Plant Pathol. J. 16(1) : 9-18 (2000)

The Plant Pathology Journal

Isolation and Differential Expression of an Acidic PR-1 cDNA Gene from Soybean Hypocotyls Infected with *Phytophthora sojae* f. sp. *glycines*

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(Received on July 26, 1999)

Using differential display techniques, a new acidic pathogenesis-related (PR) protein-1 cDNA (*GMPR1a*) gene was isolated from a cDNA library of soybean (*Glycine max* L. Merr, cultivar Jangyup) hypocotyls infected by *Phytophthora sojae* f. sp. *glycines*. The 741 bp of full-length *GMPR1a* clone contains an open reading frame of 525 nucleotides encoding 174 amino acid residues (pI 4.23) with a putative signal peptide of 27 amino acids in the N-terminus. Predicted molecular weight of the protein is 18,767 Da. The deduced amino acid sequence of *GMPR1a* has a high level of identity with PR-1 proteins from *Brassica napus*, *Nicotiana tabacum*, and *Sambucus nigra*. The *GMPR1a* mRNA was more strongly expressed in the incompatible than the compatible interaction. The transcript accumulation was induced in the soybean hypocotyls by treatment with ethephon or DL-β-amino-n-butyric acid, but not by wounding. *In situ* hybridization data showed that *GMPR1a* mRNAs were usually localized in the vascular bundle of hypocotyl tissues, especially phloem tissue. Differences between compatible and incompatible interactions in the timing of *GMPR1a* mRNA accumulation were remarkable, but the spatial distribution of *GMPR1a* mRNA was similar in both interactions. However, more *GMPR1a* mRNA was accumulated in soybean hypocotyls of the incompatible than the compatible interactions at 6 and 24 h after inoculation.

Keywords : Acidic PR-1 gene, soybean, *Phytophthora sojae* f. sp. *glycines*, mRNA, *in situ* hybridization.

Plants, unlike animals equipped with immune system, protect themselves by various defense mechanisms. Defense mechanisms are related to local cell death known as hypersensitive reaction (HR) (Smith et al., 1991), deposition of callose (Stumm and Gessler, 1986), lignification of cell wall (Vance et al., 1980), antifungal materials like phytoalexins (Bailey and Deverall, 1971), and pathogenesis-related (PR) proteins (Van Loon, 1985; Dixon and Lamb, 1990).

PR proteins have been first reported in tobacco leaves that exhibited a hypersensitive reaction to TMV (Van Loon and Van Kammen, 1970). PR proteins are characterized by selective extraction at low pH, relatively low molecular weight, accumulation in the intercellular space, a high resistance to proteolytic activity, easy resolving by electrophoresis in polyacrylamide gel, and extreme isoelectric points. Acidic and basic PR proteins with low molecular weights have been isolated from monocotyledonous plants (Zhy and Lamb, 1991) as well as dicotyledonous plants (Bol and Van Kan, 1988). It has been reported that the PR proteins accumulated in plants upon infection by pathogenic organisms such as virus, bacteria and fungi (Matthieu et al., 1989; Strauch et al., 1990; Linthorst, 1991; Stinitzi et al., 1993; Tomero et al., 1994; Kim and Hwang, 1996; Lee and Hwang, 1996) and by treatments with abiotic elicitors, UV-light and some chemicals (Green and Fluhr, 1995). Many researches have been performed to characterize PR proteins in relation to resistance, especially systemically-induced resistance (Van Loon, 1985; Carr et al., 1987; Linthorst, 1991).

Proteins belonging to the family now called PR-1 were first identified in TMV-infected tobacco plants (Van Loon, 1976). These serologically related proteins have molecular weight of 14-17 kDa and accumulate upon pathogen infection and external stimuli in many different plant species. Many isoforms of PR-1 have been isolated, and many genes encoding them have been cloned and sequenced (Linthorst, 1991; Stinitzi et al., 1993). One of the most prominent PR proteins appearing in the intercellular spaces of tomato leaves upon infection by viroids, viruses or fungi is P14 (Granel et al., 1987; Fischer et al., 1989). This 14-kDa protein was purified from potato spindle tuber viroid-infected tomato leaves and its amino acid sequence was determined (Lucas et al., 1985). P14 appeared to be serologically related to PR-1a, -1b, and 1c from tobacco and to a PR protein from cowpea (Nassuth and Sanger, 1986). Joosten et al. (1990) showed that apoplastic fluid obtained from *Cladosporium fulvum*-infected tomato leaves contains three basic PR proteins (designated P2, P4 and P6) of approximately 15 kDa. Surprisingly, very little is known concerning the biological activity and function of the first discovered protein PR-1. No resistance was found against

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viruses in transgenic tobacco plants expressing PR-1a and PR-1b (Cutt et al., 1988; Linthorst et al., 1989). Recently, transgenic tobacco plants expressing high levels of protein PR-1a exhibited increased resistance, significantly reducing disease symptoms caused by infection with two oomycete pathogens i.e., *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al., 1993). This represented the first evidence, although indirect of antifungal activity of a PR-1 protein. Niderman et al. (1995) reported antifungal activity of PR-1 against *Phytophthora infestans in vitro*. These observations indicate that proteins of the family PR-1 function directly and/or indirectly in defense of plants against oomycete pathogens, although the mechanism for this is still unknown.

Most of PR genes involved in defense responses of plants was analyzed at the transcription level. Brederode et al. (1991) demonstrated that the genes encoding the acidic PR proteins were highly inducible by TMV infection, but only moderately inducible by ethephon and not inducible by wounding. However, the genes for the basic PR proteins were expressed more strongly after treatment with ethephon than after wounding and TMV infection. The genes encoding these basic proteins were also constitutively expressed during flower formation and in roots of tobacco plants (Neale et al., 1990).

