Identification of *Phytophthora sojae* genes upregulated during the early stage of soybean infection

Xiaoren Chen, Gui Shen, Yonglin Wang, Xiaobo Zheng & Yuanchao Wang

Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China

Correspondence: Y.C. Wang, Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China. Tel.: +86 25 84399071; fax: +86 25 84395325; e-mail: wangyc@njau.edu.cn

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Abstract

To explore the molecular mechanisms that are involved in the pathogenicity of *Phytophthora sojae*, a suppression subtractive hybridization method was developed to screen for *P. sojae* genes that are differentially expressed in the early stage of *Glycine max* (soybean) infection. A cDNA library enriched for upregulated parasite genes was generated; of the 73 genes that were found to be upregulated, 66 are significantly similar to sequences in the *P. sojae* genome, and seven have no significant similarities in the databases examined. These sequences are predicted to encode proteins involved in protein biosynthesis, energy production, cell signaling, cell-wall biogenesis, and transcription regulation. Virtual Northern assay of random selected seven genes revealed that they are all highly expressed in plant infection. Reverse transcriptase polymerase chain reaction was used to further examine the expression pattern of these genes during soybean infection. These results provide an important insight into the genes expressed during *P. sojae* infection of soybean, which may be involved in oomycete pathogenesis.

Introduction

Phytophthora root rot caused by *Phytophthora sojae* Kaufmann and Gerdemann, currently the most devastating disease of soybean (*Glycine max* [L.] Merr), causes severe losses in the soybean harvest worldwide (Erwin & Ribeiro, 1996; Tyler *et al.*, 2006). *Phytophthora sojae* can infect and kill soybean plants throughout much of the growing season. As a hemibiotrophic pathogen, *P. sojae* colonizes the surfaces of host plants, followed by formation of the infection-related structure and penetration into host tissues (Enkerli *et al.*, 1997). In incompatible interactions, soybean cultivars carrying a resistance gene (*Rps*) that corresponds to an attacking *P. sojae* strain rapidly induce a hypersensitive response within hours of pathogen infection. In susceptible interactions, *P. sojae* is able to colonize host cells in an initial biotrophic phase of growth that lasts for *c.* 12 h (Ward, 1990). After this time, the pathogen enters a necrotrophic growth mode, spreading quickly throughout host tissues, causing large, water-soaked, necrotic lesions, and leaving dead host cells.

To develop improved methods for the control of *Phytophthora* infection, it is essential to understand the mechanisms by which *P. sojae* breaks down the plant's defenses. Many of the crucial interactions between the pathogen and the host occur at the earliest time points, including prepenetration. Prior to soybean infection, *P. sojae* recognizes host signals and forms infection-related structures; it must also confront preformed or induced toxic secondary metabolites in the host plants. In addition, the fungus must adapt to nutrient-deficient conditions in the early stage of host infection. The pathogen regulates the expression of its own genes to deal with the host and environmental stress, and to avoid recognition (e.g. Ham *et al.*, 1997; Connolly *et al.*, 2005).

The different phases of *P. sojae* infection require the upregulation and downregulation of numerous genes, which may be involved in the pathogenesis. Moy *et al.* (2004) used microarray analysis to identify genes whose expression changes throughout the interaction between *P. sojae* and soybean. Most of these changes in expression occur during the later stages of infection, and the role of these genes in pathogenicity has not yet been determined. To learn more about gene expression patterns during susceptible interactions between hosts and pathogens, suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996) we used to identify *P. sojae* genes that are differentially expressed in the early stages of soybean infection. SSH has been useful in a number of different systems to identify novel or specific genes (e.g. Thara *et al.*, 2003; Cramer & Lawrence, 2004; Bulman *et al.*, 2006).

In the screen described here, an *in vitro* experimental system was developed for isolating *in-planta*-induced genes

of *P. sojae*, which has the advantage that genes of the pathogen do not have to be discriminated from the host genes. The results of this study will allow a greater understanding of the infection strategies of this economically important hemibiotrophic pathogen, and may lead to novel control strategies for soybean root rot caused by it.

Materials and methods

Growth of *P. sojae* and soybean

Phytophthora sojae strain PS2 was isolated in China from soybean tissues showing root rot, and maintained on 10% V8 agar (Erwin & Ribeiro, 1996) at 25 °C in darkness. Hyphal tip plugs were used to inoculate 30 mL of sterile-clarified 10% V8 broth in 90-mm Petri dishes. Stationary mycelial cultures were incubated at 25 °C in the darkness and harvested for inoculation at 3 days postinitiation.

