Inhibition of Phosphoinositide-Specific Phospholipase C Results in the Induction of Pathogenesis-Related Genes in Soybean

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Abstract: **The inositol 1,4,5-trisphosphate (IP_3) content is decreased in soybean cells following infection with *Pseudomonas syringae* pv. *glycinea (Psg)*. In this investigation, a differential display approach was applied to isolate soybean genes that are transcriptionally up-regulated by the inhibition of phosphoinositide-specific phospholipase C (PI-PLC) activity and to study if the transcription of those genes is altered following *Psg* infection. Four genes, transcriptionally activated following treatment with the PI-PLC-specific inhibitor U-73122, were cloned. Three of the four genes were induced following infection with *Psg*. The transcripts of a hydrolase homologue *(GmHy)* were induced in the incompatible but not compatible soybean-*Psg* interaction. The transcripts of a putative ascorbate oxidase gene *(GmAO)* were induced in both compatible and incompatible interactions. *GmHy* and *GmAO* may represent new classes of pathogenesis-related genes. In addition to these two novel genes, homologues of *PR-10* and polygalacturonase inhibitor protein (*GmPR10* and *GmPGIP*, respectively) were identified. These two genes have previously been reported as pathogenesis-related. Transcripts of *GmPR-10*, but not *GmPGIP*, were induced in both compatible and incompatible soybean-*Psg* interactions. Induction of these genes, except for *GmPGIP*, following inhibition of PI-PLC by either the U-73122 treatment or bacterial infection suggests that PI-PLC may negatively regulate the expression of defence genes.**

Key words: **Differential display, IP_3, PI-PLC, PR genes, soybean, U-73122.**

Abbreviations:

GmAO:	soybean ascorbate oxidase
GmHy:	soybean hydrolase
GmPGIP:	soybean polygalacturonase inhibitor protein
IP_3:	inositol 1,4,5-trisphosphate
PIP_2:	phosphatidylinositol 4,5-bisphosphate
PI-PLC:	phosphoinositide-specific phospholipase C

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Footnotes: **The nucleotide sequences reported in this paper have been submitted to GenBank under accession numbers of AF529300 *(GmAO)*, AF529301 *(GmHy)*, AF529302 *(GmPGIP)*, and AF529303 *(GmPR10)*.**

Introduction

Phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) and generates cytosolic inositol 1,4,5-trisphosphate (IP_3) and plasma membrane-associated 1,2-diacylglycerol (DAG), both of which serve as second messengers in response to growth factors, hormones, and other extracellular signals in mammals (Berridge, 1993). Five PI-PLC isoforms, named β, γ, δ, ε, and ζ have been identified in mammalian cells (Rhee and Choi, 1992; Berridge, 1993; Song et al., 2001; Lopez et al., 2001; Saunders et al., 2002). The enzymatic activities of each type of PI-PLC isoform are regulated by distinct mechanisms (Cockcroft and Thomas, 1992; Feng et al., 1996; Lopez et al., 2001; Song et al., 2001).

A number of PI-PLC genes have been cloned from plants, including *Arabidopsis thaliana,* potato, and soybean (Hirayama et al., 1995; Kopka et al., 1998; Shi et al., 1995). Analysis of the genome sequences indicates that there are nine PI-PLC genes in *Arabidopsis* (Made et al., personal communication). Structurally, plant PI-PLCs carrying conserved X, Y, and C2 domains are similar to the mammalian PI-PLC ζ isoform (Saunders et al., 2002). Plant PI-PLCs are regulated at the transcriptional and/or post-transcriptional level. For example, *Arabidopsis AtPLC1* is activated by environmental stresses (Hirayama et al., 1995). Three potato PI-PLC isoforms are expressed in various tissues and are affected by drought stress in a gene-specific manner (Kopka et al., 1998).

The aminosteroid U-73122 reduces the accumulation of intracellular IP_3 in both animal and plant systems (Bleasdale et al., 1990; Shigaki and Bhattacharyya, 2000; Shigaki and Bhattacharyya, 2002). It also blocks the activity of purified recombinant tobacco PI-PLC (Staxén et al., 1999), whereas its inactive analogue U-73343 has no effect on the activity. PI-PLC inhibitors (U-73122, neomycin, and Compound 48/80) have been ap-

plied in discovering physiological responses regulated by plant PI-PLCs, including Nod factor-induced gene expression, calcium spiking, and root hair deformation (Engstrom et al., 2002; Pingret et al., 1998; den Hartog et al., 2001), ABA-induced closure of stomata (Lee et al., 1996), light-induced leaf movements (Morse et al., 1987, 1989), gravi-stimulated growth of the pulvinus (Perera et al., 1999, 2001), tracheary element differentiation in *Zinnia elegans* (Zhang et al., 2002), osmoregulation (Takahashi et al., 2001), and plant defences (Munnik et al., 1998, Munnik, 2001).

