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A Novel Rice (*Oryza sativa* L.) Acidic *PR1* Gene Highly Responsive to Cut, Phytohormones, and Protein Phosphatase Inhibitors

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A novel rice acidic pathogenesis-related (PR) class 1 cDNA (*OsPR1a*) was isolated from jasmonic acid (JA)-treated rice seedling leaf. The *OsPR1a* cDNA is 830 bp long and contains an open reading frame of 507 nucleotides encoding 168 amino acid residues with a predicted molecular mass of 17,560 and p*I* of 4.4. The deduced amino acid sequence of *OsPR1a* has a high level of identity with acidic and basic PR1 proteins from plants. Southern analysis revealed that *OsPR1a* is a member of a multigene family. The *OsPR1a* gene was found to be cut-inducible, whereas the phytohormones JA, salicylic acid (SA), 3-indoleacetic acid, gibberellin, and ethylene (using ethylene generator ethephon, ET) enhanced accumulation of *OsPR1a* transcript, as well as the protein phosphatase inhibitors cantharidin (CN) and endothall (EN). Induced expression of *OsPR1a* gene by JA, CN or EN, and ET was light/dark- and dose-dependent and was almost completely inhibited by cycloheximide. Dark downregulated CN-, EN-, and ET-induced *OsPR1a* gene expression, whereas it was further enhanced with JA. SA and abscisic acid blocked JA-induced *OsPR1a* transcript. Simultaneous application of staurosporine (ST) enhances CH- or EN-induced *OsPR1a* transcript, but not with JA. This is the first report on cloning of a rice acidic *PR1* gene (*OsPR1a*), which is regulated by phytohormones, phosphorylation/dephosphorylation event(s), and light.

***Key Words: OsPR1a*; rice; phytohormones; protein phosphatase inhibitors; phosphorylation/dephosphorylation.**

Our interest in rice (*Oryza sativa* L.), an important socioeconomic crop in Southeast Asia, is focused on the rice plant self-defense mechanism(s), which started with identification of defense-related and cellular protectant proteins from stressed rice plants (1). The HR response characterizing plant-pathogen interactions (2), is not only associated with synthesis of low molecular weight secondary metabolites (e.g., toxic phytoalexins; 3), cell wall rigidification as a result of callose, lignin and suberin deposition, but also with the induction of a variety of novel proteins, collectively referred to as the "pathogenesis-related proteins" (PRs) (4). It has often been suggested that the collective set of PRs may be effective in inhibiting pathogen growth, multiplication and/or spread, and be responsible for the state of the systemic acquired resistance (SAR; 5). Moreover, the induction of PRs has been found in many plant species belonging to various families (6), suggestive of a general role for these proteins in adaptation to biotic stress conditions (4). Among these PRs, the PR1 class is a dominant group that is induced by pathogens, and is commonly used as a marker for SAR, with only the PR1 members from dicots, like tobacco and tomato being well characterized (4).

The importance of the phytohormones salicylic acid (SA) and JA (7) as critical signals in induced resistance responses in plants is recognized (8, 9, 10). Our previous studies on JA effecting rice secondary metabolite production and induction of a variety of defense-related proteins, provide additional evidence for the importance of JA in rice plant self-defense, though the pathways of JA action and its interaction with other phytohormones remain to be clarified (11, 12, 1). Although, JA was shown to induce some members of the PRs in rice, we were not able to identify any proteins with homology to the PR1 protein family (1). Considering the importance of PR1 family of proteins in plant self-defense (10), and the role of JA as a signaling molecule

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in rice plants (8, 9), it was reasoned that JA should also have an ability to effect expression of the *PR1* gene(s) in rice. Thus, we planned and conducted these present experiments with a defined aim in mind: to identify JA-induced rice *PR1* gene(s).

Here, we described the cloning and characterization of a novel acidic *PR1* gene from JA-treated rice (cv. Nipponbare) seedling leaf, which we designated as *OsPR1a*. This *OsPR1a* gene comprises a multigene family in rice, and encodes a highly acidic low molecular weight protein of p*I* 4.4. Other than JA-inducibility, this *OsPR1a* gene was found to be highly responsive to cutting, exogenous application of other phytohormones and protein phosphatase inhibitors. Furthermore, cytoplasmic *de novo* protein synthesis was shown to be required for *OsPR1a* gene activation by these compounds. On the other hand, accumulation of *OsPR1a* transcript was enhanced by using a potent protein kinase inhibitor staurosporine (ST) simultaneously with protein phosphatase inhibitors, but not with JA. Results presented in this study indicate that *OsPR1a* gene expression is regulated by an interaction of phytohormones with light signals, which is most likely mediated via a protein phosphorylation/dephosphorylation event(s). This is the first report on cloning of a novel acidic *PR1* (*OsPR1a*) gene member from rice.

MATERIALS AND METHODS

Plant materials. Rice (*O. sativa* L. cv. Nipponbare) seedlings were cultivated in the greenhouse (at 25°C, 70% relative humidity, and a 12 h light period/day), and at 14 days old the fully expanded leaves were used as the experimental material. Approximately 2-cm-long leaf segments were cut with clean scissors and floated on solutions (10 ml final volume) of the test compounds in sterile Petri dishes. For observing the direct effect of ethylene gas, rice leaf segments were floated on Milli Q water in petri dishes, containing a 2.5 ml solution of ET in a small 15 ml Falcon tube cap. There was no direct physical contact between either the two solutions or the ET solution and rice leaf segment. Milli Q water-treated leaf was used as a control. Incubation was done under continuous light or under darkness as indicated in the figure legends. Leaf was harvested at the times indicated, and used immediately or stored at −80°C till total RNA was extracted.

