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ORIGINAL PAPER

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Resistance locus pyramids alter transcript abundance in soybean roots inoculated with *Fusarium solani* f.sp. *glycines*

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Abstract Soybean Sudden Death Syndrome (SDS) is caused by *Fusarium solani* f.sp. *glycines* (Fsg). Six quantitative trait loci (QTLs), each conferring partial resistance to SDS, have been discovered in an Essex × Forrest recombinant inbred line (RIL) population, but their mode of action is not clear. This study aimed to identify genes (ESTs) whose mRNA transcripts were altered in abundance in soybean roots following inoculation of Fsg. Roots of the soybean variety Forrest (four resistance alleles) were inoculated with Fsg, and 14 days later RNA sequences that were di erentially expressed relative to uninoculated roots were enriched using suppression subtraction and di erential display. The abundance of these RNAs was quantified in inoculated and non-inoculated roots by macroarray hybridizations. A unigene set of 135 ESTs was identified and used in a further macroarray analysis. The abundance of 28 cDNA fragments was increased more than two-fold in inoculated compared to uninoculated roots of RIL 23 (six resistance alleles). In Forrest and Essex (two resistance alleles), the level of only one mRNA was increased two-fold in inoculated roots compared to the uninoculated roots. In Essex most of the mRNAs analyzed decreased in abundance (61/135 showed a two-fold decrease), while in Forrest most mRNA abundances did not change. Among the 28 cDNAs that revealed a two-fold or higher increase in mRNA abundance in RIL 23, 14% code for proteins known to be involved in plant defense, 21% in metabolism, 14% in cell structure and 4% in transport. Unannotated ESTs accounted for 43% of the genes, and 4% of the sequences were previously unknown. The plant defense-related genes that showed a di erential response to Fsg inoculation suggested a role for the phenylproponoid pathway in soybean defense against Fsg. In Essex, genes involved in plant defense, cell wall synthesis, ethylene synthesis and metabolism were expressed at lower levels in inoculated roots. The di erence in response between the 2-, 4- and 6-gene pyramids suggests that QTLs for SDS resistance serve to delay symptoms or confer resistance by maintaining or increasing the expression of specific genes after inoculation/infection.

Keywords cDNA macroarray · Resistance loci pyramid · Soybean sudden death syndrome · *Fusarium solani*

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Introduction

Sudden Death Syndrome (SDS) of soybean [*Glycine max* (L.) Merr.] is caused by *Fusarium solani* f.sp. *glycines* (Fsg) (Roy et al. 1989; Roy 1997). In susceptible varieties, roots are infected within 14 days after planting (dap). The infected roots show reduction in size, browning of vascular tissues, root rot and crown necrosis (Rupe 1989; Njiti et al. 1997). The initial foliar symptoms that occur 28–60 dap are interveinal chlorotic spots and these develop into necrotic or chlorotic streaks by the intermediate stages of the disease (60–100 dap) (Melgar et al. 1994). Genetic studies in two di erent populations have determined that resistance to SDS is quantitatively inherited and controlled by six loci present on di erent linkage groups (Iqbal et al. 2001; Njiti et al. 2002). A region containing one quantitative trait locus (QTL) on linkage group G

identified by the AFLP marker ATG4 (Meksem et al. 1999; 2001) was shown to contain a candidate receptor-like kinase gene for resistance to soybean cyst nematode and candidate metabolic genes for resistance to Fsg [Hauge et al. 2001; Lightfoot et al. 2001, available at http://patft.uspto.gov/netahtml/search-bool.html; Triwitayakorn 2002). Therefore, SDS resistance probably does not conform to the gene-for-gene theory of the plant resistance response (Flor 1956; Meksem et al. 2001). There may be more factors involved in addition to resistance genes, which in turn confer partial or complete resistance to Fsg infection (Bonas and Lahaye 2002; Triwitayakorn 2002).

In earlier studies (Iqbal et al. 2002), a di erentially abundant *myo*-inositol 1-phosphate synthase (MIPS) cDNA was identified by di erential display of mRNA (DDmRNA) from soybean roots inoculated with Fsg. The MIPS mRNA, the corresponding enzyme activity and the end product of the activity was down-regulated in partially resistant and susceptible varieties but up-regulated in cultivars that contain resistance alleles at the six SDS QTLs. However, DDmRNA identifies many false positives (50–75%) and one therefore needs a high-throughput method to screen for the truly differentially expressed cDNAs (Pascolo et al. 1999). There are few techniques available with which to semi-quantitatively measure the expression of hundreds of genes. Molecular techniques such as subtraction hybridization, macro- and microarray hybridization (Rockett et al. 1999) can be used as independent tests to analyze the di erential response of plants to biotic and abiotic stresses. Dot-blot hybridization to cDNAs on a macroarray with signal evaluation by a phosphorimager is suitable for the quantification of mRNA abundance for 100–1000 genes or ESTs. cDNA macroarrays are currently being used in animal systems and a large number of pathway-specific gene sets are commercially available (http://www.superarray.com/). Subtraction hybridization of two mRNA populations followed by suppression PCR has proved to be quite e ective in enriching for mRNAs that are di erentially expressed in the experimental RNA population (Diatchenko et al. 1996; Kuang et al. 1998) and, if combined with cDNA macroarray analysis, can circumvent the limitations of DDmRNA. Suppression subtraction hybridization has been widely used in human and animal cell lines (Lappin et al. 2002; Rajkovic and Matzuk 2002) since its introduction, but has rarely been used in plants (Nuccio et al. 1997; Fourgoux-Nicol et al. 1999).

In this study, clones identified by suppression-subtraction hybridization and DDmRNA (Iqbal et al. 2002) were used in a cDNA macroarray analysis. Membranes bearing these cDNAs were hybridized to probes synthesized from RNA samples isolated from Fsg-inoculated and non-inoculated soybean roots. The response of soybean genotypes carrying di erent numbers of resistance alleles of the SDS QTLs was then analyzed.

Material and methods

Plant material

Soybean genotypes used in the study were Forrest (*Rfs*; *Rfs1*, *Rfs2*, *Rfs3*, *rfs4*, *rfs5*) and Essex (*rfs*; *rfs1*, *rfs2*, *rfs3*, *Rfs4*, *Rfs5*) varieties, and the recombinant inbred line (RIL) 23 (*Rfs*; *Rfs1*, *Rfs2*, *Rfs3*, *Rfs4*, *Rfs5*) generated from a cross between Essex and Forrest (Iqbal et al. 2001; Njiti et al. 2001). Seeds were germinated in sterile soil, and roots were collected 14 days after inoculation, as described earlier (Iqbal et al. 2002). The root samples were immediately frozen in liquid N_2 and kept at –80°C until RNA isolation.