PI-PLC activation has been demonstrated during the oxidative burst of suspension-cultured soybean cells following treatment with the elicitor polygalacturonic acid (Legendre et al., 1993). It was observed that intracellular IP_3 increased in elicitor-treated pea epicotyl tissue (Toyoda et al., 1992, 1993) and cell suspensions of tobacco (Kamada and Muto, 1994), lucerne (Walton et al., 1993), and soybean (Legendre et al., 1993). The PI-PLC signal transduction pathway has been suggested to play an important role in the establishment of hypersensitive response in *Citrus limon* following infection with *Alternaria alternata* (Ortega and Petrez, 2001). In contrast to these earlier reports, decreased cellular IP_3 levels were observed when soybean suspension cells were infected with either virulent or avirulent strains of *Psg* (Shigaki and Bhattacharyya, 2000). The decrease in IP_3 contents was consistently larger in cells infected with an avirulent than in cells infected with a virulent *Psg* strain. It was proposed that the signals from plant or bacterial origins suppress PI-PLC following infection so that plant metabolites are utilized (i) in the synthesis of defence-related compounds at the expense of housekeeping functions and/or (ii) in supplementing the growth of plant pathogens. Transcriptional activation of plant PI-PLC genes and conflicting reports on activation of the enzyme in elicited and infected tissues may support the notion that the animal model of phosphoinositide signalling is not directly applicable to plants (Rudd and Franklin-Tong, 2001).

The goal of this investigation was to isolate genes whose transcript levels increased following the inhibition of PI-PLC activity, and investigate whether these genes are pathogenesis-related. In this paper, we report the cloning of three putative pathogenesis-related (PR) genes, transcripts of which are induced in response to treatment with the PI-PLC-specific inhibitor U-73122.

Materials and Methods

Cell suspensions, bacterial pathogens, infection, and chemicals

Suspension cell cultures of soybean (*Glycine max* L.) cultivar Williams 82 were maintained at 25 °C in the dark on an orbital shaker (130 rpm) in Murashige and Skoog medium (Murashige and Skoog, 1962). Cultures were transferred every 7 days by diluting five-fold in fresh medium; experiments were performed 5 days after transfer. The bacterial pathogen *Psg* carrying either *AvrA* or *AvrC* was prepared according to Keen and Buzzell (1991). The soybean suspension cells were inoculated with the bacterial pathogen at a final concentration of $A_{600} = 0.1$, which corresponds to about 1×10^8 cells/ml. U-73122 and U-73343 were purchased from Calbiochem-Novabiochem (San Diego, USA). U-73122 and U-73343 were dissolved in 0.1% dimethyl sulfoxide (DMSO).

Differential display

Differential display was performed using a kit purchased from GenHunter (Nashville, USA). Soybean (cultivar Williams 82) suspension cells were treated with 0.1% DMSO, 100 µM U-73122, or 100 µM U-73343 for 30 min and then immediately frozen in liquid nitrogen. We used a high concentration of U-73122 to reduce the IP_3 concentration to the lowest level possible, and the cells were treated for only 30 min to avoid any downstream non-specific effects that may arise from the treatment. The treatment did not appear to have caused significant lethality under our experimental conditions. The integrity of isolated RNAs from U-73122- or U-73343-treated cells was examined by running a denaturing gel. The RNA samples did not show any signs of detectible degradation.

RNA extraction and first-strand synthesis were performed according to Ausubel et al. (1998), and the cDNA was PCR-amplified and labelled with ^{33}P according to the instructions supplied by GenHunter. The labelled cDNA was analyzed on polyacrylamide gels and exposed to X-ray films. PCR fragments that exhibited differential expression patterns were excised from the gel and purified. The purified DNA was reamplified using the same primer set used in the original amplification.

RNA and DNA gel blot analyses

RNA and DNA gel blot analyses were carried out essentially as described by Ausubel et al. (1998). RNA samples were prepared using the RNAwiz isolation reagent (Ambion, Austin, USA). Five micrograms of RNA were loaded in each lane of a 1.5% denaturing gel, electrophoresed, and transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, Boston, USA). ^{32}P-labelled DNA probes were prepared using a random priming kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were first blocked in a prehybridization buffer containing 7% SDS, 1% BSA, 1 mM EDTA, and 250 mM Na_2HPO_4 (pH 7.4) and then hybridized with a probe at 65 °C for 16 h. The blots were then washed with 2 × SSC three times (10 min each) and then with 0.2 × SSC three times (10 min each) at 65 °C. Hybridization was visualized by autoradiography.

