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Identification of Differentially Displayed Genes of a *Pseudomonas* Resistant Soybean (*Glycine max*)

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In Korea, a local soybean (*Glycine max*) genotype 561 was found to be strongly resistant to a virulent bacterial strain of a *Pseudomonas sp.* SN239. Specific genes involved in the resistance of the soybean genotype 561 were identified and the pattern of gene expression against the *Pseudomonas* infection was analyzed using differential-display reverse transcription PCR (DDRT-PCR). More than 126 cDNA fragments representing mRNAs were induced within 48 hours of bacteria inoculation. Among them, 28 cDNA fragments were cloned and sequenced. Twelve differentially displayed clones with open reading frames had unknown functions. Sixteen selected cDNA clones were homologous to known genes of other organisms. Some of the identified cDNAs were pathogenesis-related (PR) genes and PR-like genes. These cDNAs included a putative calmodulin-binding protein; an endo-1,3-1,4-β-D-glucanase; a β-1,3-endoglucanase; a β-1,3-exoglucanase; a phytochelatin synthetase-like gene; a thiol protease; a cycloartenol synthase; and a putative receptor-like serine/threonine protein kinase. Among them, four genes were found to be putative PR genes induced significantly by the *Pseudomonas* infection. These included a calmodulin-binding protein gene, a β-1,3-endoglucanase gene, a receptor-like serine/threonine protein kinase gene, and pS321 (unknown function). These results suggest that the differentially expressed genes may mediate the strong resistance of soybean 561 to the strain SN239 of *Pseudomonas sp.*

Keywords : Differential display, disease resistant soybean, gene expression, *Glycine max*, pathogenesis-related (PR) genes, *Pseudomonas*.

Plant disease resistance is determined by gene-for-gene interaction involved in plant-pathogen interactions (Flor, 1971). In the gene-for-gene interaction, plants contain dominant resistance (R) genes that specifically recognize pathogens which contain complementary avirulence genes. When plants recognize the products of avirulence genes directly or indirectly from a pathogen, the defense-related gene expression is induced and the result is resistance to pathogen attack (Keen, 1990; Staskawicz, 2001). However, when plants lack R genes or their products, the plants display characteristics of disease. These molecular mechanisms of plant resistance against specific pathogen have been well established (Cook, 1998; Czernic et al., 1999; Staskawicz, 2001). Based on the advancements of host-pathogen interaction at the molecular level, disease-resistance genes are useful for disease control strategies in agriculture. Therefore, understanding the interaction between the bacterial elicitors of individual pathogen and the corresponding response genes of host plant is a preliminary knowledge for disease control.

Gram-negative bacteria *Pseudomonas syringae* pv. *glycinea* (Psg) strains are the major pathogen of many soybean cultivars. Research on the resistance of soybean plants against Psg strains has been focused on understanding the mechanism of host-pathogen interactions (Keen, 1990; Seehaus and Tenhaken, 1998; Whalen et al., 1991). Further progress has elucidated the function of disease-resistance genes of soybean plants to pathogens, which produce elicitors that turn on resistance genes (Ji et al., 1998). For example, elicitor syringolides are glycolipid elicitors produced by the *P. syringae* avirulence gene D. These elicitor syringolides mediate the gene-for-gene complementarities, inducing the hypersensitive response (HR), only in soybean plants carrying the Rpg4 disease resistance gene (Ji et al., 1997). Recently, a better understanding of plant defense mechanisms against specific pathogens has been achieved using a variety of *Arabidopsis* defense response mutants (Shah et al., 1997; Yang et al., 1997). However, since these mutants are not common in most plants including soybeans, a mutant-based genetic analysis for a specific host-pathogen interaction is not feasible. Therefore, the comprehensive differential gene expression analysis of host-pathogen interactions is more effective. Differential display has advantages in speed and in the identification of cDNA, which is the differential gene expression in cell growth and development during the different growth phases and in

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specific environments (Liang and Pardee, 1998; Yi and Hwang, 1998; Zhang and Zhang, 1996).

This study isolated a strain of *Pseudomonas sp.* from soybean seedlings that caused severe rot of many soybean cultivars. Furthermore, a soybean cultivar showing strong resistance to this species of *Pseudomonas* was identified. Using the differential display, this study investigated the differential gene expression of resistant soybean that had been inoculated with the isolated *Pseudomonas*. Further analysis of cloned genes induced differentially during pathogen infection showed large numbers of cloned genes that had unknown functions. Some of the identified cDNAs had high similarity to pathogenesis-related (PR) genes and PR-like genes. Interestingly, cDNA fragments of genes including a cycloartenol synthase, a putative calmodulin-binding protein, a β-1,3-glucanse, and a receptor-like serine-threonine protein kinase were expressed abundantly. In this report, differentially induced novel genes of a newly identified pathogen resistant soybean were also discussed.