The soybean cultivar Hefeng35, which is susceptible to PS2, and 14 soybean differential lines carrying single *Rps* (Table S1) were used. Plants were grown axenically in a greenhouse and maintained as described by Moy *et al.* (2004).

Inoculation of soybean

The seedling hypocotyls-inoculation method was previously described by Hass & Buzzell (1976).

The leaf-inoculation method was developed in this study. Leaves were excised from 10-day-old plants of Hefeng35 and placed on a moist filter paper in Petri dishes with the adaxial surface up. First, 0.5 mL of 10% V8 juice medium containing 0.05% Tween 20 was placed on the detached leaves, and mycelia were added to the solution using forceps. Next, the excess solution was removed carefully with a pipette, and the mycelia were covered with another soybean leaf. The Petri dishes were sealed with Parafilm and placed at 25 °C in the darkness. The dishes were incubated for 1, 3, or 6 h, and the induced mycelia were carefully picked from infection sites using forceps, pooled to create the 'tester' sample. The 'driver' sample was obtained by harvesting mycelia directly from V8 broth.

Differential screening

Total RNA of the 'tester' and the 'driver' samples obtained using leaf inoculation method were extracted from mycelia using the TRIzol Reagent kit (Invitrogen) as directed by the manufacturer. The integrity of the RNA was confirmed using agarose gel electrophoresis. The SMART cDNA synthesis system (Clontech), a PCR-based amplification system that allows the creation of cDNA from very small amounts of total RNA, was used to generate the two cDNA populations. Prior to cDNA synthesis, contaminating DNA was removed from all RNA samples by treatment with DNase I (TaKaRa, Japan) following the manufacturer's protocol.

Suppression subtraction hybridization was performed with the PCR-Select cDNA Subtraction Kit (Clontech). The PCR amplification products enriched for differentially expressed sequences from the tester population were fused into the pMD18-T vector (TaKaRa), and transformed into *Escherichia coli* JM109 cells for blue–white selection. The forward-subtracted library contained 2304 white clones, and all white colonies were picked for identification and characterization.

DNA sequence analysis

To determine the proportion of the clones in the SSH library that represents transcripts that accumulate in the presence of soybean leaves, a dot-blot assay was performed according to the procedure described by Wang *et al.* (2006).

Sequencing of selected clones was performed with an ABI 3730XL DNA sequencer at Beijing Sunbiotech Co. Ltd (http://www.sunbiotech.com.cn). Initial similarity searches were performed using the BLASTX program at the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997). Only expectation values less than 1e-4 were considered to be significant, and only the most significant matches were examined. The Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Phytopathogenic Fungi and Oomycete EST Database version 1.5 (http://cogeme.ex.ac.uk), and the *P. sojae* Genome Database release version 1.0 (http://genome.jgi-psf.org/sojae1/sojae1.home.html) were also searched. Searches were performed with the BLAST algorithms available at the respective web sites using a cut-off *E* value of 1e–4 and the BLOSUM62 scoring matrix.

Virtual Northerns

Virtual Northern blots, also called cDNA Southerns, can yield information similar to that from Northern blots, and were created to confirm the expression patterns of the individual clones (Endege *et al.*, 1999). Each blot contained two samples: the driver and the tester cDNAs. Ten micrograms of double-stranded cDNA generated from new extractive RNA by the SMART procedure as described for SSH screening were loaded and blotted onto a Hybond-N^+ nylon membrane (Amersham Biosciences). Individual cDNA clones generated from the subtraction procedure were labeled using the DIG DNA Labeling and Detection Kit (Clontech) and used as probes. The control probe *actinA* (GenBank accession no. AF085345) was generated from genomic *P. sojae* DNA using the primers 5′-GTACTGCAA CATCGTGCTGTCG-3′ and 5′-TTAGAAGCACTTGCGGT GCACG-3′. The membranes were then hybridized, washed,

and stained as described for dot-blot-assay. All virtual Northern blots were repeated at least three times.