For DNA blot analysis, genomic DNA was extracted from mature leaves of soybean cultivars Williams and Williams 82, and blotted to a nylon membrane, following restriction digestion and gel electrophoresis. Hybridization and autoradiography were performed as described for RNA blot analysis.

Construction and screening of cDNA library

The cDNA library was constructed from soybean cell suspensions (cultivar Williams 82) using a Uni-ZAP XR Vector kit (Stratagene, La Jolla, USA). Fragments identified via differential display were analyzed by Northern blotting. The DNA fragments of interest were cloned in the pGEM-T vector (Promega, Madison, USA) and used for screening the cDNA library according to the standard protocol (Ausubel et al., 1998). Positive clones were isolated and sequenced.

Results

Isolation of genes induced by the PI-PLC-specific inhibitor U-73122

A PI-PLC-specific inhibitor U-73122 reduces the content of intracellular IP_3 in soybean cell suspensions. In contrast, U-73343, a near identical analogue of U-73122, exhibits no effect on *in vivo* IP_3 content (Shigaki and Bhattacharyya, 2002). IP_3 levels were consistently reduced in U-73122 but not in U-73343-treated cells (data not shown). Therefore, treatment of soybean cell suspensions with PI-PLC-specific inhibitor U-73122 and its inactive analogue U-73343 was considered for this investigation. In differential display analyses, RNA samples prepared from soybean cells treated with (i) 100 µM U-73122, (ii) 100 µM U-73343, or (iii) 0.1 % DMSO (the solvent used for the inhibitor and its analogue) were compared. Among more than 10 000 RT-PCR DNA fragments compared in differential display gels, 52 showed up-regulation in U-73122-treated cells.

PCR-amplified DNA fragments up-regulated by U-73122 were analyzed by RNA gel blotting. In RNA gel blot analyses, 10 µM U-73122 were used to avoid any possible cell death caused by high concentrations of U-73122 (Shigaki and Bhattacharyya, unpublished result). It was observed that 10 – 20 µM U-73122 were sufficient to reduce the IP_3 contents to 25 % of the background level (Shigaki and Bhattacharyya, 2002) without causing any cell death (Shigaki and Bhattacharyya, unpublished result). RNA blot analysis of 52 candidate PCR fragments isolated from differential display gels showed that transcripts of only four genes were significantly induced by the inhibitor treatment. These fragments were used to screen a cDNA library. The cDNAs for these genes were cloned and sequenced. The sequences of these four genes were deposited in GenBank (Table **1**).

General features of these U-73122-induced genes, along with their GenBank accession numbers, are presented in Table **1**. Based on sequence comparison, these U-73122-induced genes may encode (i) a hydrolase *(GmHy),* (ii) an ascorbate oxidase *(GmAO),* (iii) a *PR-10* protein *(GmPR10),* and (iv) a polygalacturonase inhibitor protein *(GmPGIP).* Interestingly, the latter two proteins were previously reported to be pathogenesis-related (Crowell et al., 1992; Favaron et al., 1994).

Transcripts of all four genes significantly increased following treatment of soybean cells with 10 µM U-73122 (Fig. **1**). However, the inactive analogue U-73343 also showed some effect on the induction of *GmPR10* and *GmPGIP.* This may suggest that U-73122 and U-73343 may have a mechanism other than through regulation of IP_3 contents for induction of these genes because, at 10 µM concentration, U-73343 does not reduce the IP_3 contents (Shigaki et al., 2002).

Expression of U-73122-induced genes following infection

To determine whether U-73122-induced genes are also induced by pathogen infection, RNA was isolated from soybean suspension cells (prepared from the cultivar Williams 82) infected with *Psg* carrying either the *avrA* or *avrC* gene. Infection of the soybean cultivar Williams 82 with *Psg* carrying *avrA (PsgA)* results in an incompatible interaction, while *Psg* carrying *avrC (PsgC)* produces a compatible interaction (Keen and