PR proteins, such as β-1,3-glucanase and chitinase, in soybean hypocotyls infected with compatible or incompatible *P. sojae* f. sp. *glycines* races were purified and characterized (Yi and Hwang, 1996 and 1997). More recently, Yi and Hwang (1998) isolated and characterized a full-length cDNA clone encoding a putative basic peroxidase from cDNA library of soybean hypocotyls infected with *P. sojae* f. sp. *glycines*. In addition, they showed that a putative pathogen-induced peroxidase gene may play a significant role in induced resistance of soybean to *P. sojae* f. sp. *glycines* and in response to various external stresses.

The objectives of this study are to screen the full-length cDNA clone encoding putative PR 1 protein from a cDNA library made with the mRNAs of soybean hypocotyls infected with an incompatible race of *P. sojae* f. sp. *glycines*, to determine and analyze nucleotide and deduced amino acid sequences of *GMPR1a*, to detect the time course expression pattern of the mRNAs for *GMPR1a* in soybean hypocotyls infected with *P. sojae* f. sp. *glycines* or treated with several elicitors, and to determine *in situ* localization of mRNA *GMPR1a* in soybean hypocotyls infected with the compatible or incompatible races.

Materials and Methods

Plant growth, fungus inoculation, and elicitor treatment. Seeds of soybean (*Glycine max* L. Merr, cultivar Jangyup) were sterilized in 0.5% sodium hypochloride for 5 min and were germinated on kitchen towel saturated with distilled water at 25±2°C, 100% humidity for 2-4 days They were sown in a plastic tray (5×15×10 cm) containing steam-sterilized soil mix (peat moss, perlite and vermiculite, 5:3:2, v/v/v), sand, and loam soil (1:1:1, v/v/v). They were raised in a growth chamber at 25±2°C with a 16 h photoperiod at 5,000 lux for 5 days.

Races 4 and 2 of *P. sojae* f. sp *glycines*, which were virulent and avirulent to soybean cultivar Jangyup, respectively, were used in this study. The fungus was maintained at room temperature on V-8 juice agar slants. To prepare the fungal inoculum, *P. sojae* f. sp. *glycines* was incubated on V-8 juice agar plates for 2-3 weeks at 27°C. The mycelium pieces (0.5×0.5 cm) were used as inoculum, which were obtained by eliminating agar portion from the cultures of *P. sojae* f sp *glycines* in V-8 juice agar plates. The inoculum was inserted into a 0.7 cm-longitudinal slit on the 5 day-old hypocotyls. The inoculated plants were maintained in a growth chamber at 25±2°C with 5,000 lux illumination for 16 hr a day. After incubation for 4, 10, 20, 30, and 40 hr, the hypocotyl tissues of inoculated plants were harvested and stored in 70 °C for further experiments.

In the experiments to examine the induction of pathogenesis-related (PR1) protein mRNAs by abiotic elicitors, 5 day-old hypocotyls of soybean were wounded or treated with various elicitors such as salicylic acid, ethephon, and DL-β-amino-N-butyric acid. Hypocotyl tissues were wounded by slitting with a sterile razor or by rubbing with 0.3% celite. Whole plants were sprayed with 0.1% salicylic acid (w/v), 10 mM ethephon, and 0.1% DL-β-amino-N-butyric acid (w/v). Ethephon-treated plants were covered with a clear plastic bag. The hypocotyl tissues treated were harvested after 18 and 36 hr, and stored in 70°C until used.

For *in situ* hybridization, the mycelium pieces of *P. sojae* f. sp. *glycines* were separated from agar portion. These pieces were ground and diluted with sterilized distilled water. The mycelial suspension was used as inoculum. Soybean seeds were sterilized and germinated for 4-5 days. Soybean hypocotyls were incubated within microtubes (1.5 ml) containing mycelial suspension at 29°C, 100% humidity under the darkness condition. At different time intervals of incubation, the hypocotyl tissues infected were harvested for *in situ* hybridization experiment.

RNA isolation. Total RNAs from hypocotyls and mycelia of *P. sojae* f. sp. *glycines* were isolated by guanidine-HCl extraction buffer (pH 7.0) (Logemann et al., 1987) and by guanidine-isothiocyanate buffer (pH 7.0) (Chomczynski and Sacchi, 1987), respectively. Total RNAs were extracted several times with guanidine/acid-phenol and precipitated with isopropanol. The total RNAs isolated were dissolved in diethyl pyrocarbonate (DEPC)-treated distilled water or formamide and stored at -70°C.

Differential display. DNA-free RNA (0.2 μg), which was isolated from healthy hypocotyls or hypocotyls inoculated with compatible race 4 or incompatible race 2 of *P. sojae* f. sp. *glycines*, was reverse-transcribed by RNA image (Gene-hunter Corporation, Brookline, USA) using one-base anchored primer, $HT_{11}C$(5'-AAGCTTTTTTTTTTTC-3'). Reverse transcription reactions (20 μL total volume containing 2 μM HT11C and 250 μM dNTP) were carried out in a thermocycler with the following conditions :

heated at 65°C for 5 min, then brought to 4°C until used for PCR reactions. Ten percent of the reverse transcription mixture was used as the template in a PCR reaction containing a $HT_{11}C$ primer in combination with an arbitrary 13-base primer (AP18 primer) in the presence of ^{35}S-labeled dATP. The PCR conditions were as follows: 94°C, 30 sec; 40°C, 2 min; 72°C, 30 sec for 40 cycles, followed by 72°C for 5 min.

Aliquots of PCR reactions were electrophoresed through a 6% acrylamide sequencing gel to separate the amplified cDNAs. The gel was then dried and exposed to X-ray film (Agfa) for autoradiography with an intensifying screen for 2-4 days. The gel regions that corresponded to the bands differentially amplified were excised from the dried gel.

