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ORIGINAL PAPER

Expression patterns in soybean resistant to *Phakopsora pachyrhizi* reveal the importance of peroxidases and lipoxygenases

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Abstract Soybean rust caused by *Phakopsora pachyrhizi* Sydow is a devastating foliar disease that has spread to most soybean growing regions throughout the world, including the USA. Four independent rust resistance genes, *Rpp1–Rpp4*, have been identified in soybean that recognize specific isolates of *P. pachyrhizi*. A suppressive subtraction hybridization (SSH) complementary DNA (cDNA) library was constructed from the soybean accession PI200492, which contains *Rpp1*, after inoculation with two different isolates of *P. pachyrhizi* that result in susceptible or immune reactions. Both forward and reverse SSH were performed using cDNA from messenger RNA pooled from 1, 6, 12, 24, and 48 h post-inoculation. A total of 1,728 SSH clones were sequenced and compared to sequences in GenBank for similarity. Microarray analyses were conducted on a custom 7883 soybean-cDNA clone array encompassing all of the soybean-rust SSH clones and expressed sequence tags from four other soybean cDNA libraries. Results of the microarray revealed 558 cDNA clones differentially expressed in the immune reaction. The majority of the upregulated cDNA clones fell into the functional category of defense. In particular, cDNA clones with similarity to peroxidases and lipoxygenases were prevalent. Downregulated cDNA clones included those with similarity to cell-wall-associated protein, such as extensins, proline-rich proteins, and xyloglucan endotransglycosylases.

Keywords Soybean rust · *Phakopsora pachyrhizi* · Resistance · Immune reaction · Microarray analysis

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Introduction

Soybean rust caused by *Phakopsora pachyrhizi* Sydow is an aggressive foliar fungal pathogen that was first described in Japan in 1902 (Hennings 1903). The pathogen has since spread to most soybean-growing countries in Asia, Africa, and South America (Miles et al. 2003a), and in 2004, it was discovered for the first time in the continental USA (Schneider et al. 2005). Yield losses ranging from 40% to 80% have been reported in countries where this disease has become endemic (Bromfield 1984; Patil and Basavaraja 1997). Fungicides are effective in managing soybean rust; however, the application of fungicides increases production costs (Miles et al. 2003b). In addition, there are concerns about fungicide resistance developing in the pathogen population and the potential negative impact to the

environment if fungicides are applied to large acreage (Sconyers et al. 2006).

Several germplasm screens have been conducted to identify soybean accessions with resistance to *P. pachyrhizi* (Hartman 1996; McLean and Byth 1976; Miles et al. 2006; Mo et al. 1994; Patil and Basavaraja 1997; Singh et al. 1974; Tan et al. 1996; Tschanz et al. 1986). Four single dominant genes, *Rpp1*, *Rpp2*, *Rpp3*, and *Rpp4*, have been described that confer resistance to specific isolates of *P. pachyrhizi* (Bromfield 1984; Bromfield and Hartwig 1980; Hartwig 1986; Hartwig and Bromfield 1983; McLean and Byth 1980). Three infection types have been described on soybean accessions after inoculation with *P. pachyrhizi*: (1) Tan, a susceptible reaction characterized by tan lesions with many uredinia and prolific sporulation, (2) RB, a resistant reaction typified by reddish-brown lesions with few uredinia and little to moderate sporulation, and (3) immune, a resistant reaction with no visible lesions or uredinia (Bromfield 1984; Bromfield and Hartwig 1980). Soybean accession PI 200492, the Japanese cultivar Komata, contains *Rpp1* and produces an immune reaction after inoculation with the *P. pachyrhizi* isolates Australia 79-1, India 72-1, and Hawaii 94-1, while other isolates of *P. pachryhizi*, including Taiwan 72-1, result in a fully susceptible Tan reaction (Bonde et al. 2006; Bromfield and Hartwig 1980; Hartwig and Bromfield 1983). The location of *Rpp1* has been mapped between Sct187 and Sat064 on linkage group (LG) G (Hyten et al. 2007). Recently, the Japanese cultivar Hyuuga was found to produce an RB reaction when inoculated with *P. pachyrhizi* urediniospores collected from field-grown soybean plants and kudzu in Georgia in 2005 (Monteros et al. 2007). The resistance gene in Hyuuga maps between Satt460 and Sat307 on LG-C2 (Monteros et al. 2007). It is not known if the resistance gene in Hyuuga is a new *Rpp* gene or whether it is *Rpp2*, *Rpp3*, or *Rpp4*.

Microscopic observations of susceptible and immune reactions in the cv. Komata (*Rpp1*) did not show any difference in the initial penetration of the fungus through the epidermis (Keogh and Deverall 1980; McLean and Byth 1981). Spore germination percentage, germ tube length, appressoria formation, and cell penetration were not different in the susceptible and immune reactions. However, unlike the susceptible reaction, secondary haustoria did not develop in the immune reaction (Keogh and Deverall 1980). Differences in the rate of lignification and the presence of a papillar structure in a resistant soybean rust interaction have also been reported (Fei et al. 1996).

Although *Rpp1* has been mapped to LG-G, the function of the gene(s) involved is still unknown. Suppression subtractive hybridization (SSH; Diatchenko et al. 1996) has been used successfully in several plant–pathogen interactions to identify changes in host gene expression after infection (Hu et al. 2006; Kurkcuoglu et al. 2007; Lin et al. 2007; Ray et al. 2003; Li and Asiegbu 2004; Verica et al. 2004; Zhulidov et al. 2004). Similarly, microarrays are an ideal method for surveys of gene expression across thousands of genes both known and unknown, giving a broad sample to mine for new resistance candidates (Alkharouf et al. 2006; Golkari et al. 2007; Schenk et al. 2000; Zabala et al. 2006). In this study, we constructed SSH complementary DNA (cDNA) libraries from messenger RNA (mRNA) extracted from *Rpp1*-susceptible and immune reactions and used cDNA microarrays to identify transcripts that are differentially expressed in the immune reaction at 6, 12, 24, and 48 h post-inoculation (hpi).

Materials and methods

Biological materials

The soybean cvs. Komata (USDA germplasm accession PI200492) and Williams were grown two per 10.2-cm-diameter clay pot filled with Sunshine LC1 mix (Sun Grown Horticulture Products, Belleview, WA) in a greenhouse under natural light. Urediniospores of the two *P. pachyrhizi* isolates Taiwan 72-1 (TW72-1) and Hawaii 94-1 (HW94-1) were harvested approximately 14 days after inoculation onto the soybean cv. Williams using a mechanical harvester (Cherry and Peet 1966) and stored in liquid nitrogen at the USDA-ARS Foreign Disease-Weed Science Research Unit Biological Safety Level-3 Plant Pathogen Containment Facility at Fort Detrick, MD, USA (Melching et al. 1983) under the appropriate USDA Animal Plant Health Inspection Service permit. Before inoculating soybean plants, urediniospores were removed from liquid nitrogen, heat shocked at 40°C for 5 min, and hydrated at 100% relative humidity at room temperature for 16 h. Spores were suspended in sterile distilled water containing 0.01% (*v*/*v*) Tween 20 and adjusted to a concentration of 2×10^4 spores ml^{-1} with a hemacytometer. Twenty-two-day-old plants of the soybean cv. Komata were inoculated at the V2 growth stage (Fehr and Caviness 1977) with 2 ml per plant of either *P. pachyrhizi* isolate HW94-1 or isolate TW72-1 using an atomizer attached to an air compressor at 20 psi. Mock inoculations were conducted by inoculating soybean cvs. Komata and Williams plants with distilled water/Tween 20 as described above. In addition, control inoculations were performed with the two *P. pachyrhizi* isolates onto the susceptible soybean cv. Williams to verify infection and virulence of both isolates. Plants were incubated in a dew chamber at 20°C overnight (approximately 16 h) and placed in a greenhouse at 20°C to 25°C under a 16-h photoperiod. Supplemental lighting was provided using 1,000-W Metalarc lights (Sylvania, Danvers, MA, USA). For

the soybean-rust SSH library, trifoliates were pooled from two plants at 1, 6, 12, 24, and 48 hpi, immediately frozen in liquid N_2, and stored at −80°C. For the microarray hybridization probes, leaflets were pooled from four to six plants at each time point in each of three independent inoculations. The 1 hpi sample was omitted from the microarray study to keep the experiment to a manageable size.

RNA extraction

Total RNA was extracted from soybean leaflets using a Trizol/guanidinium isothiocyanate protocol (Chomczynski and Sacchi 1987) with an additional lithium chloride purification step. For the SSH library construction, 20 μg of RNA from each of the time points were pooled (100 μg total), incubated with 16 units of DNaseI (Ambion, Austin, TX, USA) for 30 min at 37°C and purified using the RNeasy mini protocol (Qiagen, Valencia, CA, USA) according to manufacturers' directions. The mRNA was isolated using an Oligotex mRNA kit (Qiagen) following the manufacturer's protocols. All purified RNA was quantified using a spectrophotometer (BioRad Laboratories, Hercules, CA, USA) and separated on a 1.2% (*w*/*v*) agarose-formaldehyde gel to verify integrity.

Soybean-rust SSH library

SSH libraries were constructed using the polymerase chain reaction (PCR)-select cDNA subtraction kit (BD Biosciences Clonetech, Palo Alto, CA, USA) according to the manufacturer's protocol with minor modifications. The first-strand cDNA synthesis was performed using 3 μg of mRNA. The forward library's tester was made using mRNA from cv. Komata inoculated with *P. pachyrhizi* isolate HW94-1 (immune reaction), and the driver was prepared using mRNA extracted from cv. Komata inoculated with *P. pachyrhizi* isolate TW72-1 (susceptible reaction). The reverse subtraction library was constructed as above except that the mRNA used for the tester and driver was reversed. A control subtraction was performed using skeletal muscle cDNA provided in the kit. The cDNA fragments were ligated into the vector pT7Blue using a Perfectly Blunt cloning kit (Novagen, San Diego, CA, USA) and transformed into Novablue Singles Competent Cells (Novagen) following the manufacturer's protocol.