Chemicals. Cantharidin (CN), JA (raecemic mixture), and SA, were purchased from Sigma (St. Louis, MO). CN was prepared in acetone as a 100 mM stock solution, whereas JA was dissolved in methanol as a stock solution of 100 mM, and used at concentrations described in the figure legends. Endothall (EN) was obtained from BIOMOL Research Laboratories Inc. (PA), and used from a stock solution of 100 mM in Milli Q water. The protein synthesis inhibitors, CHX and tetracycline (TET) were from Sigma, and used at 10 μM concentrations. The phytohormones, abscisic acid (ABA), kinetin (KN), gibberellin (GA_3), 3-indoleacetic acid (IAA) were obtained from Wako Pure Chemicals (Tokyo, Japan), and used at 100 μM concentrations. ST, a potent serine/threonine protein kinase inhibitor was from Sigma, and dissolved in DMSO as a 100 μM stock solution. All other chemicals used in this study were of analytical grade.

Construction and screening of a rice cDNA library. Poly(A)$^+$ RNA (5 μg) was prepared from rice leaves which had been treated with 100 μM JA for 24 h and used for construction of a cDNA library. The library was constructed in λ ZAPII (Stratagene, La Jolla, CA) according to the manufacturer's instructions. To obtain a hybridization probe, a rice cDNA fragment corresponding to the maize acidic *PR1* gene was amplified by PCR on the basis of the maize nucleotide sequence published previously (accession number U82200 database GenBank; 13). Sense and antisense degenerate primers used for PCR were as follows: Consensus I (5′-GTGGACCCGCACAACGCG-3′) and Consensus II (5′-GCCGATCGCCGTCGAGTC-3′). The first-strand cDNA synthesized using 10 μg total RNA isolated from 100 μM JA-treated rice seedling leaf for 24 h under light, with a StartaScript RT-PCR Kit (Stratagene) following the manufacturer's protocol, was used as a template. The resultant PCR product of 321 bp was ligated into a pGEM-T vector (Promega, Madison, WI) and sequenced. The PCR product was labeled with [α-^{32}P]dCTP by the Multiprime DNA labeling system (Amersham, Buckinghamshire, UK), and used as a probe for screening the cDNA library. Phagemid vectors (pBluescript SK-) were excised from selected positive clones using an *in vivo* excision system (Stratagene).

DNA sequencing and sequence analysis. Plasmids were isolated according to the standard protocol (14), and sequenced (15) using a Dye-terminator cycle sequencing kit (Perkin Elmer, Foster City, CA). All the sequencing data were analyzed using Genetyx software (SDC Software Development, Tokyo, Japan). Searches for information and homology of nucleotide and amino acid sequence was analyzed using homology search with BLAST against the sequences in the GenBank and EMBL DNA database.

Northern blot analysis. RNA was isolated from rice seedling leaf using the RNeasy Plant Kit as per the protocol provided by the manufacturer (Qiagen, GmbH, Germany). Total RNA (20 μg) was separated on a 1.2% formaldehyde-denaturing agarose gel as described (14), blotted onto a nylon membrane (Hybond-N$^+$, Amersham), and hybridized with a ^{32}P-labeled PCR-amplified partial *OsPR1a* cDNA probe (602 bp; using primer pairs OsPR1a-F and OsPR1a-R, Fig. 1), using a Multiprime DNA labeling system (Amersham). Hybridization was carried out as described previously (16). Equal loading was confirmed by ethidium bromide staining, or after blotting by staining of RNA with methylene blue. The membrane was washed with 2× SSC and 0.1% SDS at 65°C for 1 h, and exposed to an X-ray film (Kodak, Tokyo, Japan) for 18 h at −80°C.

Southern blot analysis. Rice genomic DNA (1 μg) from rice leaves was digested with *Hin*dIII, *Eco*RI, *Xba*I, *Bam*HI, or *Pst*I, separated by electrophoresis on a 0.8% agarose gel and blotted onto a nylon membrane (Hybond-N$^+$, Amersham) by an alkaline transfer method. The membrane was hybridized with a ^{32}P-labeled PCR-amplified *OsPR1a* partial cDNA probe (OsPR1a-F1: 5′-GTCGGAGAAGC-AGTGGTACG-3′, and OsPR1a-R1: 5′-GGCGAGTAGTTGCAGGT-GAT-3′; 168 bp; Fig. 1), washed with 2× SSC and 0.1% SDS at 65°C for 1 h, followed by an additional washing with 0.2× SSC and 0.1% SDS at 65°C for 30 min, and exposed to an X-ray film (Kodak) for 18 h at −80°C.

