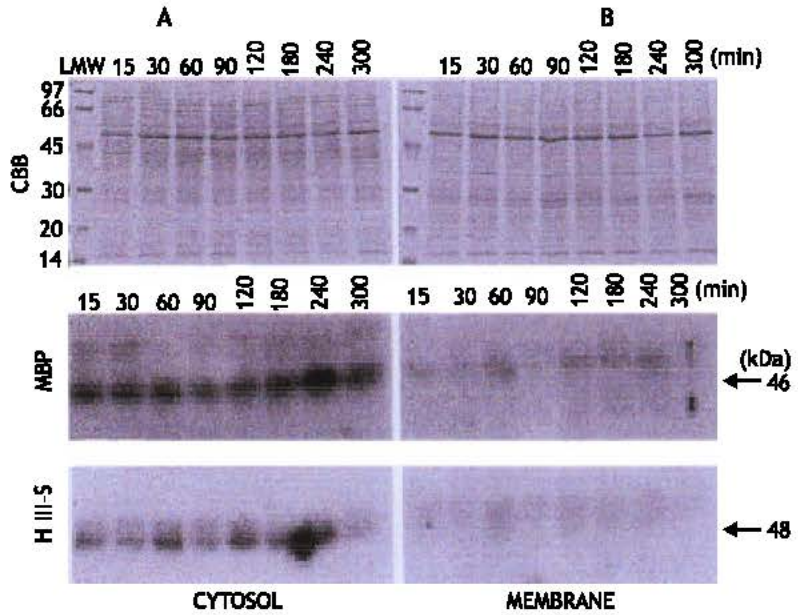

Fig. 3: Localization of the MBPK and putative CDPK activities in the cytosol fractions. Samples of leaf sheaths treated with 100 μM JA were taken at the time periods mentioned at the top of each lane in min. Proteins were separated into the cytosolic (A) and membrane (B) fractions and in-gel kinase assay was performed as described in Fig. 2. The position of the 46 and 48 kDa proteins is marked on the right

JA elicits changes in the MBP kinase and histone III-S phosphorylating activity in leaf sheaths

From the above results it was speculated that Ca^{2+} might influence certain identifiable changes in the components of the kinase-signaling cascades. In order to identify the specific kinase activities affected by JA treatment, a time-course experiment from 15 min to 6 h, with 100 μM JA with an appropriate cut control was carried out using in-gel kinase assay (Fig. 2). The CBB stained gels show almost an equal loading of the crude protein samples in both the cut control (Fig. 2A, upper panel) and after JA treatment (Fig. 2B, upper panel). Results revealed that cutting (CONTROL) caused a rapid increase in the MBP kinase (MBPK) activity of an approximately 46 kDa protein, which was identical to that seen after JA treatment. This enhanced kinase activity was observed as early as 15 min after treatment, the first time period analyzed (Fig. 2, middle panels). It should be noted that this 46 kDa MBPK activity was not present in the healthy leaf sheaths at 0 h, thus further indicating a specific response to applied JA and/or cutting. Moreover, this kinase activity was not detected in gels in the absence of MBP in the SDS-gel, indicating that the observed phosphorylation activity was not due to their autophosphorylation (data not shown).

On the other hand, in the case of the MBPK activity of approximately 56 kDa (upper band), a similar change in activity was observed in a biphasic manner, first within 30 min of treatment (JA) and after cutting (CONTROL) and then at 3 h in the case of JA and at 4 h with cut. These results present the first evidence for JA in affecting an MBPK activity in rice seedling tissues.