DNA sequencing and data analysis of soybean-rust SSH library

Before sequencing, all white colonies were checked for the presence of an insert by colony-PCR using the SP6 and T7 promoter primers. PCR products were separated by electrophoresis using 1.5% agarose gels. Clones from the forward subtraction and reverse subtraction libraries were sent to the USDA Agricultural Research Service (ARS), Eastern Regional Research Center, Nucleic Acids Facility in Wyndmoor, PA, USA for plasmid preparation and sequencing. DNA was prepared for sequencing reactions using a Qiagen BioRobot 9600 and a Beckman Biomek 2000. Single-pass sequencing was performed from the 5′ end using the plasmid DNA as template and either the T7 promoter primer or the M13 reverse primer using an Applied Biosystems (ABI; Foster City, CA, USA) PRISM big dye terminator kit and an ABI 3700 DNA analyzer. Nucleotide sequences were manually edited using Chromas v2.23 (Technelysium Pty Ltd., Helensvale, Australia). An in-house script "Scanseq" was used to identify redundant expressed sequence tags (ESTs) based on nucleotide overlap >50 nt, with e-value $\leq 10^{-5}$, reducing the number to 979 and 555 low-redundant ESTs (EST^{lr}) from the forward and reverse subtraction libraries, respectively. ESTs were identified by comparing their predicted amino acid sequences to the Entrez Protein database (http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=search&db=protein) using the BLAST X search algorithm (Altschul et al. 1997) as part of the Genetics Computer Group computer software package (version 10.3, Accelrys, San Diego, CA, USA) at the Advanced Biomedical Computing Center of the National Cancer Institute, Frederick, MD, USA. ESTs that achieved e-values $\leq 10^{-3}$ were retained for further analysis, and ESTs without similarity to any proteins in the database at the e≤ 10^{-3} cutoff were annotated as "unknown." Corresponding Uniprot identifiers were queried using PIR-PSD (Wu et al. 2004, http://pir.georgetown.edu/) to determine the Gene Ontology biological process identifier (Ashburner et al. 2000). The ESTs were classified into functional categories based on their Uniprot and PFam annotations following the rationale detailed in Table 1, which was adapted from Zabala et al. (2006).

cDNA microarray clones and microarray fabrication

Inserts from the SSH libraries were amplified by PCR using the primers U19 and T7 in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) in a 50-μl reaction containing approximately 5 ng of the purified plasmids, 1.5 mM $MgCl_2$, 0.2 μM each primers, 0.02 U *Taq* in a 1× concentration reaction buffer (Promega, Madison, WI, USA). Cycling conditions were 94°C denaturation for 2 min, 35 cycles at 94°C for 45 s, 55°C for 45 s, 72°C for 2 min, followed by an extension at 72°C for 7 min. PCR products were precipitated with isopropanol, centrifuged and resuspended in 6 μl 50% (*v*/*v*) dimethyl sulfoxide (DMSO)/Tris-EDTA (TE) to a final concentration of 80–200 ng/μl. PCR products were checked by gel electrophoresis to identify clones that produced no or multiple

Table 1 Description of categories used to separate genes found to be differentially expressed by custom microarray analysis of immune vs. susceptible soybean leaves inoculated with *P. pachryhizi* and sampled at four times after inoculation into functional groups

Category	Selection criteria[a]	Examples	Number of genes	Percent
Cell growth and maintenance	Associated with cell wall structure, protein synthesis, cell division, cytoskeleton, membrane-related	Actin binding proteins, lipases	102	9
Defense	Defense and stress-related, including abiotic stress, pathogenesis related (PR) proteins, protease inhibitors, elicitor-induced, apoptosis or HR related, R gene homologs, and secondary metabolites known to be involved in defense	Heat shock proteins, PR 10, b-glucosidase, isoflavone reductase, phenylalanine ammonia lyase	282	25
Energy	Photosynthesis and electron transport	Protein disulfide isomerase, chlorophyll a/b binding protein	34	3
Metabolism	Use of carbohydrates, amino acids, and nucleotides nucleotide synthesis, amino acid metabolism	Serine carboxy peptidase, ubiquitin conjugating enzyme	35	3
Oxidation	Related to oxidative stress	Lipoxygenase, catalase	37	3
Signaling	Signal transduction-related	Kinases, phosphatases, Leucine Rich Repeat	25	2
Transcription	Related to transcription	Transcription factors, zinc finger-containing, DNA binding	43	4
Other	Do not fit into the other categories, or fit into too many categories	Kinases, phosphatases, ion channels, transporters	114	10
Unknown	Matched a hypothetical or unknown gene, biological function unknown	Hypothetical protein P0620H05.6, protein E6	440	40

[a] Transcripts were classified based upon their Gene Ontology terms (Ashburner et al. 2000) available from The Arabidopsis Information Resource (Rhee et al. 2003), by inspection of their UniProt record (Wu et al. 2006), Pfam record (Bateman et al. 2004), or by motifs identified in the Conserved Domain Database (Marchler-Bauer et al. 2005).

amplicons. Only those with single PCR products were randomly re-racked into 384-well plates and stored at 4°C until printing.

Each of the 1,728 clones from the forward and reverse SSH libraries along with 6,155 ESTs from other soybean EST libraries were printed on each microarray slide (7,883 total). We realized that the 1,728 SSH clones produced for this study would not fill the microarray, and so we were able to include additional EST libraries that had been produced for other projects and were available in the laboratory. The clones from additional cDNA libraries used in assembling the microarray were as described by Alkharouf et al. (2006). Briefly, one library was constructed from cvs. Peking and PI437654, soybean genotypes resistant to soybean cyst nematode (*Hetrodera glycines*) population NL1-RHp. A cv. Peking Lambda ZAP II cDNA library was made from the roots and shoots of cv. Peking plants, 48 h after nematode infection as described (Alkharouf et al. 2004). Two suppressive subtraction libraries prepared from roots of PI437654, 24 h after nematode inoculation followed by a 10-h infection period, were obtained from Clemson University Genomics Institute and were constructed as described in (Tomkins et al. 1999). cDNA clones from soybean roots obtained from E.I. Dupont de Nemours, Newark, DE, USA were also used in the experiment and were described in Alkharouf et al. (2006).

Clones in the cv. Peking cDNA library duplicating those in the SSH library were identified by pairwise comparison of each clone against the EST database housed at the USDA-ARS Soybean Germplasm Improvement Laboratory, Beltsville, MD, USA (http://psi081.ba.ars.usda.gov/SGMD/Default.htm) and then removed, reducing the total number of cDNAs in the experiment. The insert from each clone was amplified by PCR using a PTC 225 thermocycler (MJ Research, Waltham, MA, USA) with the T3 and T7 universal primers in a 100-µl reaction similar to the protocol of Hegde et al. (2000). The amplified product from each clone was checked on a 1% agarose/1× Tris–borate–EDTA gel for the presence of a single band and brought to a final volume of 5 ml in 50% DMSO/50% TE solution. The clones from all libraries were randomized and re-racked before printing. A total of 7,883 PCR products were printed simultaneously in triplicate onto CMT-GAPS coated slides (Corning, NY, USA) using a Cartesian robot model PixSys 5500 PA workstation with a telechem printing head and stealth quill pins. A total of 30 slides were printed and UV crosslinked at 50 mJ/cm^2 using a UV Stratalinker 2400 (Stratagene, Cedar Creek, TX, USA).

Fluorescent probe preparation

Trifoliates were harvested from at least four plants per treatment (mock inoculation, immune reaction, and susceptible reaction) per time point (6, 12, 24, and 48 hpi). Total RNA (20 μg) from each sample was reverse-transcribed using SuperScript Indirect cDNA Labeling system (Invitrogen, Carlsbud, CA, USA) to incorporate the amino-modified nucleotides as recommended by the manufacturer. After purification, the cDNA was split equally into two tubes and labeled with either Alexa Fluor Fluorescent dye AF555 or AF647 (Molecular Probes, Eugene, OR, USA). Probes were labeled from all three biological replicates, and the absorption at 550 and 650 nm was measured using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The percentage of fluorescent dye incorporation and amount of labeled probe was calculated using the NanoDrop ND-1000 software. The labeled probes were stored at −20°C until use.

Microarray hybridization

The microarray slides were pre-washed in 0.1% (*w*/*v*) sodium dodecyl sulfate (SDS) in Coplin jars on a shaker for 1 h at room temperature (RT), rinsed in sterile water, washed in isopropanol, and dried by centrifugation at 600 rpm. Equal amounts of AF555- and AF647-labeled probes were denatured at 95°C for 3 min and added to BM hybridization buffer [5× saline-sodium citrate (SSC), 0.5% BDH Casein Hammarsten, 0.1% *N*-Lauroyl Sarcosine, 0.02% SDS, 1.0 mM dithiothreitol (DTT)], with 0.6 μg poly(A), kept at 60°C, and applied to the array under a 22× 60 mm lifter coverslip (Erie Scientific, Portsmouth, NH, USA). Slides were placed in sealed hybridization chambers containing 50 μl water and incubated at 60°C for 48 h. After hybridization, the slides were washed in 1× SSC/0.2% SDS for 10 min at 42°C, 0.1× SSC/0.2% SDS for 4 min at RT, 0.5× SSC for 10 s at RT, 0.1× SSC for 3 min at RT and dried by centrifugation at 600 rpm. A total of 30 slides were hybridized, ten for each biological replicate (inoculation experiment). For each biological replicate, there were five time points (mock, 6, 12, 24, and 48 hpi), and the corresponding dye swaps with AF555 or AF647.

Scanning and microarray data analysis

The microarrays were scanned for either AF555 or AF647 at 10-μm resolution using a ScanArray 4000 (GSI Lumonics, Meriden, CT, USA). Fluorescence intensities were extracted from the scanned images using the image processing software package SPOT (http://www.hca-vision.com/product_spot.html). Background subtraction was performed before calculating ratios. Only spots with an intensity of at least 2.0 times above the local background in both channels were used for subsequent analysis. This filtering process usually removed 1% to 10% of the elements. In addition, chimeric and redundant clones identified by sequencing were filtered out and not used in the microarray expression analysis. The extracted data from each slide was then $\log_2$-transformed and normalized using the Lowess print-tip group normalization method (Yang et al. 2002).