Table 1 Soybean genes induced by the PI-PLC-specific inhibitor U-73122

Gene	GenBank accession number cDNA deduced (a.a.)	Length (bp)/ amino acid	Best match in the databases (nr)	Score/E value (NCBI, Blastx)
GmHy	AF529301	786 bp/263 a.a.	a putative hydrolase from *A. thaliana* (AF370598)	321/7e-87
GmAO	AF529300	1484 bp/429 a.a.	L-ascorbate oxidase from *Medicago truncatula* (Y15295)	287/2e-74
GmPR10	AF529303	715 bp/127 a.a.	stress-induced protein SAM22 from soybean (P26987) (belongs to the Betvi/PR-10 family of PR protein)	233/5e-61
GmPGIP	AF529302	1665 bp/333 a.a.	Polygalacturonase-inhibitor protein from *Phaseolus vulgaris* (X64769)	423/e-107

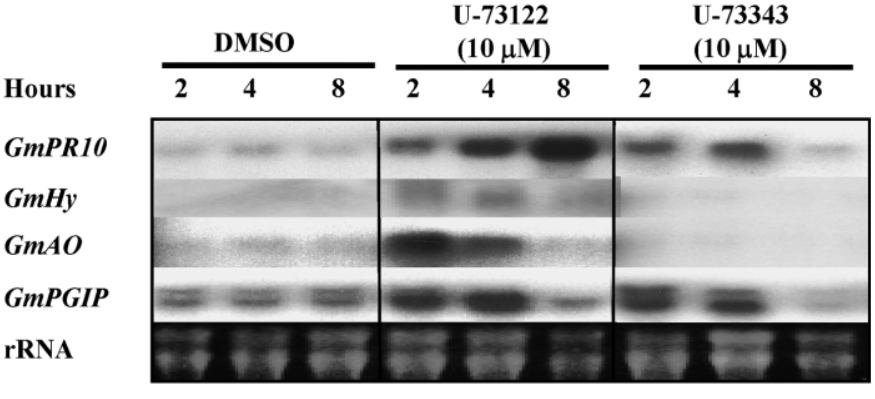

Fig. 1 Induction of soybean genes by the PI-PLC-specific inhibitor U-73122. DMSO, 0.1 % dimethyl sulfoxide, was used to dissolve U-73122 or analogue U-73343. RNA blots were analyzed for expression of *GmPR10, GmHy, GmAO,* and *GmPGIP.* RNAs were visualized by ethidium bromide staining under UV light prior to Northern blot analysis.

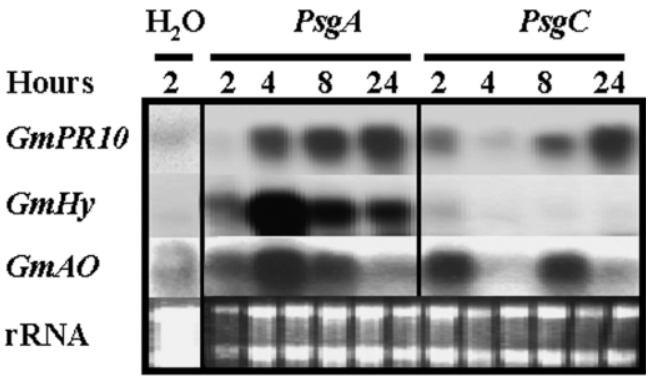

Fig. 2 Induction of soybean genes following infection. *PsgA, Psg* carrying *avrA* results in the incompatible interaction following inoculation of the cv. Williams 82. *PsgC, Psg* carrying *avrC* results in the compatible interaction following inoculation of Williams 82.

Buzzell, 1991). The results of RNA blots carrying samples prepared from infected and water control Williams 82 cell suspensions are presented in Fig. **2**.

Northern analysis showed that the expression levels of *GmHy, GmPR10,* and *GmAO* in the water control cell suspensions did not change throughout the time course of the experiment. Two hours after infection with *PsgA,* the transcription of *GmHy* increased and reached a maximum at 4 h. *GmHy* transcripts did not accumulate to detectable levels following infection with the virulent *PsgC* isolate. The accumulation of *GmAO* increased transiently following infection with both *PsgA* and *PsgC*. *GmPR10* transcripts are induced by infection with both *PsgA* and *PsgC*. Expression of *GmPGIP* was very weak and showed no induction following bacterial infection (data not shown).

U-73122-induced genes encode pathogenesis-related proteins

According to the definition of van Loon et al. (1994), PR proteins are the proteins induced in pathological or related conditions. Therefore, three genes identified in this study are likely to encode pathogenesis-related proteins.