Reverse transcriptase polymerase chain reaction (RT-PCR analysis)

The RNA samples were prepared using both leaves inoculation and hypocotyls inoculation methods. Mycelia were harvested at 0 (no-induced), 1, 3, 6, 12, 24, and 36 h postinoculation (h p.i.). RNA was extracted and treated as described above. First-strand cDNA synthesis was performed using M-MLV reverse transcriptase (RNase H Minus) and oligo$(dT)_{15}$ primer (Promega).

The primers used in the reactions are listed in Table 1. *Phytophthora sojae* genomic DNA was used as a template for the optimization of all primer pairs. PCR reactions were incubated with the following programs: for *actinA*, 95 °C for 1 min, followed by 27 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 2 min; and for all other genes, 95 °C for 1 min, followed by 26 to 35 cycles of 95 °C for 15 s, 53 °C or 55 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 2 min. All RT-PCRs were performed at least four times.

Table 1. Oligonucleotide primers used in RT-PCR expression analysis and amplicon sizes

Descriptions	Amplicon size (bp)	RT-PCR forward and reverse primers
actin A	250	5′-GTACTGCAACATCGTGCTGTCG-3′
		5′-TTAGAAGCACTTGCGGTGCACG-3′
XA8, zinc finger protein	381	5′-ATAGCCAATCTCAACAAGCAGG-3′
		5′-TTAGTCCCAGTCTTCGTCTATC-3′
XA25, glucokinase	382	5′-AGTTCCTGGCCAAGAAGTTCC-3′
		5′-AGCAGCTGATACGCGTAGAAG-3′
XA26, sugar transporter	382	5′-TCGTAAGCTTCTGCTCATCGG-3′
		5′-GCAGAAGAAGTACACGAACAC-3′
XA38, DPLP	392	5′-GCGCATCGCATGATGTATACG-3′
		5′-TACTTCCAGGGCTCAATGGTG-3′
XC46, CGI-20	418	5′-GCACTTTTTGCACTCCGAGAAC-3′
		5′-CTACTGCATGCCACCCGAAAAC-3′
XD38, Car	449	5′-ACAAGTCTTCGAGCAGCGACTA-3′
		5′-TTAGAACGCGAACTCCATCGGG-3′
XD53, BADH	372	5′-ACGATCATCACCAAGGTCACC-3′
		5′-TTATGCCTTGAGATACCAGGC-3′
XF59, glycoprotein gp2	423	5′-GAGAGTGACGAACATGCTGCAG-3′
		5′-CACATTCACTGTCGGCAGTGAG-3′
XM27, Cdc6	378	5′-CGCTGCGTATCATCAAGCATG-3′
		5′-ATACGAGCCGAGAGAAGAAGG-3′
XM37, MRG	379	5′-ATGTCCTTCATGCCGCATGTG-3′
		5′-TGCAGAACGCCAGCACATTAG-3′
XN39, UBA	591	5′-ATGGCACCAGGAGGCCGCGTGC-3′
		5′-CTAGCCAATTCTTGACAGCGCAGC-3′
Avr1b-1	417	5′-ATGCGTCTATCTTTTGTGCTTTC-3′
		5′-TCAGCTCTGATACCGGTGAAAGG-3′

DPLP, diphosphonucleotide phosphatase-like protein gene; Car, cyst germination-specific acidic repeat protein (Car) precursor gene; BADH, betaine-aldehyde dehydrogenase gene; MRG, (mortality factor on chromosome 4)-related gene; UBA, Ubiquitin-associated domain-coding region; *Avr1b-1* (GenBank accession no. AY426744), *Phytophthora sojae* elicitor gene.

Results

Creation of a subtracted cDNA library

To identify specifically expressed *P. sojae* genes involved in the early stage of soybean infection, a simple leaf-inoculation method was developed. This method assures the mechanical separation of the two interacting organisms before the SSH procedure. To test the suitability of this mimic system, pathogen infection structures were observed under a microscope (see Wilson & Coffey, 1980; Beyer *et al.*, 2002). In leaves that had been exposed to the pathogen for 6 h, colonization of the leaf by *P. sojae* could be observed (Fig. 1). To determine whether the regulation of genes occurs on stimulation with two soybean leaves similar to that seen in infection of hypocotyls, the expression of the

