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ORIGINAL PAPER

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Root response to *Fusarium solani* f. sp . *glycines*: temporal accumulation of transcripts in partially resistant and susceptible soybean

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Abstract Sudden death syndrome (SDS) of soybean is a complex of root rot disease caused by the semi-biotrophic fungus *Fusarium solani* f. sp. *glycines* (Fsg) and a leaf scorch disease caused by toxins produced by the pathogen in the roots. Development of partial rate-reducing resistance in roots to SDS was studied. The recombinant inbred line 23 (RIL23) that carried resistance conferred by six quantitative trait loci (QTL) derived from cultivars 'Essex' × 'Forrest' was compared to the susceptible cultivar Essex. Roots of RIL23 and its susceptible parent Essex were inoculated with Fsg. Transcript abundance (TA) of 191 ESTs was studied at five time points after inoculation. For most of the genes, there was an initial decrease in TA in the inoculated roots of both genotypes. By days 7 and 10 the inoculated roots of Essex failed to increase expression of the transcripts of defense-related genes. In RIL23 inoculated roots, the TA of 81 genes was increased by at least two-fold at day 3 ($P = 0.004$), 88 genes at day 7 ($P = 0.0023$) and 129 genes at day 10 ($P = 0.0026$). A set of 35 genes maintained at least a two-fold higher abundance at all three time points. The increase in TA in RIL23 was in contrast to that observed in Essex where most of the ESTs showed either no change or a decreased TA. The ESTs with an increased TA had homology to the genes involved in resistance (analogs), signal transduction, plant defense, cell wall synthesis and transport of metabolites. Pathways that responded included the protein phosphorylation cascade, the phospholipase cascade and the phenolic natural products pathways, including isoflavone and cell wall synthesis.

Keywords Sudden death syndrome of soybean · Transcript abundance · Defense related genes · Temporal accumulation

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Introduction

Plants have developed a complex of integrated defense mechanisms against fungal diseases that include preformed physical and chemical barriers and inducible defenses (Dixon et al. 1994). Within the spectrum of resistance responses other than immunity and susceptibility, the invasion of plant organs by fungal hyphae may result in the induction and sustained expression of some plant defense-related genes. Induced defenses seek to prevent or reduce pathogen ingress by activating molecules that are antimicrobial, antioxidant, involved in signaling, or cell wall strengtheners (Lamb and Dixon 1997). Partial resistance, occasionally known as rate reducing resistance, may retard the multiplication of different pathogens as observed in many crops (Parlevliet 1979; Tooley and Grau 1982; Njiti et al. 1997). Resistance may result from a reduced infection frequency, a longer latent period, reduced inoculum production by the pathogen, or a combination of these.

DNA markers have been used to dissect partial resistance to fungal pathogens into separate loci in *Zea mays* (Pe et al. 1993; Bubeck et al. 1993), *Vigna* sp. (Young et al. 1994), *Solanum tuberosum* (Leonards-Schippers et al. 1994), *Oryza sativa* (Wang et al. 1994), *Hordeum vulgaris* (Chen et al. 2003), *Glycine max* (Hnetkovsky et al. 1996) and many other crops. Each locus can potentially control one or several components of resistance. In rare cases a poor disease scoring system can confound complete and partial resistance (Meksem et al. 2001). In some cases the action of pathogen

recognition genes can be inferred (Yu et al. 2000; Gebhardt and Valkonen 2001; Piffanelli et al. 2002). However, the defense pathways that are induced by partial resistance do not share the same temporal and spatial organization observed in hypersensitive (complete) resistance (Yu et al. 2000; Piffanelli et al. 2002; Iqbal et al. 2002a; Ferrari et al. 2003; Smart et al. 2003; Schulthesis et al. 2003).

Sudden death syndrome (SDS) of soybean is caused by *Fusarium solani* f. sp. *glycines* (Fsg), recently renamed as *Fusarium virguliforme* (Aoki et al. 2003). SDS of soybean is the result of two distinct interactions; the root infection component, where the fungus causes root rot and produces toxins, and the leaf scorch component, where the toxins produced by the pathogen in the roots are translocated to the foliar parts resulting in SDS symptoms (Jin et al. 1996). The fungus is capable of semi-biotrophic growth utilizing haustoria to feed on live cells, and lytic enzymes and digestive enzymes to feed on dead cells (Roy et al. 1998). Root infection occurs prior to the appearance of foliar symptoms and both cause yield losses (Wrather et al. 2003). The root infection frequency is significantly lower and the latent period is longer for resistant varieties compared to the susceptible genotypes (Njiti et al. 1997, 1998). The rate reducing resistance response may be the result of inducible defenses (Beynon 1997).

In a recombinant inbred line (RIL) population derived from 'Essex' × 'Forrest', six loci explain about 90% of the variability in SDS disease incidence (DI). Forrest has four resistance alleles and two susceptibility alleles. Essex has two resistance and four susceptibility alleles (Iqbal et al. 2001). Linkage group G of soybean carries a cluster of disease resistance genes that include *rhg*1 (partial resistance to soybean cyst nematode race 3), *QRfs2*/*Rft* (reduces leaf scorch due to SDS), and *QRfs*1, which confer soybean root resistance to Fsg infection (Meksem et al. 1999). Genotypes that have accumulated beneficial alleles from all loci are the most resistant while those that have not accumulated any of the beneficial alleles are the most susceptible (Iqbal et al. 2001). Lines that have accumulated some beneficial alleles have an intermediate response to SDS (Njiti et al. 2001).