The gel slices were soaked in 100 μL sterile distilled water for 10 min and boiled for 10 min. After pelleting gel slices by centrifugation, the supernatants were added to 3 M sodium acetate and absolute ethanol to precipitate. The cDNA molecules that diffused from the gel fragments were reamplified by PCR and used as probes for Northern blot hybridization analysis of the total RNA samples of soybean. After RNA blot hybridization analysis, cDNAs that code for mRNAs induced only in hypocotyls infected by *P. sojae* f. sp. *glycines* were amplified by PCR and used as probes for screening the cDNA library of soybean.

Construction of cDNA library. Soybean hypocotyls (70 g) inoculated with *P. sojae* f. sp. *glycines* race 2 (incompatible) were powdered in liquid nitrogen. Total RNA was isolated from the powdered hypocotyls by guanidine-HCl extraction buffer. Poly $(A)^+$ RNA was prepared from total RNA using oligo(dT)-cellulose chromatography. Double-stranded cDNA was synthesized from poly$(A)^+$ RNA by an oligo(dT) priming method. The cDNA library was made using the lambda Zap II-cDNA synthesis and cloning kit (Stratagene, La Jolla, USA) and lambda-DNA *in vitro* packaging module (Amersham) according to the procedures described by the suppliers. The recombinant DNAs were packaged into bacteriophage particles.

Isolation of cDNA clones. A cDNA clone was isolated from a cDNA library made with the mRNAs of soybean hypocotyls inoculated with *P. sojae* f. sp. *glycines*. The cDNA library in lambda-gt11 was screened with the C18CI2 probe from the differentially displayed cDNAs. Plaque hybridization and positive signal detection were done following the instructions of the DIG DNA labeling and detection kits (Boeringer, Mannheim). About 5×10^4 plaques of the library were transferred onto duplicate nylon membranes (Hybond N^+, Amersham). The membranes were treated with denaturation solution, neutralization solution, and 2X SSC and illuminated with U.V. for membrane cross-linking. Prehybridized membranes were probed with the C18CI2 clone in hybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) at 65°C. The chemiluminescent signals were detected with CSPD as a substrate. Selected cDNA clones were subcloned to pBluescript SK(−) plasmid by ExAssist helper phage-mediated *in vivo* excision. The cDNA inserts in pBluescript SK(−) were digested with *Eco* RI and *Xho* I to analyze the size on 1% agarose gel. The cDNA clone, designated *GMPR1a*, was then screened by Southern blotting with the C18CI2 probe, as described in plaque hybridization.

cDNA sequencing and analysis. The cDNA clones screened from cDNA library were sequenced on the ABI 310 DNA sequencer (Applied Biosystem) using Thermo-cycle sequenase kit (Amersham) with T_3 or T_7 primer according the manufactures protocol. The deletion mutants were generated from the *GMPR1a* cDNA using the Erase-A-Base kit (Promega). DNA sequence data were assembled and analyzed using the BLASTN, BLASTP, BLASTX, and ORF finder e-mail server provided by NCBI databases. The full-length nucleotide sequences of *GMPR1a* cDNA was submitted to GenBank (http://www.ncbi.nlm.nih.gov/) and has been given GenBank accession number AF136636.

RNA blot hybridization analysis. Total RNAs (20 μg) was separated on 1.2% formaldehyde agarose gels in MOPS buffer. The gels were blotted onto Hybond N^+ membranes (Amersham). The transferred RNA was UV cross-linked to the membranes. Blots were hybridized with the ^{32}P-labeled *GMPR1a* probe in hybridization solution (0.25 M disodium phosphate pH 7.2, 7% SDS, 1 mM EDTA, 5% dextran sulfate) at 65°C. After hybridization, the blots were washed twice with 2X SSC, 0.1% SDS at room temperature for 10 min each and twice with 0.2X SSC, 0.1% SDS at 65°C for 10 min each. These membranes were exposed to X-ray films (Agfa) for autoradiography with an intensifying screen for 3-5 days at 70°C

***In situ* hybridization.** Soybean hypocotyl tissues cut into small pieces were fixed in 4% paraformaldehyde in PBS, pH 7.0, 0.1% Triton X-100, followed by evacuation for 5-10 min to remove air from the intercellular space. Fixation of the samples was done for 2 hr at room temperature. The samples were washed three times with PBS for 10 min each. They were then dehydrated in a graded ethanol series and cleared with xylol. Infiltration was performed by adding small droplets of liquid paraplast. The specimens were embedded in paraplast and hardened at room temperature. The embedded materials were stored at 4°C until used for *in situ* hybridization.

In situ hybridization of mRNA in the soybean hypocotyl tissues was performed according to the method of Hause et al. (1996). Tissue thin sections (10 mm thick) prepared were flatted on the microscope slide glasses coated with poly-L-lysine (Sigma), followed by baking overnight on a slide warmer set at 42°C to stick the sections on the slide. After removing the wax with xylol, the sections were rehydrated by serially diluted ethanol. The sections were rinsed in 0.01 M Tris-HCl, pH 8.0, incubated in 1% bovine serum albumin for 10 min at 37°C, and then treated with 1 mg/ml proteinase K for 30 min at 37°C. The sections were acetylated by incubation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min at room temperature and washed briefly in 2X SSC. The digoxigenin (DIG)-labeling of the insert codes for PR-1 was performed according to the manufacturers instruction (Boehringer Mannheim, Germany).

Tissue sections fixed on the slides were prehybridized for 30 min at 42°C in hybridization buffer containing 50% formamide, 2X SSC, 150 μg/ml tRNA, 0.5% blocking reagent (Bochringer Mannheim, Germany), and 40 units/ml of RNase inhibitor (Boehringer Mannheim, Germany). Hybridization was performed with the DIG-labeled probe in the same hybridization buffer at 42°C for 16 hr in a moist chamber. After hybridization, the sections

were washed twice in 50% formamide and 2X SSC for 10 min at 42°C, 2X SSC for 10 min at 42°C and then in distilled water for 5 min.