RNA isolation

Roots were ground to a fine powder in liquid nitrogen and RNA was extracted with an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were treated with DNase in-order to remove any residual DNA in the preparation. After DNase treatment, RNA was purified on RNeasy mini spin columns (Qiagen). The quantity and quality of the RNA recovered was determined by spectrophotometry at 260 nm and electrophoresis on a 1.2% (w/v) formaldehyde agarose gel. The assay was repeated before probe synthesis.

Suppression subtraction hybridization

Suppression subtraction hybridization was used to enrich for sequences that were di erentially expressed in infected Forrest roots. Subtraction hybridization used the Clontech subtraction cDNA synthesis kit (BD Biosciences Clontech) following the manufacturer's instructions. After the second cycle of amplification, the forward subtracted cDNA was cloned in the pGEM-T vector (Promega). The cDNA clones were archived in 384-well plates. A total of 259 clones were sequenced using the ABI Big Dye terminator kit (Applied Biosystems) and an ABI 377 automated DNA sequencer. The vector and adaptor sequences were removed and the sequence was BLAST searched for homology to known genes/ESTs. A unigene set of 135 was identified for macroarray analysis. Seven clones (Fi37A24, Fi39D23, Fi36H20, Fi36H18, Fi51N11, Fi53O13, Fi65E19) containing cDNA inserts previously identified by DDmRNA (Iqbal et al. 2002) were also included in the set used for macroarray analysis.

Amplification of cDNA inserts for arrays

Inserts of cDNA clones were amplified using M13 forward and reverse primers in 50-μl reaction volumes containing 10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide, 5 μM each of M13 universal forward and reverse primers, 1 U of *Taq* DNA polymerase and 10 ng of plasmid DNA. Amplification was carried out in a Perkin-Elmer 9700 thermal cycler. After initial denaturation of 94°C for 3 min, the amplification was carried out for 45 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min. The reactions were incubated for 4 min at 72°C for final extension and held at 4°C until removed from the thermal cycler. An aliquot (5 μl) of each reaction was electrophoresed on a 1.2% (w/v) agarose gel to check the amplified inserts. The PCR products were purified by ethanol precipitation, washed once with 70% (v/v) ethanol and vacuum dried.

Spotting of cDNA fragments

The vacuum-dried PCR products were resuspended in 45 μl of 0.4 M NaOH, 10 mM EDTA. Four PCRs of each sample were

combined together. The samples were denatured at 95°C for 5 min and cooled to room temperature. MilliBlot-D (Millipore) was used to transfer 20 μl of each sample onto Hybond-N$^+$ (Amersham Pharmacia Biotech) nylon membrane. The membranes were washed with 2×SSC, air dried, and stored at room temperature under vacuum.

Synthesis of [α-^{33}P]-labeled cDNA probes

Total RNA (15 μg) was used to synthesize a cDNA probe labeled with α-^{33}P. Reverse transcription of poly(A)$^+$ mRNA was carried out in a 60-μl reaction containing 1× first-strand cDNA synthesis bu er [50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$], 1.5 μg of oligo $(dT)_{12-18}$, 0.05 mM each of dATP, dGTP and dTTP, 7.5 μl of [α-^{33}P]dCTP (3000 Ci/mmol), 0.01 M DTT, and 15 μg of total RNA. The reaction mixture was heated at 65°C for 5 min and then cooled to 44 C. Exactly 2 μl of SuperScript II RNase H$^-$ reverse transcriptase (SS II RT; Gibco-BRL) was added to start the reaction. After 25 min, another 2 μl of SS II RT was added and the reaction was incubated at 44°C for an additional 35 min. The reaction was terminated by adding 7.5 μl of 0.5 M EDTA. To hydrolyze residual RNA, 6 μl of 2.5 M NaOH was added and the reaction was incubated at 65°C for 1 h. After cooling to room temperature, 12.5 μl of 2 M TRIS-HCl (pH 7.5) was added. In order to remove unincorporated nucleotides, the probe was passed through a Sephadex G-50 column.

Hybridization and post-hybridization processing

Prehybridization and hybridization were carried out in 6×SSC (1×SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 5× Denhardt's reagent [1× Denhardt's reagent consists of 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) BSA, 1% (w/v) SDS and 100 μg/ml sheared salmon sperm DNA]. Filters were briefly rinsed with 2×SSC and prehybridized for 1 h at 65 C. Probe was denatured in boiling water for 5 min, chilled on ice and added to the hybridization mixture. Hybridization was carried out at 65°C for 16 h. After hybridization, filters were briefly rinsed with 2×SSC/0.1% SDS, and then washed twice for 5 min each at 55°C with 1×SSC/0.1% SDS. If necessary, the wash step was repeated at 65°C for 5 min. In all cases, filters hybridized with probes from inoculated and non-inoculated root samples of a given genotype were subjected to otherwise identical conditions. The filters were wrapped in plastic wrap and used to expose a phosphorimager cassette. The filters representing inoculated and non-inoculated probes of a genotype were used to expose the same cassette and spot intensities were measured using a Phosphorimager 445 SI (Molecular Dynamics). Spot finding, quantification and background subtraction was done using ImageQuaNT software (Molecular Dynamics).

Data analysis

After background subtraction, the spot intensities were normalized with reference to the intensity of the β-tubulin gene present in each hybridization. Changes in mRNA abundance were calculated by dividing the spot intensity of filters hybridized to the inoculated probe by the spot intensity of filters hybridized to the non-inoculated probe of each genotype. Thus, a value of 0.5 represented a two-fold decrease and 2 represented a two-fold increase in mRNA abundance of a particular cDNA. The ratios were calculated independently for each experimantal replication and the data was subjected to analysis of variance using the general linear model (SAS Institute 1985). Orthogonal contrasts were conducted to compare the genotype e ect on changes in mRNA abundance between di erent soybean genotypes (Petersen 1994). Means were generated from ANOVA and the standard error of the means (SEM) was calculated from the standard deviation.

Results

Enrichment and identification of genes and ESTs preferentially expressed in inoculated roots

A PCR-based cDNA subtraction method was used to enrich for genes and ESTs uniquely expressed in Forrest roots 14 days after inoculation with Fsg. A total of 384 clones were randomly picked and sequenced. After sequence analysis, 259 high-quality sequences were identified and aligned. These sequences included 81 singletons, 29 sequences represented by two clones each, 10 sequences represented by three clones, three sequences represented by four clones, four sequences represented by five and four by six clones, three sequences represented by eight clones and one sequence represented by 10 clones (Fig. 1). A unigene set of 135 ESTs was identified for use on macroarrays. BLAST searches revealed that 40% of these sequences were ESTs and most have been identified in soybean (see http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html). The rest showed significant homology to known genes from soybeans or other plant species.