Potential artifacts and false positives were eliminated by selecting only for those clones that exhibited similar expression patterns between the original hybridization and the corresponding dye swap (Yang et al. 2002). These clones were determined by one-way analysis of variance (1-ANOVA) tests, which were used to detect similar expression levels across replicated slides and across the three biological samples. Only clones with an F value high enough to produce a probability of 0.1 or higher were selected as being similar across biological replicates. Structured query language (SQL) procedural scripts were written to perform t tests and 1-ANOVA tests for every clone post-filtration and normalization.

The Student's t test was used to calculate t values on $\log_2$ expression ratios to identify genes with statistically significant expression ratios. Self–self hybridized slides with equal amounts of AF555 or AF647-labeled RNA from uninfected cv. Komata (K-/K-) were used as control groups for the t test. The clones printed in K-/K- showed a ratio of −1.20 to 1.20 after Lowess print-tip normalization.

The t tests were used to analyze the expression of each clone in immune/susceptible (K:HW94–1/K:TW72-1) hybridizations using background corrected and normalized expression ratios. The results of the t tests were used to determine statistical significance ($p \leq 0.05$) of gene expression. A gene was considered differentially expressed if the t test p value was <0.05 and the average fold change for both replicates was >2.0. If the gene expression passed the t test, then a cutoff value of twofold was applied for extra stringency. The high stringency of selection kept the possibility of false positives to a minimum but at the same time increased the likelihood of false negatives. This statistical method takes into account the variability within slides and between replicated slides as well as biological samples to distinguish gene expression changes caused by treatments from gene expression changes attributable to biological and measurement variability.

SQL and *onl*ine *a*nalytical *p*rocessing (OLAP; Codd et al. 1993; Alkharouf et al. 2005) were used to produce lists of differentially expressed genes in each of the time points. In addition, the clustering function within Analysis Services (Microsoft, Redmond WA, USA) was used to find distinct expression profiles in the differentially expressed genes. A web-based user interface was developed to query the data

and post the raw and normalized data sets. The data can be accessed at http://bioinformatics.towson.edu/SGMD/MicroarrayExps/soyrust.htm.

Quantitative real-time RT-PCR analysis

Quantitative real-time reverse transcriptase PCR (RT-PCR) was carried out on total RNA from cv. Komata leaves harvested at 6, 12, 24, and 48 hpi with *P. pachyrhizi* isolates TW72-1 or HW94-1 using a Smart Cycler (Cepheid, Santa Clara, CA, USA). Four genes analyzed by RT-PCR were chosen to be representative of the differentially expressed genes. The selected genes include two defense-related genes, starvation-associated message 22 (SAM-22) and anthocyanidin synthase (ANS); a housekeeping gene, nitrate transporter 1 (NRT1–5); and an unknown gene. Ten nanograms of the same RNA that was used to create probes for the microarray analysis was used as the template for one-step RT-PCR using the Quantitect SYBR Green One Step RT-PCR kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Gene-specific primers were designed using Primer3 software (Rosen and Skaletsky 2000; http://primer3.sourceforge.net/; Table 2). Each sample was tested in triplicate for all primers. Melting curve analysis was performed on all samples to ensure amplification of a single product with the expected melting temperature and the absence of primer-dimers. The products of each primer set were tested by agarose gel electrophoresis to verify that a single product of the expected size was produced. Relative RNA quantities were determined using the ΔΔCt method (Pfaffl 2001; Pfaffl et al. 2002) by comparing the data for each gene of interest with the data for mock-inoculated control samples at each time point. The data was normalized by comparison to mock-inoculated samples.

Table 2 List of primers used in real-time RT-PCR to confirm differentially expressed genes

Genbank ID	Gene Name[a]	Orientation	Primer sequence (5′–3′)
CA851291	SAM-22[b]	Forward	TGGCTCCTGCTACCCTTTAC
		Reverse	GCTTTCTCCATCCTCAACGA
CA851866	Unknown protein	Forward	CTACGGACAAGCCACTCACA
		Reverse	CTCAACCCCATCAAAACCAG
CA851960	ANS[c]	Forward	AACCTGAAAGAAACCCCATTC
		Reverse	CACCAAAGTCCCAGAACGAT
CA852155	NRT1–5[d]	Forward	GTTCTGGTTGGTGCCTCAGT
		Reverse	GTAGCGGTGCTTCTCATGCT

[a] The microarray probes and corresponding RT-PCR primers were based on ESTs. Gene names were assigned based on homology to *Glycine max* proteins in the Entrez protein database.
[b] Starvation associated message 22
[c] Anthocyanidin synthase
[d] Nitrate transporter 1

Results

Sequencing and characterization of SSH library clones

A total of 1,056 and 672 clones from the forward and reverse SSH libraries, respectively, were sequenced. The size of the cDNA inserts ranged from 52 to slightly >600 bp. No full-length transcripts were obtained. Multiple inserts were identified in 14.7% of the clones in the forward SSH library and 12.1% of the clones in the reverse SSH library. The in-house program Scanseq was used to detect redundant sequences within the forward and reverse SSH libraries, and low-redundancy subsets (EST^{lr}) of 979 ESTs from the forward and 555 from the reverse libraries were identified. The two most abundant ESTs observed in the forward SSH library shared similarity with the "putative senescence associated protein" from pea [dbj|BAB33421.1] and "hypothetical protein" [dbj|BAF01000.1] from *Arabidopsis*, having 28 and 14 copies, respectively. The most abundant ESTs from the reverse library shared similarity with "putative senescence associated protein" from pea [dbj|BAB33421.1|] with eight copies and "NADH-dependent hydroxypyruvate reductase" [gb|AAO73867.1|] from soybean with five copies. The sequences of the EST clones were submitted to NCBI as dbEST IDs 34850316–34851929 and GenBank accession nos. DW246150–DW247763. Clones containing multiple inserts were assigned individual accession numbers for each insert.

When queried against the NCBI EST database, 94% of the clones from the forward SSH library EST^{lr} with e-value $\leq 10^{-6}$ shared identity with *Glycine max*, 5% shared identity with other species in the Fabaceae (legume) family (*Glycine soja*, *Glycine clandestine*, *Medicago*, *Phaseolus*, and *Lotus*), and 1% shared identity with other organisms. Of the EST^{lr} with e-value $\leq 10^{-6}$ in the reverse SSH library, 90% of the clones shared identity with *G. max*, 8% shared identity with other Fabaceae (*G. soja*, *G. clandestine*, *Medicago*, *Phaseolus*, *Lotus*, *Cajanus* spp.), and 2% shared identity with other organisms.

Differential gene expression

The SSH library was made by pooling RNA from five time points (1, 6, 12, 24, and 48 hpi) before subtraction. We focused on the earliest stages of the host–pathogen interaction, believing this time frame best suited for identifying genes important for the immune response. We were expecting a small number of ESTs after subtraction but instead recovered 1,728 clones. This prompted us to make a custom cDNA microarray from the ESTs to both confirm changes in gene expression observed in the SSH library and to determine when the changes occurred.

The custom cDNA microarray was used to assess the expression of the ESTs in immune and susceptible reactions at 6, 12, 24, and 48 hpi. In addition to the SSH ESTs, cDNAs from four other soybean libraries (Khan et al. 2004; Alkharouf et al. 2006) were included on the array for a total of 7,883 cDNA inserts. Boxplots were used to assess variation across three biological replicates of the microarray analysis (Fig. 1). Replicate 2 showed abnormally high levels of background fluorescence, as indicated by the wider range of values observed in the boxplot. This resulted in excluding almost all differentially induced genes when all three replicates were used in the analysis. The reason for this is not due to biological variation but rather due to experimental variation resulting from operator error. Replicate 2 was subsequently removed from the study to provide greater sensitivity to the analysis. Removing one biological replicate results in a reduction in statistical power, but as the ESTs are represented in triplicate on each microarray, it was acceptable to lose one replication of the arrays to ensure the highest quality of data. Therefore, all of our analysis reported in this study is from two biological samples in which there was at least a twofold change in the gene expression ratio of immune vs. susceptible reactions.

A total of 558 ESTs were differentially expressed over all the time points at $p \leq 0.05$. Of these, 300 ESTs were upregulated and 258 were downregulated in the immune reaction. At 6 hpi, 56 ESTs were upregulated in the immune reaction, whereas approximately 80 ESTs were upregulated at each of the other time points. Similarly, 40 ESTs were downregulated in the immune reaction at 6 hpi, and approximately twice as many were downregulated at 12 and 24 hpi (Table 3).

The other functional categories appeared in both up- and downregulated gene list and usually at more than one time point. Oxidation-related genes were upregulated at 6 hpi (lipoxygenase) and 24 hpi (superoxide dismutase, peroxidase ATP8a, and lipoxygenase) and also downregulated at 6 hpi (thyroid-stimulating hormone beta subunit, bell-like homeodomain protein 2) and 48 hpi [Zinc finger C3HC4-type RING, Homeobox protein knotted-1 like 3 (KNAT3), and WD-40 repeat family protein]. Genes related to transcription, including transcription factors and DNA binding proteins, were upregulated at 12 hpi (NtWRKY4) and 24 hpi (Myb-related transcription activator) and downregulated at 24 hpi (putative WRKY-like protein; Table 4). Genes involved in cell growth and maintenance were downregulated at 6 hpi (ribulose-1,5-bisphosphate carboxylase, small nuclear ribonucleoproetin E homolg, and nam-like protein), 24 hpi (cyclin family protein), and 48 hpi (ribosomal protein L12 family protein and ribulose bisphosphate carboxylase small chain 1; Table 4).

