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A pyramid of loci for partial resistance to *Fusarium solani* f. sp. *glycines* maintains *Myo*-inositol-1-phosphate synthase expression in soybean roots

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Abstract *Myo*-inositol 1-phosphate synthase (MIPS; EC 5.5.1.4) converts glucose 6-phosphate to *myo*-inositol 1-phosphate in the presence of NAD^+. It catalyzes the first step in the synthesis of *myo*-inositol and pinitol, and is a rate limiting step in the de novo biosynthesis of inositol in eukaryotes. Therefore, MIPS is involved in biotic and abiotic stress via Ca^{2+} signalling. Seedlings of four soybean genotypes were inoculated with *Fusarium solani* f. sp. *glycines*, the causative agent of sudden death syndrome (SDS), and differentially abundant mRNAs were identified by differential display. The genotypes carried either zero, two, four or six alleles of the quantitative trait loci (QTLs) that control resistance to SDS in an additive manner. The mRNA abundance of MIPS did not decrease following inoculation in a recombinant inbred line (RIL 23) containing all six resistance alleles of the QTLs conferring resistance to SDS of soybean. However, the abundance of MIPS mRNA was decreased in genotypes containing four, two or no resistance alleles. The specific activity of the MIPS enzyme in vitro followed the same pattern across genotypes. The IP_3 content in the inoculated roots of genotypes with two, four or six resistance alleles were higher compared to the non-inoculated root. The results suggests that a non-additive effect on transcription and translation of MIPS is established in RIL 23 roots by pyramiding six QTLs for resistance to SDS. A role of MIPS in the partial resistance or response of soybean roots to *F. solani* infection is suggested.

Keywords Gene pyramids · Gene expression · *Myo*-inositol 1-phosphate synthase · Quantitative trait loci

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Introduction

Plants are continuously exposed to pathogens. The range of plant pathogenic organisms is diverse and includes viruses, mycoplasma, bacteria, fungi, nematodes, insects, protozoa and parasitic plants (Agrios 1998). Invasion of resistant plant tissues by hyphae in the plant fungal-pathogen interactions can result in the induction and sustained expression of a varied battery of plant defenses that prevent further pathogen ingress (Dixon et al. 1994). Plants produce a wide variety of secondary metabolites, many of which have anti-microbial activities. Several defense-associated compounds such as chitinases, β-1,3-glucanase, pathogenesis-related proteins, phytoalexins, salicylic acid, active oxygen species and jasmonate have been identified (Kuc' 1995; Feys and Parker 2000).

Myo-inositol, a six-carbon-sugar alcohol, is a precursor to compounds that function not only in phosphorus storage, but also in signal transduction, stress protection, hormonal homoeostasis and cell-wall biosynthesis (Morre' et al. 1990; Loewus and Murthy 2000). *Myo*-inositol 1-phosphate synthase (MIPS) is involved in converting glucose 6-phosphate to *myo*-inositol 1-phosphate. The conversion of glucose 6-phosphate to 1-L-*myo*-inositol 1-phosphate is the first-committed and rate-limiting step in the biosynthesis of inositol in all eukaryotes (Majumder et al. 1997). MIPS is the first enzyme in a metabolic pathway converting glucose 1-phosphate to pinitol. Pinotol is a cyclic sugar alcohol involved in the tolerance of drought stress that accumulates to higher concentrations in salt-tolerant legumes (e.g., *Sesbania* spp.) and in facultative halophytes such as *Mesembryanthemum crystallinum* (Bray et al. 2000; also see the review by Bohnert and Sheveleva 1998). *Myo*-inositol, an

intermediate of the pinitol biosynthesis pathway, also acts as a precursor in the synthesis of phosphatidylinositol 4,5-bisphosphate, which in turn is hydrolyzed by phosphoinositide-specific phospholipase C (PI-PLC) and produces inositol 1,4,5-triphosphate (IP_3, a soluble messenger) and diacylglycerol (Munnik 2001). IP_3 binds to specific Ca^{2+} channels on the vacuole membrane and the rough endoplasmic reticulum, resulting in the release of Ca^{2+} to the cytosol. Cytosolic Ca^{2+} activates calmodulin and the defense response of the plant cells (for a review see Morre' et al. 1990; Loewus and Murthy 2000). IP_3 contents were decreased in a soybean cell suspension culture when challenged by *Pseudomonas syringae* pv *glycinea* (Shigaki and Bhattacharyya 2000) but increased when treated with stimulants of H_2O_2 burst (Legendre et al. 1993). In other studies, IP3 content increased 100 to 160% in *Medicago sativa* suspension cultures when inoculated with a glycoprotein elicitor from the phytopathogenic fungus *Verticillium albo-atrum* (Walton et al. 1993).

A gene coding MIPS was first isolated from *Saccharomyces cerevisiae* by genetic complementation analysis (Johnson and Henry 1989) and was used to isolate plant orthologs (Johnson 1994). Two distinct MIPS genes were identified in *Arabidopsis* (Johnson 1994; Johnson and Burk 1995; Johnson and Sussex 1995; AGI 2000). Seven sequences hybridized to a MIPS probe in maize (Larson and Raboy 1999). In soybean there are at least four MIPS genes (Hegeman et al. 2001). The multiple MIPS genes in crop plants may be used to atune differential MIPS expression to specific physiological functions.