Quantification of mRNA abundance using cDNA macroarrays

Eight out of the 142 pre-selected clones showed no amplification during PCR. Therefore, these spots acted as the ''no-DNA'' negative control on the membranes and showed no hybridization signal (Fig. 2). The majority of mRNAs (123) were decreased in abundance in the inoculated roots of the SDS-susceptible variety Essex, in contrast to the SDS-resistant RIL 23 (Fig. 3). The inoculated/non-inoculated ratio in Forrest was higher, relative to that in RIL23, for 14 cDNAs and lower for 98 cDNAs. The trend line for changes in

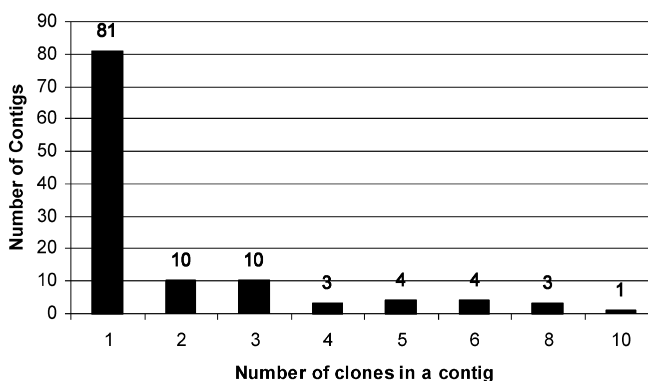

Fig. 1 Relative representation of identical or overlapping sequences in the clones of the suppression subtraction hybridization library selected for sequencing. The distribution ranges from singletons (no match with the other sequences) to a set of 10 clones derived from overlapping sequences. There was no contig of seven clones

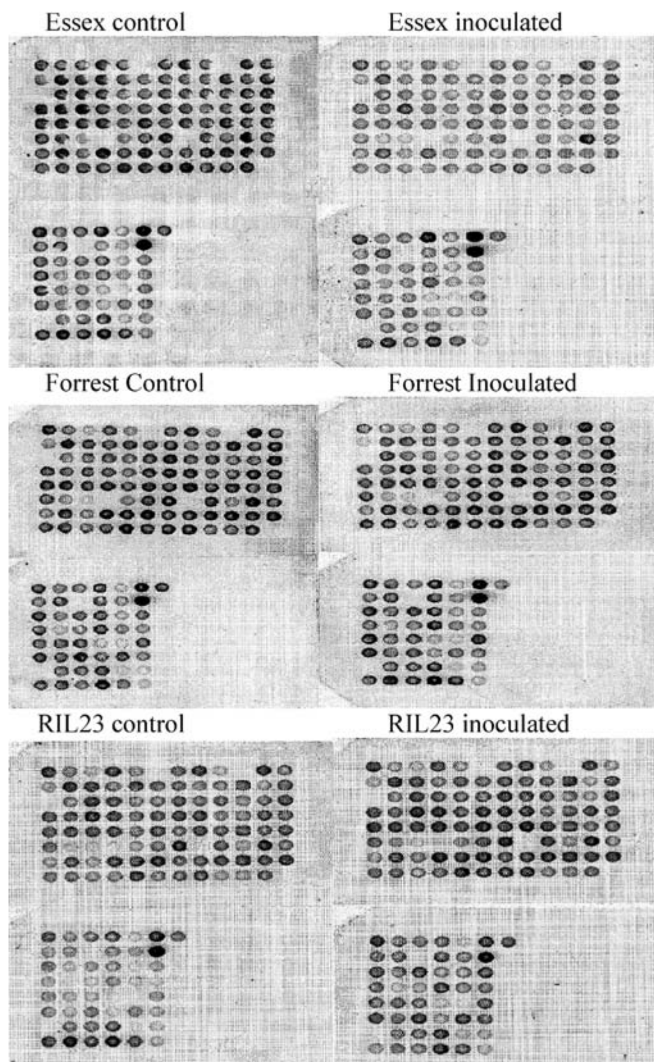

Fig. 2 Macroarray blots showing hybridization to [α-^{33}P]-labeled cDNA probes derived from uninoculated (control) and Fsg-inoculated roots. Two samples of each genotype were exposed in the same phosphorimager cassette. In each filter set, 8 spots are missing because these cDNA fragments were not amplified during PCR

mRNA abundance correlates with the number of resistance alleles at SDS QTLs present in each soybean genotype. The trend line (Fig. 3) for RIL 23 (six resistance alleles) was highest, Forrest (four resistance and two susceptibility alleles) was intermediate, and Essex (two resistance and four susceptibility alleles) was lowest in the general distribution of change in the abundance of the mRNAs (cDNAs) analyzed..

Transcripts (cDNAs) that increased in abundance in inoculated roots

In RIL23, the abundance of 28 cDNAs (mRNAs) increased by two-fold or more in roots inoculated with Fsg compared to non-inoculated roots (Table 1). Twelve of these 28 cDNAs were ESTs with no significant homology to known genes (Fig. 4) and 16 showed homology to genes of known function. The abundance ratios for the cDNA homologous to O-methyltransferase genes were 2.59± 0.20, 0.81± 0.01 and 0.35± 0.22 in RIL 23, Forrest and Essex, respectively. Orthogonal contrast analysis indicated a significant (P= 0.01) difference between RIL23 and Essex for this mRNA. The ratios for the phospholipase D (PLD) cDNA were 2.38± 0.84, 0.79± 0.01 and 0.50± 0.09 in the genotypes RIL 23, Forrest and Essex, respectively. Comparison of the change in mRNA abundance between RIL 23 (6 SDS QTL) and Essex (2 SDS QTL) indicated a 4.8-fold di erence, indicating the potential involvement of the product of this transcript in the soybean resistance response. A similar trend had earlier been observed with MIPS (Iqbal et al. 2002).

Another cDNA with high homology to soybean nodulin 22 (BM499232) gave ratios of 3.35± 1.10, 0.99± 0.08 and 0.37± 0.03 in RIL 23, Forrest and Essex, respectively. The change in abundance of this transcript indicated that, in Essex roots, Fsg infection inhibited the expression of the nodulin 22 gene. In Forrest, there was no significant change in Fsg-inoculated vs. non-inoculated roots. In RIL 23, we speculate that the metabolic activities of the roots were higher following Fsg inoculation, thus resulting in the higher rate of transcription of the nodulin 22 gene evident from the inoculated/uninoculated ratio.

The same pattern of change in mRNA abundance (2.29± 0.13 in RIL23, 1.94± 0.24 in Forrest and 0.47± 0.03 in Essex) was observed for a cDNA homologous to the *Phaseolus acutifolius* alcohol dehydrogenase-1F (ADH) gene. ADH is an important enzyme involved in the continued operation of glycolysis during waterlogging of roots (Anderson and Beardall 1991). The di erence in the response of this mRNA was highly significant between RIL 23 and Essex (P= 0.02) and between Forrest and Essex (P= 0.03).

A di erent pattern was observed for one mRNA (FiS2B2C9), which increased two-fold in inoculated Forrest roots, and one mRNA (FiS1J22) that was increased two-fold in infected Essex roots (Table 1). In both cases, the abundance of these transcripts did not change significantly in inoculated roots of RIL 23, suggesting a role for them in Fsg infection but not in soybean defense against Fsg.

