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ORIGINAL PAPER

Identification of candidate signaling genes including regulators of chromosome condensation 1 protein family differentially expressed in the soybean–*Phytophthora sojae* interaction

Narayanan N. Narayanan · Sehiza Grosic · I. M. Tasma · David Grant · Randy Shoemaker · Madan K. Bhattacharyya

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Abstract Stem and root rot caused by the oomycete pathogen, *Phytophthora sojae,* is a serious soybean disease. Use of *Phytophthora* resistance genes (*Rps*) in soybean cultivars has been very effective in controlling this pathogen. Resistance encoded by *Rps* genes is manifested through activation of defense responses. In order to identify candidate signaling genes involved in the expression of *Phytophthora* resistance in soybean, a cDNA library was prepared from infected etiolated hypocotyl tissues of a *Phytophthora* resistant soybean cultivar harvested 2 and 4 h following *P. sojae* inoculation. *In silico* subtraction of 101,833 expressed sequence tags (ESTs) originating from unstressed cDNA libraries from 4,737 ESTs of this library resulted in identification of 204 genes that were absent in the unstressed libraries. Of the 204 identified genes, seven were *P. sojae* genes. Putative function of 91 of the 204 genes could not be assigned based on sequence comparison. Macroarray analyses of all 204 genes led to identification of 60 genes including 15 signaling-related soybean genes and three *P. sojae* genes, transcripts of which were induced twofold in *P. sojae*-infected tissues as compared to that in water controls. Eight soybean genes were down-regulated twofold following *P. sojae* infection as compared to water controls. Differential expression of a few selected genes was confirmed by conducting Northern and RT-PCR analyses. We have shown that two putative regulators of chromosome condensation 1 (RCC1) family proteins were down-regulated in the incompatible interaction. This observation suggested that the nucleocytoplasmic transport function for trafficking protein and non-coding RNA is suppressed during expression of race-specific *Phytophthora* resistance. Characterization of a cDNA library generated from tissues harvested almost immediately following *P. sojae*-infection of a resistant cultivar allowed us to identify many candidate signaling genes that are presumably involved in regulating the expression of defense-related pathways for expression of *Phytophthora* resistance in soybean.

N. N. Narayanan and M. K. Bhattacharya equally contributed to the article.

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N. N. Narayanan · S. Grosic · I. M. Tasma · M. K. Bhattacharyya (✉)
Department of Agronomy, Iowa State University, Ames IA, 50011, USA
e-mail: mbhattac@agron.iastate.edu; mbhattac@iastate.edu

I. M. Tasma
e-mail: tasma12@yahoo.com

D. Grant · R. Shoemaker
USDA-ARS-CICGR, Ames, IA 50011, USA

D. Grant
e-mail: David.Grant@ARS.USDA.GOV

R. Shoemaker
e-mail: Randy.Shoemaker@ARS.USDA.GOV

Present Address:
N. N. Narayanan
Department of Plant Cellular and Molecular Biology, The Ohio State University, Columbus, OH 43210, USA
e-mail: narayanan752000@yahoo.com

Introduction

Pathogenic attack in crop species can be very devastating. The Irish potato famine and the Southern corn leaf blight in United States, among many other examples, exemplify the

necessity of crop protection from serious pathogens. Growing disease resistant cultivars has been a major and successful method of protecting crop plants from various diseases. Disease resistant plants induce a series of active defense responses in order to defend themselves from invading pathogens. These responses include rapid production of reactive oxygen and nitrogen species, changes in ion fluxes across the plasma membrane, proteolysis and regulated expression of genes for synthesis of defense compounds (Dangl and Jones 2001; Hammond-Kosack and Jones 1996).

Soybean (*Glycine max* [L.] Merr.) is a major oil seed crop and is grown throughout much of the world. In addition to human consumption, soybean is primarily used for livestock feed and numerous industrial purposes including biodiesel production. Every year a significant portion of the soybean crop is lost from various diseases. Phytophthora root and stem rot caused by the oomycete pathogen, *Phytophthora sojae* is a serious disease. Monogenic race-specific resistance encoded by a series of *Rps* genes has provided soybean reasonable protection against the pathogen over the last four decades. To date, 15 reported *Rps* genes have been mapped to four regions of the soybean genome (Sandhu et al. 2004, 2005). Despite the availability of Phytophthora resistant cultivars carrying *Rps* genes, the disease has been a consistent limiting factor for soybean seed yield due to appearance of new races or isolates that can overcome the available *Rps*-encoded resistance mechanism of cultivars. For example, in the USA the estimated average annual soybean yield suppression just from this disease is valued at 309 million dollars (Wrather and Koenning 2006).

Apart from *Rps*-encoded gene- or race-specific resistance, quantitative, partial or field resistance provides low levels of broad-spectrum resistance against *P. sojae* isolates. Two putative quantitative trait loci (QTL) have been reported to confer partial resistance in soybean (Burnham et al. 2003). Recently, by investigating a set of recombinant inbred lines developed from a cross and additional unrelated genotypes varying for the extent of partial *Phytophthora* resistance, Thomas et al. (2007) suggested that preformed suberin plays a significant role in conferring partial resistance against this pathogen. It is currently unknown if there are additional factors that regulate the expression of partial *Phytophthora* resistance.

Phytophthora sojae is a hemibiotroph. At the initial stage following infection it rapidly establishes as a biotroph in susceptible cultivars. Inoculation of etiolated hypocotyls or roots with zoospore suspensions of *P. sojae* revealed that in the compatible interaction host cells associated with the penetrated hyphae remain viable and healthy for 3–4 h following inoculation, but rapidly die in the incompatible interaction (Enkerli et al. 1997; Ward et al. 1989). The extent of hyphal penetration by virulent and avirulent races is comparable at the initial stage of infection. About 15 h following infection, virulent races start to behave like a necrotrophic pathogen and cause host cell death and necrosis (Moy et al. 2004).

Avirulence (*Avr*) genes corresponding to most *Rps* genes involved in the *Rps* gene-mediated recognition process have been mapped and *Avr1b* has been cloned (Shan et al. 2004; Tyler 2002). *Avr1b-1*, one of the two genes isolated from the *Avr1b* locus, encodes an elicitor. When infiltrated into leaves, recombinant *Avr1b-1* induced strong necrosis in soybean lines carrying *Rps1*-b and weak necrosis in lines carrying *Rps1*-k suggesting that *Avr1b-1* recognized both *Rps1*-b and *Rps1*-k alleles (Shan et al. 2004). Recently, the C-terminal conserved W and Y motifs of *Avr1b* required for avirulence function have been identified (Dou et al. 2008).

The genome of *P. sojae* has been sequenced and a superfamily of 700 proteins with similarity to avirulence genes were identified (Tyler et al. 2006). Recently, 66 *P. sojae* genes that are transcriptionally activated shortly after infection of soybean leaves were identified using a subtractive hybridization approach (Chen et al. 2007a). Further characterization of these genes will advance our knowledge of the mechanisms used by the pathogen in causing the root and stem rot disease in soybean.