GmHy

GmHy shows no similarity to known PR genes. Therefore, *GmHy* may represent a new family of PR genes. The nucleotide sequence of *GmHy* consists of 1220 bp, encoding 261 amino acids (Fig. **3**). The predicted protein does not contain a signal peptide, has a molecular mass of 29 kDa, and a pI of 5.28. A database search revealed several proteins that are highly homologous to *GmHy*. They are putative phosphatases from soybean (accession number CAD57680) and common bean (CAD57681), putative hydrolases from *Arabidopsis* (NP565738 and E84729), and sugar–starvation-induced proteins from rice (AAL83638) and maize (*ZSS3,* accession number X82617; Chevalier et al., 1995). *GmHy* also showed homology to a putative protein encoded by the ripening-related gene *Grip21* of grape berry (CAB85628) (Davies and Robinson, 2000). Fig. **3** shows the alignment of the deduced protein sequences of the *GmHy*-like genes. The functions of *Grip21* and *ZSS3* are not known.

Genomic DNA from either soybean Williams or Williams 82 was digested with various restriction enzymes and hybridized to the full length *GmHy* cDNA clone (Fig. **4**). There are no *Bgl*II, *Hin*dIII or *Pst*I recognition sites within the *GmHy* open reading frame, and all lanes showed one or two bands. Soybean is a diploidized tetrapolid, therefore, it is most likely that two copies of *GmHy* are present in the soybean genome.

GmAO

The nucleotide sequence of *GmAO* consists of 1484 bp, encoding 429 amino acids (Table **1**). The predicted amino acid sequence of *GmAO* exhibits 74% and 65% identity to ascorbate oxidase from *Medicago truncatula* (Gamas et al., 1996) and tobacco (Kato and Esaka, 1996). A number of ascorbate oxidase genes have been isolated from plants, mainly belonging to the family *Cucurbitaceae.* The deduced amino acid sequences of known ascorbate oxidase genes from higher plants share 68 – 83% identity. Therefore, *GmAO* encodes a putative ascorbate oxidase. However, the isolated cDNA is not a full-length clone and lacks the start codon and a signal peptide that have been found in most full-length cDNAs encoding ascorbate oxidase.

GmPR10

The nucleotide sequence of *GmPR10* consists of 715 bp, encoding 127 amino acids (Table **1**). *GmPR10* shares 92% identity to a soybean *PR-10* gene, *SAM22* (Crowell et al., 1992). *PR-10* genes have been reported from a number of species including soybean, alfalfa, potato, lily, and rice.

GmPGIP

The nucleotide sequence of *GmPGIP* consists of 1665 bp, encoding 333 amino acids (Table **1**). The predicted protein contains a putative signal peptide (1 – 17 a.a.). The predicted mature protein with the signal peptide has a molecular mass of 36.7 kDa and a pI of 8.05. Database searches revealed that the sequence is 66% and 68% identical to PGIPs from *Phaseolus vulgaris* and soybean, respectively (accession numbers X64796 and AF130974, respectively). Hence, *GmPGIP* encodes a putative PGIP.

Discussion

In this paper, we report the isolation of four soybean genes *GmHy, GmAO, GmPR10,* and *GmPGIP* that are transcriptionally activated in suspension culture cells following treatment with the PI-PLC-specific inhibitor U-73122. These genes are either homologues of known PR genes or represent possible novel classes of PR genes. *GmHy* may represent a new family of PR genes and has sequence similarities to putative phosphatases, putative hydrolases, sugar-starvation-induced proteins, and a ripening-related gene. '"Prints" and "Pfam" motif database analyses revealed that GmHy belongs to the superfamily of haloacid dehalogenase and epoxide hydrolase. Proteins share structural similarity, but not sequence identity (Koonin and Tatusov, 1994). Further investigation will be required to establish the importance of this novel gene in plant defences.

GmAO encodes a putative ascorbate oxidase (AO). The physiological function of ascorbate oxidase has not been clarified. Ascorbate oxidase catalyzes the oxidation of ascorbic acid to

