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ORIGINAL PAPER

Gene expression analysis in soybean in response to the causal agent of Asian soybean rust (*Phakopsora pachyrhizi* Sydow) in an early growth stage

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Abstract Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* Sydow is a potentially devastating disease posing a serious threat to the soybean industry. Understanding plant host response at the molecular level is certainly important for control of the disease. The main objective of this study was to perform a transcriptome profiling of *P. pachyrhizi*-exposed young soybean plants (V2 growth stage) using whole genome Affymetrix microarrays of soybean. Three-week-old soybean cv. 5601 T plants at the V2 growth stage were inoculated with *P. pachyrhizi*, and leaf samples were collected 72 h post inoculation with subsequent microarray analysis performed. A total of 112 genes were found to be differentially expressed from *P. pachyrhizi* exposure, of which 46 were upregulated, and 66 were downregulated. Most of the differentially expressed genes were general defense and stress-related genes, and 34 of these were unknown. Confirmational real-time reverse transcription-polymerase chain reaction was performed on a subset of 5 out of 112 differentially expressed genes. These results were congruent with the microarray analysis. Our results indicated that low and nonspecific innate response to the pathogen may account for the failure to develop rust resistance in the soybean variety studied. To our knowledge, this is the first microarray analysis of soybean in response to ASR.

Keywords Asian soybean rust · Microarray analysis · *Phakopsora pachyrhizi* · Soybean

Introduction

Soybean [*Glycine max* (L.) Merrill] is the second most important row crop in the USA, which annually contributes more than 17 billion dollars to the national economy. A prominent threat first occurred in November 2004 when the causal agent of Asian soybean rust (ASR) *Phakopsora pachyrhizi* Sydow was confirmed for the first time in southeastern America where it was thought to have arrived via hurricane Ivan (Stokstad 2005). *P. pachyrhizi* is endemic in the eastern hemisphere and has caused significant economic annual yield losses in Asia, Africa, and South America. It is believed to have spread from Asia to Africa, then to South America, and most recently to North America.

There is no commercial soybean cultivars available with known ASR resistance genes in the USA (Schneider et al. 2006). In severe cases, where fungicides are not available, ASR can devastate the crop (Rahangdale and Raut 2003). Gupta et al. (1999) observed 63% yield loss under field conditions in India by ASR. Soon after, Gaikwad and Bulden (1999) reported losses ranging from 58–91% under field conditions. Similar observations have been reported from the studies conducted in China and at the Asian

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Vegetable Research and Development Center (Hartman et al. 1991; Ramteke et al. 2003; Tschanz and Shanmugasundaram 1984). From these observations, we can infer that this is a crippling disease posing a serious threat to the soybean industry in the USA, which needs to be managed before it becomes epidemic. Development of resistant varieties is the sustainable approach for the management of such a devastating disease.

Identification of resistance genes is the first step toward this direction, and gauging endogenous upregulation of genes in response to disease-causing agents might give a clue to pertinent defense genes. Microarray analysis is a useful technology for assaying transcriptional responses for biotic and abiotic plant stresses (Bohnert et al. 2001; Kawasaki et al. 2001; Mentewab et al. 2005; Moy et al. 2004; Schenk et al. 2000). This technology has been used for such studies including soybean root rot caused by *Phytophthora sojae* (Moy et al. 2004). This technology has also been useful to identify the homologs of soybean cyst nematode in *Arabidopsis* (Puthoff et al. 2003). Realizing the usefulness of the technology, Schenk et al. (2000) performed cDNA microarray analysis in *Arabidopsis*, inoculating the plants with either *Alternaria brassicicola* treating with defense-related molecules salicylic acid (SA), methyl jasmonate, or ethylene. They found differentially expressed genes in response to one or other treatments with a coordination of gene expression at various stages as a defense mechanism.

Using this technology in peanut (*Arachis hypogoeo*), 56 resistance genes were identified for late leaf spot disease caused by *Cercosporidium personatum*, which is regarded as one of the most destructive foliar diseases of peanut (Luo et al. 2005a). They validated 17 genes, each matching with known function by real-time polymerase chain reaction (PCR). These genes showed higher expression in resistant genotype in response to *C. personatum* infection than in the susceptible genotype. A total of 42 upregulated genes were found in yet another study aiming to identify resistant genes for aflatoxin (*Aspergillus parasiticus*) in peanut using microarray and real-time PCR technology (Luo et al. 2005b). This technology has also been used to study ascochyta blight (*Ascochyta rabie*) resistance genes in chickpea (*Cicer arietinum*; Coram and Pang 2005). Microarray analysis in potato in response to late blight (*Phytophthora infestans*) detected 76 genes associated with secondary metabolites, disease defense, transcription-related functions, and others (Tian et al. 2006) These studies clearly show that microarray technology has been useful in identifying the resistance genes for fungal diseases in plants.

Transcriptional changes play a major role in plant defense process. The coordinative upregulation genes lead to defense responses such as toxin production, cell wall structure changes, programmed cell death, and so on. It is, therefore, important to study differentially expressed genes of soybean in response to *P. pachyrhizi*, which should help to understand the mechanisms of rust development and aid towards the development of resistant soybean varieties for ASR. The main objective of this study was to perform a transcriptome profiling of *P. pachyrhizi*-exposed young soybean plants (V2 growth stage) using whole genome Affymetrix microarrays of soybean. Differential gene expression patterns were analyzed and discussed. Expression patterns of selected genes were confirmed with real-time PCR experiments.