Materials and Methods

Bacterial strains, plant material, and plant infection. Colonies of bacteria were isolated on NB broth (3.0 g/L beef extracts and 5.0 g/L pepton) at 28°C from rotten soybean sprouts collected from market places in Daegu, Korea. Among them, strain number 262 showed severe soft-rot symptoms to most inoculated soybean cultivars. Further identification of strain number 262 was performed using Emax™ PRECISION Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and automated MicroLog™ Microbial Identification System (Biolog, Inc., Hayward, CA 94545, USA) according to the manufacturers protocol at the Institute of Biotechnology, Yeungnam University, Gyeongsan, Korea.

Soybean (*Glycine max*) genotypes including 511, 561, and Eunha were obtained from local farms in Korea. Seeds were surface-sterilized with 3% sodium hypochlorite for 30 minutes and germinated in Petri dishes at 26°C in the dark for 2 days. Seedlings were placed for 2 hours in NB medium with 5×10^8 cfu/mL of bacteria for *Pseudomonas* infection or without bacteria as control, and then washed with sterilized water. For plant inoculation, bacteria were cultured until 0.5 of OD number at 600 nm. The washed seedlings with or without pathogen treatments continued to grow at 26°C in the dark and were harvested every 12 hours up to 48 hours. Upon harvest, samples were frozen in liquid nitrogen and stored at -80°C. Some of the treated seedlings after 48 hours were placed under light for 48 hours to develop chloroplasts to determine the survivability after *Pseudomonas* infection.

Differential display. Total RNA was treated with DNase I before performing the reverse transcription PCR. Differential display was performed with the Differential Display Kit according to the manufacturer's protocol (Clontech, Palo Alto, CA, USA). Nine different oligo (dT)-primers were used for PCR in recombination with 10 different arbitrary 13 base primers (AP primers) using Taq DNA polymerase with [α-^{35}S] dATP. Reactions were performed on a MJ-Research thermocycler using the following parameters: 5 minutes 94°C, 5 minutes 40°C, 5 minutes 68°C for 1 cycle; 30 seconds 94°C, 30 seconds 40°C, 5 minutes 68°C for 2 cycles; 30 seconds 94°C, 30 seconds 60°C, 2 minutes 68°C for 23 cycles; and 68°C for an additional 7 minutes. PCR products were separated on a 6% denaturing polyacrylamide gel, dried under vacuum, and then exposed to X-ray film. Differentially expressed PCR products larger than 200 bp were cut from the dried gel, reamplified using appropriate primers, and cloned into a pGEM-T-Easy vector (Promega, Madison, WI, USA) for sequencing.

Northern blot analysis. Total RNA was isolated from harvested tissues using a phenol/chloroform extraction method. Northern blot analysis was carried out according to Kang et al. (2002). Briefly, 20 micrograms of total RNA were electrophoretically separated on a 1.4% agarose gel using 1 × MOPS [3-(N-morpholino)-propanesulfonic acid] buffer and transferred to membranes (Schleicher & Schuell, Keene, NH, USA) in 25 mM phosphate buffer, pH 7.0 for 12 hours. Total RNA was cross-linked to the membrane under a UV cross-linker (Stratagene, La Jolla, CA). DNA probes were synthesized and labeled with [α-^{32}P]dCTP as described by the procedures of the random labeling system (Promega Co., Madison, WI). The radioactive labeled probes were purified with a Sephadex G-25 column and denatured by boiling for 5 minutes and cooling for 5 minutes on ice. Prehybridization was performed for 3 hours at 42°C in prehybridization buffer [50% formamide, 1X Denhart's solution, 5X SSC, 10% (w/v) dextran sulfate, 1.0% SDS, and 0.1 mg/ml denatured salmon sperm DNA]. Hybridization was performed with the radioactive labeled probes for 14 hours at 42°C in prehybridization buffer. Membrane was washed twice in 2XSSC for 5 minutes at room temperature, once in 1XSSC for 20 minutes at room temperature, twice in 0.1XSSC for 20 minutes at room temperature, and once in 0.1XSSC for 5 minutes at 55°C. All SSC washing solutions contained 0.1% SDS. Washed membrane was exposed for 24 hours to X-ray film for autoradiography. Experiments including *Pseudomonas* infection and northern analysis were duplicated to verify the results.