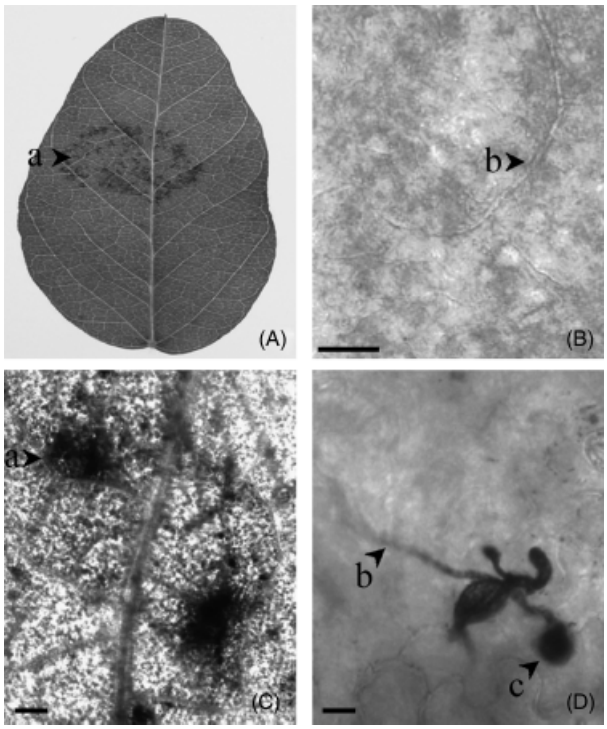

Fig. 1. Interaction between *Phytophthora sojae* and soybean leaves. (A) Lesion region in soybean leaf removing mycelium at 6 h p.i. (B) Hyphae was viewed in infected leaves at 6 h p.i. (C) The local leaf lesion stained by cleared Trypan Blue at 6 h p.i. (D) *Phytophthora sojae* infection structures visible at 16 h p.i. (a) Necrosis regions; (b) infecting hyphae; (c) appressorium or appressorium-like structure. (A) The digital photo. Bars indicate magnifications: (B) × 400, (C) and (D) × 200.

known *in-planta*-induced *P. sojae* avirulence gene, *Avr1b-1* (Shan *et al.*, 2004; Valer *et al.*, 2006), was analyzed and a clear upregulation appeared (Figs S1–S2). Furthermore, using both hypocotyl- and leaf-inoculation methods, *P. sojae* strain PS2 used in this work showed the same race specificity (Table S1; see Athow, 1987). Thus, our *in vitro* experiments closely mirror the infection process that occurred in fields, and an SSH cDNA library containing 2304 clones was generated using this method.

DNA sequence analysis

After examination of the dot blots (data not shown), 339 clones (14.7%) were selected to be sequenced because they showed hybridization signals that were clearly stronger on membranes probed with the forward-subtracted probe population than those probed with the reverse-subtracted population. Subsequently, the sequenced clones were subjected to TBLASTX searches of the *P. sojae* genome database. Hits were considered significant if the expectation value was less than 1e-4. In total, 73 putative clones were identified in 339 sequenced clones. The redundancies of the sequences identified were also counted in TBLASTX program. TBLASTX results revealed that 90% of the *P. sojae* expressed clones have potential homologs in the *P. sojae* genome. The sequences fell mainly into the functional classes of basic cellular processes, such as metabolism, protein synthesis, and energy (Table 2).

The GenBank database was also searched with the 73 sequences, and some matches were found (data not shown). However, none of the 44 unknown sequences had matches in this database. Of the 29 genes (40%) that had significant matches, only three clones were similar to sequences from fungi. However, 26 genes (90%) had significant similarities to genes from organisms other than fungi.

TBLASTX searches of the COGEME EST Database revealed that 73% (53) of the expressed *P. sojae* clones had some similarity to proteins of eukaryotic origin or microbial origin besides *Phytophthora infestans* (data not shown). Clones XA26, XB29, XF30, XL42, XR18, and XZ75 had no significant matches in the GenBank database, but significant hits in the COGEME EST database.