Following the recognition event, which is regulated by a corresponding pair of *Rps* and *Avr* genes, rapid induction of defense compounds such as glyceollin isomers occurs as a result of transcriptional activation of genes of the phenylpropanoid pathway such as *PAL* and *CHS* (Bhattacharyya and Ward 1986; Ebel and Grisebach 1988; Esnault et al. 1987). The phytoalexin, glyceollin accumulates rapidly in infected sites due to synthesis rather than the metabolism of this secondary metabolite of the phenylpropanoid pathway (Bhattacharyya and Ward 1987). RNA interference (RNAi) of genes encoding isoflavone synthase (IFS) or chalcone reductase (CHR) enzymes of the isoflavones and isoflavonoids biosynthetic pathway led to suppression in the accumulation of isoflavone 5-deoxyisoflavone and glyceollin and loss of race-specific *Phytophthora* resistance. Down-regulation of these two enzymes also resulted in loss of cell death caused by the elicitor preparation from cell walls of *P. sojae* (Subramanian et al. 2005; Graham et al. 2007).

In addition to antimicrobial compounds phytoalexins, induction of other defense-related proteins, particularly in resistant cultivars following infection with an avirulent *P. sojae* race, has been reported (Yi and Hwang 1997a, 1997b, 1998; Liu et al. 2001). These include a pathogenesis-related protein beta-1,3-glucanase (34 kDa) protein, a 36 kDa anionic peroxidase and a matrix metalloproteinase. This suggests that a number of defense strategies are activated following the recognition event encoded by *Rps* genes.

Regulatory mechanisms involved in the induction of active defenses during *Rps*-encoded race-specific *Phytophthora* resistance against invading *P. sojae* are poorly understood. The plant hormone ethylene plays a major role in *Rps*-mediated resistance. Through mutant analyses, it was shown that *Rps1*-k-specific resistance against *P. sojae* races 4 and 7 but not race 1 is significantly compromised in the ethylene insensitive mutant *etr1* (Hoffman et al. 1999). This study suggested the importance of ethylene in the expression of *Phytophthora* resistance mediated by an *Rps* gene located at the *Rps1*-k locus. Recently, the complex locus containing *Rps1*-k has been physically mapped and cloned (Bhattacharyya et al. 2005). Two functional coiled-coil nucleotide-binding leucine rich repeat (CC-NB-LRR) type genes were isolated from the *Rps1*-k locus (Gao et al. 2005). It is not yet known if soybean uses signaling pathways similar or distinct to the ones deciphered in the model plant, *Arabidopsis* (Hammond-Kosack and Parker 2003).

In this investigation, we applied a genomics approach in identifying possible signal transducing genes involved in the expression of *Rps1*-k-mediated *Phytophthora* resistance. In order to clone the candidate signal transducing genes, a cDNA library was constructed from the tissues of an incompatible interaction harvested 2 and 4 h following *P. sojae* infection of a cultivar carrying *Rps1*-k. Macroarray analyses of a selected set of cDNAs from this library allowed us to identify differentially expressed 18 candidate signal transducing genes including the ones that were subsequently shown to play important roles in the expression of disease resistance in other plant–pathogen interactions.

Materials and methods

P. sojae races and inoculation

Zoospores of *P. sojae* race 25 virulent to the soybean cultivar Williams 82, and races 1 and 4 that are avirulent to Williams 82 were used for inoculating etiolated hypocotyls. Sporangial development was induced by repeated flooding of 6-day-old mycelia with sterile distilled water as described earlier (Ward et al. 1979). Seeds of Williams 82 (*Rps1*-k) were germinated in Strong-lite vermiculite in a dark growth chamber according to Ward et al. (1979) for seven days. Twenty soybean hypocotyls were placed in glass trays and 4 drops of 10-µL zoospore suspensions containing approximately 10^5 zoospores/ml were placed on the hypocotyl surface (Ward et al. 1979). Sterile water was used as the control. Inoculated seedlings were incubated at 25°C in the dark until collection of tissues. Thin tissue layers just below the droplets of zoospores or water were excised and frozen immediately in liquid N_2.

RNA isolation

Phytophthora sojae-inoculated and water treated tissues were ground to fine powder in liquid N_2 and RNA was extracted with the RNeasy Plant Mini Kit of Qiagen (Valencia, CA, USA) according to the manufacturer's instructions.

cDNA library construction and sequencing of cDNA clones

To construct the cDNA library Williams 82 etiolated seedlings were grown for seven days in Strong-lite vermiculite in a growth chamber under dark conditions as stated earlier (Ward et al. 1979). Etiolated hypocotyls were inoculated with a zoospore suspension of *P. sojae* race 1 (10^5 spores/ml). Four 10 µl droplets of the zoospore suspension were placed on the upper 1/3rd section of the hypocotyls starting ~1 cm from the cotyledonary node. The cDNA library was constructed in the *Eco*RI–*Xho*I sites of the pBluescript II SK + vector using pooled poly(A^+) RNA samples prepared from *P. sojae* race 1-infected etiolated hypocotyl tissues excised 2 and 4 h following zoospores inoculation (Bhattacharyya 2001). Race 1 is avirulent to Williams 82. The incompatible interaction between Williams 82 and *P. sojae* race 1 was chosen for this study because we were interested in identifying candidate signaling genes essential for activation of defense genes in the resistant response. The library was transformed into electrocompetent DH10B host cells (Gibco BRL, Life Technologies, Rockville, MD, USA) through electroporation and termed Gm-c1084. Single colonies were picked into 384-well micro-titer plates for storage and sequencing. Sequencing was conducted at the Washington University School of Medicine, St. Louis.

Amplification of cDNA inserts

The cDNA inserts of 204 selected clones from the Gm-c1084 library were PCR amplified in 80-µL reaction volumes containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 5 µM each of M13 universal forward and reverse primers, 2 U of *Taq* DNA polymerase and 10 ng of plasmid DNA. After initial denaturation at 94°C for 2 min, the amplification was carried out for 40 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 3 min. The reactions were incubated for 10 min at 72°C for final extension.

Dot blotting of cDNAs

PCR amplified EST clones were denatured by boiling in 0.4 M NaOH and 10 mM EDTA for 10 min. Denatured DNA was neutralized by adding an equal volume of cold 2 M ammonium acetate, pH 7.0. Sixty ng PCR products of

each clone were blotted onto nylon membranes by using Bio-Dot® microfiltration apparatus (http://www.biorad.com/cmc_upload/Literature/12484/M1706542B.pdf). Membranes were washed with 2× SSC (1× SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), baked at 80°C for 30 min and stored at room temperature until use.