DNA sequencing and molecular genetics analysis. DNA sequencing for the differentially expressed cDNA clones in pGEM-T vector was performed with the BigDye Terminator Cycle Sequencing Kits (PE Biosystems, Foster City, CA, USA) using an automated DNA sequencer (ABI 3100, Applied Biosystems, Rockville, MD, USA) at the Institute of Biotechnology, Yeungnam University, Gyeongsan, Korea. Primers used for sequencing were SP6 (5'-TATTTAGGTGACACTATAG) and T7 (5'-TAATACGACTCACTATAGGG). DNA sequences and deduced amino acids were analyzed using the programs in DNASIS (Hitachi, Japan). The nucleotide sequences were compared with sequences deposited in public databases (GenBank, EMBL, DDBJ, PDB), and putative amino acid sequences were translated using the ORF Finder program at NCBI (http://www.ncbi.nlm.nih.gov). DNA and deduced amino acid sequences were compared with known nucleotide sequences and protein sequences in databases (GenBank CDS translations, PDB, SwissProt, PIR, and PRF) using the BLAST algorithm (Altschul et al., 1990).

Results and Discussions

***Pseudomonas* resistant local soybean genotype.** Different soybean genotypes showing resistance to *Pseudomonas sp.* were collected from farms in Korea. Among them, an indigenous soybean genotype, line 561, which showed strong resistance against a strain of *Pseudomonas sp.* was selected. The soybean seeds were then germinated and inoculated for 2 hours in NB medium with 5×10^8 cfu/mL of the bacteria under darkness. After the *Pseudomonas* treatment, the infected seedlings were washed with water and placed under light to inspect the survivability of these infected seedlings. Most *Pseudomonas* infected seedlings showed infection spots on their hypocotyls within 24 hours. The spots spread rapidly and developed into necrotic lesions. Finally, the lesions expanded throughout the whole seedlings (Fig. 1) resulting in soft-rot symptoms. Complete maceration of the seedlings occurred 3-4 days after infection. However, the *Pseudomonas* infected seedlings of soybean line 561 turned green and developed chloroplasts on their cotyledons 3-4 days after infection (Fig. 1), demonstrating that the cells were not necrotized. Within 2 days after exposure to light, the soybean line 561 grew again; the hypocotyls became elongated, shoots grew, and chloroplasts in the cotyledons and young leaves developed. The lesions on the hypocotyls of the *Pseudomonas* treated soybean 561 under light remained but did not expand, indicating that these lesions resulted in a hypersensitive response (HR) against pathogen infection. In many plant-pathogen interactions, the resistant reaction is characterized by HR, which induces local death of the host cells in the region of infection. Glutathione S-transferase genes are known as inducible genes by avirulent *Pseudomonas syringae* (Lieberherr et al., 2003). Induction of the glutathione S-transferase (GST) gene was also observed in the pathogen treated soybean line 561 (Fig. 4), supporting the hypothesis that hypersensitive response had occurred in

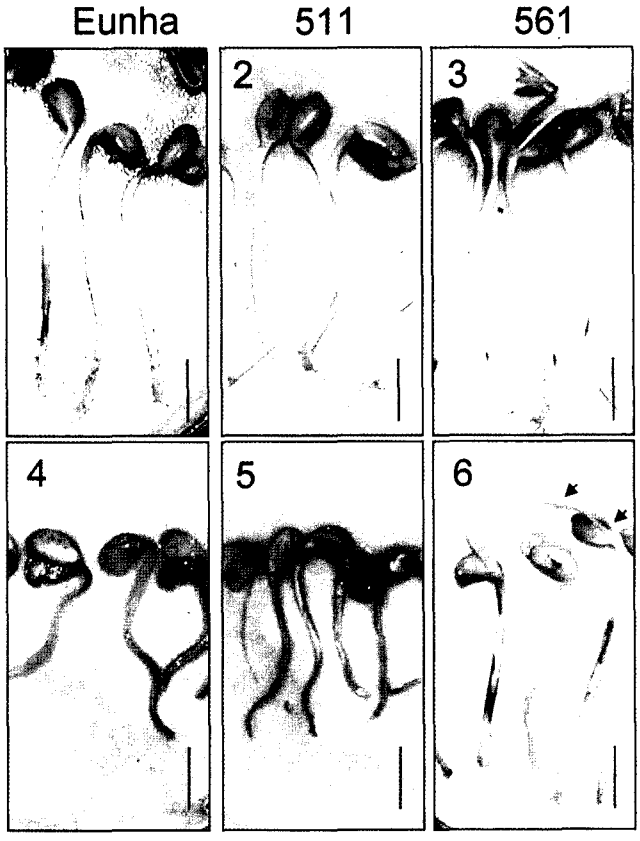

Fig. 1. *Pseudomonas* resistant soybean 561. Dark grown seedlings of soybean cultivars were inoculated for 2 hours in NB media with the *Pseudomonas sp.* SN239 and washed with sterilized water. The seedlings grown after 48 hours were placed under continuous light at 28°C for 48 hours. Soybean plants were Eunha (panels 1 and 4), 511 (panels 2 and 5), and 561 (panels 3 and 6). Seedlings in panels 1, 2, and 3 were not treated with *Pseudomonas*. Seedlings in panels 4, 5, and 6 were treated with *Pseudomonas*. Control scale bar indicates 2 cm.