Differential display of mRNA (Liang and Pardee 1992, 1995) has been used to identify and annotate pathogen/stress inducible genes in many plant species. Examples include alfalfa (Truesdell and Dickman 1997) inoculated with *Colletotrichum trifolii*; soybean cell-suspension cultures inoculated with *P. syringae* pv *glycinea* (Seehaus and Tenhaken 1998); and *Arabidopsis thaliana* inoculated with cauliflower mosaic virus and CAMV gene VI (Geri et al. 1999). In this study we used differential display of mRNA to identify genes that are differentially expressed in response to *Fusarium. solani* f. sp. *glycines* colonization of soybean roots.

F. solani f. sp. *glycines* (hereafter *F. solani*) causes sudden death syndrome (SDS) of soybean (Roy 1997). In susceptible varieties, the infection leads to root rot, leaf scorch and premature plant death (Njiti et al. 1998). In genetic studies, six quantitative trait loci (QTLs) directing SDS resistance have been identified in a cross of 'Forrest' (partially resistant) and 'Essex' (partially susceptible). Beneficial alleles at four of the loci (*Rfs, Rfs1, Rfs2* and *Rfs3*) were derived from Forrest (Prabhu et al. 1999; Iqbal et al. 2001). All four were located on linkage group (LG) G of the soybean genome. Beneficial alleles of two loci (*Rfs4* and *Rfs5)* were contributed by Essex (Chang et al. 1997; Prabhu et al. 1999; Iqbal et al. 2001). These loci were on LG C2 and I. The resistance loci were additive and showed no evidence of epistatic interaction on either root infection or leaf scorch (Njiti et al. 1998; Meksem et al. 1999).

In this study, we found that MIPS was differentially expressed in roots of soybean in response to *F. solani* infection. The specific activity of MIPS was consistent with the mRNA abundance. Both the transcript abundance and enzyme activity of MIPS were determined in a non-additive manner by the resistance alleles at the QTLs conferring resistance to SDS.

Materials and methods

Plant material

The recombinant inbred lines (RIL) were generated from a cross between Essex and Forrest (Hnetkovsky et al. 1996). Seeds were obtained from the SIUC germplasm program. The genotypes used were Forrest (*Rfs*, *Rfs1*, *Rfs2*, *Rfs3*, *rfs4*, *rfs5*), Essex (*rfs*, *rfs1*, *rfs2*, *rfs3*, *Rfs4*, *Rfs5)*, RIL 23 (*Rfs*, *Rfs1*, *Rfs2*, *Rfs3*, *Rfs4*, *Rfs5*) and RIL 85 (*rfs*, *rfs1*, *rfs2*, *rfs3*, *rfs4*, *rfs5*), (Iqbal et al. 2001; Njiti et al. 2001). Single-spore descent culture of *F. solani* f. sp. *glycines* (ST90) was used for inoculation and the pathogen was assayed as colony forming units (CFU) per g of root dry weight (Njiti et al. 2001). Briefly, seeds were germinated in a mixture of sterile sand and soil in the greenhouse. The seedlings were carefully removed from the germination trays (2 weeks after planting) and transferred to a sand and soil mixture containing 10^4 *F. solani* spores/cm^3 in styro-foam cups with holes at the bottom. The non-inoculated control seedlings were transferred to the new soil lacking inoculum (Njiti et al. 2001). The cups were set in a plastic tub in a completely randomized design. For each treatment there were 36 plants per genotype. Each tub was filled with 1.5 to 2 inches of water. About 24 samples were kept in each tub. Tubs were either inoculated or non-inoculated in order to avoid fungal transfer from inoculated to the control samples. Tubs were randomly placed on the greenhouse bench. Root samples were collected at different days after inoculation from both control and inoculated plants. For sample collection, soil was carefully washed with tap water, roots were then rinsed with distilled water, blotted on paper towels and separated from the stem tissues with scissors. The root samples were immediately frozen in liquid nitrogen and kept at –80 °C. The genotype of plants was confirmed by markers closely linked to the SDS alleles (Iqbal et al. 2001). The response of these genotypes to *F. solani* infection has been intensively studied (Njiti et al. 1998, 2001). The expected infection severity (IS) and disease severity (DS) by 28 days after inoculation was as in Table 1.

RNA isolation

Roots were ground into a fine powder in liquid nitrogen and RNA was extracted by the Qiagen RNeasy Plant Mini Kit (Qiagen, Chatsworth, Calif., U.S.A.) according to the manufacturer's instructions. RNA samples were treated with DNase in order remove

Table 1 *F. solani* root infection and SDS disease severity on soybean seedlings at 28 days after inoculation with about 5,000 fungus spores per cm^3 of plant-growth medium. Data includes four experiments and five single plant replications. DS = degree of severity of SDS leaf symptoms rated on a scale of 1–9. IS = infection severity was measured as CFU (Colony forming units per gram of root dry tissue)

Genotype	DS	IS (CFU)
Forrest	1.2	312
Essex	2.2	1,195
RIL23	1.1	107
RIL85	2.3	1,155

any residual DNA in the preparation. After DNase treatment, RNA was purified by a RNeasy mini-spin column (Qiagen, Chatsworth, Calif., U.S.A.). RNA quality was determined by electrophoresis on a formaldehyde-agarose 1.2% (w/v) gel, followed by staining with ethidium bromide. RNA was quantified by spectrophotometry at 260 nm.