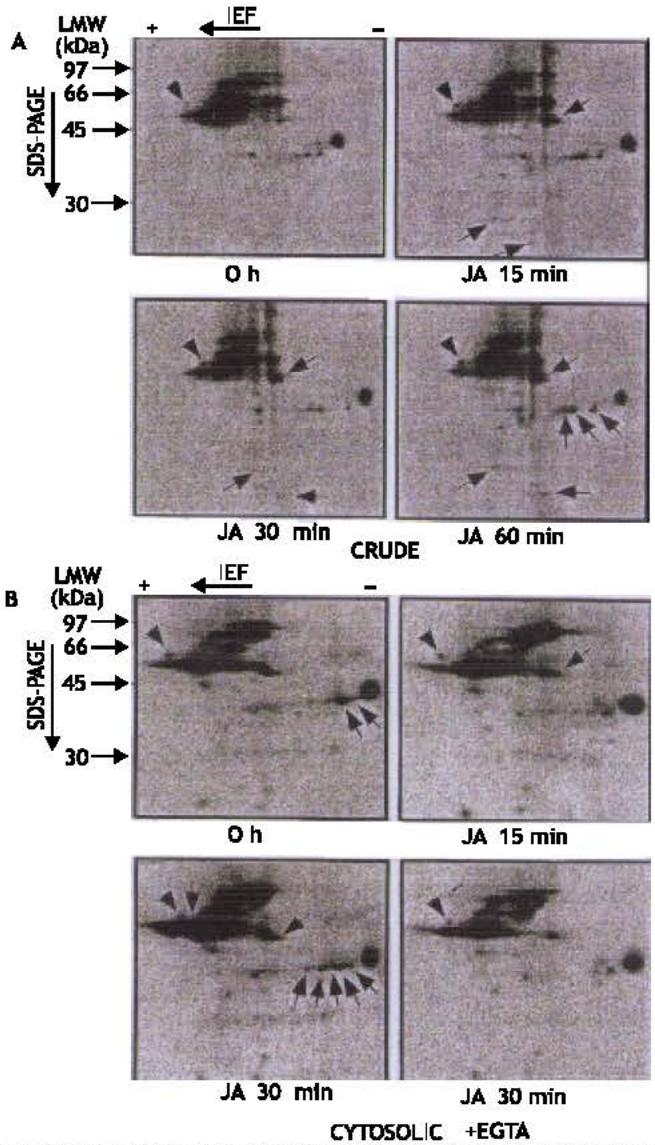

Fig. 4: *In vitro* effect of calcium upon protein phosphorylation in leaf sheath treated with 100 μM JA and 4 mM EGTA. 2D-PAGE was carried out after *in vitro* phosphorylation of crude (A) protein from leaf sheaths treated with JA for 15, 30 and 60 min and cytosol (B) protein fraction from leaf sheaths treated with JA for 15 and 30 min and JA + EGTA for 30 min. In the case of crude protein extract 0 h (which serve as cut control, CON) represents the leaf sheath placed in MQ water for 60 min, JA 15 min and JA 30 min means the samples placed in MQ water for 45 and 30 min and then in JA solution for 15 min and 30 min, respectively and JA 60 means samples placed only in JA solution for 60 min. Similarly, for the analysis of cytosolic fractions, 0 h (which serve as cut control, CON) represent the samples placed in MQ water for 30 min and JA 15 min means leaf sheath placed in water for 15 min and then in JA solution for 15 minutes and JA 30 min and JA+EGTA 30 min means the leaf sheaths placed in JA solution for 30 min and in JA+EGTA solution for 30 min, respectively. The reaction mixture contained 0.2 mM $CaCl_2$. Arrowheads mark the position of an approximately 56 kDa acidic phosphoprotein. Arrows indicate the changes in the phosphoproteins over the 0 h and the cut control, respectively