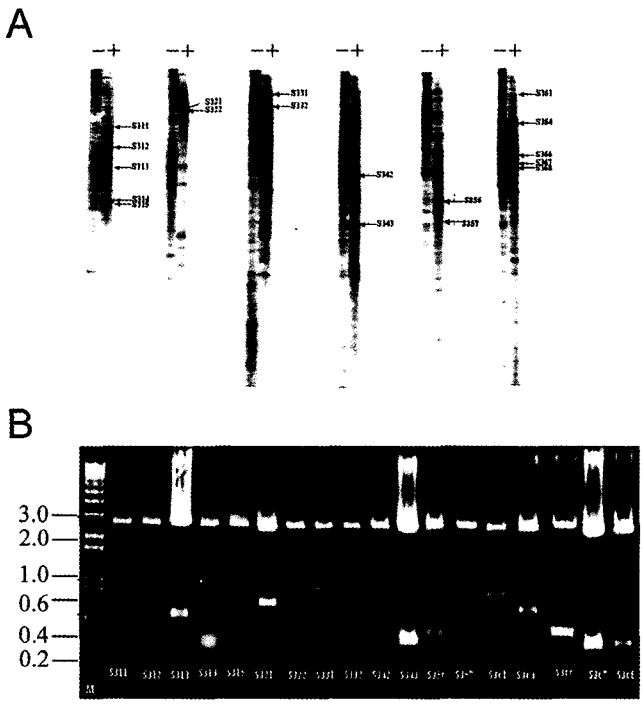

Fig. 2. A typical differential display gel and cloning of the differentially expressed cDNAs in this study. (**A**) Differential display using T2 and Pn (n=3,4,5,6,9) primer pairs were selected. Amplified cDNA fragments from differential display were separated by electrophoresis in 6% denaturing polyacrylamide gels. Total RNA extracted from soybean 561 with presence (+) or absence (−) of *Pseudomonas* was used as template for differential display. Numbers indicate selected cDNAs for further molecular analysis. (**B**) The selected cDNAs from differential display were cloned into pGEM-T easy vector. Plasmids containing cDNAs were digested with *Eco*RI. The first lane is the DNA marker.

the soybean cells attacked by the *Pseudomonas*. Therefore, results demonstrated that soybean line 561 is truly resistant against the isolated strain SN239 of *Pseudomonas sp.*

Identification of a pathogenic strain of *Pseudomonas sp.* Several *Pseudomonas* species from soybean-sprout cultures were collected from local farms and markets in Korea. Among them, one isolated *Pseudomonas* strain number SN239 showed strong pathogenicity to most inoculated soybean cultivars resulting in severe soft-rot symptoms. Further identification efforts using MicroLog™ Microbial Identification System (Biolog, Inc. Hayward, CA 94545, USA) revealed that the selected strain, SN239, is a similar strain of *Pseudomonas aeruginosa*.

P. aeruginosa is a Gram-negative bacterium and a human pathogen that causes severe systemic infections, particularly in patients with cystic fibrosis, burns, and immunosuppression (Britigan and Edeker, 1991). This pathogenic bacterium is often identified from fruits and plants and widely distributed in the natural environment (Rahme et al., 2000) and uses a shared subset of virulence factors to elicit disease in both plants and animal (Rahme et al., 1995). It is known that the Gram-negative bacteria *Pseudomonas syringae* pv. *glycinea* (Psg) strain is the major pathogen of most soybean cultivars. Resistance of soybean plants against Psg strains has been studied extensively, however, nothing is known about how this multi-host pathogen, *P. aeruginosa*, causes disease in soybean. This study is the first report of a similar strain of *P. aeruginosa* causing disease to soybean plants.

Identification of differentially expressed genes from infected soybean. Although the results of pathogen inoculation demonstrated that the soybean line 561 showed strong resistance against the *Pseudomonas sp.* strain SN239, the nature of the molecular genetics of plant-