RESULTS AND DISCUSSION

Isolation of a Rice Acidic PR1 cDNA from Jasmonic Acid-Treated Seedling Leaf

As our primary interest was in cloning the *PR1* gene(s) from JA-treated rice, especially the acidic *PR1* gene(s), of which there is no report to date, we used the previously published maize acidic *PR1* gene sequence (13) to design degenerate primer pairs, and carried out RT-PCR using total RNA extracted from 24 h JA-treated rice seedling leaf. A PCR-amplified 321 bp cDNA fragment was isolated from agarose gels, sequenced, and the nucleotide sequence was found to be

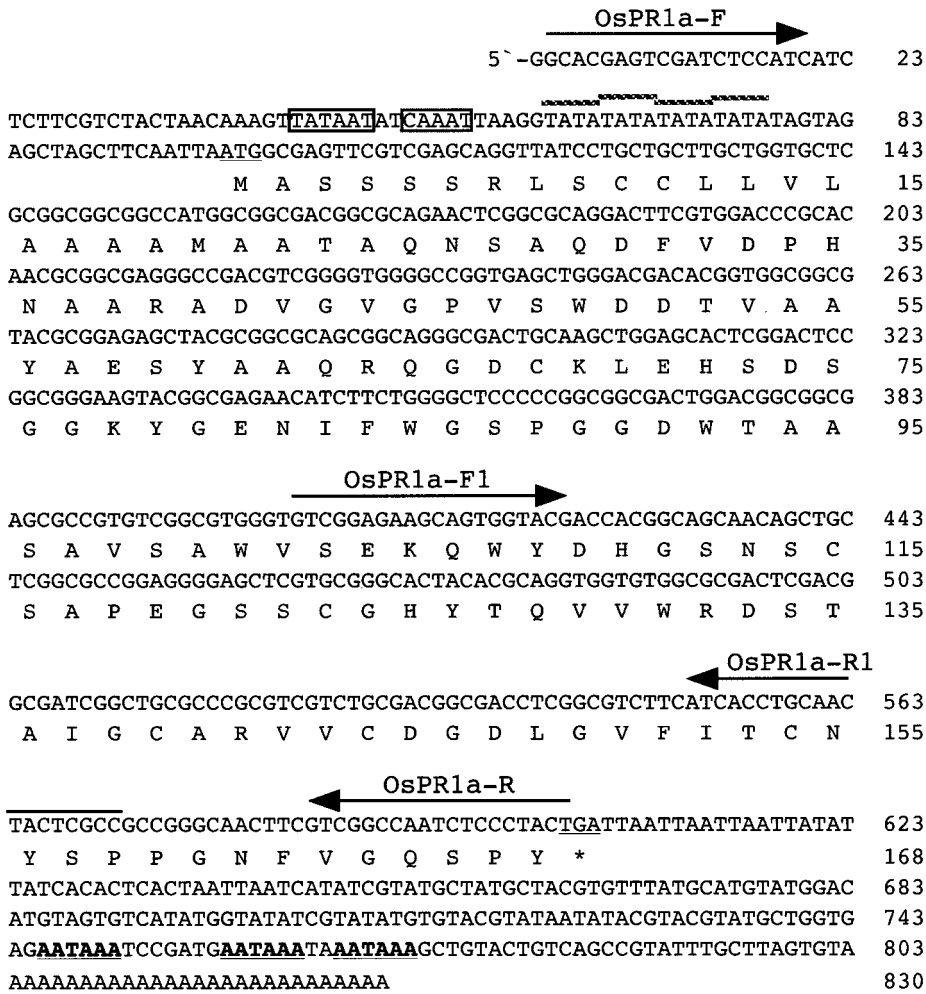

FIG. 1. Nucleotide and deduced amino acid sequences of the *OsPR1a* gene. Underlines indicate the translation initiation and termination sites. In the promoter region, the putative TATA and CAAT boxes are boxed, and top hatched lines indicate the four TATA repeat sequences. Horizontal arrows indicate the position and orientations of primers, OsPR1a-F and OsPR1a-R, and OsPR1a-F1 and OsPR1a-R1, used for PCR amplification and subsequent Northern blot and Southern blot analysis, respectively. In the 3′-untranslated region the three putative polyadenylation signals are bold underlined. The nucleotide sequence data has been submitted to the EMBL nucleotide sequence database with the Accession No. AJ278436.

highly homologous to the maize acidic *PR1* gene sequence (13). With this information in hand, we then proceeded with the isolation of a full-length cDNA clone by cDNA library screening.

To isolate a full-length rice acidic *PR1* cDNA, a cDNA library of JA-treated rice seedling leaf was prepared in λ ZAPII (Stratagene). With the above homologous cDNA fragment as a probe, a total of 20 positive clones of various lengths were obtained after screening 200,000 recombinant clones of the cDNA library using plaque hybridization. The DNA inserts were isolated from the pBluescript plasmids in the selected cDNA clones and digested with *Eco*RI/*Xho*I and the size of the cDNA inserts was determined by agarose gel electrophoresis (data not shown). The putative full-length cDNA clones which hybridized to the partial PCR amplified cDNA fragment were partially sequenced. The sequence comparisons of the cDNA clones by the BLAST program indicated that the 3 cDNA clones of about 0.8 kb are 100% identical to one another in nucleotides and encode an acidic PR1 protein. One of the 3 rice acidic *PR1* cDNA, designated as *OsPR1a*, was used for further analysis.

Nucleotide and Deduced Amino Acid Sequences of OsPR1a cDNA

The *OsPR1a* cDNA sequence (Accession No. AJ278436, EMBL) is 830 bp long and contains an open reading frame of 507 nucleotides, beginning with the first ATG initiation codon at nucleotide position 99 and ending with a TGA termination codon at position 606 (Fig. 1). The *OsPR1a* possesses a putative TATA box (TATAAT; boxed), beginning at nucleotide position 44,