Clustered mean expression profiles of ESTs sharing similar expression ratio variation over time are depicted in Fig. 3, and the corresponding genes from each of these profiles are listed in Table 5. The first profile (profile 1 in Fig. 3, Table 5) consists of ESTs downregulated at 6 and 12 hpi and upregulated at 24 and 48 hpi. This profile features ESTs that gradually increase expression over time. Members of this group have similarity to peroxidases and other proteins involved in oxidoreductase activity, such as putative senescence-associated protein. Cryptochrome 1 and pathogen-related protein, both involved in defense, were represented, as were bZIP and AP2-domain transcription factors. ESTs involved in the regulation of translation included translation initiation factor, ribosomal protein L1,

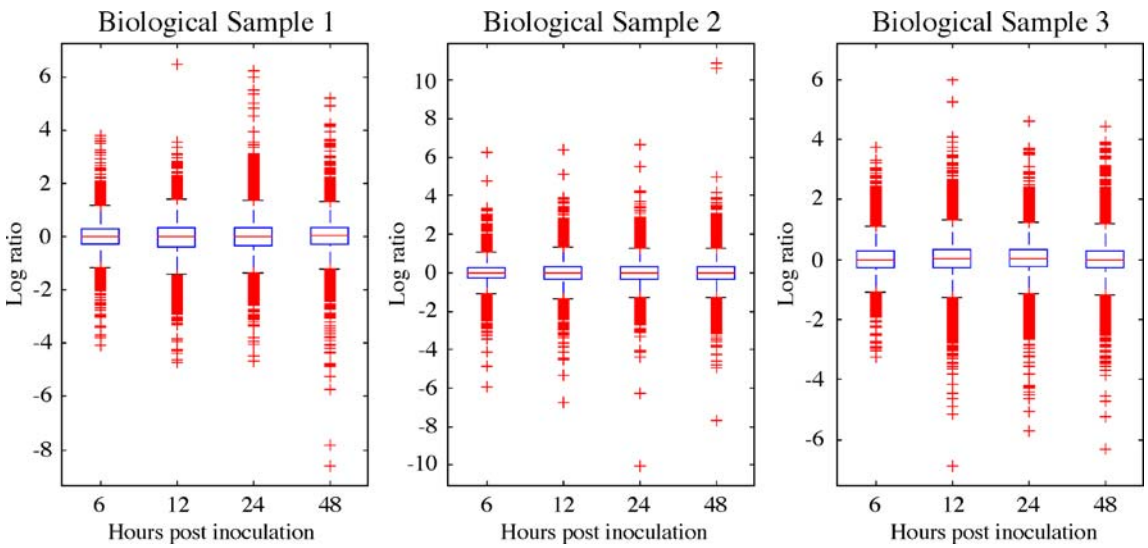

Fig. 1 Box plots showing variation in $\log_2$ expression ratios of immune vs. susceptible soybean inoculated with *P. pachyrhizi* and sampled at four time points after inoculation. Aggregate data of 6, 12, 24, and 48 h post-inoculation is shown for three biological replicates of the inoculation

Table 3 Number of EST clones found to be differentially expressed by custom microarray analysis of immune vs. susceptible soybean leaves inoculated with *P. pachyrhizi* and sampled at four times after inoculation

Library[a]	6 hpi		12 hpi		24 hpi		48 hpi		Total	
	Up	Down	Up	Down	Up	Down	Up	Down	Up	Down
Forward SSH	6	6	22	4	2	15	12	3	42	28
Reverse SSH	6	1	0	2	5	0	1	3	12	6
Other cDNA clones	44	33	60	74	76	59	66	58	246	224
Total	56	40	82	80	83	74	79	64	300[b]	258[c]

[a] EST collections from multiple sources were assembled onto one microarray (see "Materials and methods" for details).
[b] Twenty-three EST clones showed significant differential expression at two time points. Nine of the EST clones were upregulated at both time points, while 14 EST clones were upregulated at one time point and downregulated at another time point.
[c] Thirteen EST clones showed significant differential expression at two time points. Five of the EST clones were downregulated at both time point, while eight EST clones were upregulated at one time point and downregulated at another time point.

26S ribosomal RNA, and putative ATP-dependent RNA helicase. There were also 11 ESTs in this profile with unknown/no significant hits to the database.

The strength of microarray analysis is in its ability to reveal similarities in expression patterns of groups of genes, rather than in pinpointing specific expression changes for individual genes. Particularly with an array produced from ESTs, the identity and behavior of an individual spot on the array has a high degree of uncertainty, while multiple observations of similar behavior in a group of functionally related genes are far more informative (Dardick 2007). For this reason, the differentially expressed ESTs from all libraries were classified into functional groups based upon similarity to known sequences in the NCBI databases (Fig. 2). A list of selected genes from each of the EST libraries at 6, 12, 24, and 48 hpi is presented in Table 4. Several functional categories appeared at multiple time points and in both the up- and downregulated sets of genes. Defense was the most represented functional category, upregulated at 6 hpi (Bax inhibitor, caffeic acid *O*-methyltransferase, and phenylalanine ammonia lyase), 12 hpi (putative cinnamoyl-CoA reductase, β-xylosidase, Bax inhibitor, chalcone synthase 2, and heat shock transcription factor 21), 24 hpi (elicitor-inducible gene product EIG-I24 and pectinesterase), and 48 hpi [NAD(P)H dependent 6′-deoxychalcone synthase, naringenin-chalcone synthase, cytochrome P450, and 6a-hydroxymaaciain methyltransferase] (Table 4). Some defense genes were also downregulated at all time points, including xyloglucan endotransglycosylase I at 6 hpi; DnaJ protein-like, LRR receptor protein kinase, and heat shock transcription factor HSF1 at 12 hpi; heat shock transcription factor homolog T32G6.21, putative receptor-like protein kinase, and PR-4 typr protein at 24 hpi; and cytochrome P450, MYB-related protein B, and elicitor response element WRKY3 at 48 hpi (Table 4).

ESTs in the second profile (profile 2 in Fig. 3, Table 5) are downregulated at 6 hpi and continue to be downregulated over time in the immune reaction. Many of the ESTs in this group are predicted to be involved in the biogenesis of cell wall, for example, extensins, xyloglucan endotransglycosylases, and repetitive proline-rich proteins.

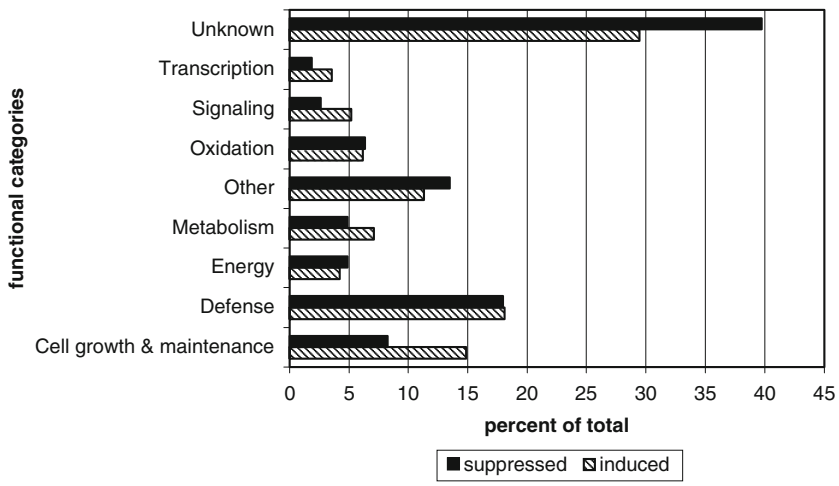

Fig. 2 Functional classification of differentially expressed genes in the immune vs. susceptible reaction of soybean cv. Komata to infection by *P. pachyrhizi* based upon homology search results

Table 4 A representative selection of transcripts found to be differentially expressed by custom microarray analysis of immune vs. susceptible soybean leaves inoculated with *P. pachyrhizi* and sampled at four times after inoculation