Differential display of mRNA

RNA samples from roots at 14 days after inoculation were used for differential display of mRNA using RNAimage kits (GeneHunter, Nashville, Tenn., U.S.A.). A total of 72 primer combinations generated with 24 random 5′ primers (H-AP1 to H-AP24) and three poly-T 3′ primers H-T11M (H-T11G, H-T11C, H-T11A) (Cat. No. G501–G503, GeneHunter, Nashville, Tenn, U.S.A.) were used to identify differentially expressed cDNAs in soybean roots following inoculation by *F. solani* under greenhouse conditions. Equal amounts (0.2 μg) of total RNA was used from *F. solani* inoculated and non-inoculated (control) samples. Reverse transcription used an H-T11M primer. After reverse transcription, a portion of the cDNA was used for PCR employing the same H-T11M primer and a random 13-nucleotide primer. Samples were separated by electrophoresis in a 5% (w/v) acrylamide gel with a 0.5× TBE buffer. Gels were transferred onto Whatman 3 MM filter paper, dried and used to expose BioMax MR films (Kodak, Rochester, N.Y., U.S.A.).

The differentially abundant transcript fragments (as judged by difference in intensity, or the presence or absence of bands) were identified. The cDNA bands were excised from the gel and re-amplified by the appropriate primer combination. The PCR products were electrophoresed on a 2% (w/v) agarose gel. Unique bands were purified by the gel purification kit (Qiagen, Chatsworth, Calif., U.S.A.), ligated into pGEM vector (Promega, Madison, Wis., U.S.A.) and used to transform DH10B electro-competent cells (Gibco BRL, Rockville, Md., U.S.A.). The recombinant clones were picked and stored in freeze-culture media.

Hybridization of fragments identified by differential display of mRNA to the Forrest-infected root cDNA library

A cDNA library of 24,500 clones was constructed from Forrest-infected roots in pGEM vector using the Clontech SMART PCR cDNA synthesis kit (Cat. # K1052-1, Clontech, Palo Alto, Calif., U.S.A.) according to the manufacturer's instructions. The cDNA fragments isolated from differential display were labeled with α-^{32}PdCTP and used as probes to identify clones from the root cDNA library. About 24,500 clones of the root cDNA library were spotted on Hybond-N$^+$ (Amersham, Piscataway, N.J., U.S.A.) nylon filters in a 3 × 3 high-density array using a Flexys Robot (Gene Machines, Ann Arbor, Mich. U.S.A.). The colonies were grown overnight on filters laid on LB agar media containing ampicillin. Bacterial colonies were lysed and DNA was fixed on the membrane according to Connell et al. (1998). The DNA was cross-linked to the membrane by the Spectrolinker XL-1500 UV cross-linker (Spectronics, Westbury, N.Y., U.S.A.) at an optimal setting before hybridization. Pre-hybridization and hybridization were carried out in Churches buffer at 55 °C (Connell et al. 1998). After high-stringency washes, the filters were used to expose BioMax-MR X-ray films overnight. The positive clones were picked from the library and sequenced.

DNA sequencing and primer design

DNA sequencing used ABI (Foster City, CA, U.S.A.) big dye terminator chemistry on an ABI377 automated DNA sequencer. Clones were sequenced by one pass using the M13 forward or reverse primer. Vector and/or adaptor sequences were removed and the rest of the sequence was used for BLAST searches. Primers were designed for the differentially abundant transcripts using "GeneFisher" primer design software available on the web (http://bibiserv.techfak.uni-bielefeld.de/~). T_m was set between 55 to 65 °C to encourage the specific amplification of the mRNAs. The primer sequences selected for MIPS amplification were forward primer 5′GGGTAGTATAGGATTCTCTTCTTTATTC 3′ and reverse primer 5′ATAGCTGAGGCTTGGGTGAG 3′ with a T_m of 59.9 and 59.2, respectively, as calculated by the primer design software. These primers encompassed a 380-bp fragment in the cDNA.

Quantitative RT-PCR

The changes in abundance of differentially expressed mRNAs were verified by quantitative RT-PCR as follows. Total RNA from roots (2 μg) was used to synthesize cDNA with random decamer primers according to the manufacturer's instructions (RETROscript First-Strand cDNA Synthesis Kit, Cat. # 1719, Ambion, Austin, TX, U.S.A.). For gene-specific amplification from cDNA, the number of cycles was optimized to stop the reaction at the exponential stage so that amplification was quantitative. The QuantumRNA 18S Internal Standard (Ambion, Austin, TX, U.S.A., Cat. # 1716) was included in all PCR amplifications. The reactions were carried out in a 50-μl volume containing 1 μl of RT reaction, 5.0 μl of 10× PCR buffer, 4 μl of 2.5 mM dNTPs, 4 μl (20 pmol total) of each gene-specific primer, 4 μl of 18S Primer:Competimer mix (1 μl of 5 μM 18S PCR primer pair was mixed with 9 μl of 5 μM 18S PCR Competimers: Ambion, Austin, TX, U.S.A., Cat. # 1716), 0.25 μl (1.25 units) of *Taq* DNA polymerase (Gibco BRL, Rockville, M.D., U.S.A.), 0.5 μl of α-^{32}PdCTP (10 μCi/μl) and water to a 50-μl final volume. PCR amplifications were carried out in a Perkin Elmer 9600 thermal cycler programmed for an initial hold of 3 min at 94 °C followed by 25 cycles of 94 °C for 20 s, 60 °C (or annealing temperature of the specific primer) for 30 s, and 72 °C for 1.5 min. The reactions were kept at 72 °C for 5 min followed by a 4 °C hold.