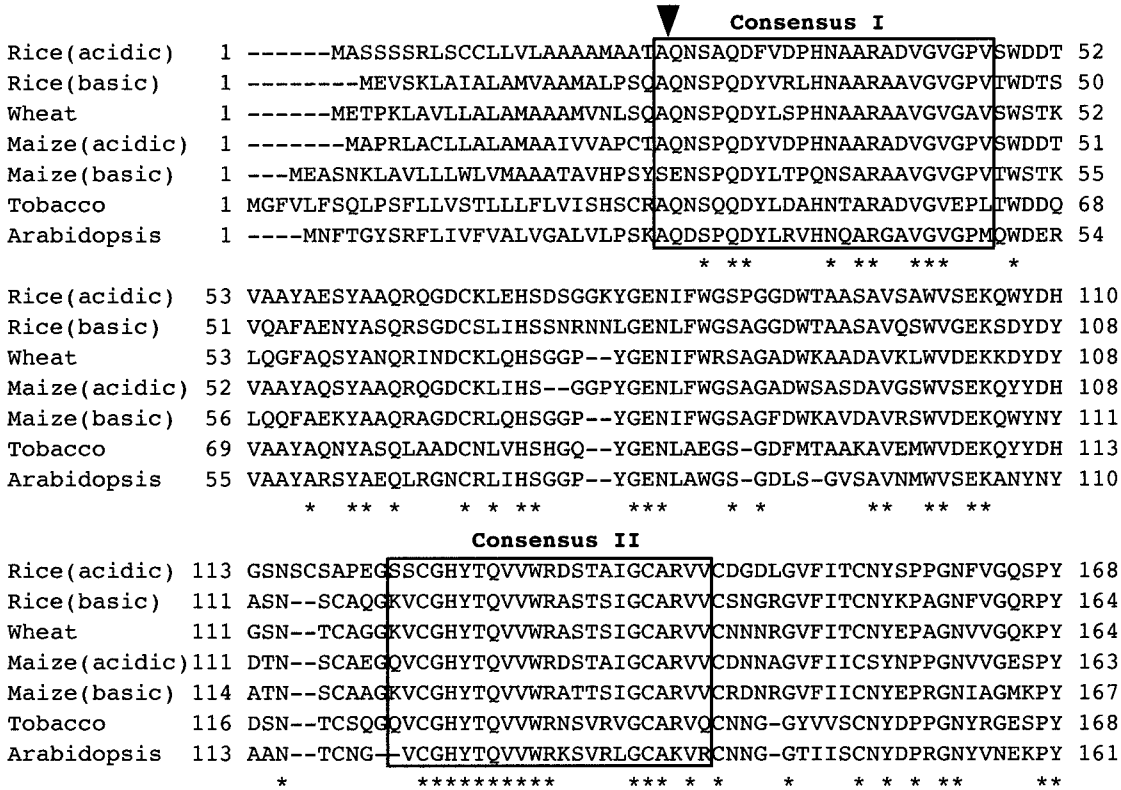

FIG. 2. Alignment of the deduced rice PR1 protein sequence (OsPR1a) with homologous sequences. The most homologous monocot sequences were identified by database searches with the Blast algorithm, whereas the *Arabidopsis thaliana* and tobacco sequences are shown as examples of dicot *PR1* genes. Origin of sequences: rice basic: U89895 (unpublished); wheat: (Molina *et al.*, 1999); maize acidic: U82200 (Morris *et al.*, 1998); maize basic: Q00008 (Casacuberta *et al.*, 1991); tobacco: X06930 (Pfitzner and Goodman, 1987); *A. thaliana:* M90508 (Uknes *et al.*, 1992). An arrowhead indicates the putative processing site and boxes indicate the consensus regions I and II. Asterisks represent identical amino acid residues and dashes indicate gaps introduced to optimize alignment.

and four TATA repeat sequences (top hatched underlines) beginning at nucleotide position 62 (Fig. 1). The presence of a TATA box just upstream of translation start point (TSP) is a potential target site for *trans*-acting factors in plants (17). This indicates that the presence of a putative TATA box, just upstream of the TSP in the *OsPR1a* leader sequence, might serve as a *cis*-element for as yet unidentified *trans*-acting factors regulating *OsPR1a* gene expression. The presence of four TATA repeat sequences between the putative TATA box and TSP in the *OsPR1a* leader sequence was not found during a survey of, at least, the *PR1* genes reported in the literature/database. It is speculated that these repeat sequences might serve as additional *cis*-acting elements. A careful observation of nucleotide sequence upstream of the TSP also revealed the presence of a CAAT box (CAAAT; boxed, Fig. 1), 47 bp upstream of the TSP. The CAAT box, a typical feature of eukaryotic genes, though not found among the reported *PR1* gene sequences, was present in the proteinase inhibitor II gene sequence from potato (18), another PR family member. Three potential polyadenylated signals, AATAAA are also located in the 3′-untranslated region at nucleotide positions 746, 759 and 767. The existence of multiple putative polyadenylation signals has been observed in maize gene encoding a basic PR1 protein (*PRms*), and for other plant genes, including *PR1* mRNA from tobacco (19, 20). The *OsPR1a* cDNA encodes a putative polypeptide of 168 amino acids with a predicted molecular mass of 17560 Da and an isoelectric point (p*I*) of 4.4 (Fig. 1).