Clone ID	GenBank ID	Description	Fold Change	Function[a]	*p* Value ≤
6 h post-inoculation					
Upregulated transcripts from the SSH libraries					
JC1_A06	GMU39567	Ribulose-1, 5-bisphosphate carboxylase	2.78	Cell growth/maintenance	0.001
RJC6_F07	AY114060	Protein phosphatase PPH1	2.45	Signaling	0.05
JC3_F05	AY380778	Bax inhibitor	2.15	Defense	0.05
RJC6_B02	AF375966	Bell-like homeodomain protein 2	2.03	Signaling	0.05
JC7_A02	AY618874	Thyroid stimulating hormone β-subunit	2.02	Unknown	0.05
Upregulated transcripts from all other libraries					
E08F09	CA852517	Galactoinase-like protein	3.53	Metabolism	0.05
DUP04B06	S22991	Phenylalanine ammonia lyase 1	3.19	Defense	0.05
E02G02	CA852073	TMV resistance protein-like	2.89	Defense	0.05
A01F17	BM107833	Ripening regulated protein DDTFR10	2.77	Transcription	0.05
DUP07A09	AJ250833	Germin-like protein	2.67	Oxidation	0.05
D11G09	CA851257	DNA-directed RNA polymerase	2.64	Cell growth/maintenance	0.05
C01E06	BM107732	WD-40 repeat family protein	2.50	Signaling	0.05
DUP05H08	Y09292	Obtusifoliol 14-α-demethylase, CYP51	2.17	Defense	0.05
A12B07	BM108290	Nitrilase 1-like protein	2.13	Other	0.05
E07A05	CA852371	Sali3-2 protein	2.12	Defense	0.005
DUP02G06	AB025926	Bax inhibitor-1	2.04	Defense	0.05
DUP01C01	U84198	Lipoxygenase	2.02	Oxidation	0.05
Downregulated transcripts from the SSH libraries					
RJC6_G10	AF509873	NAM-like protein 10	−3.46	Cell growth/maintenance	0.05
JC1_B12	GMU39567	Ribulose-1, 5-bisphosphate carboxylase	−3.40	Cell growth/maintenance	0.05
JC2_F08	AY316737	VDAC1.1, ion channel	−3.12	Other	0.05
JC11_A07	P24715	Small nuclear ribonucleoprotein E	−2.05	Cell growth/maintenance	0.05
Downregulated transcripts from all other libraries					
DUP27B06	U51192	Peroxidase precursor	−2.46	Oxidation	0.05
B13E04	CA853891	Disease resistance protein-like	−2.43	Defense	0.05
D18D08	CA851866	Probable 12-oxophytodienoate reductase CPRD8	−2.41	Defense	0.05
DUP09E01	AJ130885	Xyloglucan endotransglycosylase 1	−2.38	Defense	0.05
DUP17C03	U51193	Peroxidase	−2.33	Oxidation	0.05
B05A11	CA853165	Ripening-related protein, hydrolase	−2.21	Other	0.05
DUP22F01	U82367	UDP-glucose glucosyltransferase	−2.03	Metabolism	0.05
12 h post-inoculation					
Upregulated transcripts from the SSH libraries					
JC2_G02	AC149302	Putative cinnamoyl-CoA reductase	2.82	Defense	0.05
JC8_G06	P17957	Chalcone synthase 2	2.73	Defense	0.05
JC5_A07	AY428810	Notchless-like protein	2.49	Signaling	0.05
JC3_F05	AY380778	Bax inhibitor	2.40	Defense	0.05
JC2_G05	AY486104	β-xylosidase	2.39	Defense	0.05
JC8_G10	DW355532	Aldehyde dehydrogenase (NAD)	2.15	Metabolism	0.05
JC4_C11	NM_124313	BXL1 β-xylosidase 1, hydrolase	2.02	Defense	0.001
JC4_D11	PVU57389	Transcription repressor ROM1	2.00	Transcription	0.05
Upregulated transcripts from all other libraries					
E11D11	CA852746	Avr9/Cf-9 rapidly elicited protein 146	2.92	Defense	0.05
B06B08	CA853259	SMT3 protein homolog, ubiquitin-like protein	2.85	Metabolism	0.05
D16F06	CA851704	β-amylase/substilin inhibitor (RASI)	2.64	Other	0.05
D03D10	CA850564	Cherry-allergen PRUA1	2.48	Defense	0.05
D06A06	CA850739	WOX4 protein	2.34	Signaling	0.05
A09B02	BM108095	Sali3-2 protein	2.14	Defense	0.05
DUP20A06	Z46952	Heat shock transcription factor 21	2.12	Defense	0.05
DUP25A04	D16455	Endo-xyloglucan transferase	2.12	Defense	0.05
D14E02	CA851506	Signal recognition particle SRP54	2.09	Metabolism	0.05
DUP17E03	BAA86031	WRKY4	2.05	Transcription	0.05
E04A02	852155	NTR1-5	1.98	Cell growth/maintenance	0.05

Table 4 (continued)

Clone ID	GenBank ID	Description	Fold Change	Function[a]	p Value ≤
Downregulated transcripts from the SSH libraries					
RJC3_B11	AY085227	DnaJ protein-like	−2.61	Defense	0.05
JC6_C05	AP004053	Putative succinyl-CoA ligase	−2.40	Energy	0.05
JC6_H12	NM_116051	Splicing factor RSP31	−2.24	Other	0.05
JC10_G07	P16059	Oxygen-evolving enhancer protein 2 (OEE2)	−2.02	Energy	0.05
Downregulated transcripts from all other libraries					
SSH5B07	BM139836	2-Hydroxydihydrodaidzein reductase	−5.28	Defense	0.05
DUP09A01	AF022459	Cytochrome P450 monooygenase	−2.71	Defense	0.05
DUP14E04	S52641	Heat shock transcription factor HSF1	−2.30	Defense	0.05
SSH4F02	BM139785	Cell death associated protein	−2.26	Defense	0.05
DUP02H12	AC005170	LRR receptor protein kinase	−2.15	Defense	0.05
E10E11	CA852673	NADH dehydrogenase (ubiquinone)	−2.14	Metabolism	0.05
D17E05	CA851783	Chalcone isomerase	−2.07	Defense	0.05
DUP12G07	AC006300	Dioxygenase	−2.07	Oxidation	0.05
DUP24H12	JQ2344	Catechol *O*-methyltransferase III	−2.05	Defense	0.05
24 h post-inoculation					
Upregulated transcripts from the SSH libraries					
RJC6_E05	AC069159	Unknown protein F14G9.17	3.40	Unknown	0.05
RJC6_C12	AJ001772	Glucose-6-phosphate dehydrogenase	2.15	Energy	0.05
Upregulated transcripts from all other libraries					
SSH5D10	BM139868	Steroid 5-α-reductase family protein	4.30	Metabolism	0.05
B04F07	CA853132	Superoxide dismutase	3.54	Oxidation	0.05
DUP17H11	AC001645	Myb-related transcription activator	3.09	Transcription	0.05
A10E05	BM108215	Metallothionein 1A	2.65	Signaling	0.05
DUP20G07	T10825	Auxin-induced protein, GST family	2.42	Defense	0.05
A01N21	BM107914	Elicitor-inducible EIG-I24	2.37	Defense	0.05
D12E11	CA851330	Pectinesterase	2.26	Defense	0.05
D06H08	CA850823	Cytochrome P450 (CYP93 A1)	2.16	Defense	0.05
DUP11E10	X94945	Lipoxygenase (LOX)	2.15	Oxidation	0.05
DUP09D06	X98855	Peroxidase ATP8a	2.11	Oxidation	0.05
Downregulated transcripts from the SSH libraries					
JC2_F12	AP004168	Putative nucleolar essential protein	−2.87	Cell growth/maintenance	0.05
JC2_F03	NP_701668	Asparagine-rich protein	−2.38	Unknown	0.05
JC3_E08	NM_116156	Peptide chain release factor	−2.33	Other	0.05
JC3_B12	NM_124239	Cyclin family protein	−2.27	Cell growth/maintenance	0.05
JC3_D11	NM_105747	C2 domain-containing protein	−2.16	Unknown	0.05
Downregulated transcripts from all other libraries					
DUP12E08	L10081	Cytochrome P-450	−5.32	Defense	0.05
DUP07D11	AC005312	Receptor-like protein kinase, LRR	−4.67	Defense	0.05
E13C11	CA852899	Senescence-associated protein	−2.77	Defense	0.05
DUP25B03	AJ012693	Basic blue copper protein	−2.49	Defense	0.05
E07C11	CA852399	Abscisic stress ripening protein	−2.42	Defense	0.05
DUP19E03	AF061329	PR-4 type protein	−2.26	Defense	0.05
D14H09	CA851547	Heat shock transcription factor	−2.12	Defense	0.05
DUP21G08	AC010797	WRKY-like protein	−2.11	Transcription	0.05
DUP05B04	AJ223151	*O*-methyltransferase	−2.08	Defense	0.05
D12B07	CA851291	SAM-22	−1.82	Defense	0.05
48 h post-inoculation					
Upregulated transcripts from the SSH libraries					
JC3_D09	NM_100370	Sucrose-phosphate synthase	2.45	Metabolism	0.05
JC3_B05	NM_125017	Zinc finger (RING finger) family	2.36	Signaling	0.05
JC3_C11	NM_122431	Homeobox protein, knotted-1 like 3 (KNAT3)	2.32	Signaling	0.05
JC9_C12	AY058141	Transcription elongation factor S-II	2.25	Transcription	0.05
Upregulated transcripts from all other libraries					
E13H05	CA852946	WD-40 repeat family protein	4.71	Signaling	0.05
RJC1_D09	AY057902	Matrix metalloproteinase MMP2	2.75	Defense	0.05

Table 4 (continued)

Clone ID	GenBank ID	Description	Fold Change	Function[a]	p Value ≤
DUP20A12	JQ2249	Naringenin-chalcone synthase	2.50	Defense	0.05
B06C09	CA853272	Splicing factor SRP40	2.49	Other	0.05
DUP11E10	X94945	Lipoxygenase (LOX)	2.37	Oxidation	0.05
D06E10	CA850790	6′-deoxychalcone synthase	2.31	Defense	0.05
E11F02	CA852760	6a-Hydroxymaaciain methyltransferase	2.25	Defense	0.05
DUP17E08	JQ2344	Catechol *O*-methyltransferase III	2.22	Defense	0.05
DUP24F04	AC003105	Cytochrome P450	2.12	Defense	0.05
D08E10	CA850953	PAP24 (purple acid phosphatase 24)	2.02	Other	0.05
DUP19D02	O49859	Cytochrome P450 82A4 (P450 CP9)	2.01	Defense	0.05
D19E05	CA851960	Anthocyanidin synthase ANS	1.72	Defense	0.05
Downregulated transcripts from the SSH libraries					
JC11_H05	NM_111479	Ribosomal protein L12 family protein	−3.75	Cell growth/maintenance	0.05
RJC2_H08	AY079377	Putative salt-tolerance protein	−2.29	Defense	0.05
JC2_G06	P00865	Ribulose bisphosphate carboxylase small chain 1	−2.06	Cell growth/maintenance	0.05
Downregulated transcripts from all other libraries					
DUP05C04	X75966	Leucoantocyanidin dioxygenase (LDOX)	−3.39	Defense	0.05
SSH8H07	BM140219	PLL5 (POL-like 5); protein phosphatase type 2C	−2.83	Signaling	0.05
DUP26B03	O22176	WRKY3	−2.71	Defense	0.05
DUP11D02	F14L17	MYB-Related Protein B	−2.67	Defense	0.05
DUP14D03	S25005	DNA K-type molecular chaperone	−2.34	Defense	0.05
E04E10	CA852203	Glutathione peroxidase	−2.29	Oxidation	0.05
B10A01	CA853587	Cytochrome P450	−2.25	Defense	0.05
SSH7A05	BM139963	Abscisic acid-activated protein kinase (AAPK)	−2.08	Other	0.05

Transcripts were chosen to be a representative sample of the differentially expressed genes and to highlight interesting functional categories such as oxidation or defense. Transcripts with informative annotations rather than incomplete or non-specific annotations were preferentially chosen. The complete list of differentially expressed transcripts is presented as Supplemental Table 1.