After the completion of PCR cycles, 10 μl of gel loading dye was added to each reaction, samples were heated for 3 min at 95 °C and immediately cooled on ice. The samples were separated by electrophoresis through a 5% (w/v) denaturing acrylamide gel. The gel was dried onto Whattman 3 MM filter paper and used to expose BioMax-MR X-ray film. The films were scanned in a Kodak Digital Science Image Station 440CF (Kodak, Rochester, N.Y., U.S.A.) and band intensities were measured using Kodak 1D Image Analysis Software. MIPS band intensities were normalized to the 18 S ribosomal internal control for comparison of relative gene expression under different treatments and in different genotypes.

Protein extraction

Total soluble proteins were extracted according to Francs et al. (1985). Roots of 4–6 plants were crushed in a mortar pestle, precooled under liquid nitrogen. The fine powder (200 mg) was resuspended on ice in 1 ml of 30 mM Tris-Cl buffer (pH 7.5), 1 mM of DTT, 1 mM of ascorbic acid, 1 mM of EDTA-Na_2, 6 mg of insoluble PVP 40, and 1 mM of phenyl methyl sulfonyl fluoride (PMSF) was used to inhibit protease activity. The samples were centrifuged three-times at 20,000 g for 25 min each to clarify the supernatant. The clarified supernatant was assayed for total protein, enzyme activity and IP_3 content.

Protein estimation

A colorimetric protein assay was used according to Bradford (1976). Protein solution (100 μl) was added to 5 ml of the Bradford reagent and the absorbance was measured at 595 nm. A bovine serum albumin (BSA) standard curve was used.

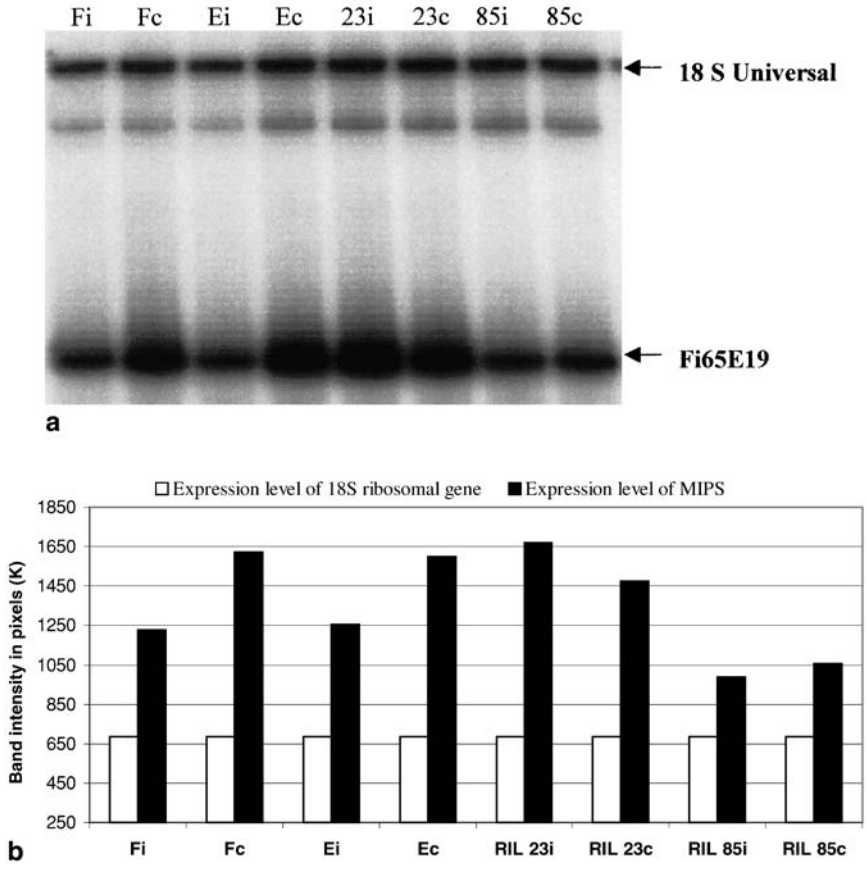

Fig. 1a, b Quantification of MI-1-P synthase mRNA abundance by RT-PCR. **a** A 380-bp cDNA fragment identified as Fi65E19 was amplified using two primers designed from the sequence of the differentially expressed mRNA. Universal primers for the 18 S rRNA gene (489 bp) were used as an internal control. *Fi* = RNA isolated from the roots of Forrest genotype that was inoculated by *F. solani*, *Fc* = RNA isolated from the roots of Forrest genotype that was not inoculated by *F. solani* but kept under the same conditions, *Ei* = Essex inoculated, *Ec* = Essex non-inoculated, *23i* = RIL 23 inoculated, *23c* = RIL 23 non-inoculated, *85i* = RIL 85 inoculated, *85c* = RIL 85 non-inoculated. **b** RT-PCR amplified fragments (380 bp) were quantified using a Kodak Digital Science Image Station 440CF and band intensities were measured using Kodak 1D Image Analysis Software. The data was normalized to a 18S ribosomal gene (489 bp) which was used as an internal control