The OsPR1a protein has a structure characteristic of the PR1 proteins. The primary translation products of the *PR1* genes contain a hydrophobic signal sequence, which is cleaved off upon entry in the endoplasmic reticulum (4). The mature PR1 proteins are mostly 135 amino acids long, contain six conserved cysteine residues forming disulphide bridges, and show a high level of sequence conservation throughout different plant families, including both monocots and dicots (4). The deduced amino acid sequence of *OsPR1a* is shown in Fig. 2, in alignments with homologous sequences. The predicted protein appears to have a leader peptide of 24 amino acid residues, which indicates that the OsPR1a protein is synthesized as a precursor protein with a leader that presumably directs the mature protein to the extracellular space. The predicted leader cleavage site (indicated by an arrowhead, Fig. 2) conforms well

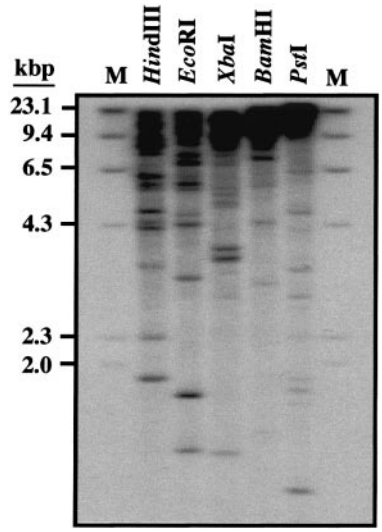

FIG. 3. Genomic Southern blot analysis of *OsPR1a*. Rice genomic DNA (1 μg) from rice leaves was digested with *Hin*dIII, *Eco*RI, *Xba*I, *Bam*HI, or *Pst*I, separated by electrophoresis on a 0.8% agarose gel, and blotted onto a nylon membrane (Hybond-N$^+$, Amersham). The membrane was hybridized with a ^{32}P-labeled PCR-amplified *OsPR1a* partial cDNA probe as mentioned under Materials and Methods. The sizes of the molecular weight marker (M) are indicated on the left-hand side.

to the published consensus sequence for eukaryotic cleavage sites and is also identical to the homologous cleavage site in the maize acidic PR1 protein (21, 13). Moreover, the OsPR1a protein sequence also contains the six conserved cysteine residues. The conservation of the PR1 protein sequences among monocots and between rice and dicots is very high, with the exception of the leader peptides (Fig. 2). The OsPR1a protein sequence shows the highest homology to the maize acidic PR1 protein (74.1%; 13). The OsPR1a protein also showed 63.1% homology to the rice basic PR1 protein (Accession No. U89895), 63.7% to the wheat PR1 protein (22), and 58% to the maize basic PR1 protein sequence (19). On the other hand, OsPR1a protein shows only 55.3% and 52.3% homology to the tobacco and Arabidopsis PR1 proteins, respectively (20, 23). A region highly homologous to the consensus region I and II of the PR1 family of proteins, is also indicated in the boxed regions (Fig. 2).

Genomic Organization of the OsPR1a

To determine the copy number of *OsPR1a* in the genome of rice, we performed Southern hybridization using as a probe, as mentioned in the materials and methods. Multiple distinct hybridizing bands were detected with four restriction enzymes, *Hin*dIII, *Eco*RI, *Xba*I, *Bam*HI, or *Pst*I (Fig. 3). This result clearly indicates that *OsPR1a* belongs to a multigene family in rice. This result is also similar with maize, where, based on gene sequence homology and southern blot analysis, a maize *PR1* acidic gene family with multiple members if proposed (13).

Induction of the OsPR1a Gene after Cut, Exogenous Jasmonate Application, and Treatment with Protein Phosphatase Inhibitors

Recent studies in rice have demonstrated that, JA possesses an ability to induce a wide variety, if not all, defense-related proteins, antifungal phytoalexins, including potential role(s) in causing increased generation of reactive oxygen species (1, 11, and 12). Using Northern blot analysis, we show that the *OsPR1a* transcript accumulates to high levels in 100 μM JA-treated leaf in a time-dependent manner, visible at 6 h, and reaching a maximum at 24 h after treatment under light (Fig. 4A). To our knowledge, there is no report on induction of an acidic *PR1* gene by jasmonates, thereby presenting first evidence in support of JA as an effective inducer of, at least, an acidic member of the *PR1* gene family in rice. Interestingly, a time-dependent accumulation of this *OsPR1a* transcript was also seen in the water-controls, indicating a highly cut-sensitive gene expression. However, the transcript levels were relatively lower than that observed after JA application (Fig. 4A). Again, there are no reports on cut-inducible acidic PR1 genes in monocots or dicots, though a recent report shows that mock (water)-inoculated maize leaf showed a weak accumulation of the acidic *PR1* transcript, that too, at 14 days after treatment (13).

Furthermore, as a variety of protein phosphatase inhibitors, including CN and EN (24), were shown to activate antifungal defense responses in soybean, and recently in rice (unpublished data), which indicated a general use for these inhibitors in investigating mechanisms of plant response to stress (25), we investigated the effect of exogenously applied 100 μM CN and EN on *OsPR1a* gene expression in rice. Upon treatment of rice seedling leaf with either CN or EN, we found that the *OsPR1a* transcript first accumulated at 6 h, and increased in a time-dependent manner up to 24 h, the last time period analyzed (Fig. 4A). Considering the effect of cut on *OsPR1a* transcript accumulation, we cannot rule out the possibility that the results obtained are not the direct effects of CN and EN. However, if we look at the levels of *OsPR1a* at 24 h, we find the strongest signals in CN and EN treated leaf, among all the treatments. This also suggests to an effect of CN and EN, most probably through hyperphosphorylation of certain signal transducing proteins, in causing an increased accumulation of the *OsPR1a* transcript. However, there are two contradictory reports in the literature on effects of a protein phosphatase inhibitor okadaic acid (OA) on tobacco acidic PR1, whereas one shows induction of a tobacco acidic PR1 protein by the protein phosphatase inhibitor OA (26), the other shows