Dot blot hybridization

Etiolated hypocotyl tissues were harvested 1, 2, 4, 8 and 24 h following water treatment or infection with *P. sojae*. RNA samples were isolated from Williams 82 hypocotyl tissues infected with either *P. sojae* race 4 or race 25, or from the control plants treated with water droplets. First strand radiolabeled cDNA probes were prepared by reverse transcribing poly(A^+) RNAs of individual RNA samples in the presence of α-^{32}P dATP. Superscript II RNase H^- reverse transcriptase (Gibco BRL, Life Technologies, Rockville, MD, USA) was used to mediate the reverse transcription reaction. Radiolabeled cDNA molecules were dissociated from RNA molecules by hydrolyzing RNAs with 6 µL of 2.5 M NaOH and incubating the reaction at 65°C for 1 h. Probe samples were then neutralized by adding 12.5 µl of 2 M Tris–HCl (pH 7.5). Unincorporated nucleotides were removed from the radiolabeled cDNAs using a Sephadex G-50 column (Bio-Rad, Inc., Hercules, CA, USA). Prehybridization and hybridization of dot blots were carried out in 6× SSC buffer, 5× Denhardt's reagent (Sambrook et al. 1989). Purified radiolabeled probes were added individually to the hybridization mixtures. After 16–20 h of hybridization at 65°C, filters were washed with 2× SSC/0.1% SDS at 65°C for 45 min, then with 1× SSC/0.1% SDS at 65°C for 45 min. The filters were wrapped with Saran Wrap and exposed to PhosphorImager screens. Spot intensities were measured using a Molecular Dynamics PhosphorImager 445 SI (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). ImageQuaNT software (Molecular Dynamics, Inc., Sunnyvale, CA, USA) was applied in identification and quantification of hybridization signals and their analyses.

Dot blot and statistical analyses

Spot intensities were normalized by dividing each spot with the mean intensity of all 204 spots of an individual treatment. To determine the changes in transcript abundance of each of the 204 genes in infected tissues, the normalized data of individual genes in infected tissues were divided by the corresponding normalized transcript abundance of the gene in water-treated control tissues. A value of 0.5 represented a twofold decrease and 2 represented twofold increase in transcript abundances in infected tissues over that in control tissues. The dot blot analyses were conducted twice using RNA preparations from two independent experiments. Two technical replicates were carried out for each RNA preparations, and data of these four replications (2 independent replications × 2 technical replications) were utilized for statistical analysis.

Resistant or susceptible host responses were analyzed for five time points 1, 2, 4, 8 and 24 h following water droplet treatment or zoospores inoculation. The null hypothesis for the data analyses was that there were no differences in transcript levels of a gene among five data points in either resistant or susceptible host responses. Thus, $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$; where μ_1 is the mean ratio of transcripts of a gene at 1 h in either resistant or susceptible response obtained from four replications (2 independent replications × 2 technical replications). The ratio for a gene in a replication was obtained, as stated earlier, by dividing the transcript level of the gene at 1 h following infection with that of the gene following water treatment at 1 h in that replication. Mean μ_1 was obtained from transcript ratios of the gene for 1 h time point in four replications. Similarly, μ_2, μ_3, μ_4 and μ_5 are mean transcript ratios for the gene for time points 2, 4, 8 and 24 h, respectively. Data were analyzed for both resistant and susceptible host responses separately for each gene by conducting analyses of variance.

RNA-blot Hybridization

Fifteen micrograms of total RNA was separated by electrophoresis in a 1.2% (w/v) agarose-formaldehyde gel, and then transferred onto nylon membranes. Blots were hybridized to ^{32}P-labeled cDNA probes, washed and exposed to X-ray films according to Sambrook et al. (1989).

RT-PCR Analyses

Prior to cDNA synthesis RNA samples were treated with RNase-free DNase I according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was conducted using a Molony Murine Leukomia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI, USA). An RNase-free oligo dT (5′-TTTTTTTTTTTTTTTTT-3′) was used to prime cDNA synthesis. The cDNA synthesis was conducted as follows: two micrograms of total RNA was mixed with 0.5 µg oligo dT primer to a total of 18 µL, incubated at 70°C for 5 min, then cooled quickly on ice. A total of 25 µL reaction was used containing 1× M-MLV reaction buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 200 µM dNTPs, 25 units RNasin (Promega), 200 units M-MLV reverse transcriptase, and RNase-free water to a final volume of 25 µL. cDNA synthesis was conducted at 42°C for 1 h. The synthesized cDNA was then diluted to tenfold in sterile double

distilled water. The cDNA molecules were used as templates in PCR reactions. Primers (Integrated DNA Technologies; Coralville, Iowa) and number of cycles used for PCR amplification are presented in Table 3. The PCR program was as follows: 94°C for initial 2 min followed variable number of cycles (Table 3) of 30 s at 94°C for denaturation, 30 s at 60°C for annealing and 1 min at 72°C s for extension with a final extension of 5 min at 72°C.

Results

In silico subtraction identified candidate genes that are transcriptionally regulated following *Phytophthora sojae* infection

To identify the soybean genes induced or suppressed immediately following *P. sojae* infection, a cDNA library from etiolated hypocotyl tissues of the resistant cultivar Williams 82 (*Rps1*-k) infected with the avirulent *P. sojae* race 1 was generated. RNAs from tissues harvested 2 and 4 h following *P. sojae* infection were pooled and used to construct the cDNA library. 4,737 expressed sequence tags (ESTs) from this cDNA library (Gm-c1084) were generated and deposited in GenBank. *In silico* subtraction (E value $\leq 10^{-4}$) of 101,833 ESTs of various soybean cDNA libraries prepared from unstressed tissues (Supplemental Table 1) from these 4,737 ESTs resulted in identification of 204 genes from the Gm-c1084 library that were absent in the unstressed libraries (Supplemental Table 1).

Of the 204 genes, seven were identified as *P. sojae* genes based on sequence homology (http://genome.jgi-psf.org/cgi-bin/runAlignment?db=sojae1&advanced=1). The *P. sojae* transcripts represented only 0.15% of the total cDNA in this library, presumably, because infected tissues were harvested at a very early stage following infection. The 197 soybean genes were individually analyzed (on September 11-13, 2007) for (1) their possible similarities to genes with known functions in NCBI's GenBank databases using the BLASTX program and (2) identical soybean ESTs or cDNAs sequences using BLASTN program (Supplementary Table 2). Of the 197 soybean ESTs, 91 did not show any matches to genes with known or putative functions. Soybean genes were classified into six categories of genes. Among the 197 soybean genes 23% encode signaling-related, 5% defense-related, 20% metabolism-related proteins, 3% transporter-related proteins, 1% transposon-related proteins, and 45% unknown proteins (Fig. 1; Supplementary Table 2). Of the 197 soybean genes, 104 showed no nucleic acid similarities to any soybean ESTs suggesting that these 104 genes are unique to the Gm-c1084 library (Supplementary Table 1). The 93 cDNAs showing similarities to ESTs of Gm-c1084 library represent sequences submitted to the GenBank database since the initial *in silico* subtraction was carried out.