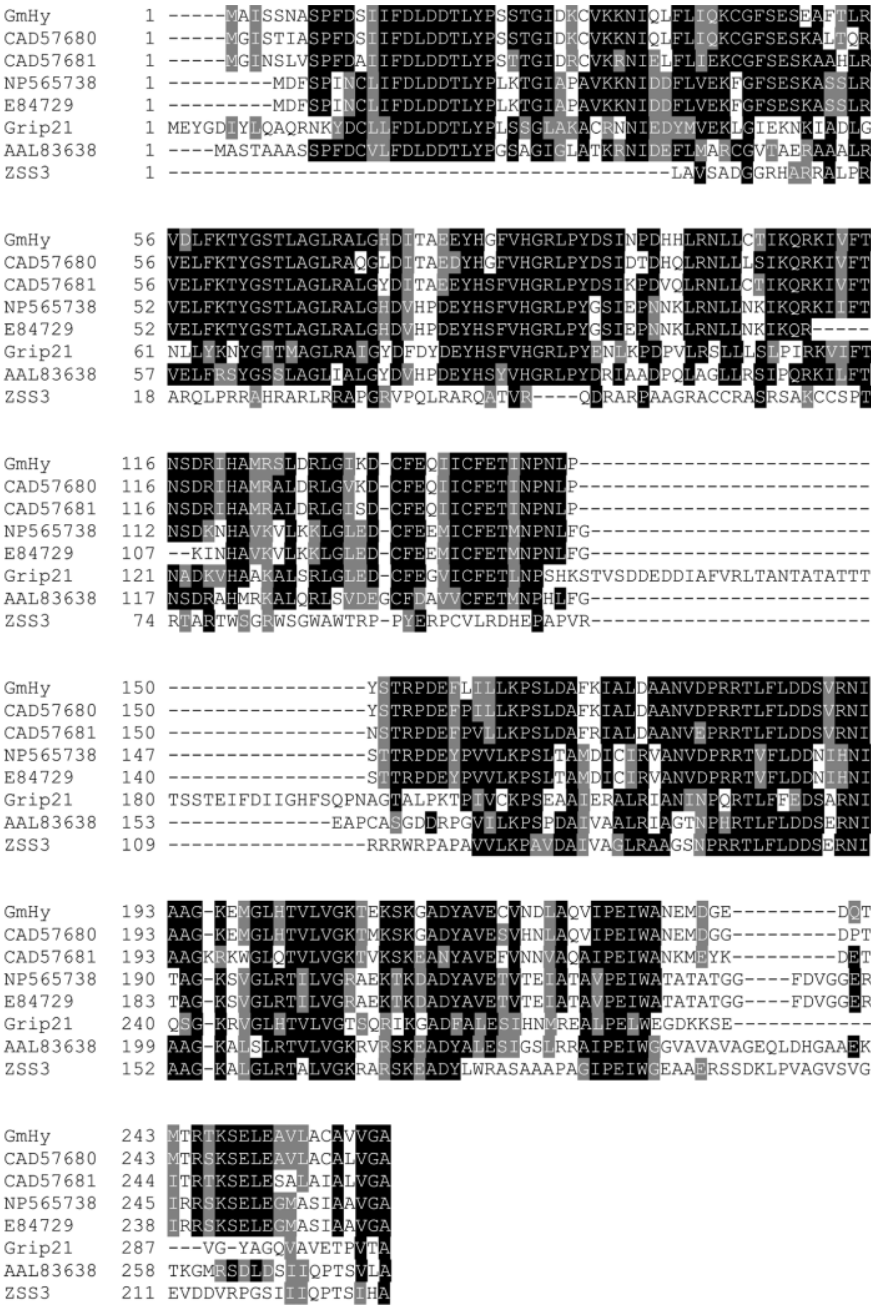

Fig. 3 Alignment of deduced amino acid sequences of polypeptides encoded by *GmHy* and its homologues from various plant species. *GmHy:* a putative hydrolase from soybean; CAD57680: a putative phosphatase from soybean; CAD57681: a putative phosphatase from common bean; NP565738: a putative haloacid dehalogenase-like hydrolase protein from *Arabidopsis thaliana;* E84729: a probable hydrolase from *A. thaliana; Grip21:* a putative ripening-related protein from grape berry; AAL83638: a sugar–starvation-induced protein from rice; *ZSS3:* a sugar starvation-induced protein from maize. Alignments were performed using the ClustalW program (Baylor College of Medicine; Thompson et al., 1994). Consensus amino acid residues are boxed in black (identical) or grey (similar). Gaps introduced to maximize the alignments residues are denoted by hyphens.

dehydroascorbic acid, both of which are thought to influence cell cycle progression in tobacco cell suspension cultures (Potters et al., 2000). An involvement of ascorbate oxidase in the reorganization of cell walls during expansion has also been suggested (Ohkawa et al., 1989; Diallinas et al., 1997; Kato and Esaka, 2000). The transcript level of melon ascorbate oxidase was induced during fruit development and suppressed during wounding (Diallinas et al., 1997). A pumpkin genomic clone encoding ascorbate oxidase has been isolated and may contain a cis-acting region responsible for auxin regulation (Kisu et al., 1997). It has been proposed that AO modifies the apoplastic redox state in order to change the activities of receptor and signal transduction pathways for the expression of disease resistance (Pignocchi and Foyer, 2003). AO can be considered as a novel class of PR genes.