Immunodetection was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany). After washing in the buffer 1 containing 0.1 M Tris-HCl, pH 7.5 for 5 min, the sections were incubated in the buffer 2 containing 1% blocking reagent (Boehringer Mannheim, Germany) for 30 min at room temperature. The sections were then incubated in an anti-DIG-alkaline phosphatase solution (1:3,000 diluted in buffer 2) for 1 hr at 32°C in a moist chamber. The slides were washed twice in buffer 1 for 15 min each and then in buffer 3 (*0.1M* Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) for 5 min at room temperature. The color reaction was developed overnight in buffer 3 with diluted nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at room temperature. The color reaction was stopped in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Hybridization signals were examined using Olympus nb BH2 (Olympus, Japan) microscope equipped with C-35AD-4 camera (Olympus, Japan). Control tests were done without DIG-labeled PR-1 probes or on the uninoculated hypocotyl tissues.

Results

Isolation of differentially expressed soybean acidic *GMPR1a* (PR-1) cDNA. Compatible or incompatible *P. sojae* f. sp. *glycines* races were inoculated on the 4-day-old soybean hypocotyls. About 30 hr after inoculation, symptom of hypersensitive reactions was visible on hypocotyls in the incompatible interaction, but not in compatible interaction. There were black regions around the infected sites on the hypocotyls of soybean inoculated with the incompatible race 2. However, there were only water-soaked and weakened symptoms on the hypocotyls of soybean inoculated with the compatible race 4.

To isolate soybean genes that function during pathogenesis, differential display was performed by using total RNA from soybean hypocotyls that were uninoculated or inoculated with the compatible race 4 or the incompatible race 2. The cDNAs were synthesized and labeled with reverse transcriptase with dNTP mixture containing ^{35}S-labeled dATP. These cDNAs were differentially displayed on the sequencing gel and exposed to X-ray film (data not shown). The cDNA fragment C18CI2 was isolated from cDNAs of soybean hypocotyl inoculated with the incompatible *P. sojae* f. sp. *glycines* race 2. The C18CI2 cDNA was used as a probe to search for the full-length cDNA clone corresponding to C18CI2 cDNA. In the Northern blot using DIG-labeled C18CI2 cDNA as a probe, there was a strong accumulation of mRNAs corresponding to C18CI2 in soybean hypocotyl inoculated with the incompatible race 2, but only a weak accumulation of C18CI2 mRNAs in the compatible interaction. However, there was no accumulation of the transcripts in healthy soybean hypocotyls and mycelia of races 2 and 4.

To isolate full-length cDNA clones, a cDNA library in lambda ZAP II (Stratagene) was constructed from hypocotyls of the 4 day-old soybean seedlings at 30 h after inoculation with *P. sojae* f. sp. *glycines* race 2. Using the DIG-labeled cDNA of C18CI2 as a probe, several positive plaques were identified from 50,000 plaques analyzed in the primary screen of the cDNA library. The positive plaques were harvested, amplified, and rescreened with the DIG-labeled cDNA of C18CI2 as a probe. Most of the reamplified positive clones were hybridized to the clone C18CI2 with different intensities (data not shown). Finally, 9 positive clones showing strong signal intensities in the secondary screen were rescued from the phagemids into pBluescript SK(−) using a helper phage. The individual plasmids with the selected 9 cDNA clones were amplified and harvested. The harvested plasmids were digested with *Eco* RI and *Xho* I to isolate cDNA corresponding to C18CI2 within the multicloning site. The size of the cDNA inserts was measured on 1% agarose gel. The cDNAs on the gel was transferred to nylon membrane and blotted with the DIG-labeled C18CI2 cDNA probe. The cDNA clone, designated *GMPR1a*, was selected for further analysis.

Nucleotide and deduced amino acid sequences of *GMPR1a* cDNA. Full-length sequencing of the selected cDNA clone *GMPR1a*, encoding a putative pathogenesis-related (PR) protein, was done using appropriate deletion derivatives of the *GMPR1a* clone. The cDNA clone has 741 nucleotides, and contains an open reading frame beginning with the first ATG initiation codon at 46 bp and ending with the TAG termination codon at 568 bp (Fig. 1). This clone has a putative signal peptide of the first 27 amino acids. In 3-untranslated region, a potential polyadenylated signal AATAAA locates at 695 bp from 5-end, and there is a 27 bp-long poly(A)$^+$ stretch at the 3-terminus. The open reading frame is capable of encoding a 174 amino acid polypeptide with a predicted molecular mass of 18,767 Da and a predicted pI of 4.23.

Comparisons of the nucleotide and the deduced amino acid sequences of the *GMPR1a* cDNA with other known sequences were conducted using the appropriate BLAST programs and the latest versions of GenBank and EMBL. The deduced amino acid sequences of the *GMPR1a* (AF136636) was 52.9-57.8% homologous to those of PR1 proteins from other plant species, such as *Brassica napus* (*BNU21849*, Hanfrey et al. 1996; *BNU64806*, Zhang and Fristensky, 1996). *Nicotiana tabacum* (*NTPRP1*, Payne et al. 1989; *NTPR1B*, Eyal et al. 1992), and *Sambucus nigra* (*SNPR1JET9*) (Fig. 2).