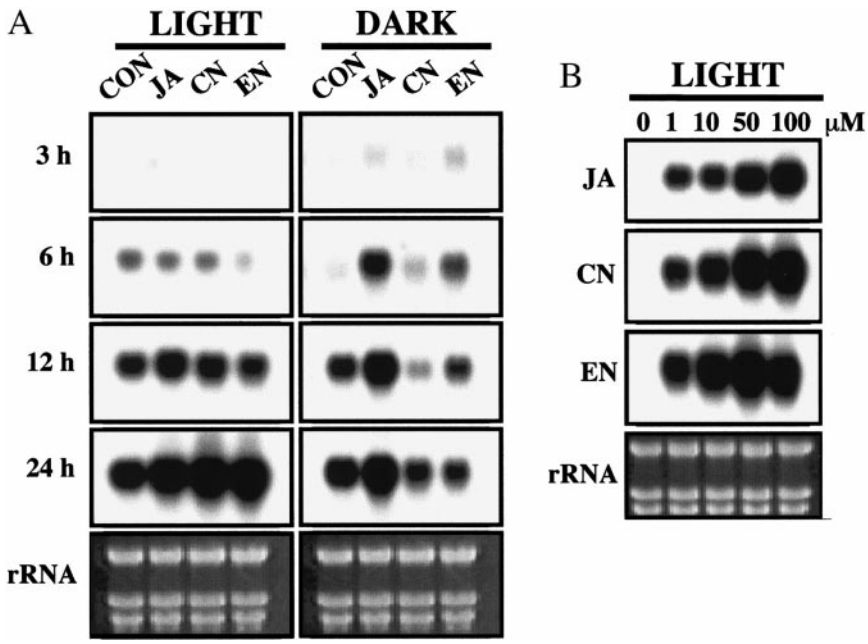

FIG. 4. Rapid induction of the *OsPR1a* gene transcript in rice seedling leaf by cut, JA, and the protein phosphatase inhibitors cantharidin and endothall. (A) Northern blot analysis of RNA from leaf treated with 100 μM JA, 100 μM CN, and 100 μM EN, and water controls (CON) in a time-dependent manner, under both continuous light (left panel) and continuous dark (right panel) incubation. Total RNA was extracted at the times indicated on the left-hand side in h. (B) Dose-dependent effect of JA, and CN or EN on *OsPR1a* mRNA accumulation in rice leaf 24 h after treatment under light. The dose in μM is indicated on the top of the lanes. Equal loading (20 μg) was monitored by ethidium bromide staining of the gels before transfer. The blots were hybridized to the ^{32}P-labeled *OsPR1a* PCR-amplified cDNA probe as indicated under Materials and Methods.

OA to effectively block salicylic acid induced acidic *PR1* gene (27).

Furthermore, the transcript levels of the cut-inducible *OsPR1a* mRNA was decreased and accumulation delayed, when the leaf segments were incubated under dark, suggesting to light signals are somehow involved in regulating *OsPR1a* gene expression (Fig. 4A). This idea received further support by the finding, that the *OsPR1a* gene expression after CN and EN treatment is significantly dependent on light, as dark incubation effectively suppressed its induction (Fig. 4A). The up-regulation of *OsPR1a* mRNA under light, suggests to an intimate interaction of light signals with the early protein kinase cascade(s) in effecting the *OsPR1a* gene expression. Surprisingly, in total contrast to a general down regulation of the *OsPR1a* transcript after cut, and CN or EN application under darkness, exogenous JA further enhanced the levels of these transcripts, as compared to JA-inducible *OsPR1a* transcript levels under light. This result suggests that JA induces *OsPR1a* in a manner somehow different from that observed after cut, or protein phosphatase inhibitor treatment. On the basis of these results, we propose a specific involvement of JA in regulating *OsPR1a* gene expression in rice, though the mechanism(s) involved therein remain unknown. Lastly, we show that the expression of this *OsPR1a* gene is dependent on the dose of exogenously applied JA, CN and EN (Fig. 4B), thus indicating to a specific effect of these chemicals in inducing this *OsPR1a* gene.

Cytoplasmic de Novo Synthesis Is Needed for OsPR1a Gene Expression

Since increased *OsPR1a* transcript levels in leaf were detectable only with a rather long time delay of 3/6 h after addition of JA or CN and EN, it was tested whether *de novo* synthesis of some protein factor(s) was required for *OsPR1a* mRNA accumulation. Therefore, the protein synthesis inhibitors, CHX or TET were applied simultaneously with JA, CN and EN to the rice leaf, and incubated under light for a period of 24 h. As shown in Fig. 5A, 10 μM CHX, a eukaryotic protein synthesis inhibitor, almost completely blocked *OsPR1a* transcript accumulation in rice leaf after all these treatments. This result is also consistent with a recent report in cultured rice cells, where 71 μM CHX essentially blocked *Pir7b* transcript accumulation if applied simultaneously with syringolin A (28). On the other hand, treatment with TET, a prokaryotic protein synthesis inhibitor, did not have any effect on induction of *OsPR1a* transcripts (Fig. 5A). These results indicate that, the requirement for protein synthesis