[a] Functional categories as defined in Table 1.

Several ESTs encode for proteins involved in transport, such as aquaporin, permeases, and translocators. In addition, an EST was identified with similarity to the oxidoreductase *fis1*, which has been shown to facilitate biotrophic relationship between rust and flax (Ayliffe et al. 2002; Roberts and Pryor 1995). Other ESTs in the functional category of defense including abscisic stress-ripening protein homolog, endochitinase, catalase, cytochrome P450 monooxygenase, heat shock protein 70, and a putative pathogenesis-related protein from *Arabidopsis* were found, as well as ESTs involved in signaling such as remorin and ADR12-2 protein, which is known to be downregulated by auxin. Ten ESTs with no significant/null hit to the database were observed in this profile.

The third profile (profile 3 in Fig. 3, Table 5) included ESTs downregulated at 6 and 12 hpi and upregulated in the immune reaction starting at 24 hpi. The majority of annotated ESTs in this group included defense-related genes such as heat shock protein 70, pectinesterase, cytochrome P450, salt-induced AAA-type ATPase, and trypsin inhibitor. Also present were transcription regulators such as IAA7-like protein and isoflavone reductase-like protein, as well as 14-3-3-like protein, which is involved in signaling. Nine members of profile 3 had unknown/no significant hit to the NCBI databases.

The final profile (profile 4 in Fig. 3, Table 5) included ESTs upregulated for the first 12 hpi in the immune reaction and then downregulated after 24 hpi. ESTs in this group had similarity to peroxidases, lipoxygenases, and to oxylase-like proteins involved in oxidoreductase activity and antibiotic synthesis. Defense-related ESTs include Bax inhibitor, NtPRp27, which is a secreted pathogenesis-related protein that is inducible by ethylene and JA (Okushima et al. 2000), peptidylprolyl isomerase ROF1 that is induced by wounding and accelerates the folding of proteins (Vucich and Gasser 1996), and diphosphomevalonate decarboxylase-like protein involved in phenylpropanoid synthesis. ESTs involved in transcription regulation include a MYB-like transcription factor and WD-40 repeat protein. ESTs involved in signaling included somatic embryogenesis receptor-like kinase-like protein and SOS2-like protein kinase. Twenty-one ESTs with no significant/null hit to the database were observed in this profile.

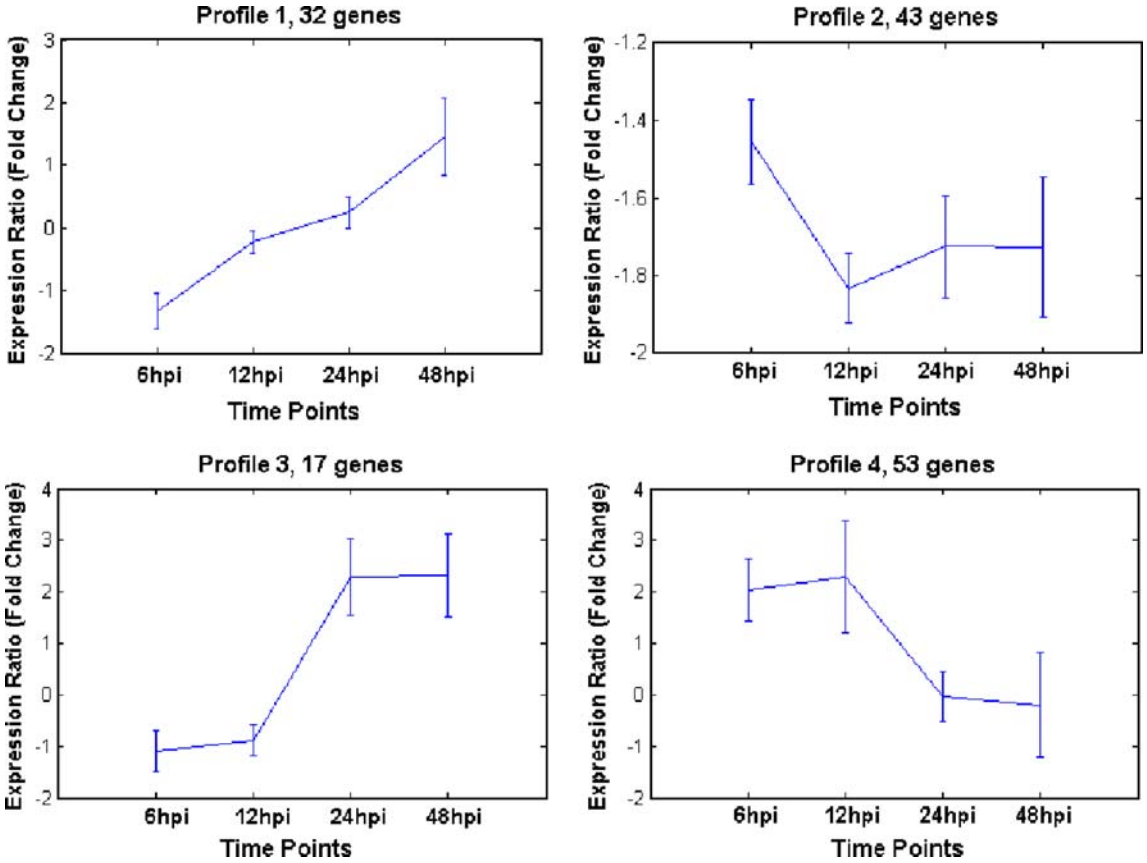

Fig. 3 Expression profiles of genes differentially expressed in leaves of the soybean cv. Komata to infection by *P. pachyrhizi*. The expression ratios of the immune vs. susceptible reactions were calculated from normalized, rescaled ratios of the fluorescent ratios of intensity in the red and green channels. Ratios represent the average from the two biological samples that were used. Error bars show the standard deviation of expression levels at each time point

Quantitative real-time RT-PCR

PCR primers were designed to 14 genes representing a range of functional categories. Of the 14 primer pairs, seven amplified single DNA products. Four primer pairs displayed linear amplification across the range of RNA concentrations tested (data not shown) and were chosen for further analysis. Three of the gene encode for known proteins, SAM-22, ANS, and nitrate transporter 1 (NTR1-5), while the fourth gene product is unknown. The fold changes were determined for each of these genes by quantitative real-time RT-PCR and microarrays and are shown Fig. 4. The RT-PCR data for the gene encoding NTR1-5 showed upregulation (a KH/KT expression ratio greater than 1) at both 6 and 12 hpi and downregulation (expression ratio less than 1) at 24 and 48 hpi. The microarray data showed a similar pattern (Fig. 4a). ANS showed upregulation at all four time points by RT-PCR and microarrays (Fig. 4b). The unknown gene was more problematic because, while both the microarrays and the RT-PCR revealed downregulation at 6 and 12 hpi, the data disagreed at 24 hpi, with the RT-PCR analysis indicating downregulation and the microarray indicating upregulation (Fig. 4c). The 48 hpi data is more consistent, with both RT-PCR and microarray analysis showing upregulation (Fig. 4c). SAM-22 showed downregulation at 6 and 24 hpi and upregulation at 12 and 48 hpi (Fig. 4d). Overall, our quantitative real-time RT-PCR data confirm the relative expression trends of the respective genes obtained by our microarray analysis.

Discussion

The gene expression of soybean after inoculation with two different isolates of *P. pachyrhizi* was analyzed using SSH and cDNA microarrays. Specifically, we inoculated the soybean cv. Komata, which contains the *Rpp1* rust resistance gene, separately with *P. pachyrhizi* isolates HW94-1 and TW72-1 and compared the gene expression patterns in the corresponding immune and susceptible reactions. This allowed us to make direct comparisons without being concerned about differences in soybean genotype influencing gene expression patterns. Unlike the

Table 5 Description, functional category, and relative fold change of genes found to be differentially expressed by custom microarray analysis of immune vs. susceptible soybean leaves inoculated with *P. pachyrhizi* and sampled at four times after inoculation