Gene activation and suppression by biotic or abiotic stimuli can be determined by measuring the transcript abundance (TA) of the genes supposed to be involved in the processes. Increased TA in response to pathogen infection has been reported in several plant species (Piffanelli et al. 2002; Ferrari et al. 2003; Smart et al. 2003; Xu et al. 2003). In soybean plants, Moy et al. (2004) reported that a total of 7.7% host genes (out of 4,000 cDNA transcripts) were upregulated during 3–48 h after inoculation with *Phytophothora sojae* zoospores. The genes that were upregulated include those encoding the enzymes of the phytoalexin biosynthesis pathway, and certain defense and pathogenesis related proteins. Strong positive correlations between increased TA, enzyme activity and metabolite flux of myo-inositol 1-phosphate synthase (MIPS) and the number of favorable alleles of SDS QTL present were observed in soybean roots in response to Fsg (Iqbal et al. 2002b). Myo-inositol is a precursor of compounds in plants that function in phosphate storage, signal transduction, stress protection, hormonal homeostasis and cell wall biosynthesis (Hegeman et al. 2001). In another study, by day 14 after inoculation, Fsg infested roots of soybean RIL23 accumulated transcripts of genes that encoded proteins related to plant defense, cell wall synthesis and lignin biosynthesis (Iqbal et al. 2002a). However, the occurrence of coordinated control of defense related genes is still unknown. The objective of this study was to measure changes in TA of known plant defense and biotic/abiotic stress related genes in soybean roots carrying different numbers of QTL for resistance to SDS of soybean. The genes selected for the study were based on our earlier enrichment (Iqbal et al. 2002a) and those known to be involved in plant defense response. These genes were selected from a soybean root cDNA library (Shoemaker et al. 2002). The soybean genotypes were selected based upon their known genetic backgrounds with one carrying a maximum number of known resistance loci present in that population. The root, being the primary target of the pathogen and the possible site of the initial defense response, was selected as the tissue to study. Change in TA of 191 known genes was monitored over a period of 10 days post inoculation.

Materials and methods

Plant materials

Seeds were obtained from the Southern Illinois University Carbondale Agriculture Research Center and were from lots increased in 2001. Two soybean genotypes were used in the study. Essex was largely susceptible to SDS (*Qrfs*, *Qrfs*1, *Qrfs*2, *Qrfs*3, *QRfs*4 and *QRfs*5) (Njiti et al. 1997; Iqbal et al. 2001). RIL23 (*QRfs*, *QRfs*1, *QRfs*2, *QRfs*3, *QRfs*4 and *QRfs*5), an $F_{5:14}$ derived line from a cross of Essex (Smith and Camper 1973) × Forrest (*QRfs*, *QRfs*1, *QRfs*2, *QRfs*3, *Qrfs*4 and *Qrfs*5) (Hartwig and Epps 1973), was very resistant to SDS (Njiti et al. 2001; Triwitayakorn et al. 2005). Seeds were germinated in 3-inch deep trays filled with sterile sand in a growth chamber set at a 16 h photoperiod with 23°C day and 18°C night temperatures and 80% relative humidity. The plants reached the second trifoliate stage (three leaves after the cotyledenous leaves) without becoming 'pot bound' by 2 weeks after sowing.

Inoculation of roots with Fsg spores

The plants were removed from the soil and the roots were washed with sterile distilled water and dipped in an Fsg spore suspension. The spore suspension, at 5,000 ± 200 spores/ml of water, was made by adding Fsg spores from several Fsg culture plates (Fsg was grown on

potato dextrose media supplemented with 80 μg/ml tetracycline) and a few drops of Tween 20 and was continuously stirred on a stir plate to keep a uniform suspension. In the case of the non-inoculated controls, plants were removed from the trays and washed with sterile distilled water but were not dipped in the spore suspension. Both inoculated and non-inoculated plants were planted in sterile sand:soil (3:1 w/w ratio) in randomized complete block design. The inoculated plants were kept in separate plastic tubs in order to avoid any cross contamination. The plants were not fertilized. Both the inoculated and non-inoculated plants were treated identically. Roots were collected from inoculated and non-inoculated plants at 1, 2, 3, 7 and 10 days after inoculation (DAI) (between 10 am and 11 am each day) as described earlier (Iqbal et al. 2002a). The root were immediately frozen in liquid N_2 and stored at −80°C.

RNA isolation

RNA was isolated separately from both inoculated and non-inoculated roots at all the time points. Roots from a minimum of five plants were ground into a fine powder in liquid N_2 and RNA was isolated using the QIAGEN RNeasy Plant Mini kit (QIAGEN, Calif., USA). RNA samples were treated with DNase and purified using the RNeasy Mini Spin columns (QIAGEN). The quality and quantity of the RNA samples was determined by electrophoresis through 1.2% (w/v) formaldehyde agarose gels and UV spectrophotometry at 260 nm.