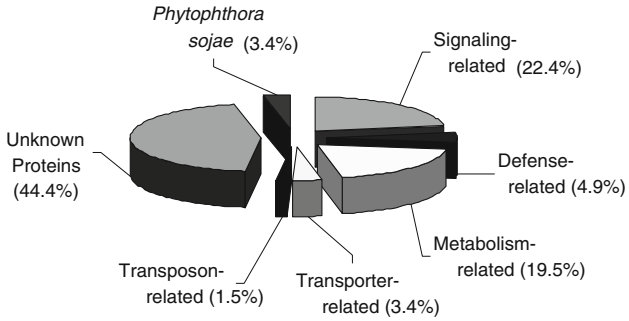

Fig. 1 Classification of 204 genes identified through *in silico* subtraction into seven putative classes

Macroarray analyses identified soybean genes showing altered transcript levels following *P. sojae* infection

It is very unlikely that changes in steady state transcript levels of all 204 ESTs of the Gm-c1084 cDNA library were induced following *P. sojae* infection. We therefore conducted macroarray or dot blot analyses for all 204 ESTs including seven *P. sojae* ESTs using radio-labeled cDNA probes prepared from total RNAs of *P. sojae*-infected or water control tissues (Fig. 2). Steady state transcript levels of 55 soybean genes and three *P. sojae* genes in infected tissues were more than twofold higher than that of the water controls with $P \leq 0.1$ (Table 1; Supplemental Table 3). Among the 55 infection-induced soybean genes, 13 were signaling-related, 2 defense-related, 10 metabolism-related, and 30

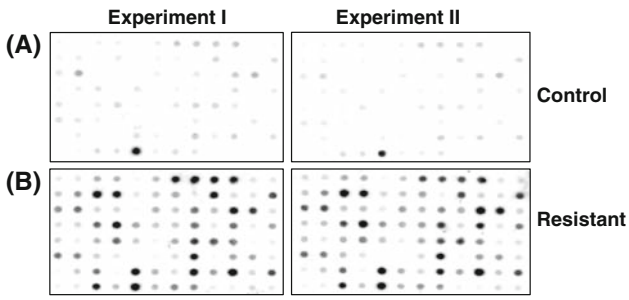

Fig. 2 Macroarray analyses of genes differentially expressed in the soybean-*Phytophthora sojae* interaction. Dot blot analyses were carried out for 204 ESTs using radiolabeled probes prepared from RNAs isolated from water control and infected tissues (see materials and methods). An example of a dot blot of 96 PCR amplified ESTs hybridized to ^{32}P-labeled cDNA probes prepared from RNA samples of **a** etiolated hypocotyls tissues harvested 24 h following treatment with water droplets and **b** the resistant hypocotyls tissues harvested 24 h following inoculation with *P.sojae* race 4 are shown here. The extent of variation between two biological replications (Experiments I and II) and transcript induction of some of the genes following *P. sojae* infection are shown (**b**)

Table 1 Twenty-seven soybean genes and three *Phytophthora sojae* genes that were induced in the soybean–*Phytophthora sojae* interaction

Accession no.	Annotation	Accession no.	*E*-value[a]	Response[b]	Post Inoculation (h)[c]					*P* value
					1	2	4	8	24	
Signaling-related										
BI972375	Ubiquitin-protein ligase	NP_190877	7.00E-24	R	0.97	1.01	1.77	1.44	2.20	0.03
				S	0.81	1.05	1.54	1.35	5.24	0.17
BQ081200	KN1-type homeobox protein	BAA76750	3.00E-11	R	0.73	1.03	1.14	1.49	1.59	0.02
				S	0.89	1.07	1.07	3.96	1.40	0.02
BI787769	Inositol polyphosphate 5-phosphatase	NP_565023	3.00E-36	R	1.01	1.15	1.53	1.53	1.99	0.11
				S	0.79	1.28	1.90	1.26	3.21	0.00
BQ080040	Receptor-like protein kinase homolog RK20-1	AAD21872	5.00E-07	R	1.13	1.53	1.66	1.26	2.54	0.10
				S	1.18	1.71	1.44	1.47	2.18	0.51
BI971693	Lectin protein kinase	ABA99273	3.00E-11	R	1.05	0.71	1.49	1.56	2.32	0.05
				S	0.84	0.67	1.96	1.18	2.39	0.03
BI787823	Auxin-responsive family protein	NP_199889	2.00E-14	R	2.03	1.35	1.18	1.30	2.84	0.28
				S	1.07	1.13	1.56	1.24	2.22	0.09
BQ079610	C3HC4-type RING finger family protein	NP_566498	4.00E-10	R	1.07	0.96	0.69	1.87	1.03	0.03
				S	1.24	1.08	1.06	2.23	1.02	0.05
BM954420	RabGAP/TBC domain-containing protein	NP_566323	5.00E-71	R	0.73	0.97	1.73	0.86	2.01	0.04
				S	1.17	1.19	1.58	0.94	1.71	0.27
BQ079861	bZIP transcription factor bZIP107	ABI34692	2.00E-84	R	0.85	1.89	1.93	0.98	1.05	0.10
				S	1.28	1.08	2.44	1.47	0.99	0.11
BI972052	RING-H2 finger protein ATL3	AAG51805	2.00E-13	R	0.77	1.81	1.15	1.66	2.65	0.05
				S	1.12	1.29	1.50	1.10	1.13	0.92
BQ080930	Leucine-rich repeat transmembrane protein kinase	NP_179911	4.00E-29	R	1.44	1.00	1.02	1.10	2.20	0.23
				S	1.01	0.99	2.09	1.27	1.59	0.03
BQ080590	Zinc finger (GATA type) family protein	NP_199525	2.00E-26	R	1.40	1.10	2.68	1.09	1.08	0.04
				S	1.22	1.09	3.04	1.19	0.93	0.04
BQ079557	TIR-NBS-LRR type disease resistance protein	ABF81464	2.00E-14	R	0.86	1.05	1.23	1.57	1.61	0.25
				S	1.09	1.02	1.34	3.01	1.61	0.05
BI972402	Wall-associated kinase	AAG50588	2.00E-14	R	1.52	0.98	1.36	1.73	1.99	0.49
				S	1.58	1.05	1.34	1.17	5.19	0.22
BI972048	Ethylene response factor ERF1	AAM63284	1.00E-16	R	1.14	1.14	1.81	1.34	2.13	0.34
				S	1.05	1.07	1.94	0.78	1.97	0.15
Defense-related										
BI972340	Cytochrome P450	CAB43505	5.00E-59	R	1.07	1.09	1.14	1.74	1.82	0.05
				S	1.64	2.84	1.15	1.67	2.31	0.78
BQ080184	PLP6 (Patatin-like protein 6)	NP_181455	2.00E-51	R	1.08	1.12	1.00	0.90	0.90	0.87
				S	1.33	1.40	2.41	0.85	1.33	0.07
Metabolism-related										
BI972374	UDP-glycosyltransferase	NP_173820	2.00E-19	R	1.33	0.88	1.78	1.45	2.64	0.06
				S	1.13	1.01	1.56	1.76	2.45	0.12
BI892849	Putative enolase	NP_177543	1.00E-82	R	1.51	1.31	1.81	1.12	2.17	0.20
				S	1.00	1.58	1.59	0.94	3.22	0.00
BQ079462	Esterase/lipase/thioesterase	NM_100704	3.00E-12	R	0.67	0.86	0.98	2.09	1.03	0.17
				S	0.97	1.17	0.97	3.08	1.02	0.02
BQ081436	RNA recognition motif containing protein	NM_100793	3.00E-54	R	1.12	1.15	1.04	1.29	1.48	0.78
				S	1.23	1.24	1.02	2.11	1.46	0.05