Materials and methods

Plant materials

Soybean [*Glycine max* (L.) Merrill] cv. 5601 T (Pantalone et al. 2003) plants were grown in a greenhouse in a potting mix at the University of Florida's North Florida Research and Education Center in Quincy, Florida. Twelve 4-l pots were planted with four seeds per pot in the fourth week of October 2005 and thinned to three plants per pot. Growing environments during day and night consisted of 28/20°C temperature and 14/10 h of photoperiod, respectively. Cultivar 5601 T was chosen because this is a USDA check for germplasm evaluation in Southeast America; although its response to ASR was unknown. All plants were kept in the same chamber of the greenhouse until the V2 growth stage (Fehr and Caviness 1977) 3 weeks after planting.

Inoculation of plants

Three-week-old (V2 stage) plants were inoculated with *P. pachyrhizi*. Inoculum of *P. pachyrhizi* was collected from a nearby soybean field in Florida that was naturally and severely infected. The identity of *P. pachyrhizi* spores were confirmed using light microscopy from field collections before inoculation. Six out of 12 pots were transferred to the adjacent chamber of the greenhouse so that no cross-contamination would occur. Otherwise, growing conditions were maintained as congruent as possible. ASR-infected leaves of field plants were collected in the morning, and the underside of the leaves was lightly touched to the upper surface of all the greenhouse plants in the six transferred pots. Precautions were taken to avoid any possible wounding while rubbing on the leaf surface. All plants were covered with plastic and watered regularly to maintain the high humidity conducive to pathogen growth. At the end of the experiment, plants had one fully expanded trifoliate leaf that was used for inoculation. The plants were about 15 cm tall by the time of inoculation.

Sample collection and RNA isolation

The experimental unit was a pot. Approximately 1 g of leaves were collected per pot by subsampling all plants in a pot in both control and *P. pachyrhizi*-inoculated treatments at 72 h postinoculation. Postinoculation time for sampling was chosen on the basis of past studies and their similarities with the present one (Puthoff et al. 2003; Schenk et al. 2000). Fully expanded and inoculated trifoliate leaves were sampled for RNA extraction. Leaves were wrapped in aluminum foil and dipped into liquid nitrogen until ground with a mortar and pestle. RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH). RNA was precipitated in cold isopropanol and washed with 75% ethanol. The general quality of RNA was determined by agarose gel electrophoresis.

DNA isolation and polymerase chain reaction

DNA was isolated from the same soybean plants (control and inoculated) by a cetyltrimethylammonium bromide method (Stewart and Via 1993). PCR was performed using *P. pachyrhizi*-specific primers (Frederick et al. 2002) to confirm the presence of *P. pachyrhizi* in the samples from *P. pachyrhizi*-inoculated plants using a Master Cycler (Eppendorf AG, Hamburg, Germany). Bands were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV light. All primers were acquired from Integrated DNA Technologies (IDT DNA, Coralville, IA).

Microarray analysis

Soybean Genome GeneChip Arrays containing more than 35,611 *Glycine max* unigenes were purchased from Affymetrix (Santa Clara, CA). The Affymetrix protocol for one-cycle eukaryotic target preparation was followed to prepare soybean probe. First, 10 μg of total RNA was converted into single-stranded cDNA using SuperScript II and a T7-Oligo (dT) primer (Affymetrix). Second-strand cDNA was synthesized using dNTPs, second-strand reaction buffer, *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and *E. coli* RNAse H (all reagents were from Affymetrix). The one-cycle cDNA synthesis was followed by a cleanup using Affymetrix GeneChip sample cleanup modules. Biotin-labeled cRNA was prepared using an Affymetrix in vitro transcription (IVT) labeling kit. After cleanup of the IVT products, the purified cRNA was fragmented to a size ranging from 35 to 200 bases using a fragmentation buffer at 94°C for 35 min. The fragmentation of the labeled cRNA was confirmed before hybridization by running samples on the Agilent 2100 Bioanalyzer using a RNA Nano Chip. Fifteen micrograms of the fragmented cRNA was mixed into a hybridization cocktail containing a hybridization buffer, B2 oligo control RNA (Affymetrix), herring sperm DNA, and BSA (Invitrogen, Carlsbad, CA). The solution was hybridized to a GeneChip at 45°C for 16 h at a setting of 60 rpm. After hybridization, the cocktail was removed from the GeneChip and stored for potential future analyses. Using the Affymetrix Fluidics 450 wash station (Affymetrix Fluidics Protocol EUKGE_WS2v5), the GeneChip was washed and stained with streptavidine–phycoerythrin (Molecular Probes), followed by a wash with biotinylated antibody goat *IgG* and another staining with streptavidin phycoerythrin. The GeneChips were immediately scanned with a GeneChip 7G high-resolution scanner. Individual scans were quality checked for the presence of control genes and background signal values. Microarray preparation and hybridization was performed at the University of Tennessee Knoxville Affymetrix Core Facility. Each microarray experiment was replicated three times with an independent biological sample for statistical analysis for a total of six chips.

Real time RT-PCR

Real-time RT-PCR as described by Yuan et al. (2006) was performed on a subset of five differentially regulated genes in three replications each of four biological samples as confirmation and to expand biological sample sizes. For RT-PCR analysis of gene expression level, the primers were designed for amplicons of about 25 bp for each gene using Primer Express 2.0 software (Applied Biosystems). A list of primers used for RT-PCR is listed in Table 1. A tubulin gene (Shen et al. 2006) was used as an internal control. Primer titration and dissociation experiments were performed to ensure that no primer dimers or false amplicons are produced that would interfere with the amplification. A standard curve was developed from three serial-diluted concentrations of cDNA.