Changes in mRNA abundance of plant defense-related genes

Plant defense-related genes whose transcripts were enriched among the suppression subtraction hybridization clones included genes for PLD, chalcone synthase (CHS), MIPS, phenylalanine ammonia lyase (PAL), a calmodulin-like protein and a clone representing cinnamic acid 4-hydroxylase (C4H). PAL and C4H catalyze important early steps in lignin phytotoxin and isoflavone

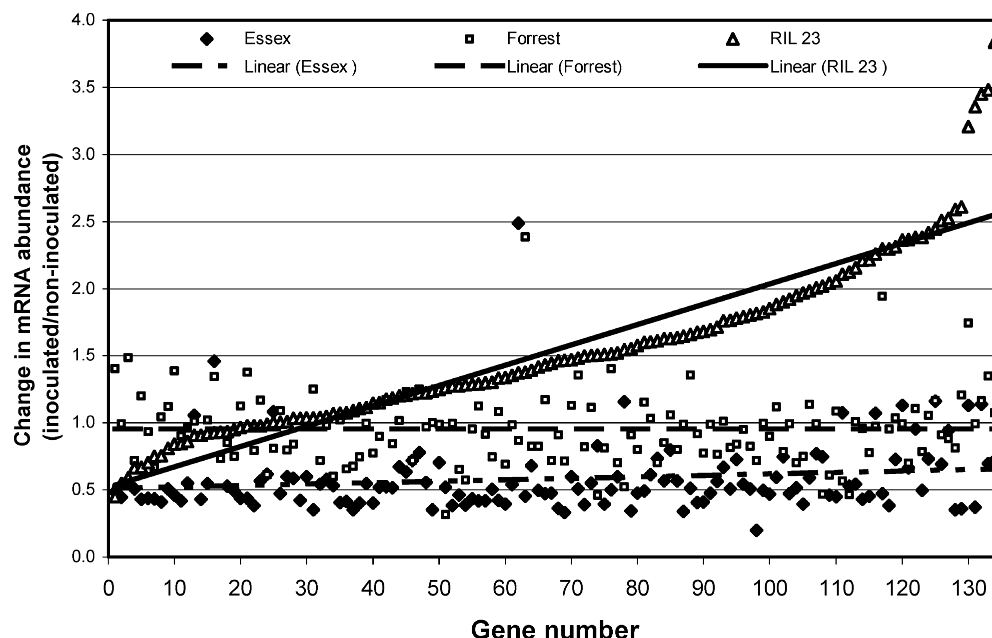

Fig. 3 An overview of the general distribution of changes in mRNA abundance (inoculated/non-inoculated) for the cDNAs used in macroarray hybridization. Gene numbers are ranked from lowest to highest change in abundance in RIL 23. The *thick lines* indicate the general trend in the change in mRNA levels for each genotype

Table 1 Transcripts that increased in abundance by two-fold or more in RIL 23 roots by 14 days of inoculation

SIU ID[a]	GenBank Accession No.	RIL23± SEM	Forrest± SEM	Essex± SEM	Homology to the known genes/ESTs
FiS2B2E4	BM499234	**3.83 ± 2.68**	1.07± 0.15	0.72± 0.33	*Glycine max* nodulin (E27)
FiS1B4	BI119570	**3.48 ± 2.18**	1.35± 0.43	0.70± 0.01	*Glycine max* EST
FiS1A4	BI119562	**3.45 ± 0.85**	1.17± 0.18	1.14± 0.26	*Glycine max* EST
FiS2B2D4	BM499232	**3.35 ± 1.10**	0.99± 0.08	0.37± 0.03	*Glycine max* nodulin 22 gene
FiS1G20	BI245413	**3.20 ± 1.13**	1.75± 0.30	1.13± 0.13	*Glycine max* SbPRP1 gene encoding a proline-rich protein
Fi51N11	BI347333	**2.61 ± 1.91**	1.21± 0.18	0.36± 0.07	Vacuolar ATP synthase
FiS1B14	BI119567	**2.59 ± 0.20**	0.81± 0.01	0.35± 0.22	*Glycyrrhiza echinata* O-methyltransferase
FiS1O8	BI245395	**2.52 ± 1.64**	0.88± 0.03	0.94± 0.24	*Glycine max* EST
Fi36H18	BI347330	**2.51 ± 1.84**	0.91± 0.18	0.69± 0.07	Putative elongation factor 1B alpha-subunit
FiS1J7	BI119563	**2.44 ± 1.55**	1.16± 0.30	1.16± 0.64	*Glycine max* EST
FiS1J17	BI273660	**2.41 ± 0.49**	1.06± 0.00	0.73± 0.05	*Glycine max* EST
FiS1i11	BI273656	**2.38 ± 1.53**	1.11± 0.49	0.95± 0.49	*Stylosanthes humilis* cinnamyl alcohol dehydrogenase (CAD1)
FiS2B2G10	BM499228	**2.38 ± 0.84**	0.79± 0.01	0.50± 0.09	*Vigna unguiculata* phospholipase D
FiS2B2B12	BM499241	**2.37 ± 1.08**	0.70± 0.12	0.66± 0.19	*Glycine max* EST
FiS1A12	BI119551	**2.36 ± 0.21**	0.99± 0.29	1.13± 0.05	*Glycine max* chalcone synthase (chs7)
FiS1A14	BI119552	**2.31 ± 1.39**	1.04± 0.03	0.73± 0.48	*Glycine max* EST
FiS1A16	BI119554	**2.30 ± 1.24**	0.96± 0.15	0.38± 0.07	*Glycine max* ADR12 mRNA
FiS1i9	BI245403	**2.29 ± 0.13**	1.94± 0.24	0.47± 0.03	*Phaseolus acutifolius* alcohol dehydrogenase-1F
FiS1N18	BI119551	**2.26 ± 0.83**	0.97± 0.38	1.07± 0.40	Chalcone synthase
FiS2B2A11	BM499233	**2.21 ± 0.78**	0.78± 0.11	0.45± 0.09	*Glycine max* SbPRP1 gene encoding a proline-rich protein
FiS1C17	BI119577	**2.21 ± 0.59**	0.96± 0.16	0.43± 0.10	*Glycine max* EST
FiS1J14	BI273643	**2.15 ± 0.11**	1.01± 0.17	0.54± 0.12	*Glycine max* EST
FiS1A17	BI119550	**2.12 ± 0.65**	0.47± 0.12	0.53± 0.04	*Glycine max* actin (Soy57) gene
FiS1B12	BI119565	**2.11 ± 1.21**	0.57± 0.18	1.08± 0.31	*Solanum tuberosum* putative membrane protein (poni2 gene)
FiS1C22	BI245396	**2.06 ± 0.41**	1.09± 0.14	0.45± 0.12	*Glycine max* EST
FiS1A10	BI119559	**2.04 ± 1.29**	0.61± 0.01	0.46± 0.05	*Glycine max* nodulin (C51)
FiS1M6	BI245393	**2.02 ± 0.85**	0.47± 0.20	0.75± 0.41	*Glycine max* EST
FiS1i13	BI273634	**2.00 ± 0.03**	0.99± 0.02	0.77± 0.11	*Glycine max* EST
FiS2B2C9	BM499236	1.38± 0.12	**2.38 ± 0.23**	0.45± 0.08	*Glycine max* gene for ubiquitin
FiS1J22	BI273637	1.37± 0.39	0.87± 0.06	**2.49 ± 1.01**	*Glycine max* EST