Three conserved regions are detected and presented with 80-90% homology in region I, 67-80% in region II, and 71-

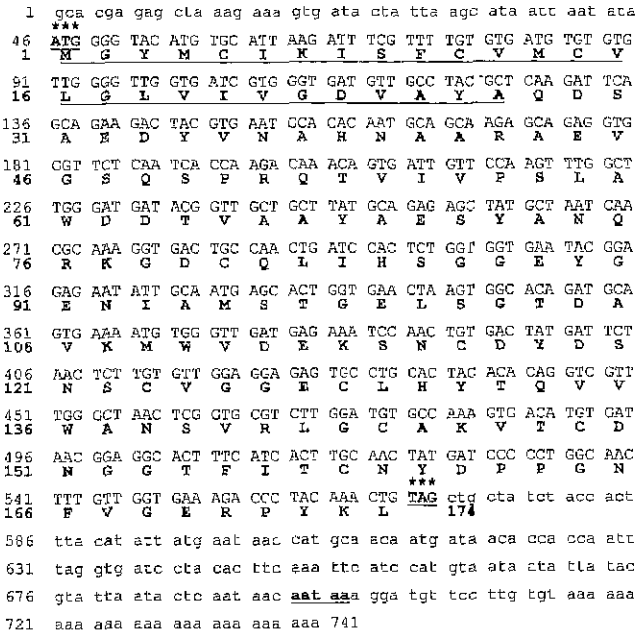

Fig. 1. Nucleotide and deduced amino acid sequences of *GMPR1a* cDNA, encoding a putative acidic pathogenesis-related (PR) protein of soybean. The open reading frame of nucleotides is indicated in uppercase letters. The predicted amino acid sequence (single letter abbreviations) is shown below the nucleotide sequence within the open reading frame. Codons for translation initiation and termination are presented by underlines and asterisk (*). The underline in amino acid sequence indicates a putative signal peptide in the N-terminal portion of the predicted protein. The underline in nucleotide sequence represents a polyadenylation site aataa in the 3-untranslated region of the cDNA.

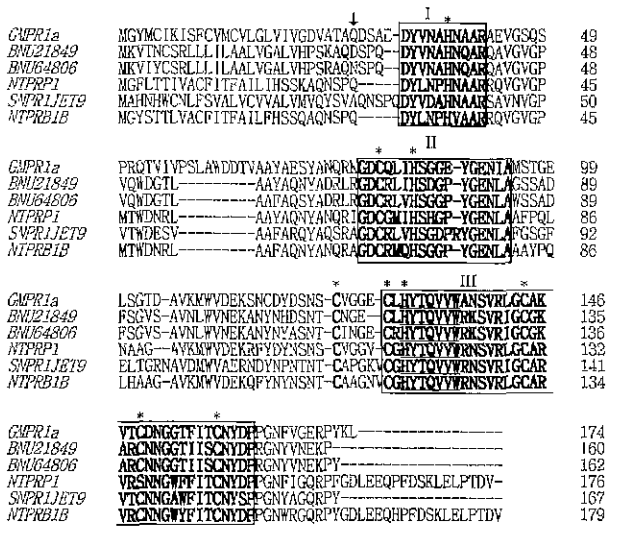

Fig. 2. Amino acid sequence alignment of *GMPR1a* cDNA (AF136636) encoding a putative acidic PR-1 protein with several PR-1 proteins from various plants *BNU21849* (*Brassica napus*, U21849, Hanfrey et al., 1996), *BNU64806* (*B. napus*, U64806, Zhang and Fristensky, 1996), *NTPRP1* (*Nicotiana tabacum*, X14065, Payne, 1989), *SNPR1JET9* (*Sambucus nigra*, Z46947), and *NTPR1B* (*N. tabacum*, X66942, Eyal et al., 1992). Highly homologous regions are boxed and labeled I, II, and III. The dashes indicate the gaps introduced to maximize alignment. The presumed cleavage site for the signal sequence of the deduced protein of the *GMPR1a* cDNA clone is indicated with an arrow. The numbers of amino acids are shown in the ends of sequences. The highly conserved histidine H and cystein C residues are represented by asterisk (*)

80% in region III (Fig. 2, boxed). There are 3 histidine and 10 cysteine residues in the deduced amino acid sequence of *GMPR1a*. Three histidines and five cysteines are conserved in all amino acid sequences of PR proteins from other plant species (Fig. 2).

Pathogen- and elicitor-dependent expression of *GMPR1a* mRNA. To test whether or not induction of *GMPR1a* mRNA in soybean hypocotyls correlates with the resistance response to *P. sojae* f. sp. *glycines*, total RNAs were isolated from soybean hypocotyls at various time intervals after inoculation with the compatible race 4 and the incompatible race 2. The total RNAs were blotted with ^{32}P labeled *GMPR1a* cDNA probe (Fig. 3A). No accumulation of *GMPR1a* mRNAs was detected in healthy hypocotyls. In the compatible interactions, accumulation of the mRNA hybridizing to *GMPR1a* was detected at 20 hr after inoculation and slightly increased at 30 hr, but remained unchanged at 40 hr. In the incompatible interactions, the *GMPR1a* mRNA was slightly detected at 4 hr after inoculation, followed by a maximum in transcript levels at 20 hr and a gradual decrease until 40 hr after inoculation. A quite different patterns of *GMPR1a* mRNA induction was observed in compatible and incompatible interactions (Fig. 3B). Some induction of *GMPR1a* mRNA was found already at 4 hr after inoculation with the incompatible race 2. The dramatic increase in *GMPR1a* mRNA occurred in the inoculated hypocotyls at 20 hr after inoculation in incompatible interaction. In contrast, the *GMPR1a* mRNA was not induced at 4 and 10 hr after inoculation with compatible race and the accumulations were at low levels at 20, 30 and 40 hr after inoculation.