Microarray data analysis

The data analysis was carried out using the ArrayAssist Software from Stratagene. The CEL file was created from the DAT image file of the chip using the GeneChip operating software from Affymetrix. Hybridization of a B2 oligo serves as a positive control by the software to place a grid over the scanned image. Noise, which is the pixel-to-pixel variations of probe cells on the array and the average background, (varying between 20 and 100) was subtracted from the signal intensity value. The control genes were analyzed (poly A controls and hybridization controls) for appropriate signal intensity. For the internal control genes, the ratio of 3′ probe sets to 5′ probe sets was analyzed with an expected variation of ratios between 1 and 3.

Table 1 List of primers used in real-time RT-PCR to confirm the differentially expressed genes

Affymetrix ID code	Gene	Primer	Primer sequence (5′-3′)
HgAffx.13168.1.S1_at	Salicylic acid (CYTB gene)	Forward	AAGAAGATGAACAAGCACAAGTACCA
		Reverse	TTCTGGCCCCTCACCTTCTAC
Gma.8514.1.S1_at	Glutathione-S-transferase (GST)	Forward	CTTGGACCCTCTAAAGATGAACAAG
		Reverse	GCTCTTCCATTACCATGATAGTTTCTTC
Gma.2826.1.S1_at	Salicylic acid induced chalcone isomerase	Forward	AGAGATGAACAAGCACAGTACCA
		Reverse	ATCTGGCCTCACCTTCTA
Gma.92003.1.S1_at	Chalcone isomerase	Forward	GTTTCCCCTGCTTTGAAAGAGA
		Reverse	GGATTGGCCTCTAACTCTTTGAAG
Gma.16812.1.S1_s_at	Peroxidase precursor	Forward	AGTTTTACAATGGATGAGATGATTAACC
		Reverse	GAGCCCCTGAAAGGATGACA
Control gene	Tubulin	Forward	CTCAGGTGATTTCATCTTTG
		Reverse	GAATTCAG TCACATCCAC

The software uses a detection algorithm to estimate the expression of each gene as present (P), marginal (M), or absent (A). This determination is based on the target-specific intensity of the probe pair relative to the overall hybridization intensity (i.e., perfect match, mismatch/perfect match, and mismatch). Each probe set consists of 11–20 probe pairs that are summarized into a signal value using the GC-RMA algorithm available in ArrayAssist. GC-RMA is a model-based algorithm that takes into consideration the multiple chips present in the experiment (Wu et al. 2004). The algorithm performs a background adjustment taking into consideration the probe sequence information to correct for nonspecific binding followed by normalization and expression measure calculation. The expression values were then converted into $\log_{10}$ or $\log_2$ scale. Probes that did not differ in expression across all the experiments by about 1.85× were filtered out. This resulted into 7,106 probe sets, which were subjected to unpaired *t* test.

The array data of the control sample was compared to the array data from the *P. pachyrhizi* inoculated sample using ArrayAssist data analysis package. The genes determined to be upregulated or downregulated in the test sample relative to the control sample (determined on the basis of unpaired *t* test) were examined by setting a cut-off value of 1.5 and less than 0.05 probability for the signal log ratio. A list of known or unknown genes was generated by performing BLASTn search http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) from Affymetrix website. Furthermore, the function of putative encoded proteins was identified by using the ExPasy protein database http://us.expasy.org/).

Real-time RT-PCR data analysis

Real-time PCR data analysis was performed with the *t* test method as described by Yuan et al. (2006). Basically, the Δthreshold cycle (Ct) for target genes and reference gene (tubulin) were obtained through subtracting the Ct value of rust inoculated sample from that of the control sample. The pairwise *t* test was then employed to derive the ΔΔCt. Parameter estimation includes the standard error, 95% confidence level, and *p* value for the ΔΔCt. The ratio and the confidence levels of the ratio were then calculated and are presented in Table 4.

Results and discussion

Quality control

All inoculated samples were PCR-positive for a unique *P. pachyrhizi* marker, which was a crucial determination as ASR symptoms were not visible when leaf samples were collected for nucleic acid extraction for transcriptomic analysis. The quality of microarray data was deemed sufficient by the observation of the normalization curve, which had a Gaussian distribution as indicated by a Kolmogorov–Smirnov test (D=0.03; p<0.01; Fig. 1). Scatter plots of signals from control vs rust inoculated samples revealed the normal distribution of data with the up- or downregulation of genes (Fig. 2). The original dataset are available at the University of Tennessee Microarray Database site http://web.utk.edu/~genome.htm). The same dataset will also be made available at the Gene Expression Omnibus of National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/geo).