Among the 73 genes, eight genes were represented by five or more ESTs, and these genes may be more important to the pathogenesis of the pathogen. So these genes were taken for further data mining and bioinformatics analysis, such as phylogenetic distribution and gene copy number in *P. sojae* and *Phytophthora ramorum*. In *P. sojae* and *P. infestans* EST databases (Randall *et al.*, 2005), the distribution of ESTs representing the clones over the various libraries were analyzed and, based on these numbers, stage-specific expression patterns and expression levels were predicted. For example, for XD53, many ESTs of *P. infestans* are found in the infection library, starvation library, and many ESTs of *P. sojae* are found in the infection library. This indicates that the XD53 gene is specifically expressed at a relatively high level in infection processes and starvation. In contrast, for XF59, there is only one EST in *P. sojae* in the zoospore library, which indicates that this gene is transcribed at a low level in zoospores. To evaluate the likelihood as to whether these genes are exclusively expressed in the infection processes of *P. sojae*, the phylogenetic distribution was analyzed and it was investigated whether the cloned genes belong to a gene family. Of the eight genes that have homologs in *P. ramorum*, only two seem to be unique for *Phytophthora* (Table 3).

Virtual Northern analysis

Bioinformatics analyses indicated that the most prevalent cDNAs encode metabolism, protein biosynthesis, energy production, cell signaling, and transcription regulation (Table 2). Using new extractive RNA, an additional experiment was performed to confirm the differential expression of these genes. Figure 2 presents virtual Northern analysis results for seven random selected clones from the forward-subtracted library, which were confirmed to be upregulated during the first 6 h of *P. sojae* infection of the soybean cultivar Hefeng35.

RT-PCR analysis

RT-PCR was used to further characterize the differential expression of seven selected genes during plant infection (Fig. 3). Interestingly, five of the selected genes were only expressed during plant infection, whereas the other two genes, betaine-aldehyde dehydrogenase (BADH) gene and mortality factor on chromosome 4 (MORF4)-related gene (MRG), were expressed *in vitro* at very low levels. The XA8, XM37, and XM27 were induced more strongly beginning at 24 h p.i., after which their expression levels began to increase. The expression of XD53, XA38, and XA25 was the highest at 3 h p.i., and the expression of XA26 peaked at 6 h p.i. Although RT-PCR is not quantitative, the use of housekeeping genes such as *actinA* as controls allows a general comparison of the transcript abundance at different infection time points.

To further confirm the suitability of the mimic system, the transcription of 11 selected genes containing the above seven genes and *Avr1b-1* were analyzed in hypocotyls-inoculation experiments (Fig. S2). The expression profiling of these 12 genes was consistent with the results obtained using the leaves-inoculation method (Fig. 3 and S1); it was confirmedly suggested that this mimic system could be used as an alternative approach to identification of the regulated genes during the pathogen infection processes.

Table 2. Summary of 73 sequenced inserts from the SSH library enriched with *Phytophthora sojae* genes expressed during the first 6 h of a compatible interaction with the *Glycine max* cultivar Hefeng35 by TBLASTX searches of the *P. sojae* genome database version 1.0