Table 1. Characteristics of cloned cDNA fragments identifid by DDRT-PCR

cDNA clone	Length (base pair)	Annotation	Accession number	% similarity of DNA sequence
pS233	~400bp	Unknown	–	–
pS234	~350bp	Unknown	–	–
pS241	~1000bp	Unknown	–	–
pS251	~800bp	Receptor-like serine-theonine protein kinase of *Arabidopsis thaliana*	NM_117222	82%
pS255	~500bp	Phytochelatin synthetase-like protein of *Arabidopsis thaliana*	AAM62930	80%
pS257	~350bp	Unknown expressed protein of *Arabidopsis thaliana*	NP_567229	81%
pS262	~500bp	Cellulose synthase-2 of *Zea mays*	AF200526	88%
pS292	~520bp	Beta-D-glucan exohydrolase of *Arabidopsis thaliana*	AY142679	89%
pS311	~593bp	Endo-1,3-1,4-beta-D-glucanase of *Arabidopsis thaliana*	BAB02778	54%
pS312	~500bp	Cycloartenol synthase of *Glycyrrhiza glabra*	AB025968	91%
pS313	~420bp	Unknown	–	–
pS314	~350bp	Unknown	–	–
pS314	~350bp	Unknown	–	–
pS315	~340bp	Thiol protease isoform A of *Glycine max*	U71380	96%
pS321	~470bp	Unknown	–	–
pS322	~550bp	Calmodulin-binding protein family of *Arabidopsis thaliana*	NP_201045	68%
pS331	~1000bp	Glucosyltransferase-like protein of *Arabidopsis thaliana*	NM_122180	82%
pS332	~650bp	Unknown	–	–
pS342	~400bp	Arginase (pAG1) of *Glycine max*	AF035671	98%
pS343	~300bp	Unknown	–	–
pS355	~400bp	Unknown	–	–
pS356	~350bp	Unknown	–	–
pS357	~300bp	Unknown	–	–
pS361	~850bp	Unknown	–	–
pS364	~650bp	Glycosyl hydrolase of *Arabidopsis thaliana*	NP_566736	59%
pS366	~500bp	DIM1 of *Arabidopsis thaliana*	NM_120912	85%
pS367	~450bp	Cycloartenol synthase of *Glycyrrhiza glabra*	AB025968	90%
pS368	~400bp	Cycloartenol synthase of *Glycyrrhiza glabra*	AB025968	89%
pS370	~560bp	1.3-beta-glucanase of *Glycine max*	AAA33946	100%

pathogen interactions in soybean has not been studied. Therefore, this study investigated the genes involved in plant-pathogen interactions of the defense mechanism at the molecular level using differential display technology.

Differential display showed that more than 126 cDNAs representing mRNAs were induced 48 hours after *Pseudomonas* infection. As shown in Figure 2, the identified DDRT-generated cDNAs were cut out of the differential display gels and reamplified. Among them, 28 cDNA fragments were cloned randomly and sequenced (Table 1 and Fig. 2). The average sizes of cDNAs were approximately 400 bp to 1 kb, indicating that the cDNAs contain enough information for analysis using bioinformatics. A BLAST search for nucleotide and amino acid sequence similarity revealed that 13 out of 28 selected cDNA clones were highly homologous to known genes in databases. However, 12 cDNA clones were unknown. The sequences of most cDNAs contained partial coding sequences (CDS) or open reading frames (ORF), demonstrating that the amplified cDNAs were representing the fragments of differentially expressed mRNAs that were up-regulated by pathogen infection (Table 1).