[a]The data show the signal ratios (inoculated/uninoculated) obtained for each of the cDNAs listed. The abundance ratios for Forrest and Essex are included for comparison. Values that are significantly di erent from the others in the same row are shown in *bold*

biosynthesis pathways, and their mRNAs increased in abundance in Fsg-inoculated RIL 23 roots compared to a significant (two-fold) decrease in Essex-inoculated roots (Fig. 5). The two clones (FiS1A12 and FiS1N18) representing CHS detected a consistently higher (more than two-fold) mRNA abundance in inoculated RIL 23 roots

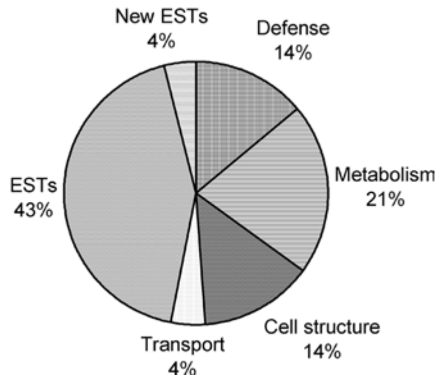

Fig. 4 Categories of genes whose transcripts showed a two-fold increase in mRNA abundance in inoculated RIL 23 roots 14 days after inoculation with Fsg

compared to non-inoculated roots. The two cDNA clones showed over 99% homology to a soybean CHS (FiS1N18 was 511 bp long and FiS1A12 was 670 bp in length). Both spots showed identical hybridization patterns to the probes from inoculated and non-inoculated samples of all the genotypes (Fig. 5). There was no change in the abundance of CHS RNA in the inoculated roots of Forrest or Essex compared to the non-inoculated roots. The sustained higher abundance of the mRNAs for O-methyl transferase (FiS1B14) (Table 1) and isoflavone O-methyl transferase, MIPS, PAL (BI273651) and the calmodulin-like protein (Fig. 5) in RIL 23 suggested that these proteins play a role in the defense of soybean against Fsg inoculation.

Decreases in mRNA abundance in inoculated roots

In inoculated RIL23 roots, only one mRNA [an EST] (FiS1H14) decreased two-fold (0.45± 0.01) in abundance compared to the non-inoculated roots. This EST decreased in Essex and did not change in abundance in inoculated Forrest roots (Table 2). In inoculated Forrest roots, there were four ESTs (3% of the total) that decreased two-fold in abundance. In Essex, 61 cDNAs (46% of the total) decreased by two-fold or more in abundance in the inoculated roots compared to the non-inoculated roots (Table 2). Among the cDNAs that reflected a significant decrease in mRNA abundance, 36% were ESTs to which no function has been assigned. The known cDNA clones with two-fold decreases in mRNA abundance include those that show high homology to isoflavone-O-methyltransferase, acidic chitinase, C4H, MIPS, pathogen-inducible- alpha-dioxygenase, calmodulin like protein and a PLD. For these defense-related genes, transcript abundance was either increased or remained unchanged in the inoculated roots compared to the non-inoculated roots of RIL23.

The change in mRNA abundance for a cDNA representing soybean sucrose synthase (FiS1C3) was 1.97± 0.56, 0.75± 0.08 and 0.39± 0.01 in RIL23, Forrest and Essex genotypes. A similar trend was observed for another cDNA representing soybean proline-rich protein (SbPRP1) where the change in transcript level was 2.21± 0.78, 0.78± 0.11 and 0.45± 0.09 for RIL 23, Forrest and Essex, respectively. Orthogonal contrast analysis revealed that the di erence in the response of SbPRP1 between RIL 23 and Essex was significant (P= 0.05). Sucrose synthase (Dennis and Blakely 2000) and PRP are important constituents of a plant's cell wall synthesis machinery (Carpita and McCann 2000). A more than two-fold decrease in the abundance of mRNAs for sucrose synthase and SbPRP1 indicates a decrease in cell wall synthesis in the roots of the SDS-susceptible genotype Essex upon infection.

A more than two-fold decrease in abundance was observed in inoculated Essex roots for mRNAs

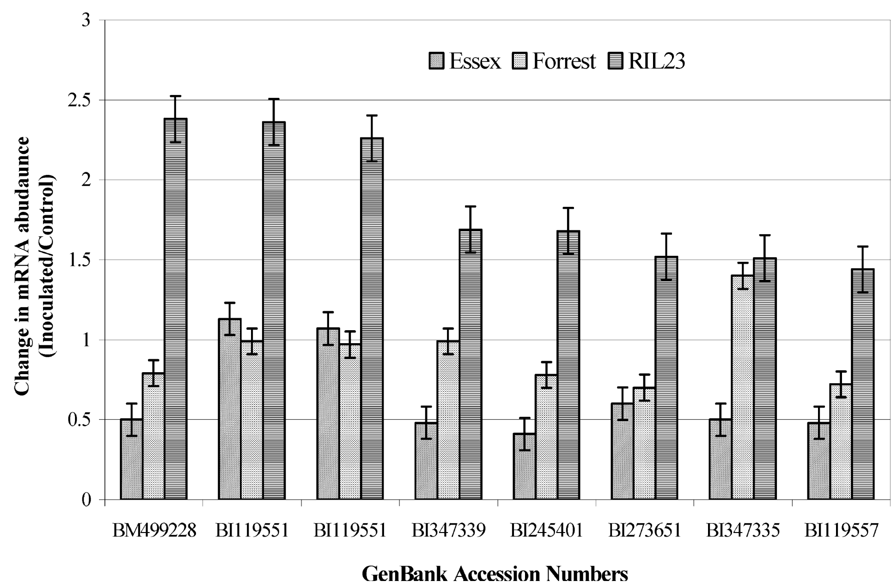

Fig. 5 Comparison of changes in mRNA abundance (inoculated/non-inoculated) of plant defense-related sequences among Essex, Forrest and RIL23. GenBank Accession No. for the sequences are shown on the X-axis. BM499228 (phospholipase D), BI119551 (SIU clone ID FiS1A12), BI119551 (SIU clone ID FiS1N18) (chalcone synthase), BI347339 (*myo*-inositol 1-phosphate synthase), BI245401, (isoflavone O-methyltransferase), BI273651, (phenylalanine ammonia lyase), BI347335, (calmodulin like protein), BI119557, (cinnamic acid 4-hydroxylase)