The levels of expression of the *GMPR1a* mRNA in the hypocotyls in response to wounding or chemical elicitors were examined by Northern hybridization analysis (Fig. 4). Wounding and salicylic acid treatment had no effect on the abundance of mRNAs corresponding to *GMPR1a*, but its transcripts were induced after treatment with ethephon. In addition, the *GMPR1a* mRNA also showed a considerable induction upon the treatment with DL-β-amino-N-butyric acid (Fig. 4). Treatment with DL-β-amino-N-butyric acid on soybean hypocotyls increased the resistance of soybean to *P. sojae* f. sp. *glycines* infection (data not shown). The expression of mRNA for *GMPR1a* in the hypocotyls was different between 18 and 36 hr after treatment with several elicitors (Fig. 3). Interestingly, *GMPR1a* mRNAs strongly

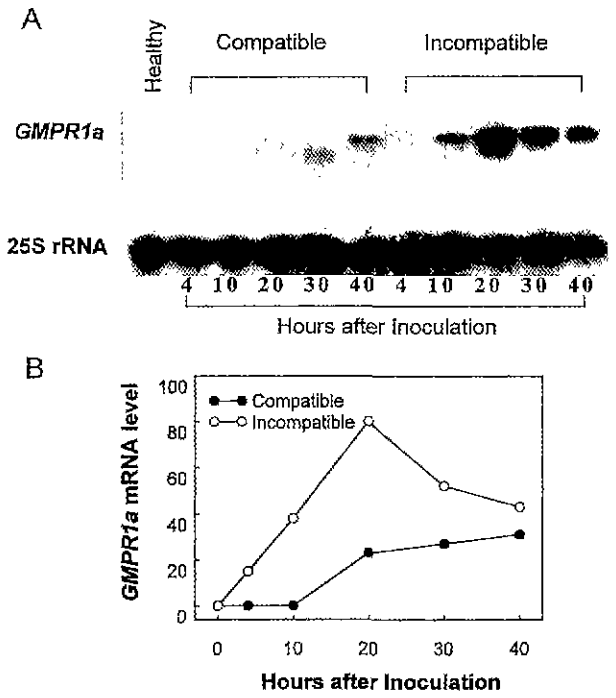

Fig. 3. A. Northern blot analysis of *GMPR1a* mRNAs in soybean hypocotyls at different time intervals after inoculation with compatible race 4 and incompatible race 2 of *Phytophthora sojae* f. sp. *glycines*. Total RNA (30 mg) from each sample was loaded in each lane. As a loading control, the gel at bottom represents nonspecific hybridization to 25S rRNA in the lanes. B. Densitometric comparison of accumulation of *GMPR1a* mRNA in soybean hypocotyls between compatible and incompatible interactions of soybean with *P. sojae* f. sp. *glycines*.

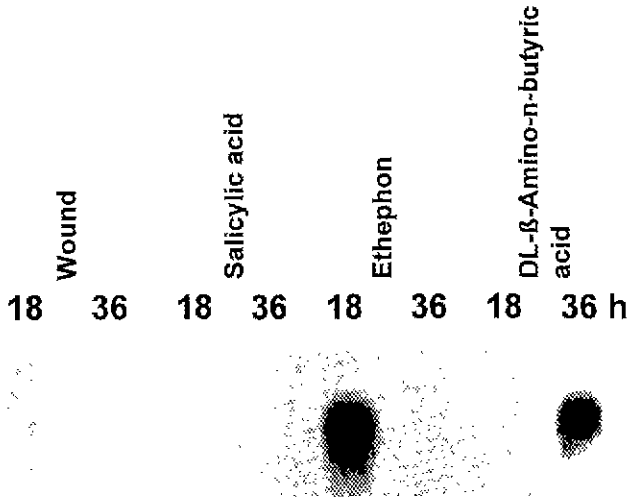

Fig. 4. Northern blot analysis of *GMPR1a* mRNAs in soybean hypocotyls at 18 and 36 hr after either wounding or treatment with abiotic elicitors such as 0.1% salicylic acid, 10 mM ethephon and 0.1% DL-β-amino-n-butyric acid. Total RNA (30 mg) from each sample was loaded in each lane.

accumulated at 18 hr, but disappeared at 36 hr after treatment of ethephon. Salicylic acid did not induce *GMPR1a* mRNAs in hypocotyls at 18 and 36 hr after treatment.

***In situ* localization of *GMPR1a* mRNA in compatible and incompatible interactions.** The differential expression of *GMPR1a* mRNA in the infected soybean hypocotyl tissues enabled us to perform a more detailed examination by cytochemical methods. *In situ* hybridization was used to determine the temporal and spatial expression of the *GMPR1a* mRNA in soybean hypocotyls infected by the compatible or incompatible races of *P. sojae* f. sp. *glycines*.

By *in situ* hybridization, *GMPR1a* mRNAs became detectable mainly within the vascular bundles of the infected soybean hypocotyl tissues. The label was found exclusively within cells around with the vascular bundles, especially with the phloems (Figs. 5C, D, E and F). However, the xylems were nearly free of labeling (Figs. 5D and F). At the early stage of the infection, very low level of labelling was visible around vascular bundles (Figs. 5C and D). At 24 hr after inoculation, the labeling was visible and more distinct than that at 6 hr after inoculation (Figs. 5E and F). However, the cortical and mesophyll cells were nearly free of labeling.

To check the specificity of the *GMPR1a* probe for *in situ* hybridization, a negative control test was performed. All the steps of *in situ* hybridization were done without the *GMPR1a* probe for cross-sections that were obtained at 24 hr after inoculation. As the results, any labeling was not visible in the cortical and mesophyll cells (Fig. 5B). *In situ* localization of *GMPR1a* mRNA in the infected soybean cells in the incompatible interaction was quite different from that in the compatible interaction. In the incompatible interaction, the *GMPR1a* mRNA expression started at 6 hr after inoculation (Fig. 5D). Accumulation of the *GMPR1a* trascripts was remarkable at 24 hr after inoculation (Fig. 5F). In the compatible interaction, the spatial expression of the PR-1 mRNA was similar to that of the incompatible interaction. However, the expression of the transcript started later in the compatible than in incompatible interaction. Less transcripts of *GMPR1a* were accumulated in the infected soybean cells, compared to that of the incompatible interaction (Figs. 5C, D, E and F). Uninfected, healthy soybean plant tissues were free of labeling (Fig. 5A).