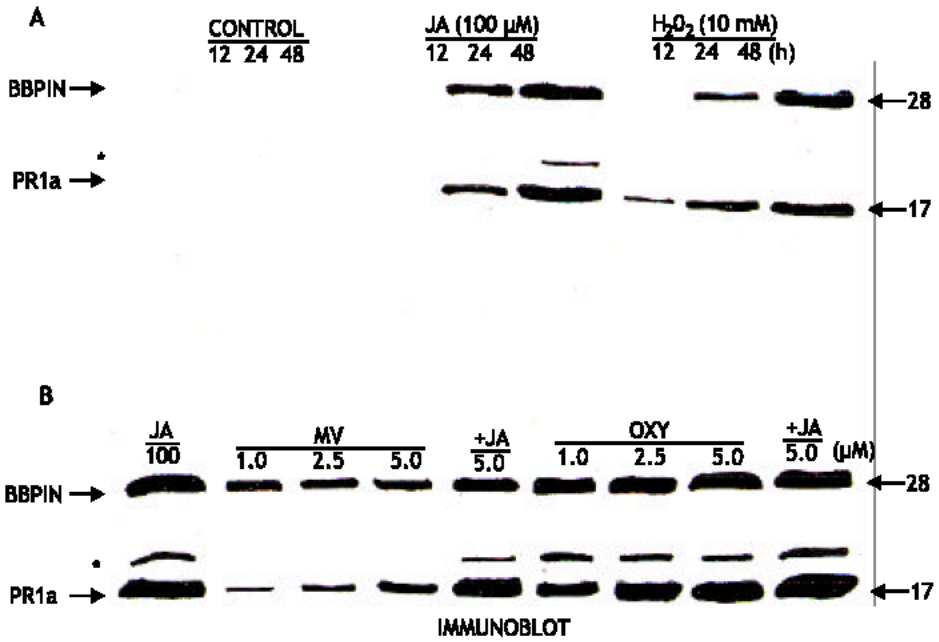

Fig. 5: H_2O_2 and free radical generating chemicals also induce accumulation of these two defense/stress-related proteins. A, Effect of 10 mM H_2O_2 on leaf sheath proteins at the respective time periods, given in h over each lane. JA (100 µM) treated leaf sheath proteins were electrophoresed as a comparative control. B, Effect of MV and OXY on the leaf sheath proteins, Leaf sheath was treated with MV (1.0, 2.5 and 5.0 µM), or with a co-application of 100 µM JA and with OXY (1.0, 2.5 and 5.0 µM), or with a co-application of 100 µM JA. Panels show the immunostained 28 kDa BBPIN and the 17 kDa PR1a proteins. The asterisk shows low molecular weight polypeptides that cross-react with the anti-28 kDa BBPIN antibody.

Recently a MAPK has been isolated from blast fungus infected and wounded rice (He *et al*., 1999). Likewise an increase in the MBP-phosphorylating activity was shown to be associated with a protein of approximately 47 kDa in the leaves of *Arabidopsis thaliana* treated with ethylene (Novikova *et al*., 2000). Further, application of JA and irradiation of wounded young tomato leaves with ultraviolet-C and UVB/UVA to induced 48 kDa MBPK activities (Farmer and Ryan, 1990; Stratmann *et al*., 2000). In another report, osmotic (NaCl) stress was shown to rapidly activate two protein kinases (35 and 46 kDa in size, respectively) that phosphorylated MBP in an in gel assay (Munnik *et al*., 1999). The estimates of the enzymes responsible for MBP phosphorylation provided by the in gel assays in these independent studies, including our present work, fall within the range of MAPKinases reported in plants (Knetsch *et al*., 1996; Zhang and Klessig, 1997 and Usami *et al*., 1995).

On the other hand, a potent activation of the 48 kDa kinase activities that phosphorylated histone III-S, in the presence of Ca^{2+} was seen after JA application and which was significant over