Table 2 Transcripts that decreased by two-fold or more in abundance in Essex and Forrest roots by 14 days after inoculation

SIU ID	GenBank Accession No.	RIL 23 (± SEM)	Forrest (± SEM)	Essex (± SEM)	Sequence homology to the known genes/ESTs
FiS1i7	BI273640	1.81± 0.51	0.72± 0.21	**0.20 ± 0.04**	*Glycine max* EST
FiS2B2G2	BM499242	1.47± 0.31	0.72± 0.07	**0.33 ± 0.03**	*Glycine max* EST
FiS2B2F4	BM499240	1.65± 0.14	0.99± 0.18	**0.34 ± 0.00**	*Glycine max* EST
FiS1N17	BI273683	1.55± 0.09	0.91± 0.10	**0.34 ± 0.04**	*Glycine max* EST
FiS1N21	BI273684	1.24± 0.11	1.00± 0.13	**0.35 ± 0.01**	*Glycine max* EST
FiS1C1	BI119575	1.09± 0.45	0.68± 0.06	**0.35 ± 0.02**	*Glycine max* thiol protease isoform B mRNA
FiS2B2A6	BM499229	1.04± 0.21	1.25± 0.09	**0.35 ± 0.03**	*Arabidopsis thaliana* homeotic protein BEL1 homolog
FiS1B14	BI119567	2.59± 0.20	0.81± 0.01	**0.35 ± 0.22**	O-methyltransferase
FiS2B2C11	BM499238	1.46± 0.74	0.91± 0.16	**0.36 ± 0.01**	*Glycine max* EST
Fi51N11	BI347333	2.61± 1.91	1.21± 0.18	**0.36 ± 0.07**	Vacuolar ATP synthase
FiS2B2D4	BM499232	3.35± 1.10	0.99± 0.08	**0.37 ± 0.03**	*Glycine max* nodulin 22 gene
FiS1A16	BI119554	2.30± 1.24	0.96± 0.15	**0.38 ± 0.07**	*Glycine max* ADR12 mRNA
FiS1H13	BI273634	0.98± 0.70	0.79± 0.11	**0.38 ± 0.09**	Alfalfa nucleic acid binding protein (alfin-1) mRNA,
FiS1A6	BI119561	1.27± 0.12	1.00± 0.26	**0.38 ± 0.16**	*Glycine max* gene for ubiquitin
FiS1C3	BI245397	1.97± 0.56	0.75± 0.08	**0.39 ± 0.01**	*Glycine max* sucrose synthase (SS)
FiS1M18	BI273679	1.50± 0.57	0.82± 0.03	**0.39 ± 0.01**	*Glycine max* EST
FiS1O2	BI273689	1.28± 0.17	0.57± 0.06	**0.39 ± 0.01**	*Glycine max* EST
FiS1N22	BI273685	1.34± 0.31	0.69± 0.09	**0.40 ± 0.02**	*Glycine max* EST
FiS2B2E10	BM499230	1.51± 0.62	0.81± 0.11	**0.40 ± 0.04**	*Glycine max* EST
FiS1D6	BI245412	1.15± 0.47	0.77± 0.16	**0.40 ± 0.04**	*Arabidopsis thaliana* putative C2H2-type zinc finger protein
FiS1J16	BI119556	1.11± 0.24	0.75± 0.00	**0.40 ± 0.08**	*Glycine max* nodulin 22 gene
FiS1i4	BI119575	1.67± 0.65	0.92± 0.13	**0.40 ± 0.11**	*Glycine max* thiol protease isoform B mRNA
FiS1i17	BI273635	0.76± 0.12	1.04± 0.01	**0.41 ± 0.00**	*Glycine max* EST
FiS1K2	BI273673	1.07± 0.17	1.02± 0.11	**0.41 ± 0.01**	*Glycine max* tefS1 gene for elongation factor EF-1a
FiS1C9	BI245401	1.68± 0.51	0.78± 0.07	**0.41 ± 0.10**	*Medicago sativa* isoflavone-O-methytransferase mRNA
FiS1F4	BI273647	0.84± 0.48	0.90± 0.20	**0.42 ± 0.04**	*Glycine max* EST
FiS1D8	BI118914	1.07± 0.24	0.66± 0.24	**0.42 ± 0.06**	*S. tuberosum* mRNA for plastidic ATP/ADP-transporter
FiS1K5	BI273687	1.33± 0.10	1.08± 0.07	**0.42 ± 0.06**	*Glycine max* actin (Soy58) gene
FiS1i1	BI273632	1.03± 0.36	1.02± 0.27	**0.42 ± 0.08**	Nuclear encoded precursor to chloroplast protein
FiS1H9	BI273631	1.29± 0.37	1.13± 0.15	**0.42 ± 0.10**	Pea histone H2A mRNA
FiS1D3	BI245410	1.30± 0.66	0.91± 0.21	**0.42 ± 0.15**	*N. tabacum* mRNA for root-specific gene
FiS1O1	BI273676	1.29± 0.12	0.95± 0.19	**0.43 ± 0.02**	Phenylalanine ammonia-lyase [soybeans, mRNA, 1427 nt]
FiS1H8	BI245400	0.75± 0.24	0.67± 0.17	**0.43 ± 0.04**	*Zea mays* plasma membrane integral protein
FiS1K17	BI273671	0.67± 0.04	1.20± 0.43	**0.43 ± 0.08**	*Glycine max* EST
FiS2B2B11	BM499231	0.91± 0.41	0.91± 0.12	**0.43 ± 0.09**	*Medicago truncatula* zinc transporter (ZIP)
FiS1C17	BI119577	2.21± 0.59	0.96± 0.16	**0.43 ± 0.10**	*Glycine max* EST
FiS1F5	BI273648	0.98± 0.56	1.38± 0.11	**0.44 ± 0.03**	*Glycine max* chib1-a gene for acidic chitinase
FiS1H11	BI273656	0.70± 0.39	0.93± 0.02	**0.44 ± 0.08**	*Pisum sativum* ubiquitin conjugating enzyme (UBC4)
FiS1i22	BI273638	0.97± 0.55	1.13± 0.06	**0.44 ± 0.08**	*Daucus carota* mRNA for AX110P
FiS2B2C9	BM499236	1.38± 0.12	2.38± 0.23	**0.45 ± 0.08**	*Glycine max* gene for ubiquitin
FiS2B2A11	BM499233	2.21± 0.78	0.78± 0.11	**0.45 ± 0.09**	*Glycine max* SbPRP1 gene encoding a proline-rich protein
FiS1F2	BI273645	0.55± 0.14	0.99± 0.37	**0.45 ± 0.10**	*Glycine max* EST
FiS1C22	BI245396	2.06± 0.41	1.09± 0.14	**0.45 ± 0.12**	*Glycine max* EST
FiS2B2A9	BM499237	1.27± 0.03	0.65± 0.16	**0.46 ± 0.01**	*Glycine max* EST
FiS1A10	BI119559	2.04± 1.29	0.61± 0.01	**0.46 ± 0.05**	*Glycine max* nodulin (C51)
Fi37A24	BI347331	0.84± 0.06	1.39± 0.23	**0.46 ± 0.16**	*Glycine max* EST
FiS1N3	BI273686	1.85± 0.28	0.90± 0.13	**0.47 ± 0.02**	*Daucus carota* poly(A)-binding protein
FiS1i9	BI245403	2.29± 0.13	1.94± 0.24	**0.47 ± 0.03**	*P.acutifolius* alcohol dehydrogenase-1F mRNA
FiS1M4	BI273682	1.00± 0.65	1.09± 0.14	**0.47 ± 0.07**	*Glycine max* putative water channel protein (Pip1)
FiS1K18	BI273672	1.92± 0.73	0.99± 0.21	**0.47 ± 0.14**	*Glycine max* EST
FiS1C18	BI119578	1.43± 0.02	1.17± 0.32	**0.47 ± 0.18**	*Glycine max* Sali3–2 mRNA
FiS1A5	BI119557	1.44± 0.56	0.72± 0.08	**0.48 ± 0.04**	*Glycine max* cinnamic acid 4-hydroxylase
FiS1H14	BI273658	**0.45 ± 0.11**	1.40± 0.59	**0.48 ± 0.04**	*Glycine max* EST
Fi65E19	BI347339	1.69± 0.92	0.99± 0.19	**0.48 ± 0.06**	Myo-inositol-1-Phosphate synthase
FiS1D13	BI245414	1.58± 0.70	0.80± 0.23	**0.48 ± 0.14**	*Glycine max* EST
FiS1G17	BI273652	1.60± 0.28	1.15± 0.06	**0.49 ± 0.01**	*Glycine max* EST
FiS2B2D11	BM499239	0.95± 0.29	0.75± 0.28	**0.49 ± 0.03**	*Nicotiana attenuata* pathogen-inducible alpha-dioxygenase
FiS1D24	BI245409	1.82± 0.58	1.00± 0.14	**0.50 ± 0.07**	*Pisum sativum* brassinosteroid biosynthetic protein LKB
Fi53O13	BI347335	1.51± 0.73	1.40± 0.21	**0.50 ± 0.07**	Calmodulin like protein
FiS2B2G10	BM499228	2.38± 0.84	0.79± 0.01	**0.50 ± 0.09**	*Vigna unguiculata* phospholipase D
FiS1K23	BI273674	1.30± 0.67	0.74± 0.13	**0.50 ± 0.11**	*Vigna radiata* 1-aminocyclopropane-1-carboxylate oxidase
FiS2B2E1	BM499235	1.26± 0.25	**0.32 ± 0.02**	0.52± 0.00	*N. tabacum* EF-1-alpha-related GTP-binding protein (SUP1)
FiS1A17	BI119550	2.12± 0.65	**0.47 ± 0.12**	0.53± 0.04	*Glycine max* actin (Soy57) gene
FiS1M6	BI245393	2.02± 0.85	**0.47 ± 0.20**	0.75± 0.41	*Glycine max* EST
FiS1B2	BI119569	1.51± 0.50	**0.46 ± 0.20**	0.83± 0.13	*Glycine max* EST