Table 1 continued

Accession no.	Annotation	Accession no.	*E*-value[a]	Response[b]	Post Inoculation (h)[c]					*P* value
					1	2	4	8	24	
BI893692	Glycosyl transferase family 1 protein	NP_001043742	4.00E-31	R	0.96	1.53	1.24	1.19	1.81	0.02
				S	0.92	1.40	1.28	1.55	1.98	0.24
BI971616	Glucosyltransferase-13	BAB86931	7.00E-48	R	1.09	0.95	1.43	1.27	1.92	0.12
				S	1.08	0.71	1.55	0.81	2.41	0.05
BQ081232	Arginine N-methyltransferase family	AAU05537	9.00E-06	R	1.07	0.85	1.36	1.31	1.32	0.49
				S	0.90	0.94	2.11	1.86	1.18	0.07
BI893851	Laccase 2	NP_180477	5.00E-07	R	1.31	0.77	1.28	1.95	1.06	0.34
				S	1.30	0.87	1.10	0.87	1.31	0.08
BQ079683	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	ABD32606	2.00E-40	R	0.85	0.88	0.93	1.37	1.44	0.18
				S	0.79	0.89	0.86	1.97	1.68	0.04
BQ081048	ATEXO70D1 (exocyst subunit EXO70 family protein D1)	NP_177391	2.00E-52	R	1.06	0.86	1.31	2.54	1.39	0.45
				S	1.28	0.90	0.93	0.77	2.11	0.05
Phytophthora sojae proteins										
BQ080022	Putative cold-induced protein (cip1 gene)	AJ272394	2.00E-41	R	1.42	2.05	2.57	1.09	19.02	0.03
				S	1.64	2.49	1.50	2.92	5.61	0.27
BI972338	Putative hydroxyproline-rich glycoprotein 1	BAD22517	2.00E-23	R	1.25	1.28	1.21	1.36	5.26	0.00
				S	1.23	1.30	1.58	2.31	1.69	0.35
BI971940	No match	NA[d]	NA	R	2.07	1.13	1.41	1.30	6.48	0.04
				S	1.07	1.14	1.74	2.60	2.63	0.07

Genes showing twofold induction in transcript levels as compared to that in water controls in at least one time point of either or both resistant and susceptible host responses are included. *P* value (probability values of F ratios) of all genes except BI972402 and BI972048 showed F ratios with $P \leq 0.1$. Thus, only 25 of 27 genes presented here showed two-fold induction and F ratios with p values ≤ 0.1

[a] E-value, BLASTX program was applied to identify the most similar sequences

[b] R, resistant host response; S, susceptible host response

[c] Post Inoculation, the details of the data can be obtained from "Dot blot and statistical analyses" of the materials and methods section

[d] *NA* not applicable because E-values were bigger than 1.00E-4

genes with unknown functions (Table 1; Supplementary Table 3). The number of *P. sojae* infection-induced defense-related genes was rather low because of the design of the experiment. The library was constructed from *P. sojae* infected tissues almost immediately following inoculation (2 and 4 h following inoculation) to avoid isolating this category of genes. Furthermore, transcripts of the phenylpropanoid biosynthetic pathway leading to glyceollin or lignin synthesis are also abundant in other unstressed tissues and were therefore subtracted from our library. For example, induction of genes encoding Phenylalanine Ammonia-Lyase, Chalcone Synthase (CHS) 7, CHS8, and Isoflavone Synthase2 for isoflavonoid synthesis in seeds has been reported (Dhaubhadel et al. 2007). High similarity of these genes with the isoforms induced in *P. sojae*-infected tissues presumably resulted in their subtraction and non detection in this study.

Of the 197 soybean genes, eight genes comprised of three signaling-related, two metabolism-related genes and three genes with unknown function showed over twofold reduction in transcript levels following *P. sojae* infection as compared to that in water control tissues with $P \leq 0.1$ (Table 2). We did not expect to identify these eight genes, of whose transcript accumulation was reduced following infection, because *in silico* subtraction should have allowed us only to isolate transcriptionally activated genes. This unusual observation could be possible because of the following reason. The *in silico* subtraction was conducted using only 101,833 ESTs of various soybean cDNA libraries (Supplemental Table 1). It is very unlikely that all transcripts of etiolated hypocotyls are present in this sample of 101,833 ESTs. When we were harvesting *P. sojae*-infected tissues for constructing the Gm-c1084 library we could not avoid co-harvesting healthy tissues. Thus, Gm-c1084 library contains ESTs originating from healthy tissues that were absent among the 101,833 ESTs of various soybean cDNA libraries. Presumably eight of the genes representing

Table 2 Nine soybean genes down-regulated following *Phytophthora sojae* infection

Gene ID	Annotation	Accession No.	*E*-value[a]	Response[b]	Post Inoculation (h)[c]					*P* value
					1	2	4	8	24	
Signaling-related										
BI971650	Putative protein kinase	AAW22874	5.00E-25	R	0.79	1.20	0.53	0.59	0.24	0.01
				S	0.85	1.13	0.53	0.50	0.17	0.01
BQ081031	Regulator of chromosome condensation (RCC1) family protein	NP_566512	1.00E-43	R	1.53	0.77	0.37	0.58	0.52	0.01
				S	1.48	0.70	0.58	1.08	0.41	0.09
BQ080005	Regulator of chromosome condensation (RCC1) family protein	NP_680156	6.00E-39	R	1.10	1.14	1.07	0.72	0.69	0.14
				S	0.98	1.04	0.84	0.64	0.50	0.03
Metabolism-related										
BQ081616	DnaJ domain containing protein	EAY79152	7.00E-58	R	0.68	1.02	0.50	0.58	0.60	0.13
				S	1.00	0.87	0.44	0.64	0.36	0.02
BI971772	Structural maintenance of chromosomes 1 protein	AAS68515	1.00E-43	R	0.82	0.94	0.54	0.58	0.24	0.26
				S	1.05	0.85	0.45	0.43	0.20	0.03
BQ081450	Proteinase inhibitor I9, subtilisin subtilisin-like protease	ABD33266	1.00E-65	R	1.15	1.15	0.77	0.63	0.61	0.13
				S	1.25	0.89	0.87	0.92	0.63	0.25
Unknown proteins										
BQ081322	No match	NA[d]	NA	R	0.99	1.20	1.22	0.89	0.81	0.00
				S	1.08	1.08	1.00	0.49	0.63	0.33
BM954314	No match	NA	NA	R	0.87	1.01	0.59	0.59	0.32	0.16
				S	1.06	1.62	0.43	0.31	0.39	0.00
BI892971	No match	NA	NA	R	1.34	0.84	0.39	0.52	0.33	0.04
				S	0.97	0.57	0.49	0.30	0.36	0.00

Of the nine genes, all but BQ081450 showed two-fold reduction in transcript levels as compared to that in water controls in at least one time point of either or both resistant and susceptible host responses. Thus, only eight of the nine genes presented here showed two-fold reduction in transcript levels

[a] E-value, BLASTX program was applied to identify the most similar sequences

[b] R, resistant host response; S, susceptible host response

[c] Post Inoculation, the details of the data can be obtained from "Dot blot and statistical analyses" of the materials and methods section

[d] *NA* not applicable because E-values were bigger than 1.00E

these healthy-tissue-specific ESTs were down-regulated following *P. sojae* infection.