Preparation of macroarray filters

A total of 191 ESTs representing 191 homologs of known genes were selected. These ESTs include 28 representing genes with altered TA identified earlier at 14 DAI (Iqbal et al. 2002a) and 163 ESTs from a soybean root EST library. The ESTs from the root library were selected on the basis of their involvement in plant defense, stress and related pathways. Exactly 4 μg of recombinant plasmid DNA was spotted on Hybond N^+ membrane (Amersham Biosciences, Piscataway, N.J., USA). The DNA was re-suspended in 20 μl of a buffer consisting of 0.4 M NaOH and 10 mM EDTA, then denatured at 95°C for 5 min, cooled to room temperature and spotted on the membranes (Iqbal et al. 2002a).

Synthesis of cDNA probes and hybridization

Total RNA (15 μg) was used to synthesize α^{33} P-labeled cDNA probes which were then hybridized to the membranes (Iqbal et al. 2002a). Probes prepared from RNA isolated from inoculated and non-inoculated roots were hybridized simultaneously to separate membranes.

Data analyses

The spot intensities from the hybridized membranes were measured using a PhosphorImager (Molecular Dynamics, Calif., USA) and the background was subtracted using ImagQuant NT software. *G. max* β-tubulin (GenBank accession AI461071) was used as a control on all filters. The data were normalized and changes in TA were inferred from the changes in hybridization intensity in the inoculated roots compared to the non-inoculated roots of each genotype, as described earlier (Iqbal et al. 2002a). Cluster analysis using QT (quality threshold) and the K-means algorithm was performed using the Gene Spring software (Silicon Genetics, Calif., USA). The mean of the ratios (change in expression) of the two replicates of Essex were compared to the mean of ratios of the two replicates of RIL23 at different time points by paired sample *T*-tests using Microsoft Excel software. Paired sample *T*-tests were also used to compare the change in TA ratios from day 1 to each of the other days for both Essex and RIL23 separately, to test the significance of all changes.

Results

Initial response

The initial response (at 1 DAI) of the roots to Fsg infection, as measured by changes in TA of the two genotypes, Essex and RIL23, was similar. The TA of most of the genes was decreased in both genotypes. However, an increase in the TA of the EST homologous to *Arabidopsis thaliana* AWI31 (AI441364) indicates a wounding stress response. Other genes in Essex that had at least a two-fold increase in TA were identified by the ESTs AI441818, BI273648, AI438014, AI440615, AI437671, AI443797, AI494734 and AI496621. In RIL23, the TA of these nine ESTs was only slightly increased. In Essex at 1 DAI a total of 72 ESTs had at least a two-fold decrease in their TA compared to the 119 showing decreased abundance in RIL23. Among these ESTs, 46 were common to both Essex and RIL23. However, this dramatic decrease in TA in both genotypes returned to normal by day 2 after inoculation. Similar observations have been made in cotton roots in response to *F. oxysporum* f. sp. *vasinfectum* (Dowd et al. 2004).

By day 2 after inoculation, 10 genes showed a greater than two-fold increase in their TA in the inoculated roots of Essex. These genes were identified as ESTs BI273648 and AI494734 (also increased on day 1), and BI273674, AI442369, AI441809, AI437703, AI443229, AI441049, AI522838 and AI495354. In RIL23, there were 12 ESTs (AI444099, AI441234, AI444097, AI442455, AI443797, AI460918, AI460471, AI443886, AI437942, AI442586, AI460777, AI437613) with at least a two-fold increase in their TA. Some of the genes represented by these ESTs include a putative

pathogenesis-related protein, gibberellin-20-oxidase, abscisic stress ripening protein 1, a jasmonate inducible protein isolog, malonyl CoA-acyl carrier protein transacylase and endo-1,4-beta-glucanase. The paired sample *T*-test revealed that the overall change (inoculated/non-inoculated) in TA based on the mean of the two replicates was significantly higher ($P = 0.015$) in the roots of RIL23 than in Essex. This significant increase in the TA of genes in the inoculated RIL23 roots indicates the initial response of a resistance genotype to the pathogen.

Mid-response

The two-fold increase in TA of the ESTs observed at day 1 and 2 after inoculation in Essex did not extend to day 7. By day 7, TA was largely similar in both the inoculated and non-inoculated roots of the susceptible genotype Essex. However, the TA of the stress-induced gene H4 was observed to be 3.3-fold higher in inoculated Essex roots compared to non-inoculated roots. Two other ESTs, orthologous to a sucrose transporter and endo-1,4-beta-glucanase (AI442455, AI442505), also maintained at least a two-fold increase in TA in Essex. Considering a two-fold change in TA as significant in this study, the rest of the 187 ESTs on the array did not show any significant change in their TA in Essex.

In RIL23 at 3 DAI, the TA of the array genes was higher in the inoculated roots compared to their level in the non-inoculated roots. This increase was significant ($P = 0.004$) compared to day 2 after inoculation. The TA of 81 ESTs was increased by at least two-fold in the inoculated roots compared to the non-inoculated roots. The increase in the TA of these 81 ESTs and the significance of the change ($P = 0.004$) from day 2 after inoculation indicates the start of several resistance responses in the roots. By day 7 after inoculation, the