Differentially expressed genes

A total of 112 genes were found to be significantly differentially expressed in leaves of V2 growth stage (21 days old) soybean plants with respect to ASR (Tables 2 and 3). Out of 112 differentially expressed genes, 46 were upregulated, and 66 were downregulated. Most of the

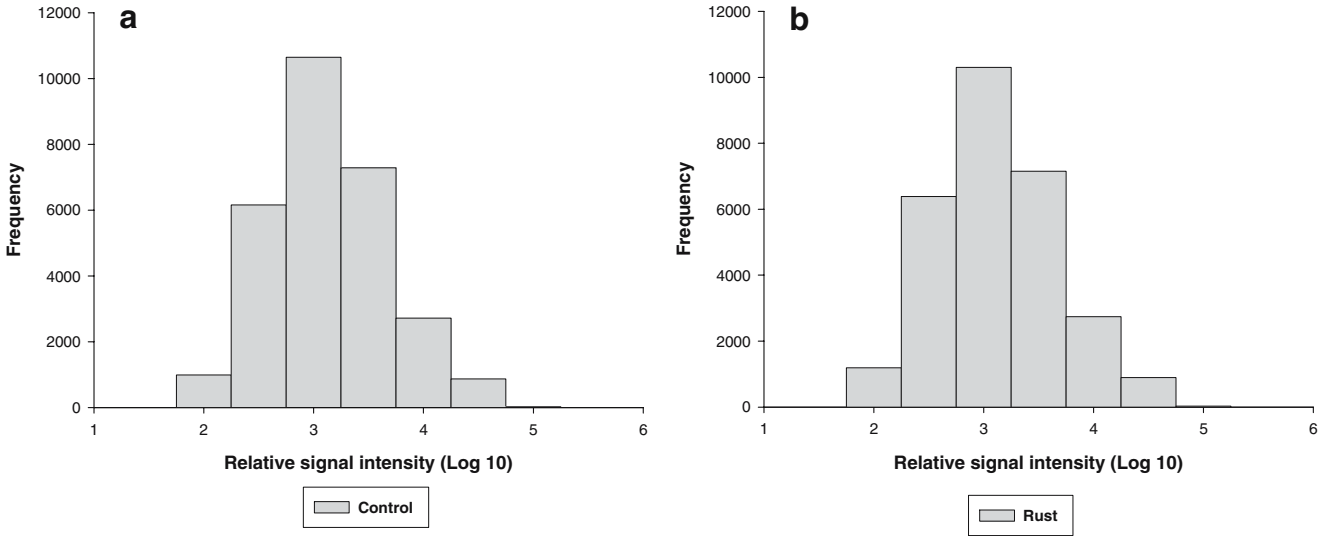

Fig. 1 Distribution of normalized relative signal intensity ($\log_{10}$) in microarray analysis in soybean cv. 5601 T at the V2 growth stage in **a** control and **b** *P. pachyrhizi*-inoculated samples. The data were found to be normally distributed as indicated by a Kolmogorov–Smirnov test (D=0.03; p<0.01)

upregulated genes had defense- and stress-related functions, which have been reported in other plant species such as *Arabidopsis*, rice, peanut, potato, pepper, and others (Bohnert et al. 2001; Kawasaki et al. 2001; Khan et al. 2004; Lee et al. 2004; Luo et al. 2005a; Moy et al. 2004; Nimchuk et al. 2003; Tian et al. 2006). There were 14 unknown upregulated genes with a fold induction ranging from 3.3 through 1.5 (Table 2). Indeed, most of the upregulated genes found in this study are general defense-related genes. The relatively low number of differentially regulated genes (with >2.0-fold induction) is to be expected in a species that has low innate resistance to this particular disease—for example, the plant cannot mount a successful defense against the ASR pathogen. Soybean cv. 5601 T has been shown to be resistant to the stem canker, soybean mosaic virus, and southern root knot nematode but is susceptible for sudden death syndrome and soybean cyst nematode (Pantalone et al. 2003) with no known host response to ASR. Thus, our results show that soybean attempts to mount a defense to *P. pachyrhizi* infection by way of upregulating a few but ineffective defense genes. The unknown genes might have ASR-specific defense or other functions; thus, we are in the process of cloning and characterizing them.

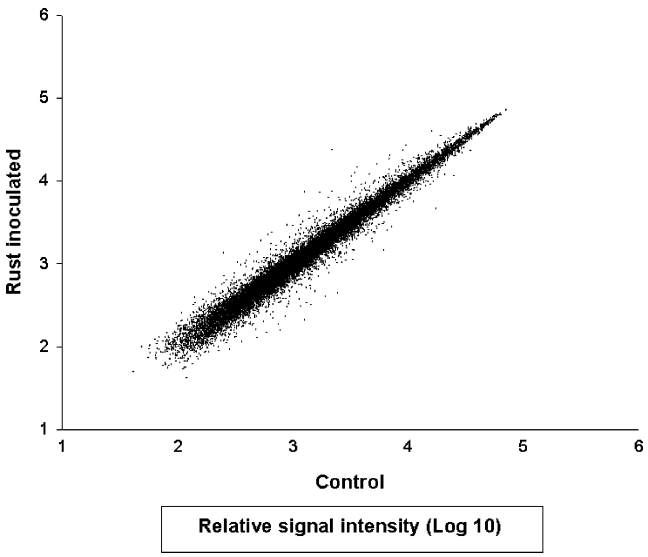

Fig. 2 Comparative scatter plot of normalized relative signal intensity ($\log_{10}$) between control vs *P. pachyrhizi*-inoculated samples in soybean cv. 5601 T at V2 growth stage

Defense genes

Among the upregulated genes are those encoding a SA-related protein, heat shock proteins (HSP), a leaf senescence-associated receptor-like protein kinase (LSRK), a glutathione S-transferase (GST), and chalcone isomerase (CI), which play roles in general defense and stress tolerance. The SA and HSP had more than seven- and fivefold induction, respectively, upon *P. pachyrhizi* inoculation in the leaves of V2 stage soybean plants. Furthermore, there was another SA-related gene with almost a 2.5-fold induction. Similarly, the GST and CI had three- and twofold induction, respectively (Table 2). It has been suggested that biotrophic pathogens tend to activate SA-dependent pathways in the defense mechanism, whereas necrotrophic pathogens induce jasmonate-dependent pathways (Nimchuk et al. 2003). SA is an important regulator of systemic acquired resistance (Durner et al. 1997). This is a simple six-carbon aromatic compound with –COOH and –OH functional groups. From the past research, evidences have been accumulated indicating that SA acts as an