Expression analyses of selected genes that are differentially regulated following *P. sojae* infection and identification of a gene encoding putative regulator of chromosome condensation protein 1 family

We applied both Northern and RT-PCR analyses to determine the expression patterns of seven selected putative soybean signaling genes, one defense-related and two *P. sojae* genes following infection. Northern blot analyses were conducted for five candidate soybean signaling genes that are induced following pathogenic infections. Results of Northern analyses are presented in Fig. 3. Transcript levels of most genes were induced 4 h after *P. sojae* inoculation. Semi-quantitative RT-PCR analyses was conducted for one candidate signaling gene that was induced following infection, two signaling and one defense-related genes that were suppressed following infection and two *P. sojae* genes induced in infected tissues (Fig. 4; Table 2).

Expression of *GmERF1* (BI972048) was induced following *P. sojae* infection. By 8 h following infection, high levels of *GmERF1* transcripts were detected in both susceptible and resistant responses (Fig. 3). *GmERF1* is most likely a homolog of Arabidopsis *ERF1*, a transcription factor regulated by the phytohormone ethylene and is involved in inducing plant responses to pathogen attack (Gutterson and Reuber 2004). Constitutive overexpression of this gene has been shown to confer resistance against several necrotrophic fungi in Arabidopsis (Berrocal-Lobo et al. 2002). Overexpression of a wheat homolog of the gene in tobacco induced several pathogenesis-related genes and resistance against a bacterial pathogen (Park et al. 2001).

Table 3 Primers and number of PCR cycles used in RT-PCR analyses

Gene ID	Forward Primer	Reverse Primer	Number of Cycles
BI972052	CAGAGTCTGCACAAACTTTACAAGCATTATG	GGCATGTGGTATGATTCTTCAACCAAGCAC	28
BQ080005	CCCCAACAACGGAACAACCTCCTTCTTC	CACCGAGTAGTGTTTACTTTCTGATACAC	32
BQ081031	CCCGACCAATTTTCAGGCCACGCCTTG	CCGCAGCAATATGAACAACAGAGCTAAG	32
BQ081450	GGTTGTCTTCCTGCTGATAGTACTCT	GGCCAGACGCCGGTGTCGAGGAGTCCA	30
BI972338	GCGGGCGGCGGCTACGGCCTTCTTGAC	CGGACGCCGGCAAGATCGCCACGATCATC	34
BQ080022	GCCCCGCGTCCGGCCGCTGGTCTGCTG	CGGCCGAGTCGCCGTTCTTGGGCTTGTTG	34
V00450	CCCCTCAACCCAAAGGTCAACAG	GGAATCTCTCTGCCCCAATTGTG	30

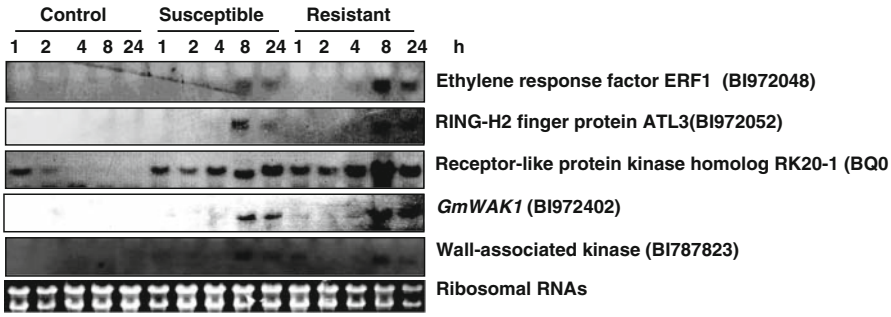

Fig. 3 Northern blots of soybean genes that were induced following *Phytophthora sojae* infection. Resistant and susceptible etiolated hypocotyls tissues were harvested at various time points following inoculation Williams 82 (*Rps1*-k) with *P. sojae* avirulent race 4 and virulent race 25, respectively. In control sterile water droplets were used in place of zoospores suspensions to inoculate the hypocotyls. Accession numbers of individual candidate soybean genes are shown in parenthesis

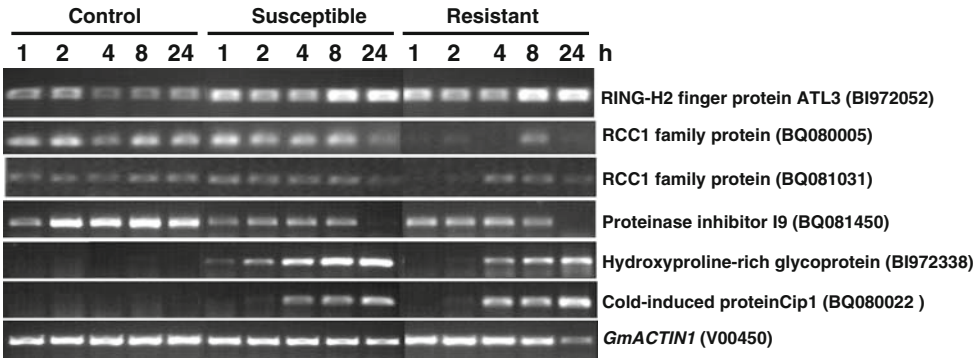

Fig. 4 RT-PCR analyses of genes that were differentially expressed in the soybean–*Phytophthora sojae* interaction. Resistant and susceptible etiolated hypocotyls tissues were harvested at various time points following inoculation Williams 82 (*Rps1*-k) with *P. sojae* avirulent race 4 and virulent race 25, respectively. In control sterile water droplets were used in place of zoospores suspensions to inoculate the hypocotyls. Accession numbers of individual candidate soybean genes are shown in parenthesis

We have also demonstrated that in *P. sojae*-infected tissues, transcripts of *GmRING-H2* (BI972052), a gene encoding a C3HC4-type RING-finger protein that binds two atoms of zinc, are induced (Figs. 3, 4). The protein showed high similarities to the tobacco ACRE132 protein induced by the interaction between Avr9 and Cf-9 proteins in tobacco cell suspensions (Durrant et al. 2000). Functional role of this putative transcription factor in disease resistance is yet to be established.