GmPR10 showed very high sequence similarity to *PR-10*. PR-10 proteins show structural similarity to ginseng ribonuclease and, therefore, ribonuclease activity has been proposed (Moiseyev et al., 1997). In fact, a PR-10-like protein from white lupin roots has been shown to have ribonuclease activity (Bantignies et al., 2000).

PGIPs have been isolated from a number of plant species (Favaron et al., 1994). PGIPs carry eight leucine-rich repeats (LRRs). LRRs are versatile structural motifs responsible for

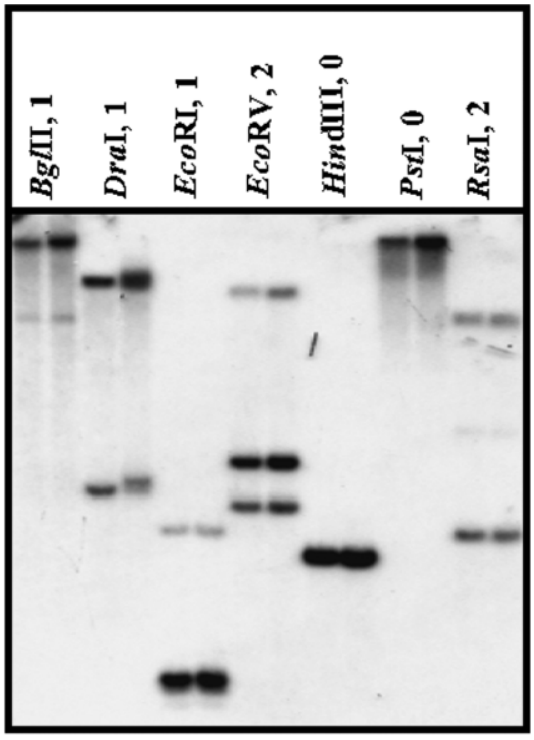

Fig. 4 Genomic organization of *GmHy*. Each lane contains 10 µg of genomic DNA from either Williams (lanes 1, 3, 5, 7, 9, 11, and 13) or Williams 82 (lanes 2, 4, 6, 8, 10, 12, and 14) digested with various restriction enzymes. The numbers following each restriction enzyme indicate the number of recognition sites for that enzyme within the *GmHy* open reading frame.

many protein-protein interactions and are involved in many different cell functions, such as receptor dimerization, domain repulsion, regulation of adhesion and ligand-binding events (Buchanan and Gay, 1996; Jia et al., 2000). PGIPs interact with fungal endopolygalacturonases and inhibit their enzymatic activity *in vitro* (De Lorenzo and Cervone, 2002; Leckie et al., 1999). Therefore, they are considered to be an important protein for expression of plant resistance against phytopathogenic fungi. Different plant PGIP genes respond differentially to stress stimuli or elicitor treatment, such as fungal pathogens and salicylic acid (De Lorenzo and Ferrari, 2002). *GmPGIP* cloned in this investigation is induced by treatment of the PI-PLC-specific inhibitor U-73122, but is not induced by infection. It will require further investigation if *GmPGIP* is induced following fungal infection.

Among the genes reported in this paper, *GmHy* is of particular interest. It represents a novel family of PR genes. *GmHy* may be associated with the expression of disease resistance because its transcripts were induced rapidly only in the incompatible interaction. Our earlier report indicated that the reduction in cellular IP_3 content was more pronounced in the incompatible than in the compatible interaction (Shigaki and Bhattacharyya, 2000). The difference in IP_3 content between compatible and incompatible interactions may explain why the gene is induced in the incompatible but not in the compatible interaction.

The cellular IP_3 level of soybean cell suspension is decreased following infection with *Psg* and stays low for several hours (Shigaki and Bhattacharyya, 2000). U-73122 is a potent and specific PI-PLC inhibitor in both plant and animal systems (Shigaki and Bhattacharyya, 2002; Bleasdale et al., 1990). Reduction in IP_3 levels, either by infection or PI-PLC inhibitor treatments, resulted in induction of transcripts of defence genes. However, it is not a universal phenomenon. Of the four U-73122-induced genes, only three are shown to be up-regulated following infection. Signal pathways induced following infection are highly complex (Hammond-Kosack and Parker, 2003). Most likely, the PI-PLC signalling pathway is inhibited by pathogen infection in order to better use the plant metabolites for synthesis of defence compounds (Shigaki and Bhattacharyya, 2000). This study shows that regulation of at least some defence genes is influenced by the levels of IP_3. Less IP_3 produced by either infection or U-73122 treatment results in induction of a subset of PR genes. Investigation of known defence genes in U-73122-treated tissues is expected to reveal useful information to further support this observation.