Enzyme assay

Inositol biosynthesis was measured by the rapid colorimetric method of Barnett et al. (1970). Crude extract (200 µl) was incubated with 200 µl of 5 mM D-glucose 6-phosphate and 100 µl of 5 mM NAD for an appropriate time at 37 °C. The reaction was quenched by the addition of 200 µl of 20% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation. Exactly 500 µl of the supernatant was incubated for 1.5 h with 500 µl of 0.2 M $NaIO_4$. One millilitre of 1 M Na_2SO_4 was added to the supernatant to remove the excess $NaIO_4$. For the measurement of inositol monophosphate, a 2 ml reagent mix [0.4 ml of 3 M H_2SO_4, 0.4 ml of 2.5% (w/v) ammonium molybdate, 0.8 ml of H_2O and 0.4 ml of 10% (w/v) ascorbic acid] was added and incubated for 1.5 h at 37 °C. The absorbance was measured at 660 nm and enzyme units were calculated. A standard curve for 0.05 to 0.7 µM of *myo*-inositiol phosphate was used to determine the amount of inositol phosphate in each sample.

D-*myo*-inositol 1,4,5-triphosphate (IP_3) [^3H] content assay

Aliquots of samples from the protein extraction step were reacted with perchloric acid by adding 0.2 vol of 20% (v/v) perchloric acid to the clarified supernatant and incubated on ice for 20 min. Proteins were sedimented by centrifugation at 6,000 g for 15 min at 4 °C. The supernatant was decanted into plastic tubes and the pH was adjusted to 7.5 with ice-cold 10 M KOH. $KClO_3$ was removed by centrifugation at 2,000 g for 15 min at 4 °C. IP_3 concentration was measured using an [^3H] IP_3 assay kit (Amersham Pharmacia Biotech, N.J., U.S.A., Cat. No. TRK 1000) following the manufacturers instructions. An IP_3 standard curve was plotted employing the 5th-order inverse logarithmic regression model using the statistical program "Data fit" version 7.1.44 developed by Oakdale engineering, Pa., U.S.A.. The standard curve was used for the IP_3 determination in the unknown samples. IP_3 concentration was converted to pmol/µg of soluble protein to compensate for extraction differences.

Results

Differential display of mRNA identifies MIPS

Differentially amplified cDNA fragments from the differential display of mRNA were used as probes to screen a Forrest cDNA library prepared from roots inoculated with *F. solani*. The BLAST search revealed that the cDNAs have significant homology to MIPS, calmodulin-like protein, glyceraldehyde 3-P dehydrogenase, vacuolar ATP synthase, a ribosomal protein, elongation factor 1 beta, a ribosomal protein L24, and four ESTs. Sequence alignment showed 100% homology to the soybean MIPS gene from cv Williams (AF293970) over 408 bp but with a 10-bp 5′ extension. The translation start codon (ATG) at nucleotide number 72 predicted a 115 amino-acid polypeptide with 100% homology to the "Williams" predicted MI3PS (AF293970). It also showed that the predicted sequence (115 amino acids) has 100% homology to soybean MI1PS (AY038802) and 95% similarity to MI1PS isolated from *Phaseolus vulgaris* (AF282263).

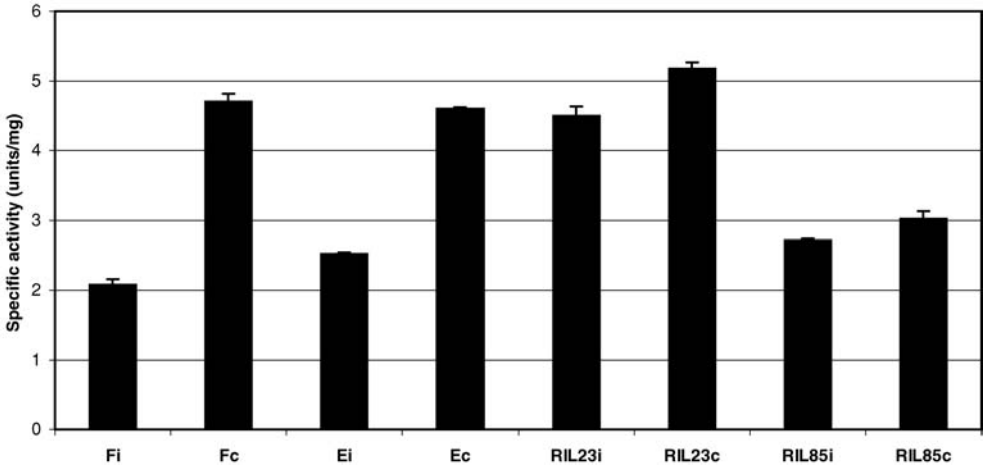

Fig. 2 Specific enzyme activity of MI-1-P synthase in soybean roots at 14 days after inoculation. The T-bar shows the standard error of the means (SEM)