A putative receptor-like protein kinase, an *RLK3* homolog, has been shown to be induced in pathogen-infected tissues (Czernic et al. 1999; Lange et al. 1999). Overexpression of cysteine-rich receptor-like kinase gene *CRK13* has recently been shown to regulate the expression of several pathogenesis-related proteins and accumulation of salycilic acid in Arabidopsis and to enhance resistance against a bacterial pathogen (Acharya et al. 2007). A homolog of *CRK13* gene, *GmCRK13* (BQ080040) was rapidly

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HsRCC1-b        MSPKRIAKRRSPPADAIPKSKKVKDTRAAASRRVPGARSCQVSHRSHSTEPG--LVLTLG 58
HsRCC1-c        MSPKRIAKRRSPPADAIPKSKKVK-----------------VSHRSHSTEPG--LVLTLG 41
GmRCC1-1        -----MLRRSFSKLHLSLRAPGLG-----------------FPTRSFSRDAGKRFAALWG 38
                     : :*  .  .   ::  :                  .. **.* :.*  :.   *

HsRCC1-b        QGDVGQLGLGENVMERKKPALVSIPEDVVQAEAGG-MHTVCLSKSGQVYSFGCNDEGALG 117
HsRCC1-c        QGDVGQLGLGENVMERKKPALVSIPEDVVQAEAGG-MHTVCLSKSGQVYSFGCNDEGALG 100
GmRCC1-1        NGDYGRLGLGNLDSQWKPVVCPAFRNKTLNAIACGGAHTLFLTEDGCVYATGLNDFGQLG 98
                :** *:****:   : *  .  :: :..::* * *  **: *::.* **: * ** * **

HsRCC1-b        RDTSVEGSEMVPGKVELQEKVVQ------------------ 140
HsRCC1-c        RDTSVEGSEMVPGKVELQEKVVQVSAGDSHTAALTDDGRV- 140
GmRCC1-1        VSESKHYSVEPLCVFGEEKKVVQISAGYNHSCAITVDGELY 139
                 . * . *      .  ::****
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Fig. 5 Identification of a putative soybean orthologue of human regulators of chromosome condensation protein 1 family. Three human RCC1 proteins, HsRCC1-a, -b, and –c together with three Arabidopsis RCC1-like sequences and two GmRCC1 sequences were initially compared using ClustalW program. (http://www.ebi.ac.uk/Tools/clustalw/index.html). From that initial analysis of HsRCC1-b, HsRCC1-c and GmRCC1-1 showing most similarity to GmRCC1 sequences were selected to conduct the final ClustalW analysis and data are presented here. RCC1 domain is underlined. At the N-terminal region of RCC1 domain, a glycine-rich motif (*boxed*) is conserved between soybean and human RCC1 proteins

induced following *P. sojae* infection (Fig. 3). As compared to water controls, rapid induction of this gene was recorded for both resistant and susceptible responses. However, in the resistant response the level of *GmCRK13* expression was higher than that in the susceptible response (Fig. 3). Although following *P. sojae* infection the induction of *GmCRK13* was observed in both resistant and susceptible responses, its higher transcript levels in the resistant response as compared to that in the susceptible response at 8 h following infection may contribute towards expression of *Phytophthora* resistance.

It has been recently demonstrated that the defense signal molecule salicylic acid (SA) suppresses auxin-related genes to inhibit auxin responses and express disease resistance (Wang et al. 2007). Contrary to this, pathogens are considered to enhance the accumulation of auxin to promote disease development in Arabidopsis (Chen et al. 2007b). Here we have shown that an auxin-responsive family protein gene, *GmARG7* (BI787823) was induced following *P. sojae* infection (Fig. 3). Whether induction of *GmARG7* is involved in the expression of *Phytophthora* resistance or development of basic compatibility will require further studies.

Steady state transcript levels of only a few soybean genes were down-regulated following *P. sojae* infection (Table 2). Subtilisin-like serine proteases are considered to be pathogenesis-related proteins (P69B) (Tornero et al. 1997). It has been shown that a Kazal-like extracellular serine protease inhibitor produced by the oomycete pathogen *P. infestans* inhibits the tomato subtilisin-like P69B serine protease (Tian et al. 2004). Significant similarities of a soybean protein (BQ081450) with the tomato subtilisin-like serine proteases P69B was observed (BLASTP, E = 1e-16) and therefore it could be a soybean homolog (*GmP69B*) of tomato P69B protein. Transcript levels of *GmP69B* were reduced following *P. sojae* infection. By 24 h following infection, no detectible levels of *GmP69B* transcripts were recorded in both resistant and susceptible responses (Fig. 4). Down-regulation of this pathogenesis-related gene may be an additional mechanism used by *P. sojae* to overcome the basic resistance mechanism conferred by this protein.

Of the genes suppressed following *P. sojae* infection, one soybean gene, *GmRCC1-1* (BQ080005) showed high identity to human regulators of chromosome condensation 1 (RCC1) proteins (Table 4). We identified two forms of this gene from soybean, *GmRCC1-1 and GmRCC1-2* (BQ081031). Only GmRCC1-1 showed high similarity (36% identity) to the human RCC1 proteins (Table 4). Comparison of GmRCC1-1 with human RCC1 proteins revealed conserved unknown motifs located at the N-terminal region of RCC1 domain (Fig. 5). *GmRCC1-1* is down-regulated in the resistant host response (Fig. 4). Both GmRCC1 sequences were used to search for similar Arabidopsis sequences in GenBank using the BLASTX program. Three distinct RCC1-like proteins, AtRCC1-1, AtRCC1-2 and AtRCC1-3 were identified. AtRCC1-2 showed high identity to GmRCC1-1 (Table 4).

Transcripts of two *P. sojae* genes, *PsCIP1* (BQ080022) and *PsHPG1* (BI972338) with high sequence identity to cold-induced protein 1 and hydroxyproline-rich glycoprotein 1, respectively were shown to accumulate following infection. Higher levels of *PsCIP1* transcripts in the resistant response as compared to that in susceptible response suggested that stress-related gene *PsCIP1* is induced in the incompatible interaction. Transcripts of *PsHPG1* but not *PsCIP1* were detected as early as 1 h following *P. sojae* infection in the susceptible response.

Discussion

Rapid induction of phytoalexins plays a critical role in restricting spread of the pathogen in infected tissues of the

Table 4 GmRCC1-1 showed high identities to the human regulators of chromosome condensation protein 1 family

	GmRCC1-2	AtRCC1-1[a] (%)	AtRCC1-2 (%)	AtRCC1-3 (%)	HsRCC1-a (%)	HsRCC1-b (%)	HsRCC1-c (%)
GmRCC1-1	NA[b]	31	65	30	36	36	36
GmRCC1-2		73	32	37	27	27	27
AtRCC1-1			29	33	25	25	25
AtRCC1-2				28	27	27	27
AtRCC1-3					27	27	27
HsRCC1-a						96	96
HsRCC1-b							96

[a] Accession numbers for AtRCC1 proteins are: AtRCC1-1, NP_566512; AtRCC1-2, NP_680156; AtRCC1-3, NP_566789

[b] *NA* Not applicable because no overlapping region for comparison was found

resistant host response or incompatible soybean–*P. sojae* interaction. Through silencing of phytoalexins biosynthetic genes by RNAi it has been shown that phytoalexins such as glyceiollin are essential for expression of *Phytophthora* resistance in soybean (Graham et al. 2007; Subramanian et al. 2005). Transcripts of genes encoding enzymes involved in phytoalexin biosynthesis are rapidly induced following infection. This suggests that one of the regulatory mechanisms for phytoalexin biosynthesis is controlled at the transcriptional level. However, it is not known how the transcriptional activation of these phytoalexin biosynthetic genes or other defense-related genes is initiated following *P. sojae* infection. Presumably, several signaling pathways are induced, as in other plant–pathogen interactions, to regulate the expression of defense-related genes in the soybean–*P. sojae* interaction (Hammond-Kosack and Parker 2003).