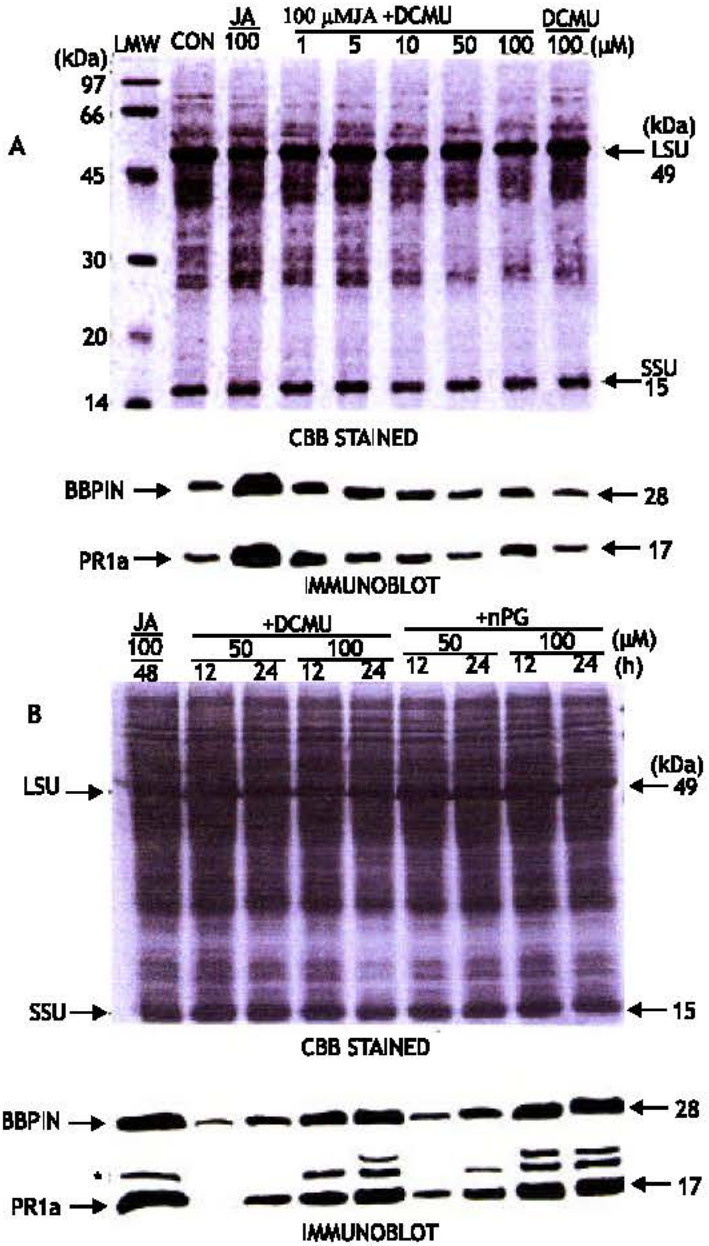

Fig. 6: Photosystem II electron transport system inhibitor considerably blocks the JA induced accumulation of the two defense/stress-related proteins in seedling leaf sheaths. A, Co-application of DCMU with JA at 48 h under continuous light. JA (100 µM) treated leaf sheath proteins were electrophoresed as a comparative control. B, Time-lag experiment with DCMU and nPG applied at 12 and 24 h after JA treatment. CBB stained 15% polyacrylamide gels are shown on the top. Lower panels show the immunostained 28 kDa BBPIN and the 17 kDa PR1a proteins

the cut control (Fig. 2B, lower panel). Moreover, this 48 kDa-phosphorylation activity was almost completely inhibited by the addition of EGTA, which indicated that this 48 kDa protein kinase is a putative CDPK (Yang and Komatsu, 2000). In the absence of substrate in the gel, the three-kinase activities were also observed in the presence of Ca^{2+}, but the 48 kDa putative CDPK had considerably reduced activity (Yang and Komatsu, 2000). This was due to autophosphorylation. This result indicates the possible presence of a JA responsive CDPK in leaf sheath tissues. However, unlike the MBPK activity, this putative CDPK activity was also detected in the healthy leaf sheaths (0, lane 1, CONTROL/JA), thus indicating a significant difference in the presence and/or activation of kinase activities in rice seedling leaf sheath tissues. Activation of CDPKs downstream of plant hormone application has been reported in dicots and monocots. For example, auxin treatment increased the CDPK gene expression in mungbean cuttings (Botella ***et al.***, 1996). Gibberellin (GA_3) was shown to affect CDPK activity in rice (Abo-El-Saad and Wu, 1995). A requirement for protein phosphorylation/dephosphorylation in the induction of ACC oxidase by ethylene (Kwak and Lee, 1997) was also demonstrated. Furthermore, changes in the protein kinase activity and phosphorylation of proteins were found in the membrane fractions from rice seeds treated with ABA (Komatsu ***et al.***, 1997).

Finally, time course experiments indicated that these 46 kDa MBPK and 48 kDa putative CDPK activities were predominantly cytosolic in nature, as demonstrated by separating the crude protein extracts into their respective cytosolic (Fig. 3A) and membrane (Fig. 3B) fractions. The CBB stained SDS-PAGE gels representing equal loading are presented in Fig. 3A (upper panels). These results further demonstrate that applied JA has the ability to modulate the kinase activities in the rice seedling leaf sheath tissues, of both the MBPK and histone III-S type and that these changes are cytosolic in nature. A previous report on rice lamina joint and associated CDPK revealed that application of a steroid-type phytohormone, brassinolide caused an increase in the activities of a cytosolic 45 kDa CDPK (Yang and Komatsu, 2000).