mRNA abundance of differentially expressed sequences

The mRNA abundance of MIPS was determined by quantitative RT-PCR (Fig. 1a). An 18S ribosomal RNA gene was used as an internal control during the RT-PCR, and the band intensities were normalized with the intensity of the control. The RT-PCR was repeated once; therefore, error bars are not shown (Fig. 1b). At 14 days after inoculation in Essex and Forrest, MIPS mRNA abundance was reduced in response to infection by *F. solani* (Fig. 1a and b) to 21–24% of the non-inoculated controls. In contrast, the MIPS mRNA abundance in the resistant recombinant inbred line (RIL 23) was increased by 13% in the inoculated sample. The expression of MIPS in the susceptible RIL 85 was decreased by 6%. However, the greatest abundance of MIPS mRNA in RIL 85 was 25–50% lower than all other genotypes and treatments.

The mRNA abundance of the calmodulin-like protein, glyceraldehyde 3-P dehydrogenase, and vacuolar ATP synthase, remains unchanged in inoculated samples when analyzed by quantitative RT-PCR at 14 days after inoculation. Earlier and later time points were not assayed.

Enzyme activity of *myo*-inositol-1-phosphate synthase

Specific activity of MIPS in the protein extract prepared from roots of Forrest, Essex, RIL 23 and RIL 85 after 14 days of inoculation was determined (Fig. 2). In non-inoculated samples, MIPS specific activity was higher in RIL 23 (5.18 ± 0.08 units/mg) compared to Forrest (4.7 ± 0.10 units/mg), Essex (4.59 ± 0.03 units/mg) and RIL 85 (3.0 ± 0.1 units/mg), correlating with the mRNA abundance and allelic complements of the genotypes.

In RIL 23 inoculated samples, there was no significant (P = 0.0518) decrease in MIPS activity (4.49 ± 0.14 units/mg) compared to the non-inoculated roots. However, in both Forrest (2.01 ± 0.083 units/mg; P = 0.0027) and Essex (2.5 ± 0.01 units/mg; P = 0.0002), the enzyme activity decreased significantly in the inoculated roots. In RIL 85 there was no significant reduction (P = 0.1174) in activity but the enzyme was constitutively low (2.7 ± 0.02 units/mg).

To relate enzyme activity to root development in RIL 23 and RIL 85 the specific activity of MIPS was measured at 3, 7, 14 and 21 days after inoculation (Fig. 3). A steady, inoculation independent increase in the MIPS activity is evident in RIL 23 roots from day 3 onwards, which may peak by day 21. In RIL 85, there was no significant change in the activity of MIPS from day 3 to day 21 regardless of the inoculation state. The differences between inoculated and non-inoculated samples were not significant for either genotype at any time.

Comparison in change of mRNA abundance and MIPS activity after inoculation

The percentage of changes in both mRNA abundance and MIPS activity in roots was calculated at 14 days after inoculation (Fig. 4). After inoculation, Essex and Forrest MIPS mRNA abundance decreased by 21–24% and MIPS specific activity decreased by 46–56%. In RIL 23, the mRNA abundance increased by 13% after inoculation but MIPS specific activity decreased by 15% compared to non-inoculated roots. In RIL 85, the mRNA abundance decreased by 6% and MIPS activity decreased by 10% compared to the non-inoculated roots.

Concentrations of IP_3 in roots

The highest IP_3 concentration was observed in RIL23 inoculated roots (Table 2). IP_3 concentration in RIL23 was 3.1 ± 0.46 pmol/μg in non-inoculated roots compared to 7.0 ± 0.24 pmol/μg in inoculated roots, i.e., 2.26-fold higher in the inoculated roots. In RIL85, IP_3 content was 3.0 ± 0.08 pmol/μg in non-inoculated roots compared to 1.9 ± 0.16 pmol/μg in inoculated roots, i.e., 1.5-fold lower in the inoculated roots. The IP_3 concentration was slightly higher in Forrest (four resistance alleles) in

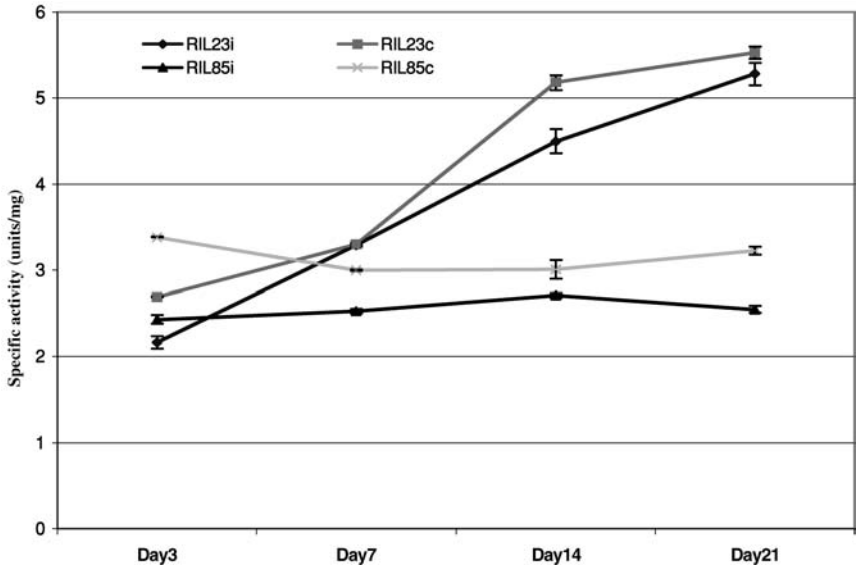

Fig. 3 Specific activity of MIPS at 3, 7, 14 and 21 days after inoculation in RIL 23 (carrying six resistance alleles) and RIL 85 (carrying six susceptible alleles). The T-bar shows the standard error of the means (SEM)