Table 2 List of upregulated genes in soybean cv. 5601 T at V2 growth stage in response to ASR (*P. pachyrhizi*) after 72 h of inoculation

Affymetrix probe ID	Genes	Fold change in function	*p* Value
HgAffx.13168.1.S1_at	Salicylic acid (CYTB gene)	7.76	0.01
Gma.2505.2.S1_×_at	Ferritin/Heat shock protein (HSP) (chloroplast protein) nuclear gene	5.42	0.02
GmaAffx.69544.2.S1_at	Class II HSP	3.35	0.03
GmaAffx.9483.1.S1_at	Unknown	3.32	0.01
GmaAffx.78978.1.S1_at	Leaf senescence-associated receptor-like protein kinase	3.30	0.03
GmaAffx.4452.1.S1_at	Leaf senescence-associated receptor-like protein kinase	3.16	0.04
Gma.8514.1.S1_at	Glutathione-S-transferase (GST)	3.02	0.01
GmaAffx.87405.1.S1_at	F9L1.11 gene	2.88	0.03
GmaAffx.20786.1.A1_at	Unknown	2.55	0.00
Gma.2826.1.S1_at	Salicylic acid induced chalcone isomerase (1B1)	2.48	0.01
GmaAffx.37813.1.S1_at	Putative receptor-like protein kinase	2.38	0.03
GmaAffx.71308.1.S1_at	Heat shock transcription factor	2.35	0.03
GmaAffx.92003.1.S1_at	Chalcone isomerase 1B1	2.10	0.04
GmaAffx.31196.1.S1_at	Subtilisin-like protease C1	2.03	0.02
GmaAffx.47228.1.S1_at	Vascular sorting receptor-like protein	2.01	0.03
Gma.7535.1.S1_at	Submurgence-induced protein, Anthocyanin 5-aromatic acyltransferase	2.00	0.02
GmaAffx.71308.2.A1_at	Transcribed locus	1.99	0.04
GmaAffx.92003.1.S1_×_at	CI 1B1	1.93	0.04
GmaAffx.45125.1.S1_s_at	Weakly similar to NP_189600.1 transferase	1.92	0.05
GmaAffx.3550.1.S1_at	Moderately similar to NP_189034.1 beta-amylase	1.88	0.03
GmaAffx.46447.1.S1_at	Unknown	1.88	0.02
Gma.8324.1.S1_at	Weakly similar to NP_201352.1 unknown protein	1.85	0.05
GmaAffx.4195.1.S1_at	Transcribed locus	1.79	0.05
Psojae_rRNA_347_at	Unknown	1.76	0.05
Gma.7535.1.S1_a_at	Weakly similar to NP_189600.1 transferase	1.76	0.03
GmaAffx.57686.1.S1_at	Unknown	1.75	0.01
GmaAffx.80998.1.S1_at	Moderately similar to NP_192950.2 aminomethyltransferase	1.74	0.04
Gma.17725.1.A1_at	Weakly similar to XP_512419.1 predicted: calreticulin (*Pan troglodytes*)	1.68	0.05
GmaAffx.92410.1.S1_s_at	Weakly similar to NP_175552.2 Alcohol dehydrogenase, aldehyde reductase	1.67	0.04
GmaAffx.66413.1.S1_at	Unknown	1.66	0.04
GmaAffx.674.1.S1_at	Weakly similar to NP_200685.1 dehydrodolichyl diphosphate synthase	1.66	0.05
Gma.17556.1.S1_at	Weakly similar to NP_172641.1 unknown protein	1.65	0.00
Gma.6763.1.A1_at	Moderately similar to NP_564821.1 ATRBOH F F; NAD(P)H oxidase	1.62	0.04
GmaAffx.62324.1.A1_at	Unknown	1.60	0.03
GmaAffx.39568.1.S1_at	Unknown	1.59	0.02
GmaAffx.56521.1.S1_at	Weakly similar to NP_189274.1 carboxylic ester hydrolase/hydrolase, acting on ester bonds	1.58	0.04
GmaAffx.51152.1.S1_at	Unknown	1.58	0.05
GmaAffx.36625.1.S1_at	Unknown	1.57	0.01
GmaAffx.62050.1.S1_at	Unknown	1.55	0.04
GmaAffx.24431.1.S1_at	F1 beta subunit of ATP synthase mRNA, partial cds; nuclear gene for mitochondrial product	1.54	0.00
GmaAffx.39479.1.A1_at	Unknown	1.53	0.02
GmaAffx.73241.1.S1_at	Weakly similar to NP_573215.1 CG5613-PA, isoform A (*Drosophila melanogaster*)	1.52	0.04
Gma.13893.1.A1_at	Weakly similar to NP_567379.1 protein binding/Ubiquitin-protein ligase/zinc ion binding	1.52	0.01
Gma.2723.1.S1_at	Unknown	1.51	0.03
GmaAffx.32011.1.S1_at	Weakly similar to NP_566629.1 ABF4 (ABRE binding factor 4); DNA binding/transcription factor/transcriptional activator	1.50	0.05
GmaAffx.67437.1.S1_at	Unknown	1.50	0.03

Table 3 List of downregulated genes in soybean cv. 5601 T at V2 growth stage in response to ASR (*P. pachyrhizi*) after 72 h of inoculation