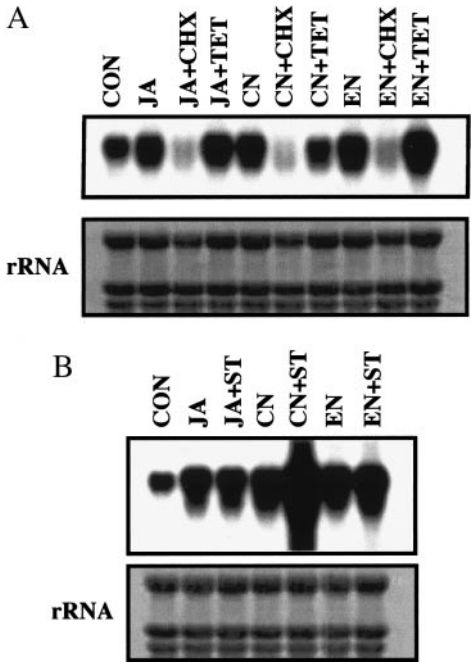

FIG. 5. Cycloheximide blocks *OsPR1a* gene expression, whereas staurosporine enhances its induction with CN or EN. (A) Northern blot analysis of RNA from leaf treated with 100 μM JA, or 1 μM CN and EN alone, or simultaneously with 10 μM CHX or 10 μM TET as indicated on the top of each lane. The water control (CON) is shown in lane 1. (B) Northern blot analysis of RNA from leaf treated with 100 μM JA (lane 1) and with a simultaneous treatment with 10 μM staurosporine (ST, lane 2). Effects of 10 μM ST on CN- and EN-induced *OsPR1a* transcript levels are shown in lanes 3 to 6. CN and EN were used at 1 μM concentrations, respectively. Total RNA was extracted at 24 h after treatment under continuous light. Equal loading (20 μg) was confirmed after blotting, by staining of RNA with methylene blue, and blots were hybridized to the radiolabeled *OsPR1a* PCR product as in Fig. 4.

precedes activation of the *OsPR1a* gene, and that this *de novo* protein synthesis is cytoplasmic in nature.

Dramatic Enhancement of the OsPR1a Transcript after a Simultaneous Application of Staurosporine with Cantharidin

The above results suggested that CN and EN effected induction of the *OsPR1a* gene is mediated via hyperphosphorylation of one or more unidentified phosphoproteins. Moreover, as the phosphorylation of a protein kinase can be blocked using protein kinase inhibitors, like ST and K252a (27), we investigated the effect of ST on JA, CN or EN induced *OsPR1a* gene expression.

ST, at 10 μM concentration when applied to rice leaf simultaneously with 100 μM JA, was observed to slightly reduce the *OsPR1a* transcript level (Fig. 5B). A plausible explanation is that JA-induced *OsPR1a* gene expression might follow a protein kinase cascade mediated via phosphorylation of a serine/threonine residue(s) of one or more phosphoproteins, which can be partially blocked by ST. On the contrary, accumulation of the *OsPR1a* transcript dramatically increased when ST was simultaneously added with CN, although enhancement was not to similar levels with EN (Fig. 5B). The observed differences between CN and EN could be due to the different sites of action for these two protein phosphatase inhibitors. As ST does not reduce, but enhances *OsPR1a* transcript levels with CN and EN, it might be possible that a serine/threonine protein kinase is associated with one or more protein phosphatase in effecting *OsPR1a* gene expression. Whether, this proposed kinase mediated signaling cascade negatively regulates *OsPR1a* gene expression is a possibility, which deserves attention in future studies. These results indicate that phosphorylation and/or hyperphosphorylation state (or levels) of a serine/threonine residue(s) of one or more unidentified phosphoproteins is responsible for regulating the *OsPR1a* gene, as was suggested for the *PR1* gene expression in tobacco (27).

OsPR1a Transcript Accumulates after Treatment with IAA and GA_3, and the JA-Induced OsPR1a Gene Expression Is Blocked by SA and ABA

It was reasoned that as SA and JA can strongly influence other processes in plant growth and development, it would not be unexpected that interactions could occur between them and with other phytohormones and signal molecules (29). A current updated model proposed for defense gene activation in tomato, confirms that SA inhibits the octadecanoid-signaling pathway downstream of JA biosynthesis in dicot species (30). Although in rice, SA has been speculated to be a constitutive defense compound, no study examines the effect of SA (or other phytohormones) on JA-inducible defense gene activation (31). SA at 100 μM concentrations caused a slight increase in *OsPR1a* transcript level, whereas, IAA and GA_3 were as effective as JA in increasing *OsPR1a* transcript levels, and ABA and KN did not have any significant effect (Fig. 6, lanes 1 to 7). We then examined the effect of some of these phytohormones on JA-inducible *OsPR1a* transcript levels. Results presented in Fig. 6, show that JA-induced accumulation of *OsPR1a* transcript was almost blocked by a simultaneous treatment with 100 μM SA (lane 8) or ABA (lane 10), though at higher concentrations of 200 μM (lanes 9 and 11), the inhibition was complete (Fig. 6). The corresponding relative mRNA abundance in percentage is given in Fig. 6B. This result provides the first evidence on exogenous SA blocking JA-inducible PR gene (*OsPR1a*) activation in monocots (rice), thereby suggesting similarities in the mechanism(s) by which SA might block JA-inducible defense gene among dicots and monocots. In addition, we also show that not only SA, but also ABA was