By analyzing transcripts of *P. sojae*-infected tissues almost immediately (2 and 4 h) after inoculation we were able to isolate several signaling genes that are induced in *P. sojae*-infected tissues (Table 1). Expression studies confirmed *P. sojae* infection-mediated differential expression of several candidate signaling-related and one defense-related gene that may play critical role in the expression of *Phytophthora* resistance in soybean. Functional analyses of these candidate genes will be required to establish their roles in the expression of *Phytophthora* resistance in soybean.

In this study we used two *P. sojae* races; race 1 was used for generating the cDNA library and race 4 to characterize the putative cDNAs that were identified through *in silico* subtraction analysis. Previously it was observed that Phytophthora resistance encoded by *Rps1*-k against race 4 and race 7 but not race 1 was compromised in the ethylene insensitive mutant *etr1* (Hoffman et al. 1999). It is therefore possible that some of the candidate signaling-related genes identified through *in silico* subtraction step did not show induction because they were not regulated by the pathway required for resistance against race 4 or race 7. Further characterization of these genes in race 1-infected hypocotyls will be required to test this possibility.

We report two genes encoding candidate regulators of chromosome condensation 1 (RCC1) family protein that were down-regulated specifically in the incompatible interaction following *P. sojae* infection (Table 2). RCC1 homologs have not been isolated in plants. The protein is highly diverged from mammalian RCC1s thus making sequence-based identification of the plant homologs unsuccessful (Meier 2007). Although several Arabidopsis proteins were putatively annotated as RCC1 proteins, clear RCC1 domains were not evident among these proteins. Sequence identity between Arabidopsis RCC1 and human RCC1 isoform C proteins ranges from 25 to 27%. Of the two soybean RCC1 proteins, GmRCC1-1 (BQ080005) showed 37% sequence identity to all three human RCC1 isoforms a, b and c. At the N-termini of RCC1 proteins, we detected an unknown glycine-rich motif (GNGDYGRLGLG), which is highly conserved between soybean GmRCC1-1 and human RCC1 proteins (Fig. 5). The glycine-rich motif is very similar to G-loop (GEGTYG) motif that contains T and Y residues, phosphorylation of which abolishes kinase activity (Hanks and Quinn 1991). High sequence conservation between this soybean and human sequences suggested that GmRCC1-1 could be orthologous to human RCC1 proteins. Three candidate Arabidopsis *RCC1* genes, *AtRCC1-1, -2* and *-3* showing high sequence identity to GmRCC1-1 and GmRCC1-2 (BQ081031) were identified (Table 4). GmRCC1-1 showed high identity to AtRCC1-2 (AT5G08710) and GmRCC1-2 showed high identity to AtRCC1-1; and therefore, the two Arabidopsis proteins could be orthologous to soybean RCC1 proteins reported here.

RCC1 plays a major role in nucleocytoplasmic transport, mitosis and nuclear-envelop assembly in mammals (Hetzer et al. 2002). Nucleocytoplasmic trafficking of protein and non-coding RNA molecules is carried out by Ran protein mediated pathway, whereas Ran-independent pathway is involved in exporting mRNA molecules (Cullen 2003). The cycle of nucleocytoplasmic trafficking mediated by Ran begins in the cytoplasm following binding of

importin proteins, importins α and β to the cargo in presence of RanGDP. The cargo is imported into nucleus through the nuclear pores. RanGDP is imported into nucleus by NTF2 protein. In nucleus RanGDP is phosphorylated to RanGTP by RCC1. In presence of RanGTP, importins dissociate from the cargo. Importin α binds to the nuclear export receptor, CAS in the nucleus and forms a complex with RanGTP. Importin β interacts with RanGTP. Both importins are then exported from the nucleus to the cytoplasm where they dissociate following conversion of RanGTP into RanGDP. In cytoplasm, RanGTP is converted to RanGDP through its intrinsic GTPase activity, activated and catalyzed by Ran GTPase-activating protein, RanGAP1 and Ran binding protein, RanBP1 (Fassati 2006; Görlich and Kutay 1999). Recently, the coiled-coil nucleotide-binding leucine rich repeat (CC-NB-LRR)-type resistance (R) protein, Rx has been shown to interact with the RanGAP protein, NbRanGAP2 (Tameling and Baulcombe, 2007). Silencing of NbRanGAP2 resulted in partial loss of Rx-mediated resistance against the potato virus X.

Recent work suggested that nucleocytoplasmic trafficking plays an essential role in the expression of race- or gene-specific disease resistance. Members of (1) CC-NB-LRR and (2) TIR-(domain with similarity to domains found in Toll and mammalian interleukin-1 receptors) NB-LRR type R proteins have been shown to partition between cytoplasm and nucleus (Shen and Schulze-Lefert 2007). Through fusion of nuclear export signal (NES) to one member of each CC-NB-LRR and TIR-NB-LRR classes of R proteins it was shown that nuclear localization of disease resistance (R) proteins is essential for expression of their resistance function (Burch-Smith et al. 2007; Shen et al. 2003). Furthermore, components of nuclear pore complex for nucleocytoplasmic trafficking, MOS3 and MOS6, are shown to be essential for expression of innate immunity (Zhang and Li 2005; Palma et al. 2005). MOS6 is the importin α homolog of *Arabidopsis*.

RT-PCR analyses showed that transcript levels of both *GmRCC1-1* (BQ080005) and *GmRCC1-2* (BQ081031) were strongly reduced in the resistant host response as compared to that in water controls or the susceptible host response (Fig. 4). If GmRCC1-1 and GmRCC1-2 are orthologous RCC1 proteins in soybean, nucleocytoplasmic trafficking is most likely suppressed in the *Rps1*-k-encoded resistant response immediately following *P. sojae* infection. In the susceptible response against a virulent *P. sojae* race, such a response is probably absent (Fig. 4). Recently, it has been demonstrated that an orthologue of RCC1 is required for virulence of the protozoan parasite *Toxoplasma gondii* in mice (Frankel et al. 2007).

Of the 197 soybean genes identified through in situ subtraction, 91 showed no matches to any genes with known function. Only 11 of these genes showed significant similarities to genes of other organisms with unknown function (Supplementary Table 2). Of the rest 80 ESTs showing no matches to the genes with known or putative functions, 60 ESTs contain high quality sequences. Failure to observe matches of these 60 high quality ESTs sequences with any gene sequences could be explained by any one or more of these reasons: (1) these genes are highly diverse from genes of other plant species and as a result identity can not be established from sequence comparison; (2) soybean-specific genes; and (3) some of the sequences are from 5′- or 3′-untranslated regions of genes and they do not contain open reading frames for identification. Macroarray analyses showed that of the 91 genes with unknown functions, 40 were induced twofold and three genes were down-regulated twofold at $P \leq 0.1$ in infected tissues as compared to that in water controls (Supplemental Table 3 and Table 2). Therefore, a significant proportion of these genes could play important roles in the expression of disease resistance in soybean and other crop species. RNA interference of these and other *P. sojae* infection-induced genes should facilitate confirming the roles of these genes in the expression of *Phytophthora* resistance in soybean. Overexpression studies for the down-regulated genes using a promoter unaffected by pathogenic infection are expected to reveal useful information for understanding the roles of these genes in the establishment of the root and stem rot diseases or expression of *Phytophthora* resistance in soybean.

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