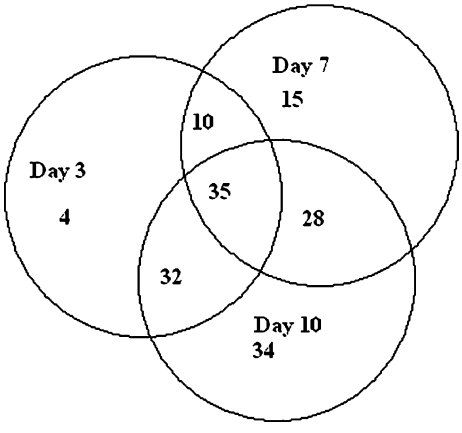

Fig. 1 Number of genes that had at least a two-fold increase in TA at different days after inoculation in RIL 23. There were 81, 88 and 129 ESTs that showed at least a two-fold increase in TA at days 3, 7 and 10 after inoculation. However, there are 35 which are common to all three time points

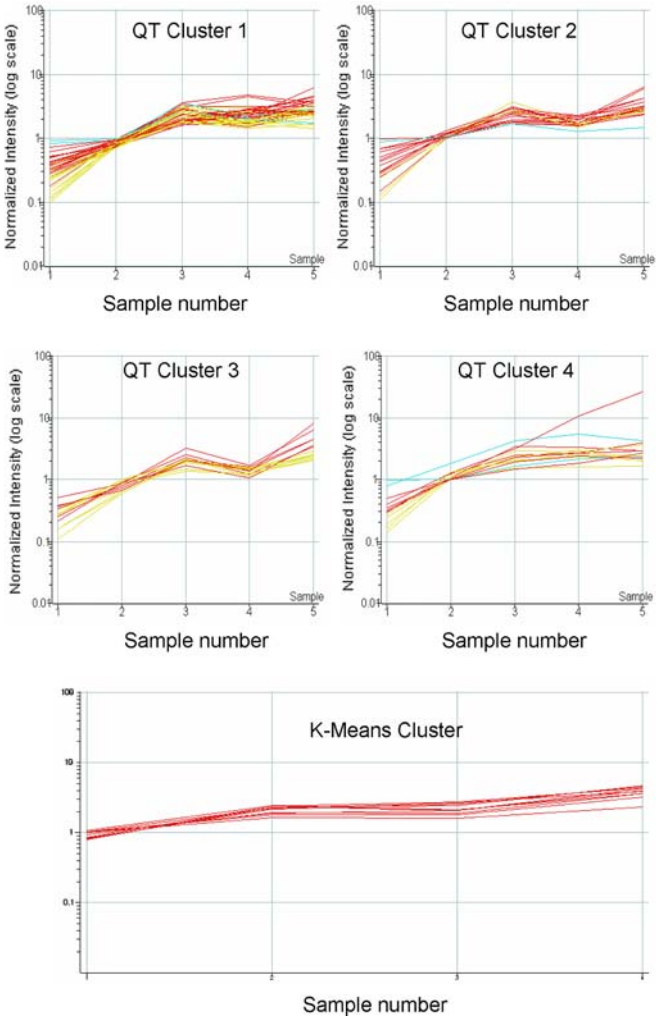

Fig. 2 QT Cluster 1 contains 37 genes represented by ESTs AI440931, AI437501, AI507864, AI460590, AI438104, AI437999, AI442530, AI443448, BI245409, AI495526, AI444067, AI442658, AI441049, AI443936, BI273648, AI441586, BI119576, AI461073, AI437968, AI442204, AI442612, AI437774, AI437899, AI443638, AI443953, AI522838, AI507793, AI495119, AI494672, BI273686, BI245412, AI441027, AI442632, BI273656, BI347333, AI437820, AI522819. QT Cluster 2 contain 17 genes represented by ESTs BI347337, AI443896, AI443299, AI441021, BI347335, BI347329, AI494734, BI347339, BM499228, AI437977, BI347330, AI437544, AI461110, AI443454, AI442425, AI416617, AI437985. QT Cluster 3 contain 14 genes represented by ESTs BI273674, AI495655, AI437704, BI119557, AI437773, AI441895, AI438014, AI437531, AI495493, BI245400, AI443881, AI443819, BI273673, AI443248. QT Cluster 4 contain 13 genes represented by ESTs AI440582, AI442296, AI461033, AI437671, AI496621, AI494845, AI443609, BM499239, AI495627, AI460654, AI443974, BM499236, AI443142. There were 111 genes that did not fall in any of these clusters (see the remaining genes in the Table in the Electronic Supplementary Material). K-means clustering grouped 10 ESTs representing glyceraldehyde-3-phosphate dehydrogenase, inosine-5′-monophosphate dehydrogenase, myo-inositol-1-phosphate synthase, NAD(P)H-dependent-6′-deoxychalcone synthase, phenylalanine ammonia lyase, phospholipase D, putative receptor-like protein kinase, repetitive proline-rich cell wall protein 1 precursor, ubiquitin and ubiquitin conjugating enzyme (UBC4). In the K-means analysis, the data for day 1 after inoculation was removed. The rest of the ESTs did not cluster