GenBank	Clone ID	Category and putative function*	*E value*	Protein ID†	Redundancy
DV738011	XA3	Unknown protein	0	158148	1
DV738012	XA5	NA	0	NA	
DV738013	XA8	Zinc finger (C2H2 type) family protein	1.9e–26	135721	1
DV738014	XA15	60S ribosomal protein L38	4.8e–18	117827	1
DV738015	XA16	Unknown protein	4.1e–21	130314	1
DV738016	XA17	Unknown protein	0	133126	2
DV738017	XA18	Unknown protein	5.9e–43	128447	1
DV738018	XA20	Hypothetical protein FLJ14153	0	130057	1
DV738019	XA25	Glucokinase	0	138629	8
DV738020	XA26	Monosaccharide transport protein MST1	0	109253	2
DV738021	XA28	Ribosomal protein L28-like	8.6e–24	136764	2
DV738022	XA32	GLP_741_36440_38116	0	133972	1
DV738023	XA37	Hypothetical protein UM03873.1	9.3e–28	144051	1
DV738024	XA38	Diphosphonucleotide phosphatase-like protein	0	142906	1
DV738025	XA43	Hydroxysteroid (17-beta) dehydrogenase 4	0	120335	3
DV738026	XA49	Putative tyrosinase	2.1e–21	133890	3
DV738027	XA51	Hypothetical protein UM06507.1	0	142932	1
DV738028	XB29	Putative storage protein LPV	0	120460	9
DV738029	XC46	Chromosome 7 ORF 20; CGI-20 protein; H_NH1244M04.5	0	141633	12
DV738030	XD27	Acyl-Coenzyme A dehydrogenase, short/branched chain	0	109319	3
DV738031	XD38	Cyst germination-specific acidic repeat protein (Car) precursor	0	140883	1
DV738032	XD39	Cystathionine beta-synthase	0	109455	1
DV738033	XD53	Betaine-aldehyde dehydrogenase	0	108817	9
DV738034	XD74	Orotidine 5′-phosphate decarboxylase-orotate phosphoribosyltransferase	0	135354	1
DV738035	XE73	ENSANGP00000007211	3.5e–15	136725	1
DV738036	XF23	Conserved hypothetical protein	5.7e–40	137590	2
DV738037	XF27	No significant matches	NA	NA	
DV738038	XF30	Hypothetical protein MG01607.4	0	144022	2
DV738039	XF34	Hypothetical protein AN1657.2	7.6e–37	137070	2
DV738040	XF36	Putative ribosomal protein L19	0	109394	2
DV738041	XF59	Glycoprotein gp2	0	129547	5
DV738042	XF63	No significant matches	NA	NA	
DV738043	XF64	Ribosomal protein S15 isoform	9.5e–35	128815	1
DV738044	XF85	No significant matches	NA	NA	
DV738045	XF89	Predicted protein	1.3e–27	129426	1
DV738046	XG18	Hypothetical protein D10Ertd667e	4.4e–28	141071	1
DV738047	XG64	tRNA synthetase class I (W and Y) family protein	8.9e–29	136606	1
DV738048	XH40	ENSANGP00000011088	0	140041	1
DV738049	XH44	Heat shock protein Hsp90	0	108777	1
DV738050	XJ40	No significant matches	NA	NA	
DV738051	XJ50	Unnamed protein product	0	140775	1
DV738052	XK25	Similar to capillary morphogenesis protein-1	5.0e–32	130594	1
DV738053	XK51	Unknown protein	0	145014	3
DV738054	XL7	OSJNBb0116K07.9	0	132232	7
DV738055	XL42	Similar to SNRPF (Small nuclear ribonucleoprotein) protein	1.1e–29	133236	1
DV738056	XL49	Unknown protein	4.5e–13	130534	1
DV738057	XM27	Cdc6-related protein	0	139531	1
DV738058	XM33	Hypothetical protein FG01918.1	0	136359	2
DV738059	XM34	Unknown protein	5.8e–42	131293	2
DV738060	XM37	(Mortality factor on chromosome 4)-related gene family protein	0	144750	1
DV738061	XM62	Putative phosphoethanolamine cytidylyltransferase	0	123290	1
DV738062	XM77	AAA-type ATPase family protein	3.3e–39	132189	5
DV738063	XN8	Translocated promoter region protein	0	133408	1
DV738064	XN39	Ubiquitin associated domain	0	142734	1
DV738065	XN88	No significant matches	NA	NA	
DV738067	XP10	Unknown protein	0	144429	1

Table 2. Continued.

GenBank	Clone ID	Category and putative function*	*E value*	Protein ID†	Redundancy
DV738068	XP78	No significant matches	NA	NA	
DV738069	XR8	Unknown protein	0	129982	1
DV738070	XR10	Unknown protein	0	136434	1
DV738071	XR18	No significant matches	NA	NA	
DV738072	XR19	Hypothetical protein FG05384.1	0	127296	1
DV738073	XR63	DR1549 conserved hypothetical protein	2.1e–38	137590	3
DV738074	XR81	19 kDa protein having G-X-X-X-Q-X-W motif	0	142672	5
DV738075	XT12	Predicted NADH: ubiquinone oxidoreductase, subunit RnfC	4.9e–41	137250	1
DV738076	XU91	MesA	0	127631	1
DV738077	XV4	Ribosomal protein S17	0	138845	1
DV738078	XW22	Expressed protein	1.6e–41	142095	1
DV738079	XW33	MGC68616 protein	0	108387	1
DV738080	XW54	Adaptor-related protein complex (AP)-3 delta subunit	0	133171	1
DV738081	XW85	Heat shock transcription factor 1a	0	132607	1
DV738082	XX44	Protein kinase family protein	0	138194	1
DV738083	XZ57	60S ribosomal protein L2 (L8)	0	108787	4
DV738084	XZ75	Unnamed protein product	1.4e–37	129889	1

*Putative identification is based on TBLASTX searches of the *P. sojae* Genome database.
†The protein ID number according to the *P. sojae* Genome database.