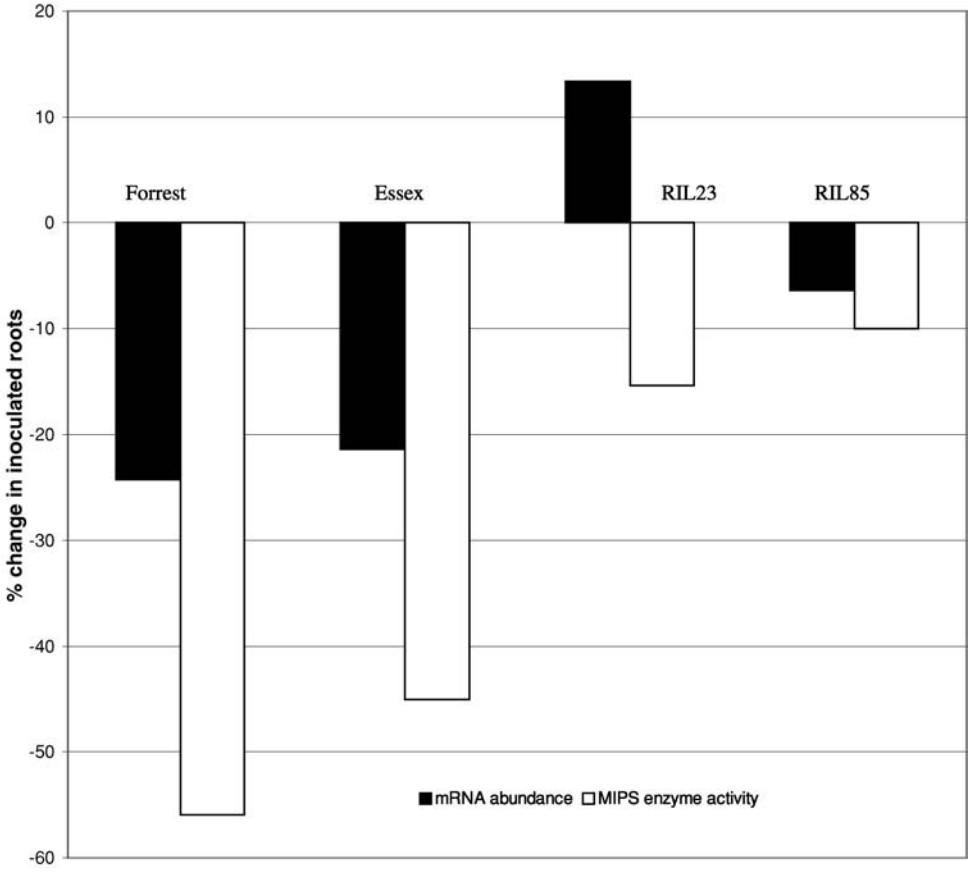

Fig. 4 A comparison of the percentage change in MIPS mRNA abundance and MIPS specific enzyme activity at 14 days after inoculation

the inoculated root, 3.6 ± 0.06 pmol/μg compared to 2.4 ± 0.14 pmol/μg in the non-inoculated roots. In Essex (two resistance alleles), the IP_3 content was 2.8 ± 0.13 pmol/μg in non-inoculated compared to 6.1 ± 0.24 pmol/μg in the inoculated roots, i.e., 2.18-fold higher in the pathogen inoculated roots. The mean IP_3 content in all the genotypes were significantly higher (1.64-times) in inoculated compared to the control root samples ($P = 0.0001$). Only in the case of RIL85, did IP_3 content decrease after inoculation.

Discussion

The slight, but non-significant, increase in mRNA abundance of MIPS in the inoculated samples of RIL 23 (con-

Table 2 D-*myo*-inositol 1,4,5-triphosphate (IP_3) assay. IP_3 concentrations were determined using the IP_3 standard curve. IP_3 was quantified as pmol/μg of soluble protein present in the sample

Sample		IP_3 pmol/ml	Total protein μg/ml	IP_3/total protein (pmol/μg) ± SEM
RIL23	Control	473.5	153.35	3.1 ± 0.46
RIL23	Inoculated	631.0	90.44	7.0 ± 0.24
RIL85	Control	170.5	56.15	3.0 ± 0.08
RIL85	Inoculated	280.3	144.5	1.9 ± 0.16
Forrest	Control	401.5	164.0	2.4 ± 0.14
Forrest	Inoculated	325.5	90.4	3.6 ± 0.06
Essex	Control	401.5	144.9	2.8 ± 0.13
Essex	Inoculated	635.0	103.35	6.1 ± 0.24

taining resistance alleles of the six QTLs) contrasted with the parent varieties (which each contained either four or two resistance alleles) where the MIPS mRNA significantly decreased. The above effect in RIL23 appears to be the product of interaction of at least two QTLs, with one or more being derived from each parent. It is noteworthy that, in the roots without fungal inoculation, the mRNA abundance is similar in Essex, Forrest and RIL 23 (Fig. 1a and b). Additional evidence that at least two of the resistance alleles control MIPS non-additively was that both mRNA abundance and enzyme activity were significantly lower in RIL85 by 14 days after transplanting, irrespective of inoculation.