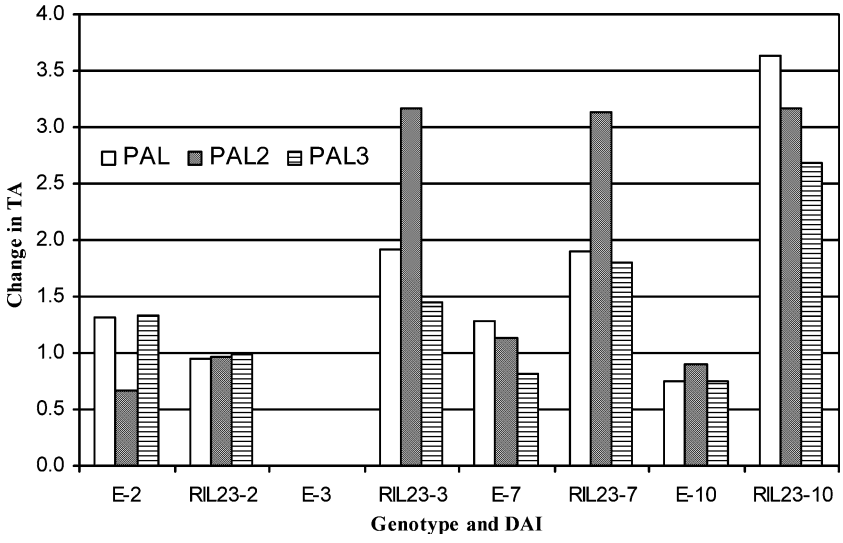

Fig. 3 A comparison of the change in TA of phenylalanine ammonia lyase (*PAL*), PAL class II (*PAL2*), PAL class III (*PAL3*). Note that the data for Essex at day 3 after inoculation was missing. *E-2* changes in TA in Essex at 2 days after inoculation (*DAI*), *RIL23-2* changes in TA in RIL 23 at 2 DAI and so on

abundance of 45 of these 81 ESTs was still at least two-fold greater than the baseline abundance in the inoculated roots compared to the non-inoculated roots. In addition to these 45 ESTs, there were 43 new transcripts that had a greater than two-fold increase in TA. The increase in TA from day 3 to day 7 was also significant ($P = 0.0023$). A total of 88 ESTs showed an increased TA in the roots of RIL23 compared to just 3 in Essex.

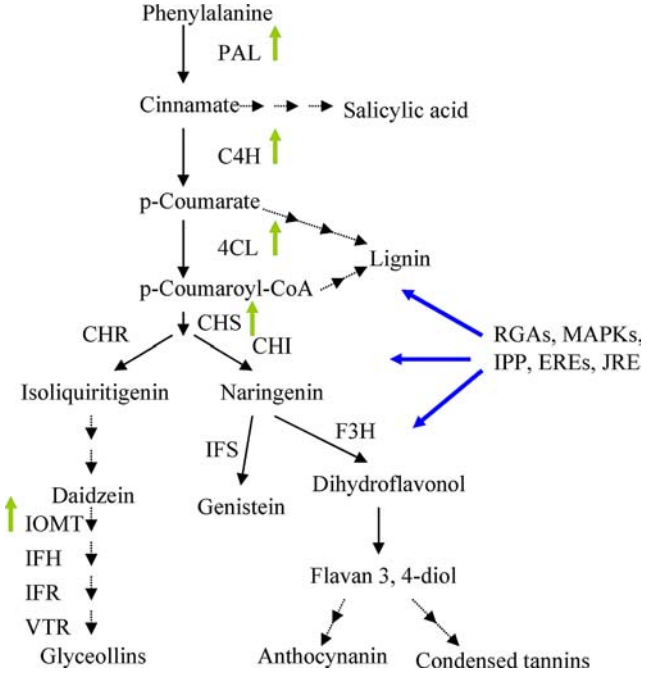

Fig. 4 Enzymes involved in the biosynthesis of some important secondary metabolites that play a significant role in plant defense. *PAL* Phenylalanine ammonia lyase, *C4H* cinnamic acid-4-hydroxylase, *4CL* 4-coumarate-CoA ligase, *CHS* chalcone synthase, *CHR* chalcone reductase, *CHI* chalcone isomerase, *IFS* isoflavone synthase, *F3H* flavanone-3-hydroxylase, *IOMT* isoflavone o-methyltransferase, *IFH* isoflavone hydroxylase, *IFR* isoflavone reductase, *VTR* vestitone reductase. *Green arrows* indicate the increase in TA for the EST encoding the enzyme. *Blue arrows* represent the signals inferred to potentially be involved. Signals included resistance gene analogs (*RGAs*), protein kinases (*MAPKs*), ispenenyl pyrophoshatases (*IPPs*), ethylene response (*EREs*), jasmonate response (*JRE*). IFH and VTR were not present on the assay