Table 3. Analysis of selected clones (redundancy ≥ 5) using data mining and bioinformatics

Clone ID	Protein ID of *P. sojae**	Protein size	Transcripts in *P. sojae* EST database†	Transcripts in *P. infestans* EST database†	SwissProt BLAST hit of *P. sojae*	Phylogenetic distribution‡	Genes in *P. sojae*§	Genes in *P. ramorum*§
XA25	138629	352	IN(1)	SP(3) ZO(1) CY(1) MY(1) ST(4)	GLK_SYNY3(Q55855)	Other species	6	6
XB29	120460	74		IN(3) ST(1)		Only in *Phytophthora*	5	3
XC46	141633	316		SP(1)IN(1)		Only in *Phytophthora*	2	1
XD53	108817	494	IN(5)	IN(6) ST(5) MY(1) SP(1) ZO(1) CY(1)	DHAB_ARATH(Q9S795)	Other species	> 10	> 10
XF59	129547	1065	ZO(1)		TRHY_HUMAN(Q07283)	Other species	> 10	5
XL7	132232	309	IN(1) MY(1)		CPPM_ARATH(O49290)	Other species	5	3
XM77	132189	1138		IN(1)	NSF_ARATH(Q9M0Y8)	Other species	3	> 10
XR81	142672	2584	–	–	FMN2_MOUSE(Q9JL04)	Other species	4	5

**Phytophthora sojae* hits with *E* value $< 1E\text{-}50$ and identity $> 99\%$ are listed.
†The tissue types from which the EST libraries are derived are zoospores (ZO), germinated cysts (CY), sporangia (SP), mycelia (MY), infection (IN), and starvation (ST). The numbers in brackets indicate the number of ESTs present in the various libraries (Randall *et al*., 2005).
‡Homologues in species other than *Phytophthora* were considered as homologues when the BLAST *E* value was less than 1E-3 and the similarity $> 30\%$.
§*Phytophthora sojae* and *Phytophthora ramorum* whole-genome sequences and gene annotation at the JGI website (http://www.jgi.doe.gov/genomes) were used for analysis. Genes with BLAST similarity higher than 50% were considered to be members of the same gene family. Numbers indicate the size of the family.

Discussion

Despite the economic importance of *P. sojae*, little is known about the pathogenicity mechanisms of this organism. The purpose of this study was to identify *P. sojae* genes that are specifically expressed during soybean infection, because genes upregulated in the early infection stages may be important for infection and nutrient absorption. At 1, 3, and 6 h p.i., samples were collected and total RNA was extracted for use in SSH because, at these stages, the

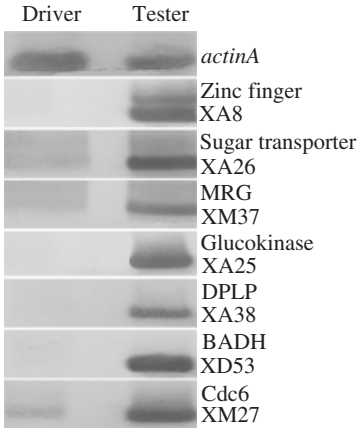

Fig. 2. Virtual Northern analysis of *Phytophthora sojae* gene expression. Sample loadings were normalized with respect to *actinA* expression. Therefore, 30 μg of mycelia cDNA (Driver), and 20 μg of samples blending 1, 3, and 6 h p.i cDNA (Tester) were loaded. cDNA was electrophoresed and blotted to the membranes and probed with DIG-labeled cDNA probes respective to each clone. MRG, (mortality factor on chromosome 4)-related gene; DPLP, diphosphonucleotide phosphatase-like protein gene; BADH, betaine-aldehyde dehydrogenase gene.