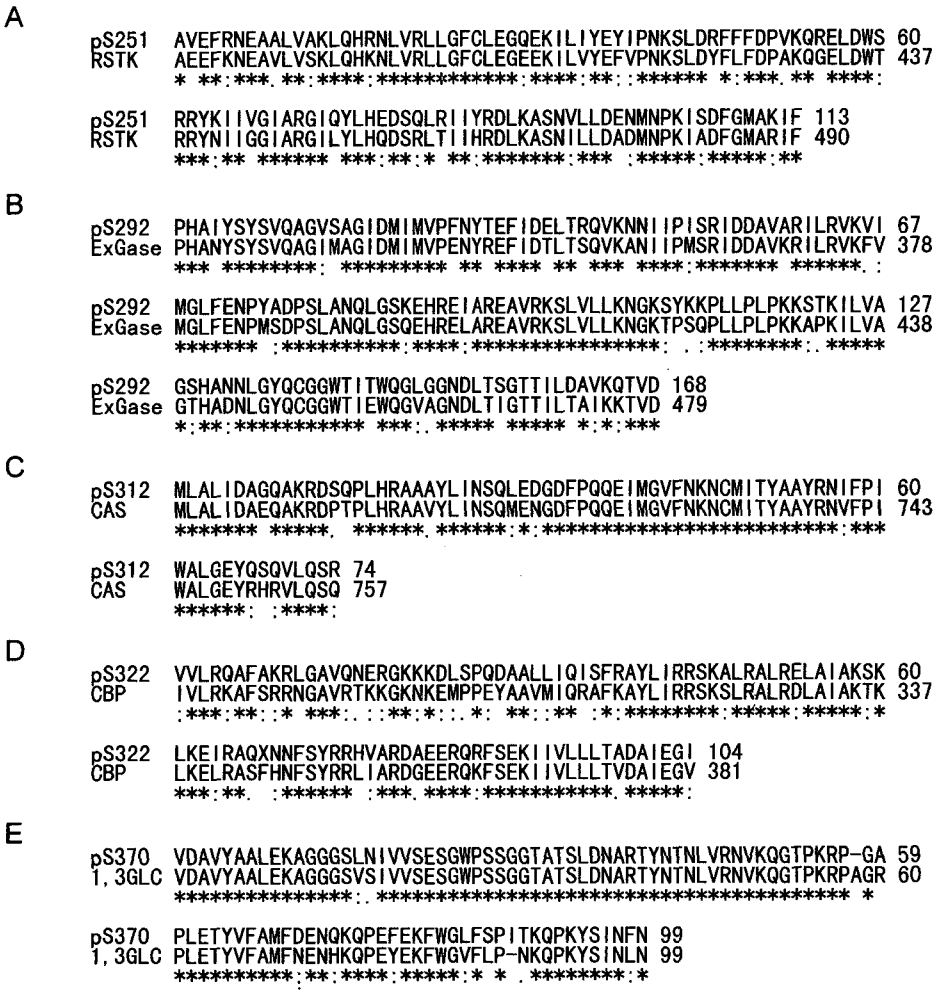

Fig. 3. Alignments of the amino acid sequences of differentially displayed cDNA fragments with those of known genes in the GenBank. (**A**) The amino acid sequence of the translated fragment pS251 was aligned with a protein from a receptor-like serine/threonine kinase (RSTK) of *Arabidopsis thaliana* (NM117222). Aligned amino acid sequence is a catalytic domain of the serine or threonine-specific kinase subfamily. (**B**) The amino acid sequence of the translated fragment pS292 was aligned with β-glucan exohydolase (ExGase) of *Nicotiana tabacum* (BAA33065). (**C**) The amino acid sequence of the translated fragment pS312 was aligned with cycloartenol synthase (CAS) of *Glycyrrhiza glabra* (BAA76902). (**D**) The amino acid sequence of the translated fragment pS322 was aligned with calmodulin-binding protein of *Arabidopsis thaliana* (NP201045). (**E**) The amino acid sequence of the translated fragment pS370 was aligned with a β 1,3-glucanase (1,3 GLC) of *Glycine max* (AAB03501). Alignments were performed with the Clustal program (Thompson et al., 1994). Identical amino acids are marked with an asterisk (*) and equivalent amino acids are marked with dots (: and .). Gaps (–) were introduced for maximum homology. GenBank accession numbers for the listed nucleotide sequences are AY352266 for pS251, AY352268 for pS292, AY352269 for pS312, and AY352271 for pS370.

Analysis of differentially expressed genes from infected soybean. This study analyzed the possible roles of selected soybean cDNAs in the pathogen resistance response.

The fragment pS251 cDNA showed 89% similarity with a receptor-like serine-threonine protein kinase of *Arabidopsis thaliana* (Table 1 and Fig. 3). This the first report of identification of a receptor-like serine-threonine protein kinase (named *GmRLK1*) from *Glycine max*. The putative receptor-like protein kinase gene (*At-RLK3*) of Arabidopsis was identified as differentially regulated during various plant-pathogen interactions (Czernic et al., 1999). In tomato, the *Pto* gene, which is involved in resistance to *Pseudomonas syringae* pv. tomato, coded a serine-threonine protein kinase for a signal transduction pathway (Czernic et al., 1999). This study also found that the putative receptor-like serine-threonine protein kinase of soybean (pS251) was differentially induced in the *Pseudomonas* treated hypocotyls compared with non-treated control (Fig. 4, pS251). Therefore, it is highly possible that the identified receptor-like serine-threonine protein kinase may function as a signal receptor for *Pseudomonas* infection. Further investigation with northern blot analysis showed that the levels of transcripts were almost the same in all parts of the whole soybean plant (Fig. 4, pS312), indicating that this gene is constitutively expressed.

The differential display DNA fragment pS322 encodes part of a putative calmodulin-binding protein (CBP) (Table 1). The deduced amino acid sequences showed 68% similarity to calmodulin-binding protein (accession number NM 125633) of Arabidopsis (Fig. 3). Interaction between Ca^{2+} and calmodulin (CaM) functions is a key role of the Ca^{2+} sensor in eukaryotes. Recently, Reddy et al. (2003) found that the expression of the *CBP* gene in Arabidopsis was highly induced in response to avirulent *Pseudomonas syringae* pv. tomato carrying avrRpm1. Further analysis suggested the role of CBP in Ca^{2+}-mediated defense signaling and cell-death. Similarly, this study found that expression of a putative *CBP* (pS322) gene was dramatically induced in the hypocotyls of a *Pseudomonas* infected seedling (Fig. 4, pS322). In addition, it was observed that this putative soybean *CBP* gene was expressed relatively high in stems and leaves, but less in roots, nodules, flowers, and pods. However, the gene did not express in the seeds (Fig. 4, pS322). These results strongly suggest that CBP in soybeans is involved in Ca^{2+}-mediated defense signaling and cell-death during the hypersensitive response (HR) induced by the *Pseudomonas* sp. SN239.