The plant PI-PLC signalling pathway is yet to be understood completely. The PI-PLC-specific inhibitor U-73122 and its inactive analogue U-73343 have been extensively used in studying the function of PI-PLCs in both mammals and plant species (Shigaki and Bhattacharyya, 2002 and references therein). It has been suggested that U-73122 may uncouple the G protein involved in PI-PLC activation (Smith et al., 1990). However, a direct inhibitory effect of U-73122, but not U-73343, on an *E. coli*-expressed plant PI-PLC has been reported (Staxén et al., 1999). Therefore, in inhibitor-treated cells, reduction in IP_3 contents could arise due to inhibition of PIP_2 metabolism by PI-PLCs. This may, however, lead to increases in PIP_2 contents which can trigger effects by regulating various enzymes for cellular functions such as vesicle trafficking (De Camilli and Takei, 1996) or ion channel activity (Ashcroft, 1998). Therefore, PIP_2, in addition to IP_3, could be involved in the activation of defence genes.

In infected tissues of intact plant organs, transcripts encoding a putative inositol polyphosphate 5′-phosphatase enzyme that degrades IP_3 are induced (Hermsmeier et al., 2000; Narayanan and Bhattacharyya, unpublished). However, in the experimental conditions used in this study, degradation of IP_3 in infected cells was found to be actually lower than in uninfected cells (Shigaki and Bhattacharyya, 2000). Therefore, it is possible that reduced IP_3 levels in infected tissues may result in down-regulation of either PI-PLC activities and/or degradation of IP_3 by induced expression of an inositol polyphosphate 5′-phosphatase.

The transcripts of three of the four genes cloned in this investigation by applying a random approach are also induced in infected tissues, where the amount of IP_3 stays low for the course of the experiment. The data suggest a negative association between levels of cellular IP_3 and induction of defence genes. In this investigation, we failed to observe induction of *GmPGIP* in infected cells. It is possible that the gene is not induced in *Psg*-infected cells because, in infected cells, the IP_3 levels are reduced to only about 50%, as opposed to 25% of the background level in cells treated with 10 µM U-73122 (Shigaki and Bhattacharyya, 2000; Shigaki and Bhattacharyya, 2002). *GmPGIP* may require a much lower concentration of IP_3 for its induction.

Such a low IP_3 concentration may be possible in interactions of soybean with other pathogens such as fungi. Different plant PGIP genes respond differentially to stress stimuli or elicitor treatment (De Lorenzo and Ferrari, 2002). Alternatively, pathogens and PI-PLC-specific inhibitors do not regulate the expression of at least some defence genes through down-regulation of IP_3 contents.

Regulation of defence gene expression is highly complex. Diverse signalling molecules can regulate induction of defence genes during infection (Hammond-Kosack and Parker, 2003). Considering the complexities involved in defence gene expression, the observed negative correlation between IP_3 contents and induction of defence genes can be considered significant, and suggests a putative role of IP_3 for regulation of defence genes. We hypothesize that IP_3 negatively regulates some of the defence genes. Global transcript profiling experiment using *pi-plc* mutants would assist us in testing our hypothesis.

The reduction in IP_3 contents is much more pronounced in the incompatible than in the compatible interaction (Shigaki and Bhattacharyya, 2000). The difference in IP_3 contents between compatible and incompatible interactions may explain why *GmHy* is induced in the incompatible but not in the compatible interaction. We observed that the incompatible interaction resulted in rapid and strong induction of defence genes within the timecourse of our experiments. A large part of the differences between compatible and incompatible interactions is quantitative in nature; and the plant responses to avirulent pathogens are much faster as compared to that against the virulent pathogens (Tao et al., 2003).

Earlier, we reported that the reduced IP_3 content was correlated with decreased DNA replication in soybean suspension cells (Shigaki and Bhattacharyya, 2002). Reduced IP_3 contents following infection for a sustained period (Shigaki and Bhattacharyya, 2000) most likely signal the inactivation of genes for DNA replication or other growth-related processes and activation of defence genes. Identification of PR genes induced by a PI-PLC-specific inhibitor supports such a hypothesis, and also opens up the possibility of demonstrating the importance of IP_3 in defence gene expression.

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