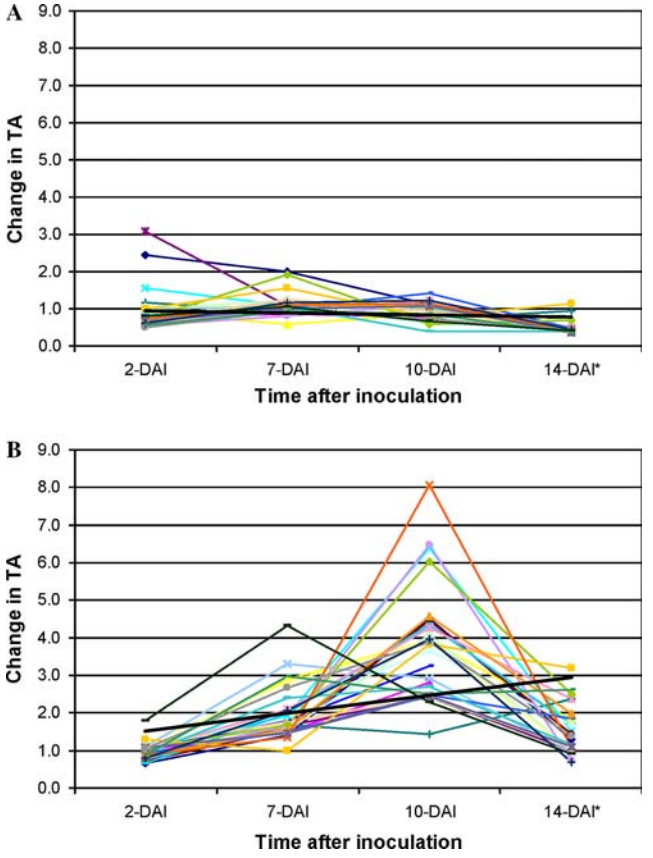

Fig. 5 Changes in TA in Essex (*Panel A*) and RIL23 (*Panel B*) are compared for the genes that were in common to this study and in Iqbal et al. 2002a. The results from this study are for 2, 7 and 10 days after inoculation (DAI). The results from 14 DAI are from Iqbal et al. 2002a. The two panels are shown on the same scale for easy comparison

This increase in TA in the inoculated roots of RIL23 was highly significant ($P < 0.001$) compared to that in Essex. In Essex, at day 7 after inoculation, these 88 ESTs showed either a decreased or unchanged TA.

Late response

In Essex there was no significant increase in the TA of most of the genes in the array at day 10 after inoculation. However, there were 11 genes that showed an at least two-fold decrease in TA. These include ESTs AI437572, AI441758, AI437618, AI460635, AI440721, AI460578, AI442485, AI443444, AI443209, BI245412 and AI442287. A total of 116 genes on the array showed a decrease in TA that was less than two-fold. For another 23 genes, the change in TA was 1.1- to 1.7-fold. When the change in TA of all the genes at day 10 was compared to that at day 7, there was a slight but significant decrease ($P = 0.0005$).

At 10 days after inoculation, in RIL23 there were 129 genes with at least a two-fold increase in TA in the inoculated roots compared to non-inoculated roots. Among these, 63 showed at least a two-fold increase in TA at day 7 and continued this trend at day 10. However, when days 3, 7 and 10 were compared, the TA of 35 of these genes was found to be consistently at least two-fold or greater in RIL23 (Fig. 1). The increase in the TA of the genes on the array was highly significant ($P > 0.0001$) in RIL23 compared to Essex. In RIL23 the changes in TA from day 7 to day 10 were also significant ($P = 0.0026$).

The ratios of gene expression in RIL23 were used to generate clusters of genes with similar patterns of transcript accumulation (hereafter clusters) at days 1, 2, 3, 7 and 10 after inoculation (Fig. 2). Four clusters were generated representing different patterns of change in TA in RIL23. Cluster 1 had a set of 37 ESTs grouped together. Some of these ESTs represent a Ca^{2+}/calmodulin dependent protein kinase, a Ca^{2+}-ATPase, a calcium binding protein isolog, 7-o-methyltransferase, phenylalanine ammonia lyase I and II, chalcone flavanone isomerase, chalcone isomerase, the *chib 1-a* gene for acidic chitinase and a chitinase. Cluster 2 contained 17 ESTs. These ESTs represented genes that include a calmodulin-like protein, a putative receptor-like protein kinase, a calcium-dependent protein kinase, myo-inositol-1-phosphate synthase and a phospholipase D. The third cluster grouped 14 ESTs and represented genes including cytchrome P450, cinnamic acid-4-hydoxylase, catechol-o-methyltransferase, cellulose synthase, a repetitive proline-rich cell wall protein and a gene for elongation factor EF-1. The fourth cluster contained 13 ESTs and the genes represented include a disease resistance protein homolog, a TMV resistance protein homolog, a stress related protein, a calmodulin like protein, isoflavone reductase and phenylalanine ammonia lyase class III. Using the K-means clustering algorithm, when the program was asked to generate a cluster without setting any preconditions, 10 genes were clustered (Fig. 2). The rest of the genes did not fall into any category under these analysis conditions. To generate clearer trends in expression changes, the RIL23 data was also analyzed with a rival penalized competitive learning (RPCL) clustering algorithm and presented as Supplementary Fig. 1 http://dx.doi.org/10.1007/s00122-005-1969-9.

There were three ESTs in the array representing different members of the phenylalanine ammonia lyase (PAL) gene family. PAL regulates one of the first steps in both the phenylpropanoid and lignin biosynthesis pathways. The genes included were *PAL*, PAL class II (*PAL2*) and PAL class III (*PAL3*). *PAL* and *PAL2* had a similar expression pattern in RIL23 (Fig. 3) and were grouped in cluster 1. The change in expression of all the *PAL* genes was higher in RIL23 inoculated roots compared to Essex inoculated roots, indicating a sustained higher activity of the phenylpropanoid and lignin biosynthesis pathway leading to better resistance by its roots.