The differential display DNA fragments, pS312, pS367, and pS368, coded a cycloartenol synthase of *Glycyrrhiza glabra* (Table 1 and Fig. 3). Interestingly, cycloartenol synthase in soybean has not been identified yet. Thus, this is the first report of the identification of a soybean cycloartenol synthase (*CAS*) gene. Cycloartenol synthase converts 2,3-oxidosqualene to cycloartenol, the well-known first cyclization product of triterpenoid saponins and steroid biosynthesis in plants and algae (Meyer et al., 2002; Shi et al., 1994). Triterpenoid saponins are synthesized via the isoprenoid pathway by cyclization of 2,3-oxidosqualene to give primarily oleanane (β-amyrin) or dammarane triterpenoid skeletons. The natural role of triterpenoid saponins mediated by cycloartenol synthase in plants is likely to be in defense against attack by pathogens and pests (Haralampidis et al., 2001). The northern blot analysis showed that the levels of transcripts between the *Pseudomonas* treated or untreated samples were almost the same (Fig. 4, pS312). Surprisingly, however, the cDNA for the putative cycloartenol synthase of soybean was found in 3 out of 27 cloned cDNAs (Table 1). This abundant occasion of cDNAs for cycloartenol synthase in the differential display of *Pseudomonas* treated soybean line 561 suggests that the cycloartenol synthase may be related to defense system against *Pseudomonas* infection. Although it is premature to

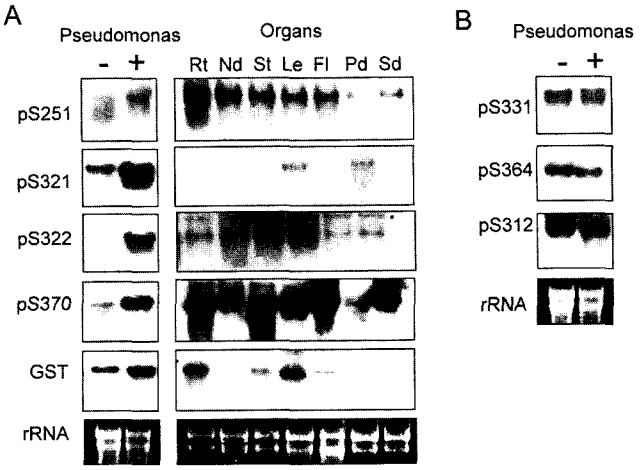

Fig. 4. Northern blot analysis with the differential display cDNA fragments. Total RNA was isolated from seedlings placed for 24 hours under light after *Pseudomonas* infection (+) or without (–) infection. (**A**) Expression patterns of differentially induced cDNAs 12 hours after *Pseudomonas* infection (+) or control (–). Each radioactively labeled cDNA probe was encoded for a protein: pS251 for receptor-like serine-threonine protein kinase; pS321 for unknown protein; pS322 for putative calmodulin-binding protein; pS370 for a putative 1,3-β-endoglucanse; and GST for glutathione S-transferase of soybean. GST was used as a pathogen inducible gene control. Total RNAs were isolated from the roots (Rt), nodules (Nd), stems (St), leaves (Le), flowers (Fl), pods (Pd), and seeds (Sd) from healthy plants. In each lane, 10 μg of total RNA was separated by electrophoresis and hybridized with appropriate probes. (**B**) Expression patterns of differential displayed cDNAs but constitutive expression in 24 hours after *Pseudomonas* infection: pS312 for cycloartenol synthase; pS331 for glucosyltransferase-like protein; and pS364 for glycosyl hydrolase. Ribosomal RNA is shown as a loading control.

make an assumption that cycloartenol synthase is directly involved in the defense system, this enzyme may be activated to produce more saponins to defend against pathogens and pests.

The differential display DNA fragment pS315 encodes a putative thiol protease with 96% similarity to that of *Glycine max* (Table 1). This study could not detect any signal for pS315 in the northern blot analysis using total RNA, indicating that this thiol protease gene is expressed at a relatively low level. Thiol proteases are the most proteolytic enzymes that function to degrade proteins in senescent plants (Granell et al., 1992). A form of programmed cell death by proteolytic activity is one of the major hypersensitive responses during plant-pathogen interactions (Heath, 2000). Therefore, this suggests the role of thiol protease in hypersensitive responses by *Pseudomonas*, although there is no report that thiol proteases are involved directly in plant defense.