Affymetrix probe ID	Genes	Fold change in function	*p* value
GmaAffx.77432.1.A1_at	Peroxidase like protein: cationic peroxidase isozyme 40K	−4.09	0.01
GmaAffx.30537.1.S1_at	Phosphate induced protein 1 (PHI-1)	−2.93	0.03
GmaAffx.12305.1.S1_at	B1047H05.38 gene (CF-2.1 protein)	−2.72	0.03
Gma.14554.1.S1_at	Unknown	−2.68	0.04
Gma.899.1.A1_at	At5 g11970 gene	−2.37	0.04
GmaAffx.89697.1.S1_s_at	Peroxidase precursor	−2.35	0.01
GmaAffx.70713.1.S1_at	Putative membrane transporter	−2.17	0.03
Gma.8077.2.S1_at	Brassinosteroid-regulated protein (BRU1)	−2.16	0.02
Gma.16812.1.S1_s_at	Peroxidase precursor	−2.03	0.02
GmaAffx.71803.1.S1_at	Unknown	−1.99	0.05
GmaAffx.82860.1.S1_at	Unknown	−1.88	0.01
GmaAffx.17665.1.S1_at	Weakly similar to NP_190006.1 ATP binding/kinase/protein kinase/protein serine/threonine kinase/protein–tyrosine kinase	−1.87	0.05
Gma.1326.3.S1_at	Moderately similar to NP_567707.1 lyase/pectate lyase	−1.83	0.00
GmaAffx.47230.2.S1_at	AUX1-like permease (lax1 gene)	−1.81	0.00
GmaAffx.23381.1.S1_at	Unknown	−1.80	0.00
GmaAffx.71051.1.S1_at	Unknown	−1.78	0.03
GmaAffx.82564.1.S1_s_at	Transcribed locus	−1.77	0.00
Gma.1486.2.S1_at	Weakly similar to NP_563941.1 RNA binding/Endoribonuclease	−1.77	0.03
GmaAffx.77432.2.S1_at	Weakly similar to NP_194904.1 peroxidase	−1.77	0.01
GmaAffx.24449.1.A1_at	Unknown	−1.75	0.01
GmaAffx.69813.1.A1_at	Transcribed locus	−1.73	0.03
GmaAffx.54724.1.S1_at	Moderately similar to NP_175853.1 ATP binding/kinase/protein kinase/protein serine/threonine kinase/protein–tyrosine kinase	−1.73	0.04
GmaAffx.92741.1.S1_s_at	Unknown	−1.73	0.05
Gma.5267.1.S1_at	Weakly similar to XP_467525.1 unknown protein [Oryza sativa (japonica cultivar group)]	−1.73	0.02
GmaAffx.53860.1.S1_at	Unknown	−1.72	0.05
Gma.7448.1.A1_at	Unknown	−1.71	0.01
GmaAffx.1824.1.A1_at	Unknown	−1.70	0.00
Gma.12710.1.A1_at	Weakly similar to NP_196419.1 DNA binding/nucleic acid binding	−1.70	0.05
GmaAffx.75004.1.S1_at	Unknown	−1.70	0.03
Gma.16796.1.S1_at	Weakly similar to NP_186796.1 ATHB-1; transcription factor	−1.69	0.00
GmaAffx.34243.1.S1_at	Unknown	−1.69	0.00
Gma.9598.1.A1_at	Weakly similar to NP_196335.1 Arabidopsis thaliana At5 g07180 gene	−1.68	0.02
Gma.13139.1.S1_at	Moderately similar to NP_849712.1 transcription regulator	−1.68	0.04
Gma.14323.1.A1_at	Moderately similar to NP_566413.1 unknown protein	−1.68	0.05
GmaAffx.47684.1.A1_at	Mitotic cyclin a1 type	−1.67	0.00
Gma.16718.1.A1_at	Transcribed locus	−1.66	0.02
GmaAffx.37013.1.S1_at	Transcribed locus	−1.65	0.00
Gma.6159.1.S1_at	Transcribed locus	−1.65	0.01
GmaAffx.68011.1.S1_at	Unknown	−1.63	0.05
Gma.3026.1.S1_at	Transcribed locus	−1.61	0.02
GmaAffx.68853.1.S1_at	Unknown	−1.60	0.00
GmaAffx.24074.1.S1_at	Weakly similar to NP_917279.1 OSJNBb0032K15.9	−1.59	0.02
Gma.12279.1.A1_at	Weakly similar to NP_568850.1 DNA binding/transcription factor	−1.59	0.05
GmaAffx.18845.1.S1_at	Partial mRNA for hypothetical protein (ORF1)	−1.59	0.04
GmaAffx.34720.4.S1_s_at	Unknown	−1.58	0.03
GmaAffx.67477.2.S1_at	Moderately similar to XP_470717.1 alpha-expansin	−1.58	0.05
GmaAffx.62323.1.S1_at	Unknown	−1.58	0.02
Gma.12733.1.A1_at	Transcribed locus	−1.57	0.01
GmaAffx.51421.2.S1_at	Weakly similar to NP_171713.1 unknown protein	−1.57	0.04
Gma.1907.1.S1_at	Weakly similar to NP_566387.1 carboxylic ester hydrolase/hydrolase, acting on ester bonds	−1.56	0.02

Table 3 (continued)