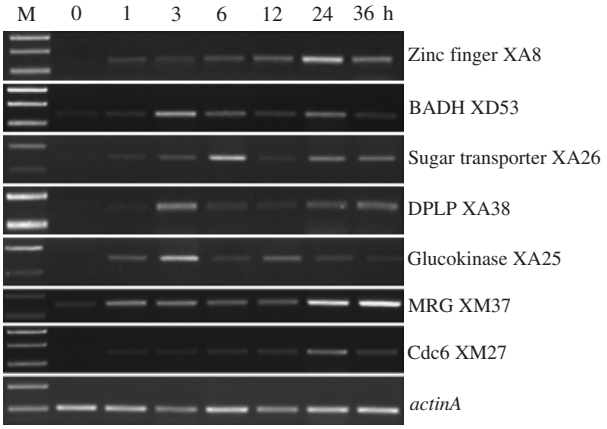

Fig. 3. Changes in transcript level over time of the differential clones in response to coincubation with two soybean leaves. BADH, betaine-aldehyde dehydrogenase gene; DPLP, diphosphonucleotide phosphatase-like protein gene; MRG, (mortality factor on chromosome 4)-related gene; M, 2000 bp DNA size marker.

pathogen has formed some infection-related structures, and has entered soybean tissues without yet triggering the appearance of significant disease symptoms (Fig. 1). Thus, this initial period of infection should be an excellent starting point for identifying genes involved in soybean infection and nutrient absorption. There have been few studies on the early stages of *P. sojae* infection of soybean owing to the difficulties involved in obtaining RNA from the pathogen. In general, zoospores have been used to inoculate plants, but with this method it is difficult to extract sufficient *P. sojae* RNA from infected soybean tissues, and the RNA is readily contaminated with host sequences. To overcome this obstacle, here, a simple mycelium leaf inoculation method was developed that was useful for characterizing the genes involved in the early stages of host infection.

Bioinformatics searches of three databases, the nonredundant GenBank database, the COGEME EST database, and the *P. sojae* genome database, revealed that the 73 identified sequences had at least one significant match in these databases. Perhaps due to gaps or insufficient annotation of the pathogen sequences, 10% (7) of the sequences identified had no significant matches in the databases. Not surprisingly, given the relative lack of available oomycete sequences annotation, 37 of the sequences identified are unknown proteins.

Zinc finger protein is an important gene family of putative transcription factors that have diverse functions in various cellular processes of eukaryotes and prokaryotes (Chomczynski & Sacchi, 1987; Liu *et al.*, 1999; Thiel & Cibelli, 2002; Krishnal *et al.*, 2003). XA8 encodes a putative zinc finger (C2H2 type) peptide that may have a crucial role in *P. sojae* pathogenicity. This gene is not expressed in an uninduced mycelium but appears to be expressed at a relatively high level by 24 h p.i. (Fig. 3 and S2). However, when exposed to H_2O_2 (1 mM), the expression level of this gene gradually increases over time (data not shown).

An interesting gene identified in the SSH library, represented by clone XD38, shows significant similarity in the genome database with the cyst germination-specific acidic repeat protein (Car) (see Gornhardt *et al.*, 2000) precursor. Another clone, XF59, shows significant homology to equine herpesvirus 1 glycoprotein gp2, an unusual mucin-like protein of high molecular mass, which may be important for the adhesion of the pathogen to the host surface (refer Hardham, 2001).

SSH was used to identify a number of *P. sojae* genes that are upregulated in the early stages of the host plant infection. These genes may be involved in *P. sojae* pathogenesis and represent an excellent resource for future studies of the mechanisms of pathogenicity and virulence. These studies will include the isolation of the full-length cDNA sequences of these genes, and use overexpression, antisense, or RNA interference (see Tyler, 2007) to test their functions in *P. sojae* pathogenesis.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. RT-PCR analysis of selected genes in response to coincubation with leaves. UBA, ubiquitin-associated domain-coding region; CAR, cyst germination-specific acidic repeat protein (Car) precursor gene; CGI20, chromosome 7 ORF 20 or CGI-20 protein gene; GP2, glycoprotein gp2 gene; M, 2000 bp DNA size marker.

Fig. S2. Changes in transcript level over time of the differential clones in response to coincubation with hypocotyls. UBA, ubiquitin-associated domain coding region; CAR, cyst germination-specific acidic repeat protein (Car) precursor gene; CGI20, chromosome 7 ORF 20 or CGI-20 protein gene; Gp2, glycoprotein gp2 gene; BADH, betaine-aldehyde dehydrogenase gene; DPLP, diphosphonucleotide

phosphatase-like protein gene; MRG, (mortality factor on chromosome 4)-related gene; M, 2000 bp DNA size marker.
Table S1. Comparison of the hypocotyl- and leaf-inoculation methods using 14 soybean differential lines

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