Three cDNA fragments coding for glucan hydrolases were identified including a β-1,3-D-glucan exohydrolase (pS292), a 1,3-1,4-β-D-glucanase (pS311), and a β-1,3-glucanase (pS370) (Table 1). The β-1,3-glucanase hydrolyses the 1,3-β-D-glucosidic linkages in β-D-glucans and other glucans which make up the cell walls of the fungus (Cline and Albersheim, 1981). The plant β-1,3-glucanase is induced by potential pathogen attack and classified as pathogenesis-related protein (PR-2) (Benhamou, 1995; Dong et al., 1991). The amino acid sequence of pS370 showed 100% similarity to the β-1,3-endoglucanase of soybean (accession number AAA33946.1). As shown in Figure 3, the deduced amino acids of pS370 also shared high similarity (88%) to a pathogen inducible β-1,3-glucanase of soybean (accession number AAB03501) (Reyes and Lerner, 1983). Northern blot analysis showed that the β-1,3-glucanase (pS370) transcripts were dramatically accumulated in the hypocotyls treated with *Pseudomonas* compared with those of control seedlings (Fig. 4, pS370), indicating that a soybean β-1,3-glucanase (pS370) is induced by the pathogenic *Pseudomonas* strain SN239. The β-1,3-glucanase gene was expressed in high levels in most plant parts except pods. However, the transcripts highly accumulated in flowers and immature seeds, indicating that this gene is actively involved in flower and seed developments (Fig. 4, pS370). The 1,3-1,4-β-D-glucan endohydrolase is required for the depolymerization of 1,3-1,4-β-D-glucans in germinated grain or the partial hydrolysis of the polysaccharide in elongating vegetative tissues (Hrmova et al., 1995; Slakeski and Fincher, 1992). The pS292 coded β-1,3-exoglucanase shared 75% similarity to that of *Nicotiana tabacum* (accession BAA33065) (Fig. 3). This enzyme was detected in the secreted proteins of tobacco cultured cells with pathogen (Okushima et al., 2000). These suggest that these glucanases are actively involved in the degradation of β-glucan elicitors and 1,3-β-D-glucans in the cell walls of the pathogen. Unfortunately, this study could not detect any signal in the northern blot analysis for pS292 and pS311. This implies that these transcripts may be too rare to be detected by northern blot analysis using total RNA as template.

As shown in Table 1, 12 differentially displayed clones did not have any similarity with known genes in the databases. However, it was found that the level of pS321 transcripts showed drastically differential accumulation in the *Pseudomonas* treated seedlings compared with untreated seedlings (Fig. 4, pS321). Therefore, it should be considered that pS321 is a novel pathogen-inducible gene. Although further analysis of these unknown genes could not be performed, some of them are related possibly to the defense system against the isolated *Pseudomonas*. Furthermore, the transcripts of this unknown gene were detected in the stems, leaves, and pods (Fig. 4, pS321), indicating that this gene may be developmentally controlled. Therefore, further research of these differentially expressed unknown genes, especially pS321, are currently being developed.

Some of the differentially displayed DNA fragments encode metabolic enzymes (Table 1), including cellulose synthase (pS262), glucosyltransferase-like protein (pS331), arginase (pS342), and glycosyl hydrolase (pS364). Interestingly, the differentially displayed DNA fragment of the pS255 clone showed 80% similarity to a phytochelatin synthase-like protein of *Arabidopsis* (Table 1). Phytochelatin synthase synthesizes phytochelatins (PCs), metal-binding cysteine-rich peptides in plants, and yeasts from glutathione in response to heavy metal stress (Vatamaniuk et al., 2000). The pS366 cDNA encoded a putative DIM-like protein (Table 1) known as a cell cycle controller of yeast (Berry and Gould, 1997). *Dim1* is a small evolutionarily conserved protein essential for G2/M transition that has recently been implicated as a component of the mRNA splicing machinery and defined as a novel branch of the thioredoxin fold superfamily involved in cell cycle (Zhang et al., 1999). The transcript levels of these cDNAs were constitutive in hypocotyls both treated and untreated with *Pseudomonas* (Fig. 4), indicating that these may not be directly related to defense system.

In summary, this study found a soybean genotype line 561 which showed strong resistance against a strain of *Pseudomonas*, which is severely pathogenic to most soybean cultivars. Results of differential display showed that more than 126 cDNA fragments representing mRNAs were induced 48 hours after *Pseudomonas* infection. The identified cDNAs were novel or highly homologous to pathogenesis-related (PR) genes and PR-like genes. These results suggest that these differentially expressed genes induced by

the pathogen may mediate the strong resistance function of soybean line 561.

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