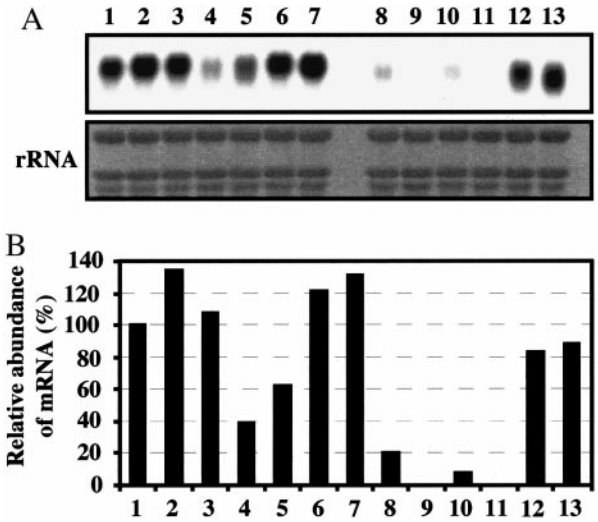

FIG. 6. Phytohormones GA_3 and kinetin induce *OsPR1a*, whereas SA and ABA completely block JA-induced *OsPR1a* transcript accumulation. (A) Northern blot analysis of RNA from leaf treated with 100 μM JA, SA, ABA, KN, IAA, and GA_3 for 24 h under light (lanes 2 to 7). Lane 1 represents the water control. The next 6 lanes show Northern blot analysis of rice leaf treated with 100 μM JA in combination with 100 or 200 μM SA (lanes 8, 9), ABA (lanes 10, 11), and KN (lanes 12, 13), respectively. (B) The histogram shown below is the relative abundance of mRNA in percentage, and is a representative of two independent experiments. Total RNA was extracted 24 h after treatment, equal loading (20 μg) was confirmed, and hybridization was carried out as indicated in Fig. 4.

equally effective in blocking JA-inducible *OsPR1a* gene expression, a first report. On the other hand, KN was also showing some inhibitory effect on accumulation of the *OsPR1a* transcript, when applied simultaneously with JA. These results indicate to a complexity in the cross talk between phytohormones in regulating defense gene activation in plants, and more studies will be needed to generalize about the signal pathway cross talk for plants (10).

Regulation of OsPR1a by the Ethylene Generator Ethephon

The phytohormone ethylene regulates many different processes in plants and has been implicated in defense responses (32). JA and ethylene have also been shown to co-operate in regulating the expression of many genes, and at least some JA-inducible genes are not inducible in plants unable to produce or sense ethylene (10 and references therein). Our aim was to investigate, whether ethylene has any effect on the expression of the *OsPR1a* gene, without going into the synergies of ethylene with JA. We used the ethylene generator ET (33) to treat leaf segments, and found that ET causes a rapid time- and dose-dependent increase in *OsPR1a* transcript levels over control (Figs. 7A and 7C). Furthermore, accumulation of the *OsPR1a* transcript by ET was dependent on light (Fig. 7A), as was found for CN and EN (see Fig. 1A), and was blocked by CHX, but not TET, indicative of an involvement of *de novo* cytoplasmic protein synthesis (Fig. 7D). To confirm that the effect of ET was through generated ethylene gas, we prevented any direct contact between the ET solution and leaf segments by a simple experimental setup as described in materials and methods. It was found that the observed enhancing effect of ET on *OsPR1a* gene expression is indeed due to the ethylene gas, which followed a similar time-dependent expression as shown in Fig. 7B. This result is also consistent with a recent report on an ethylene (through ET)-inducible pepper basic *PR1* gene (34). Thus, in addition to other phytohormones responsive *OsPR1a* gene expression, we also provide evidence for a role of ethylene in regulating *OsPR1a* gene expression in rice.

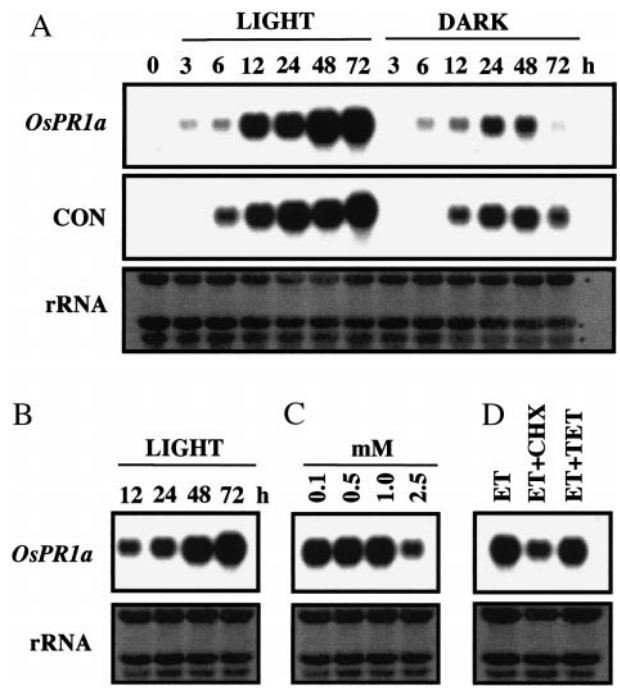

FIG. 7. Light- and dose-dependent induction of *OsPR1a* gene by ethephon. (A) Northern blot analysis of RNA from leaf treated with 1 mM ET, under both continuous light (left panel) and continuous dark (right panel) incubation, in a time-dependent manner, as given above each lane in hours (h). In parallel, a control experiment was also performed with cut leaf segments floated on only Milli Q water. The rice seedling leaf at 0 h was used as a control. (B) Effect of ethylene generated by ET, without any direct contact between ET and the rice leaf segments on *OsPR1a* transcript, in a time-dependent manner under light. (C) Dose-dependent effect of ET on *OsPR1a* gene analyzed at 48 h after treatment. The dose in mM is indicated on the top of each lane. (D) *OsPR1a* transcript levels after treatment with 1 mM ET alone, and simultaneously with 10 μM CHX or 10 μM TET. Sampling was done at 48 h in light. Equal amounts of total RNA (20 μg) loaded for each sample were confirmed, and the blots were hybridized as in Fig. 4.

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