Profile, functional category, gene ID	GenBank ID	Putative protein name	Hours post-inoculation, fold change			
			6	12	24	48
Profile 1		31 genes total[b]				
Cell growth and maintenance						
RJC6_A02	1307148	Tubulin alpha	−1.12	−0.07	0.06	1.11
B13F12	CA853911	Uroporphyrinogen decarboxylase	−1.21	−0.38	0.26	1.10
SSH3F10	BM139701	Repetitive proline-rich protein	−1.01	−0.04	0.04	1.32
RJC7_H02	X76932	Ribosomal protein L1	−1.05	−0.11	0.09	1.09
SSH6E11	BM139921	26S ribosomal RNA	−1.27	−0.01	0.12	1.81
DUP18A10	Unavailable	Putative ATP-dependent RNA helicase	−1.22	−0.53	0.03	4.54
DUP10E04	Unavailable	Translation initiation factor eIF-4A.9	−1.24	−0.22	0.03	1.27
Defense						
SSH3H04	BM139705	Cationic peroxidase	−1.29	−0.45	0.05	1.56
DUP27F12	Unavailable	Peroxidase	−1.32	−0.28	0.03	1.65
DUP23G11	Unavailable	Peroxidase	−1.41	−0.10	0.05	1.18
DUP17C03	Unavailable	Peroxidase	−2.33	−0.04	0.32	1.34
JC8_G07	AB049723	Putative senescence-associated protein	−1.09	−0.19	0.56	1.05
JC11_H03	AB049723	Putative senescence-associated protein	−1.17	−0.33	0.15	1.45
RJC4_B10	AY351861	Cryptochrome 1	−1.15	−0.10	0.11	1.29
DUP11B04	Unavailable	Pathogen-related protein	−1.22	−0.26	0.89	1.37
Metabolism						
DUP28D03	Unavailable	Indole-3-acetate beta-glucosyltransferase	−1.22	−0.76	0.06	1.06
Transcription						
DUP10F05	Unavailable	AP2 domain transcription factor	−1.33	−0.04	0.27	1.19
DUP14E09	Unavailable	bZIP transcription factor	−1.04	−0.20	0.24	1.59
Other						
DUP24F08	Unavailable	Putative ADP-ribosylation factor	−1.09	−0.09	0.32	1.92
Unknown						
B11H12	CA853762	CG13482 gene product	−1.32	−0.42	0.18	1.66
E13H07	CA852948	contains ‘An’ repeat	−1.76	−0.30	0.42	1.27
DUP15F12	Unavailable	DNA binding protein isolog	−1.39	−0.50	0.04	1.03
JC3_E02	S19977	Gene GA protein	−1.38	−0.12	0.20	1.42
Profile 2		42 genes total				
Cell growth and maintenance						
DUP09G05	Unavailable	Class IV endochitinase	−1.20	−2.01	−1.06	−1.57
D18G12	CA851904	Extensin	−1.02	−1.65	−1.69	−1.12
A13A04	BM108366	Extensin class I	−1.15	−1.65	−1.83	−1.06
DUP28H01	Unavailable	Putative xyloglucan endotransglycosylase	−1.30	−1.50	−1.16	−1.12
DUP15G04	Unavailable	Xyloglucan endotransglycosylase 1	−1.38	−1.17	−1.09	−3.22
SSH1F08	BM139907	Repetitive proline-rich protein	−3.18	−1.38	−1.85	−10.35
Defense						
E12B10	CA852804	Abscisic stress ripening protein homolog	−1.31	−1.32	−2.00	−1.23
D19D07	CA851952	Catalase	−1.61	−1.14	−1.17	−2.21
DUP09A01	Unavailable	Cytochrome P450 monooxygenase 71D10p	−1.76	−2.90	−1.46	−1.16
DUP06A10	Unavailable	Heat shock protein 70 cognate	−1.13	−1.84	−1.22	−1.45
DUP15H12	Unavailable	Probable aldehyde dehydrogenase fis1	−1.09	−9.55	−1.13	−1.68
D18C05	CA851851	Putative pathogenesis-related protein	−1.72	−3.57	−1.26	−1.12
DUP10F04	Unavailable	40S ribosomal protein S8	−1.18	−1.28	−1.17	−3.64
D18H04	CA851908	Oligosaccharyl transferase 9.5 KD subunit	−1.67	−1.54	−1.66	−1.30
JC1_A01	NM_119787	Serine carboxypeptidase S28 family protein	−1.33	−1.12	−4.58	−1.73
C01F01	BM107738	E3 ubiquitin protein ligase UPL1—F14J16.14	−1.11	−2.00	−1.55	−1.14
SSH6F10	BM139933	Translationally controlled tumor protein MSTCTPMR	−1.27	−1.47	−1.06	−1.17

Table 5 (continued)

Profile, functional category, gene ID	GenBank ID	Putative protein name	Hours post-inoculation, fold change			
			6	12	24	48
Metabolism						
DUP09B07	Unavailable	Putative acyl-CoA synthetase	−1.09	−1.18	−1.24	−1.10
Transcription						
D18D11	CA851869	Zinc finger DNA binding protein	−1.21	−1.44	−1.42	−1.51
A09G01	BM108152	Squamosa-promoter binding-like protein 1	−1.24	−1.51	−1.55	−1.04
RJC4_C05	ATAC011623	Chromatin remodeling complex ATPase chain (ISW2-like)	−1.18	−1.11	−1.01	−1.08
SSH1D02	BM139676	Glial cells missing gene homolog (mGCM1)	−1.12	−1.19	−4.67	−1.34
Signaling						
DUP02H12	Unavailable	Putative LRR receptor protein kinase	−1.41	−2.15	−2.11	−3.09
D17A03	CA851736	Remorin	−1.01	−1.28	−1.31	−1.28
B11H04	CA853754	ADR12–2 protein	−1.58	−1.15	−1.95	−1.62
Other						
B10E12	CA853643	Aquaporin 2	−1.16	−1.24	−1.06	−1.33
JC2_F05	AP008231	Permeases—major facilitator superfamily	−6.41	−1.41	−1.60	−1.15
B13G10	CA853921	Translocase 7 K chain TOM7, mitochondrial	−1.44	−1.32	−1.19	−1.08
JC2_G11	P21727	Phosphate translocator (CTPT)	−1.11	−1.28	−1.15	−1.15
JC9_D09	NM_125229	Exocyst subunit EXO70 family protein	−1.17	−1.39	−1.46	−1.68
B11G08	CA853747	Mannitol-permesase IIA component	−1.25	−1.20	−1.19	−1.30
Unknown						
B08C03	CA853436	Putative 14-kDa proline-rich protein	−1.11	−1.32	−1.55	−1.96
Profile 3		17 genes				
Defense						
DUP20D11	Unavailable	Heat shock protein 70	−1.15	−1.36	1.85	4.00
B09D03	CA853534	Pectinesterase homolog F14M4.24	−1.19	−0.87	2.85	1.58
DUP19D02	Unavailable	Putative cytochrome P450	−1.94	−0.87	1.73	2.02
SSH3C05	BM139639	AAA-type ATPase	0.00	−0.06	2.06	2.19
D03G05	CA850582	Trypsin inhibitor	−0.69	−1.61	6.29	2.33
Transcription						
B06B02	CA853253	IAA7 like protein	−0.05	−1.15	1.65	1.55
A10E07	BM108217	Isoflavone reductase-like protein	−1.35	−1.76	1.75	1.97
Signaling						
B07A08	CA853335	14-3-3-like protein	−0.68	−0.61	1.69	2.81
Unknown						
D16D07	CA851683	Putative DNA-binding protein F16J13.120	−0.20	−1.47	1.53	1.57
Profile 4		52 genes				
Cell growth and maintenance						
E08C06	CA852482	Mitochondrial 60S ribosomal protein L16	1.87	3.26	−0.03	−1.67
B06E06	CA853291	Putative tyrosyl-tRNA synthetase	2.71	1.52	0.01	−0.02
RJC5_B09	AF117339	FtsH-like protein Pftf precursor	1.80	1.51	−1.71	−0.19
A05G03	BM108008	Putative RING zinc finger protein	1.68	1.78	−0.21	−1.46
D18B06	CA851840	UBL5 ubiquitin-like protein	2.10	4.23	−1.00	−0.28
RJC1_E11	S72485	Peptidylprolyl isomerase ROF1	1.60	1.71	0.55	1.08
E11A11	CA852712	Putative RNA-binding protein	1.58	2.26	−0.27	0.03
DUP24A07	Unavailable	RNA helicase	1.54	2.11	−0.06	−2.00
DUP15C10	Unavailable	Germin-like protein	1.57	1.61	−1.15	−0.08
Defense						
SSH8D07	BM140177	Lipoxygenase	2.57	2.18	−0.20	−2.83
DUP01C01	Unavailable	Lipoxygenase	2.15	5.76	0.08	−1.57
DUP07B08	Unavailable	Lipoxygenase-1	1.77	1.71	0.02	−2.24
DUP09F04	Unavailable	Lipoxygenase-3	1.71	1.87	1.25	2.32
E14F03	CA852979	NtPRp27	1.82	2.38	−2.03	−1.53
D16D12	CA851687	Oxylase-like protein	1.67	1.57	0.27	0.07
DUP23H12	Unavailable	Peroxidase 5 precursor	1.96	1.57	−1.28	−1.73
DUP11A06	Unavailable	Peroxidase 5 precursor	1.56	1.97	−1.77	−0.03

Table 5 (continued)

Profile, functional category, gene ID	GenBank ID	Putative protein name	Hours post-inoculation, fold change			
			6	12	24	48
Energy						
JC7_B09	AC135460	Chlorophyll a/b-binding protein CP26 precursor	1.80	1.76	0.25	0.37
Transcription						
JC3_F05	AY380778	Bax inhibitor	2.16	2.62	−1.29	0.00
DUP02G06	Unavailable	Bax inhibitor-1	2.11	5.10	−3.60	−0.26
DUP12A02	Unavailable	Putative transcription factor	2.09	7.43	−0.16	0.77
DUP28A12	Unavailable	Putative WD-40 repeat protein, MSI4	1.52	1.59	1.14	1.10
Metabolism						
DUP22A03	Unavailable	12-oxo-phytodienoate reductase	2.97	1.83	−2.19	2.66
E05G08	CA852289	Diphosphomevalonate decarboxylase-like protein	2.12	2.49	2.22	−0.10
Signaling						
DUP13B01	Unavailable	Receptor-like kinase-like protein	3.30	1.61	1.53	−0.09
A05F01	BM107995	SOS2-like protein kinase	1.80	1.70	−1.39	−0.81
Other						
C01H06	BM107763	SNARE protein	1.61	2.21	1.22	−1.08
B12F12	CA853823	Putative plasma membrane intrinsic protein	1.53	1.55	−1.92	−0.04
JC11_G08	Q36795	Preprotein translocase secA precursor	1.86	1.62	2.46	−1.90
Unknown						
E10F07	CA852681	CG13721 gene product	2.68	1.57	1.78	−0.17
DUP13A11	Unavailable	Eukaryotic protein kinase domain	2.24	5.68	−0.22	−0.11
SSH5A08	BM139826	Metallothionein-like protein PPE243532	5.61	3.80	1.89	0.50

Genes with an expression ratio greater than 2.0 were clustered by expression profile (see footnote a). Graphical representations of the profiles are depicted in Fig. 2.

[a] Similar expression profiles were grouped into clusters using Online Analytical Processing (OLAP, Alkharouf et al. 2005).

[b] Genes annotated as unknown, no significant hits, or hypothetical protein have been removed for brevity.