A comparison of the cDNAs that were also studied at 14 DAI (Iqbal et al. 2002) indicate that day 14 is very late in the infection process (Fig. 4, 5). A trend line indicate a continued decrease in TA in the inoculated roots of Essex. Only 3 ESTs (BI245413, BI347330 and BI119576) had a non-significant increase at day 14 compared to day 10. Comparison of the two experiments in RIL23 indicate that 10 DAI was the time when there was a maximum response as measured by change in TA. By day 14, only BI119576, representing cinnamyl alcohol dehydrogenase (CAD1), showed a two-fold increase in TA in the inoculated roots. The other ESTs that maintained a two-fold higher level of TA in inoculated roots included BI347333, BI347330, BM499228. However, for rest of the ESTs, the increase was less than two-fold. A general trend line indicates the initial pattern while a close look at the individual genes indicate that 7–10 DAI was the time where the maximum changes in TA were observed.

Discussion

The results of this study indicate a temporal response of genes to Fsg infection of the roots of a resistant soybean genotype. This change in TA (at days 3, 7 and 10) was consistent with those observed in earlier studies (Iqbal et al. 2002a), where the TA of 28 cDNAs identified by differential display and subtractive hybridization were significantly higher in partially resistant roots compared to a susceptible genotype at 14 DAI. The root response, as determined by changes in TA, is similar to reactions observed in partial resistance to fungi in *Arabidopsis*, barley, potato and tomato (Yu et al. 2000; Piffanelli *et al*. 2002; Ferrari et al. 2003; Smart et al. 2003) in terms of the pathways and signals induced. However, the response of the roots of the resistant soybean in terms of accumulating higher amounts of transcripts was slow

compared to both the classic HR response (Moy et al. 2004) and during partial resistance to other fungi.

In RIL23 the significant increase ($P = 0.004$) in TA of the array genes by day 3 after inoculation indicates that recognition of the pathogen by the soybean roots may occur between day 2 and day 3. This increase is later than that reported for comparable partial resistance interactions (Piffanelli et al. 2002; Ferrari et al. 2003; Smart et al. 2003). The Fsg macroconidia that were used as the inoculum must first convert to chlamydospores in the soil, which then infect the roots (Melgar et al. 1994), provoking a delayed response in the roots. Once the resistant genotype detects the pathogen, the genes and pathways involved in preventing disease become active and their transcripts increase.

Pathogen recognition

Plant cells often use receptors at the cell surface to sense environmental changes then transduce this information via activated signaling pathways to trigger adaptive responses (Morris and Walker 2003). In case of both pathogenic and symbiotic relationships, receptor like kinases (RLKs) plays a role in the plant's recognition of the microbe (Yu et al. 2000; Piffanelli et al. 2002; Morris and Walker 2003). The EST that represented a *G. max* RLK showed a two-fold increase in TA in RIL23 at day 3 after inoculation, and no change in TA in Essex. Another EST representing a sub-class of *G. max* leucine rich repeat/receptor protein kinases had a two-fold increase in TA at days 7 and 10 after inoculation in RIL23. It has been observed that Fsg hyphae grow both intercellularly and intracellularly (Roy et al. 1998). Therefore the increase in the TA of these RLKs at different times after inoculation suggests their involvement in pathogen recognition during both intercellular and intracellular growth. As intracellular growth of the hyphae leads to cell death and release of the cellular contents, more RLKs are upregulated during the later stages of the infection.

Signal transduction

The TA of two ESTs (AI442296, BI347335) representing calmodulin-like proteins was consistently higher in RIL23 compared to Essex. Similarly, ESTs homologous to Ca^{2+} -ATPase, a calcium-dependent protein kinase and a phospholipase D (PLD) also had a higher TA in RIL23 roots compared to those of Essex. Calcium is known to be a part of the cellular signal recognition system and, therefore, the increase in the TA of Ca^{2+}-activated genes may be involved in pathogen elicitor recognition, or triggering the defense response (Keates et al. 2003; Piffanelli et al. 2002). The increase in PLD transcripts also suggest defense gene activation through jasmonic acid biosynthesis rather than the calcium ionophore-dependent activation in plant cells or the oxidative burst of animal cells. The gradual increase in expression of the PLD transcript was also consistent with the RIL23 root response to SDS pathogen at 14 DAI (Iqbal et al. 2002a).

Metabolic response

The phenylpropanoid pathway provide lignins, lignans, suberin, flavonoids, coumarins and stilbenes and is strongly activated upon infection by many pathogens (Pakusch and Matern 1991; Jaeck et al. 1992; Moy et al. 2004; Dowd et al. 2004). PAL, the first and rate-limiting enzyme of the phenylpropanoid pathway, is increased in response to pathogen infection (Hahlbrock and Scheel 1989; Constabel 1999). It also leads to the synthesis of salicylic acid which in turn can trigger systemic acquired resistance. The clusters inferred from this study suggest that the *PAL* genes (*PAL*, *PAL2*, *PAL3*) are controlled by at least two different regulatory mechanisms, as in raspberry (Kumar and Ellis 2001) and *Phaseolus vulgaris* (Cramer et al. 1989). The early increase in the TA of *PAL2* in the inoculated roots of resistant soybeans indicates its involvement in the early defense response. It may include fungal elicitor recognition between day 2 and 3 after inoculation, similar to the *P. vulgaris* response to *Cladosporium fulvum* infection (Cramer et al. 1989). The overall increase in the TA of all the ESTs representing the *PAL* family of genes suggests their involvement in the resistance mechanism. In contrast, the inability of the roots of Essex to increase the transcript of the *PAL* genes and the possible consequent lack of increase in the production of phenolic compounds in response to infection may ultimately lead to the onset of disease. The other enzymes of the phenylpropanoid pathway that showed at least a two-fold increased in TA include chalcone synthase, C4H and IOMT, further implicating the involvement of several of the phenylpropanoid pathways in the processes of resistance (Fig. 4). The coordinate increase in C4H and PAL may participate in the reinforcement of cell walls by increased deposition of the lignin building units (monolignols) and coumarates in the infected plants and thus the restriction of pathogen invasion (Dixon et al. 1995). Coordinate regulation was observed between PAL, chalcone isomerase and isoflavone reductase. Together these data suggest that fungicidal isoflavones may also participate in the restriction of pathogen invasion (Shadle et al. 2003).