The compatible race 4 of *P. sojae* f. sp. *glycines* was heavily colonized in the cortical and mesophyll tissues of soybean hypocotyls. However, no fungal growth was found in mesophyll tissues, but some fungi grew in cortical tissues of soybean hypocotyls at 24 hr after inoculation of the incompatible race 2 (data not shown). Nevertheless, there was little labeling of *GMPR1a* mRNAs in the cortical and mesophyll tissues of soybean at 24 hr after inoculation of both compatible and incompatible races. However, there

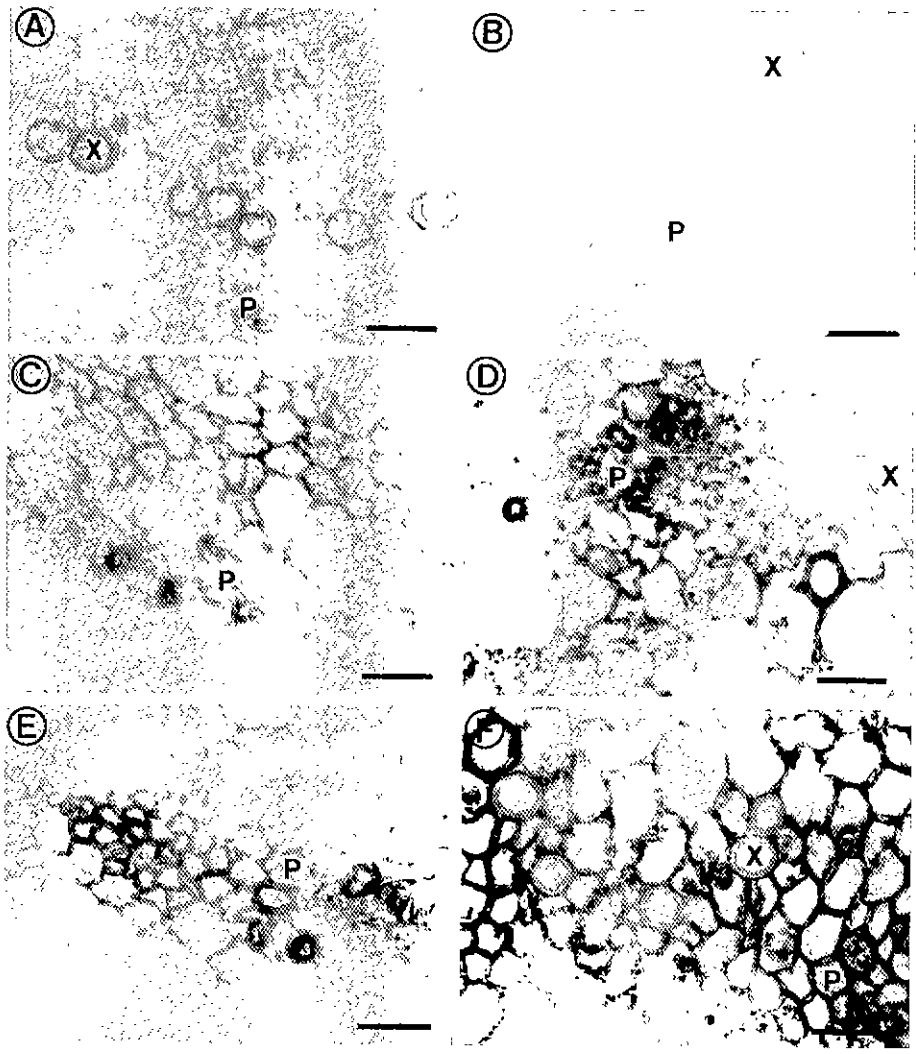

Fig. 5. *In situ* localization of *GMPR1a* mRNA in the vascular bundle area of soybean hypocotyl tissues after inoculation with *Phytophthora sojae* f. sp. *glycines*. Samples were taken from the infected hypocotyls of the compatible and incompatible interactions at the indicated time intervals (A: 0 hr, B: 24 hr, C and D: 6 hr, E and F: 24 hr after inoculation). (A) Uninfected, control hypocotyl tissues. Cross-section of the hypocotyl tissue is free of labeling. Labeling also is not seen in xylem and phloem cells. (Bar size = 1 mm). (B) Negative control. Cross-section of hypocotyl tissues at 24 hr after inoculation of the compatible race 4. *In situ* hybridization was done without *GMPR1a* probe. No labeling is visible in all xylem and phloem cells of hypocotyls. (Bar size = 1 mm). (C) Compatible interaction between soybean and *P. sojae* f. sp. *glycines* at 6 hr after inoculation. Very weak labeling is seen in phloem cells. (Bar size = 1 mm). (D) Incompatible interaction between soybean and *P. sojae* f. sp. *glycines* at 6 hr after inoculation. Densly labeling is seen in the phloem cells. The labeling density is stronger than that in compatible interaction. (Bar size = 1 mm). (E) Compatible interaction between soybean and *P. sojae* f. sp. *glycines* at 24 hr after inoculation. The transcripts accumulated mainly in vascular bundles of hypocotyl tissues, especially within phloem cells. The labeling density gradually increases during the pathogenesis. (Bar size = 1 mm). (F) Incompatible interaction between soy- bean and *P sojae* f. sp. *glycines* at 24 hr after inoculation. Very strong labeling is seen in vascular bundle of hypocotyl tissue. The transcripts accumulate mainly within phloem cells. The labeling density increases strongly at 24 hr after inoculation. (Bar size = 1 mm). Abbreviations used in figures; P, phloem; X, xylem.

was dense labeling in the phloem cells of soybean hypocotyls in compatible and incompatible interactions (Figs. 5E and F).