[a]The data show the signal ratios (inoculated/uninoculated) obtained for each of the cDNAs listed. The abundance ratios for RIL 23 are included for comparison. Values that are significantly di erent from the others in the same row are shown in *bold*

representing vacuolar ATP synthase, ATP/ADP-transporter and alcohol dehydrogenase (Table 2), indicating a decrease in metabolic activity in the infected roots. In RIL 23, the abundance for these cDNAs was either increased significantly (alcohol dehydrogenase) or remained unchanged. Another mRNA representing 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase; 4e-43) was decreased by two-fold (0.5± 0.11) in inoculated Essex roots, whereas its abundance in RIL 23 inoculated roots remained unchanged (1.30± 0.67). ACC oxidase converts 1-aminocyclopropane-1-carboxylic acid to ethylene. A decrease in the level of this transcript can be attributed to a decrease in ethylene synthesis and in turn to a decreased response to wounding, senescence and biotic stress caused by Fsg infection.

Discussion

The invasion of plant tissues by a fungus results in the induction and sustained expression of a varied battery of plant defenses that prevent further pathogen ingress in cases of resistance responses (Dixon et al. 1994). The quantitatively inherited resistance of soybeans to SDS suggests the involvement of several metabolic pathways or responses in the process of resistance to the fungal infection. RIL 23 contained resistance alleles at all six QTLs (Njiti et al. 2001) that have been identified for resistance to SDS and, therefore, was a good candidate for an analysis to identify all components of the root response to Fsg inoculation. When sequences identified by subtraction hybridization were compared to a unigene set of 4000 ESTs, only nine were found to be in both collections. The changes in mRNA abundance for the nine common sequences were similar pattern to those detected in preliminary microarray experiments (Yaegashi 2002), confirming the validity of macroarray analysis. However, in any such analysis, it is important to have as many genes/ESTs as possible representing that particular response. Enrichment by subtraction hybridization of unique sequences expressed during a particular response is a viable way to identify a representative set of genes and ESTs. A general set of ESTs isolated from a particular plant organ such as the root may not be very useful in gene expression analysis unless the number of ESTs represents the whole genome. Typically EST libraries under-represent rare transcripts (Drawid et al. 2000) and genes for membrane proteins.

Resistance locus pyramids

In RIL 23, 20% of the cDNAs blotted onto the nylon membrane showed a two-fold increase in mRNA abundance in the Fsg-inoculated roots compared to less than 1% in inoculated Forrest and Essex roots. Although Forrest contains four resistance alleles, and is partially resistant (tolerant) to SDS, only one mRNA showed two-fold increase in abundance upon infection. However, the general pattern of mRNA abundance for all the cDNAs was intermediate in Forrest compared to the resistant and susceptible genotypes. The low overall abundance of all the cDNAs in inoculated Essex roots suggested that the resistance alleles of this variety alone had very little e ect on resistance until 14 days after inoculation. Relatively little change in mRNA abundance in inoculated Forrest roots suggests that Forrest resistance alleles alone can probably help the plant survive an Fsg infection beyond 14 days after inoculation, but may not provide stable resistance at later times after inoculation. However, when combined by gene pyramiding, the Essex alleles do contribute significantly to resistance, as is evident from the higher mRNA abundance of all the cDNAs in inoculated RIL 23 roots. A strong interaction among the loci contributed by both parents is implied and can be measured in a substitution mapping population.