***In vitro* protein phosphorylation affected by JA**

The next sets of experiments were carried out in order to assess the accompanying changes, in the protein phosphorylation status after JA treatment. For this, phosphorylation of the crude and cytosolic protein fractions were carried out in an ***in vitro*** protein phosphorylation experiments. The phosphorylated proteins from both of these protein fractions were subsequently separated by 2D-PAGE and visualized by autoradiography (Fig. 4). The results with the crude protein fraction are presented in figure 4A. A number of proteins in JA treated samples (spots labeled by arrowheads and arrows) in the molecular mass range of approximately 25 to 40 kDa and 45 to 60 kDa were phosphorylated in the presence of calcium, in general slightly over the cut control (0 h). In particular, a protein that was acidic in nature (approximate pI 4.3 and an approximate molecular mass of 56 kDa) was strongly phosphorylated after JA treatment (indicated by an arrowhead). It should be noted that this particular acidic protein was weakly phosphorylated in the 0 h control and this result was consistently observed in repeated experiments. It would be interesting to investigate this 56 kDa phosphoprotein (and other phosphoproteins) for its involvement in phytohormone signaling and Ca^{2+} homeostasis in future

studies. Moreover, the changes in the phosphorylation status of these proteins were quite rapid, within 15 to 30 min after application of JA. This was in good correlation with the rapid modulation of kinase activities observed in Fig. 2 and 3. In a recently published report, a similar change in the phosphorylation status of proteins was found after brassinolide treatment of rice lamina joint (Yang and Komatsu, 2000), which suggests there are specific changes downstream of phytohormone application in rice seedling tissues.

As the protein kinase activities were further found to localize predominantly in the cytosolic fractions obtained from the crude protein extract, we also investigated the phosphorylation status in the cytosolic fractions (Fig. 4B). It was again confirmed that JA treatment caused a more rapid and stronger phosphorylation of many proteins (marked by arrowheads and arrows) over the cut control, at similar time periods. It was again observed that although the approximately 56-kDa acidic protein was phsophorylayted in the cut controls (upper left panel), the intensity of its phosphorylation was stronger after JA treatment within 15 min of application. Most of the protein phosphorylation in both the crude and the cytosolic protein fractions was also strongly inhibited in the presence of 4 mM EGTA in the reaction mixture (Fig. 4B, lower right panel), strongly suggesting a Ca^{2+}-dependent phosphorylation of these proteins. It is possible these phosphoproteins may constitute some of the endogenous substrates for the putative CDPKs affected by JA. Although the nature of these phosphoproteins remains to be clarified, they may be involved in the JA signaling in the rice seedling leaf sheath.

Exogenously applied H_2O_2 also elicits accumulation of the JA-induced defense/stress-related proteins

We next investigated the effect of the ROS (i.e., ROS itself, chemicals that generate the ROS and ROS scavengers) in influencing JA action on induction of the two defense/stress-related proteins in rice seedling (Fig. 5). H_2O_2 (widely used at exogenous concentrations ranging from 10 to 50 mM) was used at 10 mM to examine its effect on accumulation of BBPIN and PR1a in the leaf sheaths. Results presented in Fig. 5A show that H_2O_2 alone is indeed able to activate the accumulation of these two defense/stress related proteins in leaf sheath tissues and the levels of these two proteins were significantly higher than cut in cut control and only slightly lower than that seen after JA treatment. These results showed that H_2O_2 application can elicit the accumulation of BBPIN and PR1a.