Affymetrix probe ID	Genes	Fold change in function	*p* value
Gma.15869.2.S1_at	Weakly similar to XP_306569.1 ENSANGP00000014631 [Anopheles gambiae str. PEST]	−1.56	0.05
GmaAffx.56750.1.S1_at	Unknown	−1.55	0.04
Gma.5799.2.S1_at	Weakly similar to NP_564354.1 unknown protein	−1.55	0.05
Gma.16796.2.S1_at	Weakly similar to XP_469610.1 putative transcription activator	−1.55	0.05
Gma.592.1.S1_at	Weakly similar to NP_566273.1 metal ion binding	−1.54	0.05
Gma.16815.1.A1_at	Ribulose-1,5-bisphosphate carboxylase small subunit	−1.54	0.00
GmaAffx.20328.1.A1_s_at	Unknown	−1.53	0.01
Gma.6991.1.S1_at	Moderately similar to NP_913365.1 P0665D10.16	−1.53	0.01
GmaAffx.88183.2.S1_at	Unknown	−1.52	0.05
GmaAffx.67879.1.S1_at	Weakly similar to XP_474410.1 OSJNBa0088H09.6	−1.51	0.05
Gma.5290.1.S1_at	Transcribed locus	−1.51	0.01
GmaAffx.80346.1.S1_at	Weakly similar to NP_909852.1 putative thiamin biosynthesis protein	−1.51	0.05
Gma.3066.1.S1_at	Moderately similar to XP_479493.1 AP2 domain transcription factor EREBP; flavonoid 3′-hydroxylase	−1.50	0.05
GmaAffx.85063.1.S1_at	Unknown	−1.50	0.03
GmaAffx.93331.1.S1_at	Weakly similar to NP_172556.2 unknown protein	−1.50	0.04
GmaAffx.76868.1.S1_at	Weakly similar to NP_566600.1 oxidoreductase	−1.50	0.00

endogenous signal for plant defense responses. Resistant hosts produce higher concentration of SA upon challenged by pathogen, which has been shown in a number of experiments including the production of transgenic mutants (Mauch-Mani and Metraux 1998). As *P. pachyrhizi* is an obligate parasite and biotroph, it follows that we would observe mainly SA-related upregulated genes in the present study, which did occur. HSP genes have been reported to be cell defense-related genes (Luo et al. 2005b; Patel et al. 2004).

Puthoff et al. (2003) found 12 genes with differential expression pattern in response to soybean cyst nematode (SCN) caused by *Heterodera glycines* in *Arabidopsis*. Similar to the findings of the present study, those genes were not specific to the SCN rather were involved in general defense; furthermore, soybean has low innate resistance to *H. glycines*. In yet another experiment by Schenk et al. (2000) using cDNA microarray analysis in *Arabidopsis* in response to fungus *Alternaria brassicicola*, or defense-related molecules SA, methyl jasmonate, or ethylene, they found 705 genes with differential expression in response to one or other treatments. One of the common genes identified in their experiment and the present one was a GST. Luo et al. (2005b) have also reported a GST as an upregulated gene in response to *A. parasiticus* in peanut. GSTs are an important protein family that play a detoxification role in plant cells. In a different study, HSP genes were found to be upregulated in wheat and *Arabidopsis* in response to powdery mildew (*Blumeria graminis* fsp. *tritici*; Michel et al. 2006). Another upregulated gene identified in the present study, CI, has also been shown to be defense-related in soybean (Ebel and Grisebach 1988).

There were 66 genes with repressed expression, most of them were peroxidase-related, and only one was a membrane transporter (Table 3). The peroxidase genes were repressed by as high as fourfold. There were 20 unknown genes with 2.7 through 1.5 times repressed expression in response to ASR in the soybean. Peroxidase-related genes found in the present study have also been reported as differentially expressed in soybean in response to SCN (Khan et al. 2004; Lee et al. 2004). Peroxidases are a group of enzymes that catalyze oxidation–reduction reactions (Passardi et al. 2004). They are also believed to have constitutive function and are downregulated by the host to divert resources to more pressing needs (Moy et al. 2004) when plants are attacked by pathogens.

As in the present study, Peroxidase, GST, and LSRK were found to be differentially regulated genes in pepper when challenged by the soybean pustule pathogen (*Xanthomonas axonopodis* pv. *glycines*; Lee et al. 2004). They found the three most upregulated genes involved in phytoalexin biosynthesis, which are common plant defense-related compounds and act as toxins to the attacking organisms. Khan et al. (2004) conducted a cDNA microarray experiment in soybeans in response to SCN. The major differentially expressed gene categories were stress, cell wall maintenance or development, synthesis of secondary metabolites, metabolic pathways, signal transduc-

tion, and membrane transport among unknown genes. It should be noted that more than 50% of the total genes expressed differentially were unknown; whereas in our study, we found about one-third unknown genes.

We used a 1.5-fold induction level as a cut-off point at $p=0.05$ level determined by the unpaired t test. The 1.5 cut-off level for gene expression has been used by several researchers even without any statistical test for its significance (Khan et al. 2004; Mentewab et al. 2005; Patel et al. 2004). Even in some studies, they have used >1.0 as a cut-off point (Luo et al. 2005a,b). However, recently, there has been a tendency of reporting only statistically significant regulated genes (Puthoff et al. 2003; Troyanskaya 2005). This approach is more stringent in the sense that all genes with more than a 1.5-fold increase in expression may not differ significantly in all cases from the control.