Signal recognition

From the cDNA macroarray analysis, several potential genes/sequences in common pathways related to signal recognition were identified. PLD encodes an enzyme that hydrolyzes phospholipids. Comparison of the change in abundance of this RNA between RIL23 and Essex indicates a 4.8-fold di erence. In plants, PLD plays an important role in fruit ripening, stress and wound responses (Katagiri et al. 2001; Lee et al. 2001; Munnik 2001; Zien et al. 2001). PLD is part of the plant cell's signal recognition system and, therefore, is potentially involved in pathogen elicitor recognition which, in turn, helps trigger the defense response. The inoculated/non-inoculated ratio for the mRNA for calmodulin like protein (Fi53O13) was 1.51± 0.73 in RIL23. There was no significant di erence in the behavior of this mRNA between RIL23 (1.51± 0.73) and Forrest (1.40± 0.21). However, Essex showed a significant decrease in abundance (0.50± 0.07). Therefore, RIL23 and Forrest have approximately three-fold more mRNA for this calmodulin like protein in inoculated roots compared to Essex. The abundance of MIPS mRNA determined in this study was consistent with that identified earlier (Iqbal et al. 2002); further indicating the involvement of elicitor-induced defense responses of soybean through PLD, IP3 and calmodulin in RIL23.

Defense

Macroarray analysis identified genes/sequences in common pathways related to soybean defense against Fsg inoculation/infection. The important genes involved in lignin, flavonoid and isoflavonoid biosynthesis pathways, like PAL and C4H, showed an increase in their transcript level in inoculated RIL23 roots but a significant decrease in their transcript level in inoculated Essex

roots. PAL, the first and rate-limiting enzyme of the phenylpropanoid pathway, increases in response to pathogen infection (Hahlbrock and Scheel 1989) as a result of transcription of one of several PAL genes (Constabel 1999). C4H is an important enzyme which converts cinnamate into 4-coumarate in the phenylalanine/hydroxycinnamate pathway. Accumulation of PAL and C4H will result in the accumulation of substrate for the downstream steps in the phenylpropanoid pathway, because these two enzymes are key enzymes in the upstream segment of the pathway. PAL and C4H exhibit increased activity in resistant wheat lines at very early stages of infection and this continued to increase until 6–7 days after inoculation with *Puccinia graminis* f.sp. *tritici*. In contrast, in a compatible interaction, it either remained constant or decreased as compared to the control samples (Moerschbacher et al. 1988). Similar trends in the expression of genes involved in lignin biosynthesis observed in this study suggest that Fsg inoculation suppresses lignin biosynthesis activity in susceptible varieties compared to the resistant soybean RIL. The exact pattern of changes in expression can be determined by a time-course expression analysis of these genes.

The first enzyme in the downstream segment of the phenylpropanoid pathway is CHS. CHS is encoded by a multigene family in soybeans (Akada and Dube 1995). CHS is the first rate-limiting step of the flavonoid pathway which produces C_{15} aglycone skeletons for phytoalexin synthesis (Strack 1997) and the second rate-limiting enzyme in the phenylpropanoid pathway. It also separates the biosynthesis of the antimicrobial flavonoid phytoalexins from that of lignin precursors. CHS is known to play important roles in the plant defense response. Transcripts of the CHS gene family showed a more than two-fold increase in abundance (2.36± 0.21) in RIL 23 upon inoculation. Essex and Forrest, in contrast, showed signal ratios of 1.13± 0.05 and 0.99± 0.29, respectively. Therefore, the change in total mRNA abundance of the CHS family does not di er significantly between Essex and Forrest. However, gene-specific changes cannot be detected by the method we used, so a di erential response is not excluded. Combining the resistance alleles in RIL23 may cause CHS mRNA to increase in abundance compared to either of the parents. The response in RIL 23 suggests the involvement of CHS-derived isoflavonoids (e.g. glyceollin) in resistance to SDS (Lozovaya et al. 2001).

The signal ratio for the isoflavone O-methyltransferase RNA in RIL23 was 1.68± 0.51. Forrest showed a ratio of 0.78± 0.07. In contrast, in the susceptible cultivar, Essex, the signal ratio was 0.41± 0.10. The accumulation of IOMT may result in the accumulation of the fungal inhibitor glyceollin (Lozovaya et al. 2001) in RIL23 and Forrest roots. Since glyceollin is not abundant in susceptible plants, low concentrations of IOMT were expected to in Essex. Among phytoalexins, hydroxycoumarins and hydroxycinnamate conjugates are important compounds that contribute to disease resistance mechanisms in plants (Strack 1997). Therefore, the seven plant defense genes discussed here (Fig. 5) may have an important role in soybean defense against Fsg.

Cell wall and metabolism

In the soybean genotype Essex, in addition to a decrease in the abundance of transcripts of defense-related genes, there was a significant decrease in mRNAs encoding sucrose synthase and SbPRP1 in the inoculated roots. The cell wall is the primary defense of plant cells against invading pathogens (Ride 1980) and a decrease in the levels of transcripts involved in cell wall synthesis indicates the inability of the susceptible genotype to protect itself from the fungal infection. Moreover, the decline in transcription of genes coding for proteins involved in metabolism, such as vacuolar ATP synthase, ATP/ADP-transporter and alcohol dehydrogenase, further indicates the weakening of the cell wall in the infected roots of the susceptible variety as compared to the response of cultivar with a resistance gene pyramid. The decrease in the abundance of ACC oxidase mRNA also indicated a low level of ethylene biosynthesis, suggesting that the roots become less responsive to biotic stress and wounding in Essex.

In this study, we used an EST representing soybean β-tubulin as a constitutively expressed internal control for the purpose of normalizing any quantitative variation occurring during probe synthesis. Ozturk et al. (2002) has reported no change in mRNA abundance for an EST representing tubulin when it was used in microarray and quantitative RT-PCR analyses of drought- and salt-stressed barley roots. A β-tubulin sequence has also been used as a quantitative control to document the response of *Arabidopsis* to infection by *Pseudomonas syringae* pv. *tomato*, wounding and infiltration of water into leaves (Kohler et al. 2002). Therefore, any increase or decrease in mRNA abundance of documented genes/ESTs was suggested to be due to the roots' response to the pathogen.

The results of this study strongly support the hypothesis that pyramiding of resistance alleles helps maintain strong expression of genes/ESTs involved directly or indirectly in plant defenses against fungal pathogens. It has an additive e ect on maintaining a steady level of mRNA transcripts of the key genes involved in the phenylpropanoid pathway, the flavonoid pathway, cell wall biosynthesis, ethylene biosynthesis, and of key metabolic genes, in addition to those involved in signal transduction or induced by fungal elicitors in a genotype having six QTLs for resistance to SDS. The results support our earlier report (Iqbal et al. 2002) on MIPS mRNA abundance and enzyme activity in soybean roots. In order to further elucidate the onset of resistance responses in soybean roots, multiple experiments at di erent times after inoculation are required. These objectives are under study using these enriched clones, together with a unigene set of an EST library, for microarray analysis (Yaegashi 2002).

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