Growth regulators/hormones

The mitogen-activated protein kinase (MAPK) cascade(s) are important regulators of the plant defense/stress responses such as the phenylpropanoid pathways (Liu et al. 2003). The global signals jasmonic acid (JA), salicylic acid (SA), ethylene (ethephon), and abscisic

acid, the hydrogen peroxide fungal elicitor chitosan, drought, high salt and sugar, and heavy metals can all induce the MAPK cascades (Meindl et al. 1998; Kim et al. 2003). The arrays in this study included five ESTs representing *G. max* MAP kinases (AI440721, AI443953, AI437948, AI460578, AI441087). The transcript of the MAP kinase-like protein (AI443953) was increased three-fold in RIL23 inoculated roots from day 3 to day 10. Other kinases showed a significant increase in their TA by day 7. In contrast, MAPK transcripts were either decreased or unchanged in Essex. An involvement of many kinases in the root resistance response of RIL23 to Fsg infection was inferred.

The role of plant growth regulators in resistance is complex due to their overlapping biochemical, physiological and morphological effects (Ferrari et al. 2003; Liu et al. 2003). The auxin and jasmonate signaling pathways share common components (Tiryaki and Staswick 2002). An EST ortholog of an auxin-induced protein 22D (AI437500) increased in TA from 3 to 10 DAI. An EST ortholog of a jasmonate-inducible protein (AI460918) increased in TA from 2 to 10 days after inoculation. The ESTs representing an ethylene-forming enzyme and the ethylene-insensitive-like3 enzyme increased in TA at 7 and 10 days after inoculation. The phytohormones auxin and ethylene have earlier been implicated in the response of cotton seedling roots and seedling hypocotyls to *F. oxysporum* f. sp. *vasinfectum* infection (Dowd et al. 2004), therefore, their change in TA in this study is not surprising.

Pathogenesis related proteins

Pathogenesis related (PR) proteins showed expression in soybean roots in response to *P. sojae* (Moy et al. 2004). In this study, the TA of soybean *PR1* (AI507793) and *PR5* (AI460954) did not change in Essex inoculated roots. The upregulation of PR proteins in RIL23 inoculated roots may be the result of resistant root's active defense response. The increase in the TA of *PR1* in RIL23 roots is consistent with the increase observed in the *PR1* transcript in both the compatible and incompatible interactions of late-blight fungus *P. infestans* with *S. tuberosum* (Taylor et al. 1999).

Conclusion

The study focused on the response of soybean genotypes of known genetic backgrounds to the fungal pathogen Fsg. The results clearly demonstrated that the responses of known genes of the plant defense, signal recognition and transduction, and metabolic processes were different in the partially resistant and partially susceptible soybean roots. This study identified a set of genes that had at least a two-fold increase from day 3 to day 10 after inoculation in RIL23. These genes thus form the basis of a robust molecular fingerprint that can be used to assay SDS resistance in soybean germplasm. Four of the ten genes clustered by K-means algorithm; myo-inositol-1-phosphate synthase, PAL, PLD and NAD(P)H dependent-6′-deoxychalcone synthase have earlier been identified as showing an altered TA at 14 DAI (Iqbal et al. 2002a). The results of this study were consistently reproducible and support our earlier identified genes and transcripts. In order to explore the involvement of these upregulated genes in the resistance response, their protein abundance, activity of their enzymes and flux of metabolites need to be further studied in a variety of soybean germplasm.

The action of loci underlying partial resistance to SDS has been inferred to be additive in QTL mapping studies (Bubeck et al. 1993; Pe et al. 1993; Young et al. 1994; Leonards-Schippers et al. 1994; Wang et al. 1994; Hnetkovsky et al. 1996; Chen et al. 2003). Essex is not as susceptible as some other cultivars. However, the results of this study of roots indicate the interaction was not additive as Essex did not mount any resistance response as determined by an increase in the TA of these genes. Field studies have identified Essex as partially susceptible (Njiti et al. 1997), while mapping studies in an Essex × Forrest RIL population have indicated the presence of resistance alleles at two of the six QTL conferring resistance to SDS. Soybean SDS consists of root infection and leaf scorch. Therefore, studies with foliar/above ground parts for TA analysis and RILs containing up to five resistant alleles of QTL conferring resistance to SDS will enable us to explore the contribution of individual QTL and their additive/non-additive nature in the processes of resistance to SDS. With limitations in the study of gene interactions, definite conclusions about the nature of the resistance response to Fsg infection cannot yet be made. The genetically characterized populations described here provide an excellent platform for studying gene/QTL interactions with resistance mechanisms.

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