Discussion

In the primary structure of all the PR proteins, three conserved regions (boxes I, II and III) are presented in Fig. 2. Each conserved region contains one histidine residue in highly conserved sites. Moreover, in putative mature protein of *GMPR1a*, five of ten cysteine residues are located at highly conserved sites in the three conserved regions (Fig. 2). The positional relationships among the three conserved regions and the locations of the cycteine residues in the PR-

l proteins indicate that these six proteins have a similar tertiary structure. In conserved region III, "HYTQVVW" sequence exists in over 40 PR-1 proteins from various plant species such as *Arabidopsis thaliana* (Uknes et al., 1992), *Hordeum vulgare* (Stevens et al., 1996), *Lycopersicon esculentum* (Tornero et al., 1994), *Medicago truncatula* (Szybiak-Strozycka et al., 1995), *Nicotiana tabacum* (Cutt et al., 1988; Payne et al., 1989; Ohshima et al., 1990; Pfitzner et al., 1990; Eyal et al., 1992) and *Zea mays* (Casacuberta et al., 1991). The existence of this highly conserved amino acid sequence suggestes that PR-1 proteins in these plants have some common characters and functions.

In soybean plants, the research of PR1 proteins at molecular levels has not been yet reported. The mRNAs corresponding to the *GMPR1a* cDNA were induced and accumulated in soybean hypocotyls infected by compatible and incompatible *P. sojae* f. sp. *glycines* races. There was difference between the incompatible and the compatible interactions in expression patterns of *GMPR1a* mRNAs. The *GMPR1a* mRNAs accumulated more strongly in the incompatible interaction than in the compatible one. This result was similar to expression patterns of PR1 protein genes in other plants (Granell et al., 1987; Eyal et al., 1992; Tornero et al., 1997), suggesting that the gene corresponding to *GMPR1a* was activated to a greater extent in defense response than in the disease development. The symptom on the hypocotyls was restricted only at the inoculation site by 40 hr after inoculation with the incompatible race 2. However, in compatible interaction, the hypocotyls infected by compatible race 4 show the typical symptoms of Phytophthora rot disease, such as dampping-off, water-soaked and wilt, at 40 hr after inoculation. Therefore, it is suggested that soybean PR *GMPR1a* may play an important role in defense against pathogens at initiation of pathogen invasion.

In soybean hypocotyls, the expression of *GMPR1a* gene encoding PR-1 protein was also stimulated by treatment with ethephon and DL-β-amino-n-butyric acid (Fig. 4). In particular, ethylene-releasing ethephon caused strongly an enhancement of the level of *GMPR1a* mRNA at 18 hr after treatment, but *GMPR1a* mRNA was not detectable at 36 hr after treatment. DL-β-amino-n-butyric acid caused induction of *GMPR1a* expression at 36 hr after treatment. It is assumed that each of abiotic elicitors required different times to turn on a signal for activation of the genes encoding PR-1 protein of soybean. Interestingly, treatment with salicylic acid, which was known as an endogenous signal for systemic acquired resistance (SAR) in tobacco, did not induce the accumulation of the *GMPR1a* mRNAs. Therefore, salicylic acid may not play a role as an endogenous resistance signal in soybean-*Phytophthora* interaction.

The localization of PR-1 (*GMPR1a*) mRNA in *P. sojae* f. sp. *glycines*-infected soybean hypocotyl tissues was studied by using *in situ* hybridization technique. Usually, the transcripts of PR-gene were not or little detected in control plants, but were induced by pathogen infection or treatment with abiotic elicitors. However, some PR proteins were detected in healthy root. In soybean-*Phytophthora* system, there were no transcrips of *GMPR1a* gene in healthy, control soybean hypocotyl tissues (Fig. 3A and Fig. 5A). However, a marked accumulation of the *GMPR1a* mRNA was detected in soybean hypocotyls infected by incompatible race 2. This result is well consistent with the well documented increase in PR-1 protein upon pathogenic attack. (Granell et al., 1987).

Using northern blot analysis and *in situ* hybridization, we have demonstrated that the acidic PR-1 *GMPR1a* mRNA accumulated more rapidly in incompatible interaction than in compatible interaction (Fig. 3). The transcript accumulation was very rapid in the incompatible interaction, but their spatial distribution was similar in both interactions. However, *GMPR1a* mRNA accumulation started already in incompatible interaction at 4 hr after inoculation (data from northern blot analysis, Fig. 3), and in compatible interaction 6 hr after inoculation (data from *in situ* hybridization, Fig. 5). In incompatible interaction, the transcript levels reached a maximum at 20 hr after inoculation, but in compatible interaction at 40 hr after inoculation. These results strongly suggested that the PR-1 genes might be induced in defense against fungal pathogens and in cell damage by disease development (Linthorst, 1991; Ryals et al., 1996; Sticker, 1997). However, there were little transcripts of *GMPR1a* in cortex and mesophyll tissue where the hyphae of *P. sojae* f. sp. *glycines* would exist. In cortex and mesophyll tissue, some cells would be damaged by pathogen, but the transcripts of *GMPR1a* gene were not detected, which suggested that the *GMPR1a* gene may not be induced in cell damage by disease development in soybean.

Our study shows that the PR-1 (*GMPR1a*) mRNA accumulated mainly in the vascular bundle, especially phloem (Fig. 5). Some other PR-protein genes also were localized in vascular bundles (Eyal et al., 1993; Büchter et al., 1997). Hause et al. (1996) have demonstrated that JIP-23 synthesis occurred in phloem cells during the developmentally caused stress. They suggested a possible protective effects of *JIP-23* on cell constituents during stress period. Pathogen attack may be a strong stress to the plant, thereby producing unknown signal molecules that trigger the several defense mechanisms. In this case, the signal molecules may make a dramatic change of metabolic pathway in the infected plant. The unknown signal molecules seem to be transported from cell to cell or through the intercellular spaces, and finally to phloems. They could also affect the PR-1 gene expression in vascular tissue. Thus, *in situ* local-

ization of PR-1 mRNA in vascular bundles (phloem) of soybean hypocotyl tissue suggests specific functions of the gene products in these cells. This function might also be related to defense against *P. sojae* f. sp. *glycines*, because the phloem part could be a profitable area for fungal nutrition uptake.

Acknowledgement

This work was in part supported by the special research grant of Korea University to B. K. Hwang.

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