Furthermore, the effect of H_2O_2, like that observed for JA, was dependent on time, thus suggesting a time-dependence effect of its action. To further clarify the induction of BBPIN and PR1a, which might be due to the ROS, an additional experiment that involved treating leaf sheath segments with two ROS generating/lipid-peroxidizing chemicals (MV and OXY) was carried out (Fig. 5B). It was found that OXY had a particularly strong effect on the accumulation of these two proteins, whereas MV was only partially effective. However a co-application of JA with either MV or OXY only slightly further enhanced the induced accumulation of these two proteins (Fig. 5B, JA+MV/OXY), over both MV and OXY applied alone. As this accumulation was not synergistic, it is suggested that, at least, both JA and the ROS may induce these two defense/stress-related proteins independently. These findings can be better explained by using ROS (particularly H_2O_2)

overproducing rice mutants in future studies. It was previously shown that JA (by using methyl JA) can cause ROS accumulation in rice through lipid peroxidation and which is one of the causes for the senescence in rice leaves (Hung and Kao, 1998). In our present report, it is suggested that ROS accumulation downstream of JA might be partly involved in influencing the induced accumulation of these defense/stress-related proteins and which also might depend on the type of the elicitor used.

DCMU and nPG block the JA induced accumulation of BBPIN and PR1a proteins

The preceding results on ROS affecting accumulation of the two defense/stress-related proteins suggests an involvement of the ROS in affecting the induction of BBPIN and PR1a. In this final set of experiments, we used a potent inhibitor of the photosystem II electron transport system, DCMU that was previously used to suggest that JA has the ability to potentiate ROS generation in the chloroplasts (Rakwal and Komatsu, 2001a). DCMU was shown to have an inhibitory effect on JA action because it completely blocked the necrotic lesion formation in leaves and browning of the leaf sheaths (Rakwal and Komatsu, 2001a). To test whether DCMU application could also reverse the JA induced accumulation of the BBPIN and PR1a, leaf sheaths were treated with DCMU that was co-applied at different doses with 100 μM JA. It was found that the DCMU considerably, though not completely, suppressed the JA induced accumulation of these two proteins in a dose-dependent manner (Fig. 6A). This result suggests that JA treatment affects the photosynthetic apparatus and might influence the levels of the ROS that may be in some way affecting the induction of the BBPIN and PR1a. Moreover, 100 μM DCMU itself did not significantly alter the accumulation of these two proteins, over the cut control.

Finally, a time-lag experiment was carried out using DCMU and another free radical scavenger, nPG to investigate the time periods during which these inhibitors are effective in blocking JA action. Both DCMU and nPG were applied at 12 and 24 h after treatment with JA and the levels of BBPIN and the PR1a were checked at 48 h after incubation under continuous light (Fig. 6B). Surprisingly, both DCMU and nPG were more effective at lower concentrations of 50 μM, than at 100 μM concentrations. When these inhibitors were applied 24 h after treatment with JA, the reduction in the levels of the BBPIN and PR1a were not as significant as when applied with 12 h after application with JA. In fact the BBPIN and PR1a protein amounts after nPG treatment were similar to or more than that seen with JA treatment alone at 48 h. This compelled us to look back at our data in fig. 5A and a further examination revealed that co-applied DCMU at 100 μM concentrations was not so effective as a 50 μM solution. It is quite possible that at higher concentrations, these inhibitors are slightly toxic (possibly toxic at lower levels also) to the cells and thus lead to the discrepancy in the results at 100 μM concentrations. However, these results suggest that certain early events involving the ROS are indeed affecting the accumulation (or the levels) of the BBPIN and the PR1a proteins in leaf sheath tissues.

Results presented in this study show that Ca^{2+} chelators/antagonists effectively block induction of the BBPIN and PR1a proteins upon co-application with JA and second, that, JA indeed elicits a rapid activation of the kinase activities of the MAPK (shown by using MBP as a substrate) and CDPK (putative, using histone III-S as a substrate) type and specific increases in

the phosphorylation status of certain proteins. These results clearly demonstrate that JA treatment to rice seedling leaf sheaths results in activation of the kinase-signaling cascades, of which the phosphorylation of certain target proteins may be actively involved in mediating through subsequent downstream signaling cascades the activation of the defense/stress-related proteins, the 28 kDa BBPIN and the 17 kDa PR1a investigated in this report. Furthermore, that the disturbances in the electron transport chain of the photosystem II and the subsequent damage to the photosynthetic machinery and generation of ROS may be linked to the JA elicited accumulation of the defense/stress-related proteins. This study suggests the involvement of chloroplastic generated ROS and as the chloroplast provides essential energy to the plants, it also plays an important role in the defense/stress responses in rice.

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