RB-resistant reaction that occurs on soybean cvs. containing *Rpp2*, *Rpp3*, or *Rpp4*, there are no visible macroscopic symptoms on cv. Komata in an immune reaction (Bromfield 1984). The initial infection process by *P. pachyrhizi* is similar in the *Rpp1* immune and susceptible reactions. Spore germination, appressorium formation, and penetration of the epidermal cell occur within 24 h (Keogh and Deverall 1980; McLean and Byth 1981). In the susceptible reaction, haustoria are visible by 48 h and hyphal growth proceeds intercellularly. However, in the immune reaction, fungal growth does not continue beyond the formation of the penetration peg (Keogh and Deverall 1980; McLean and Byth 1981). Therefore, we chose to examine gene expression during the first 48 hpi. By analyzing gene expression at 6, 12, 24, and 48 hpi, we were able to construct temporal expression profiles.

We initially created a SSH library enriched for sequences significantly more or less abundant in the immune reaction and sequenced the library to identify novel genes important for soybean rust resistance. We were surprised to find that the most common EST in both the forward and reverse libraries shared similarity to the same protein, a "putative senescence-associated protein" from pea [dbj|BAB33421.1]. This suggests that the SSH enrichment was not particularly successful. It is also plausible that these two ESTs represent distinct members of a gene family. Soybean has been shown to have several gene families whose members are highly similar at the sequence level while exhibiting different expression patterns (Grandbastien et al. 1986; D'ovidio et al. 2006; Nelson and Shoemaker 2006; Webb et al. 2007). The small size of the ESTs, an unavoidable feature of the SSH construction method, makes it problematic to unambiguously identify members of gene families. The large number of clones in the SSH library was unpredicted, and we were unable to identify ESTs playing a key role in soybean rust resistance without additional information. In addition, pooling RNA from multiple time points did not allow for resolution of temporal changes in gene expression. Therefore, we chose to construct a cDNA microarray to confirm differentially expressed clones and ascertain changes in gene expression over time.

Our overall goal in this experiment was to identify soybean genes involved in the early stages of the immune response to *P. pachyrhizi*. We were successful in this endeavor, measured by the fact that so many of the differentially expressed genes had similarilty to defense-related genes identified in other

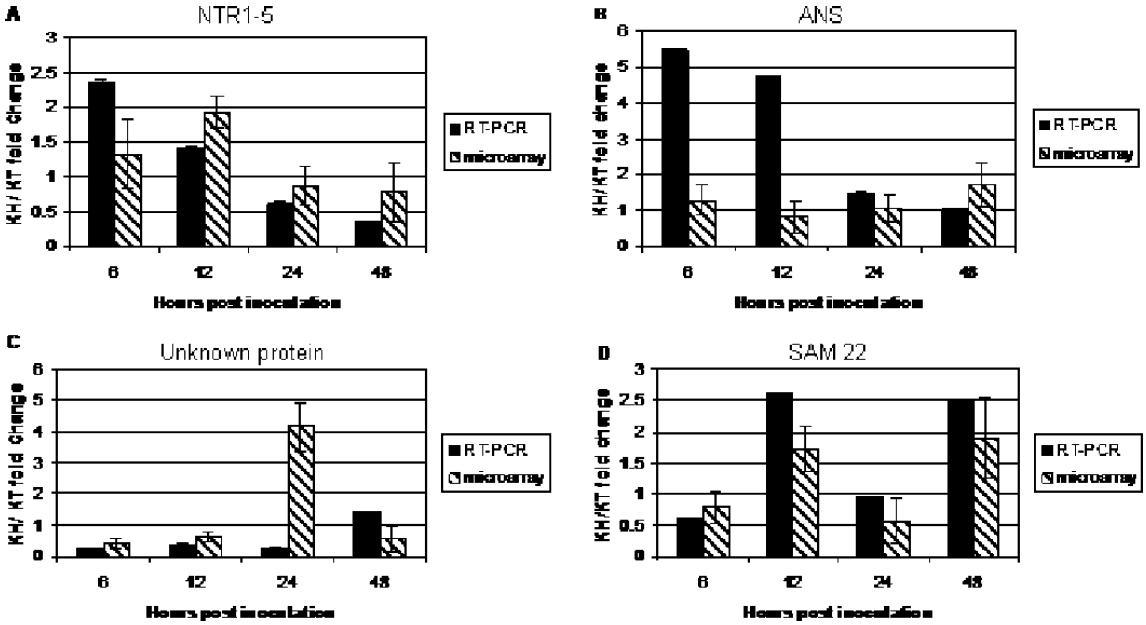

Fig. 4 Differential expression ratio of selected genes in microarray analysis and quantitative real time RT-PCR in the soybean cv. Komata to infection by *P. pachyrhizi*: **a** nitrate transporter 1 (*NTR1-5*), **b** anthocyanidin synthase (*ANS*), **c** unknown protein, and **d** starvation associated message 22 (*SAM-22*)

plant–pathogen interactions. The differentially expressed gene list at each time point contained many defense-related genes, and these genes often had complementary functions. While no single gene was upregulated at every time point, classes of genes encoding oxidizing enzymes, such as lipoxygenases and peroxidases, were upregulated at all time points. In addition, antioxidant genes that help the cell protect itself against the damaging effects of oxidative stress (Camera et al. 2004; Foyer and Noctor 2005; Pinto et al. 2006) were also upregulated. Reactive oxygen species (ROS) play numerous roles in defense, from catalysis of cell wall fortification (Bradley et al. 1992), to signaling cascades (Levine et al. 1994), to toxic free radical production (Brisson et al. 1994). In addition to the protective role of ROS, oxidative damage of the host cell can also lead to necrosis. It has been suggested that a key indicator of the cell's ability to survive pathogen attack is the oxidative balance in the cell (Foyer and Noctor 2003, 2005; Kawano 2003; Levine et al. 1994; Winkel-Shirley 2001). Soybeans (cv. Williams 82) inoculated with *Pseudomonas syringae* had an initial weak transient H_2O_2 burst followed approximately 3 hpi with a second massive burst of O_2 production that was sustained for several hours (Levine et al. 1994). H_2O_2 was shown to function as both a diffusible signal triggering expression of defensive compounds in cells surrounding the infection site and as a programmed cell death (PCD) trigger in the infected cell (Levine et al. 1994). Peroxidases and lipoxygenases are both highly represented in profiles 2 and 4, suggesting that a complex interplay of oxide concentrations/levels may play an important role in the ability of the host cell to resist further infection (Passardi et al. 2005; Shah 2005). Lipoxygenases in profile 4 are induced early, returning to basal levels 12 hpi. Their expression could be responsible for an early oxidative burst resulting in cell-wall modification and induction of defensive compounds such as glutathione S-transferase (GST) at the site of infection and in the neighboring cells. Peroxidases in profile 1 begin with low expression in resistant soybeans but, by 48 hpi, are induced in resistant cells and could provide a second oxidative burst in infected cells that might trigger PCD. Additional enzymes related to the oxidative state of the cell, including senescence-associated protein (an ACC oxidase, profile 1) and isoflavone reductase (high expression at 24 and 48 hpi, profile 3) are also coordinately regulated. The lack of visible lesions in *Rpp1* resistant soybeans could indicate that PCD is not occurring in *Rpp1* plants, or perhaps only the initially infected cell becomes necrotic, resulting in a lesion so small as to be invisible to the naked eye. Further investigation into the nature of the *Rpp1*-mediated immune response will surely shed light on the reason for the lack of a visible lesion.

Genes in the phenylpropanoid synthesis pathway are among the most powerful antioxidants in the cell and are significantly upregulated by soybean in response to *P. syringae* (Zabala et al. 2006; Zou et al. 2005). Phenylpropanoid synthesis also leads to the production of defensive phytoalexins and cell-wall-reinforcing lignin (Winkel-Shirley 2001). Several genes similar to those involved in phenylpropanoid synthesis were differentially expressed in this study, suggesting a role of phenyl-

propanoids in maintaining the oxidative balance of soybean cells and in rust resistance. Recently, a commercial oligonucleotide microarray was developed containing approximately 36,500 soybean genes (GeneChip Soybean Genome Array, Affymetrix, Santa Clara, CA, USA). The Soybean Genome Array was used to analyze gene expression in *Rpp2*-mediated rust resistance (van de Mortel et al. 2007). That study also identified the phenylpropanoid synthesis pathway as important in the RB resistance reaction (van de Mortel et al. 2007). It will be interesting to see whether additional studies can identify which consequence of phenylpropanoid synthesis (antioxidant production, phytoalexin production, or lignin accumulation) is most important for the immune reaction.

In addition to the importance of phenylpropanoids in rust resistance, van de Mortel et al. (2007) also found that WRKY transcription factors were regulated in the *Rpp2*-mediated resistance response of soybean rust. Similarly, MYB and bHLH transcription factors are induced in nitrogen-stressed *Arabidopsis* (Unni et al. 2007). These families of transcription factors were also differentially regulated in the immune response to soybean rust, with MYB transcription factors upregulated at 12 and 24 hpi and downregulated at 48 hpi. WRKY transcription factors were upregulated at 12 hpi and downregulated at 24 and 48 hpi. Differential expression of multiple transcription factor family members suggests that a complex positive and negative regulation pattern of defense pathways allows the host to prevent rust infection.

A recent study of susceptible soybeans infected with *P. pachyrhizi* (Panthee et al. 2007) reveals moderate induction of general defense-related genes, including GSTs, heat shock proteins, and salicylic-acid-dependent genes, while these same genes and defense pathways are induced earlier and much stronger in the *Rpp2* resistant reaction (van de Mortel et al. 2007). This suggests that rust resistance may be more a matter of timing and degree of induction of innate immunity pathways rather than the result of specific induction of a few genes.

This study identified 538 differentially expressed ESTs using a custom-designed cDNA microarray. By extending our study with the use of the Affymetrix GeneChip Soybean Genome Array, we anticipate the discovery of additional differentially expressed genes that would allow for a more comprehensive understanding of *Rpp1*-mediated resistance. In addition, a comparison could be made to the differentially expressed genes identified from the *Rpp2* resistant reaction. Such a comparison would provide insight as to whether *Rpp1* and *Rpp2* share similar resistance mechanisms.

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