Real time RT-PCR

We chose five genes (four upregulated genes and one downregulated gene) for real-time RT-PCR to confirm the differential expression and to increase biological sample size. For each gene, four biological replicates and three technical replicates were used. The total number of replicates is, therefore, increased to 12. The microarray and real-time PCR data correlated very closely (Table 4 and Fig. 3). Although one gene related to SA synthesis that had a different value between methods, the trend of expression was still the same. Validation of differentially expressed genes detected in microarray analysis by other methods (like RT-PCR) has a biological significance from a practical point of view. Although results between two methods correlated, there was a difference between microarray and RT-PCR expression ratios, but similar results have been reported by several researchers in the past (Mentewab et al. 2005; Moy et al. 2004; Patel et al. 2004; Ralph et al. 2006).

Our study was a first step in investigating the soybean transcriptome in response to *P. pachyrhizi* infection. Additional studies might utilize *Glycine tomentolla*, a known ASR resistant relative of soybean (Hartman et al. 1992), to identify genes that might be useful for conferring ASR resistance in soybean. A priori, one might expect that resistant germplasm would have an orchestrated upregulation of resistance genes, and that many of these genes might have a currently unknown function. The appearance of relatively recent (hence less studied) genes would be consistent with the 'evolutionary arms race' in which host and pathogen mutate relatively rapidly in response to each other (Laine 2006; Miller et al. 2005). Resistance genes are expected to be specific for certain pathogens. In this example of soybean and ASR, soybean has been, thus far, apparently unsuccessful in evolving resistance against ASR.

From prior studies, it is unclear whether there is a relationship between host reaction to pathogens and the number of upregulated genes, which includes qualitative and quantitative traits (Khan et al. 2004; Moy et al. 2004; Schenk et al. 2000). It has been reported that there are four putative major genes (uncloned and uncharacterized) that

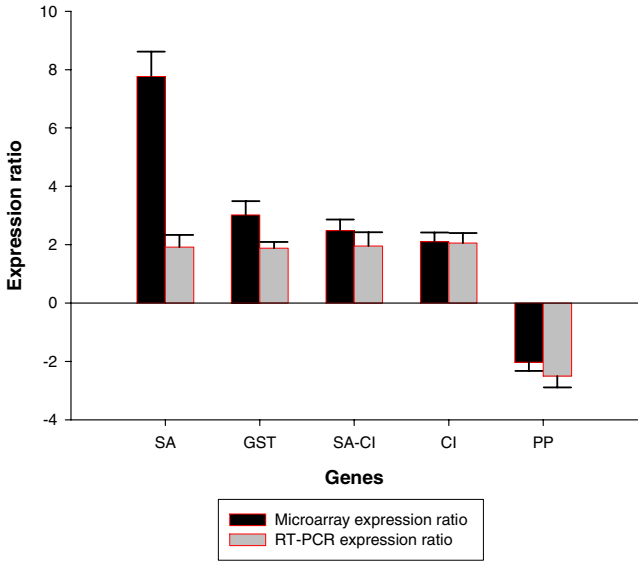

Fig. 3 Differential expression ratio of selected targets in microarray analysis and real time RT-PCR. (*SA*, *GST*, *SA–CI*, *CI*, and *PP* [peroxidase precursor]) in soybean at the V2 growth stage

Table 4 Validation of microarray-based gene expression by real-time RT-PCR in soybean at V2 growth stage

Gene	ΔΔCt	Standard deviation	Microarray expression ratio	p Value for microarray	RT-PCR expression ratio	p Value for RT-PCR	LCL	UCL
SA	−0.94	0.83	7.76	0.01	1.92	0.0009	1.38	2.67
GST	−0.91	0.43	3.02	0.01	1.88	<0.0001	1.53	2.30
SA–CI	−0.97	0.95	2.48	0.01	1.95	0.10	1.22	3.13
CI	−1.04	0.67	2.10	0.04	2.06	0.0002	1.53	2.77
Peroxidase precursor	1.33	0.77	−2.03	0.02	−2.50	0.0002	−3.86	−1.64

Ct Threshold cycle, *LCL* lower confidence limit, *UCL* upper confidence limit, *SA* salicylic acid, *GST* glutathione-S-transferase, *SA-CI* salicylic acid induced chalcone isomerase, *CI* chalcone isomerase

confer low-to-moderate ASR resistance in soybean (Hartwig 1986; Rahangdale and Raut 2004; Ramteke et al. 2003). Cloning and characterization of unknown genes from the present study may be able to provide a clue whether these same genes were differentially expressed.

The results from the present study indicated that, unlike in the case of other diseases and plant species, there are limited number of genes involved in the interaction between early growth stages soybean tissue and *P. pachyrhizi*. This could be because of three reasons. First, the genotype did not have any strong ASR resistance genes. This is possible because no US commercial cultivars have known resistance genes for ASR. Second, ASR typically infects at V4 or R1 growth stage under natural conditions. However, our study was at an earlier stage of plant development. Third, these are the only genes involved in host–pathogen interaction. In any case, the findings from the present study are valuable for two reasons. First, if any of the upregulated genes could be demonstrated to show resistance, they could be very important for the development of an ASR-resistant soybean. Second, even if none of them confers resistance, our results will still provide important information about how soybean attempts to mount a defense against ASR.

One or more of the defense-related candidate genes might be cloned and overexpressed in a transgenic soybean plant in hopes of conferring increased resistance, which is largely lacking in the species. Only one soybean variety was tested in this study, but there could be intervarietal differences in transcriptional response. As ASR research progresses, there might be other data gaps that need filling to gain a more complete picture of how soybean responds to *P. pachyrhizi*. In either cases, the present study is expected to serve as a foundation for further studies.

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