The specific activity of the MIPS enzyme decreased significantly in all the soybean lines when infested with the pathogen. The percentage decrease in enzyme activity was double the decrease in mRNA abundance in the Forrest (56%) and Essex (45%) varieties (Fig. 4). However, in inoculated RIL 23, the MIPS specific activity decreased by just 15%. These results suggest that factors other than mRNA abundance determine MIPS activity. Among the factors controlling MIPS activity may be fungal inhibition of epigenetic factors, allosteric effectors, and/or expression of isozymes of *Gm*MIPS1 (Hegeman et al. 2001). These factors are related to the bi-allelic contrast between RIL 23 and the other genotypes.

The IP_3 contents were significantly increased in Essex (two resistance alleles) and RIL 23 (six resistance alleles). The increase in Forrest was less compared to the RIL 23 and Essex genotypes (Table 2). In RIL 23, the resistance alleles were contributed by both Forrest and Essex varieties, while the genes contributing toward IP_3 accumulation may be contributed by Essex. These genes could be the elicitor recognition elements, toxin resistance elements (Njiti et al. 1998) or other elements directly or indirectly involved in the resistance response. Overall, the higher level of IP_3 content in RIL 23 seems to be an additive effect of the QTL from Essex and Forrest in response to pathogen inoculation. The additive nature of the increase in IP_3 content was further confirmed by a decrease in IP_3 content in the inoculated roots of RIL85, a genotype with no resistance allele. In field and greenhouse studies, it was observed that the QTL on LG C2, when contributed by Essex, has a significant impact on SDS resistance to leaf scorch but not on root resistance (Njiti et al. 1998; Triwitayakorn, unpublished data). The quantitative, additive inheritance of the soybean resistance to SDS suggests that several biochemical pathways or metabolic responses are involved in the continuum between resistance and susceptibility to the fungal infection (Iqbal et al. 2001).

Potentially, MIPS plays an important role in initiating and/or maintaining the soybean defense response to *F. solani* infection. IP_3 was increased in the fungus-inoculated genotypes containing two, four or six resistance alleles of the loci conferring resistance to SDS. The increase in IP_3 content in intact roots after inoculation with the fungal pathogen was consistent with earlier studies where the glycoprotein elicitor from the phytopathogenic fungus increased the IP_3 content in cell-suspension cultures. The co-relation of IP3 content with gene pyramids was further predicted by its decrease in the genotype containing no allele of the loci conferring resistance to SDS. Gene pyramiding also helped maintain MIPS activity even after inoculation. It further helped generate genotypes producing higher levels of IP3 content, a known activator of cellular-defense response.

There has been extensive physiological and biochemical evidence for the importance of inositol in plants including its role in seed germination, membrane formation, cell-wall biogenesis and stress response (Loewus 1990; Laxalt et al. 2001; Munnik 2001). The sustained higher expression of MIPS in RIL-containing resistance alleles of the six QTLs for SDS may be indicative of a stress response that produces strengthened cell walls and increased signal transduction. The MIPS when measured in soybean roots responded to the stage of development, *F. solani* infection and the plant genotype (Fig. 3), all factors that affect the extent of SDS disease progression.

MIPS1 and MIPS2 were sequenced from root ESTs libraries, but Northern hybridization and immuno-detection assays were unable to detect *Gm*MI1PS1 expression in soybean roots (Hegeman et al. 2001). We detected the expression of *Gm*MIPS1 in roots by quantitative RT-PCR which is more sensitive than Northern hybridization. We also confirmed that the enzyme activity was correlated with the mRNA abundance. The isoform analyzed by Hegeman et al. (2001) was abundant in immature cotyledons.

Pyramiding beneficial alleles has been a widely applied practice by plant breeders for the development of varieties with traits that are affected by multiple genes (Stuber et al. 1999). DNA markers have been used to increase the efficiency of pyramiding genes for yield, disease resistance (Kelly et al. 1995; Kloppers and Pretorius 1997; Iqbal et al. 2001), pest resistance (Web et al. 1995; Porter et al. 2000) and abiotic stress (Flowers et al. 2000; Cho et al. 2002). Constructing large gene pyramids with DNA markers is arduous, error prone and associated with low yields. Biochemical markers for gene pyramids

are potentially useful tools for breeders. However, biochemical and metabolite analyses of the effects of gene pyramiding have not been reported.

Further studies will include determining the complete sequence of the gene and its alleles, determining its position in the integrated genetic and physical map in relation to known resistance QTLs (Zobrist 2000) and analysis of protein abundance using isoform-specific antibodies. We also aim to elucidate its role in resistance response and its relationship with QTL gene pyramiding, and a potential germplasm screening tool for gene pyramids.

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References

Agrios G (1988) Plant pathology. Academic Press, San Diego, California, USA

Barnett JEG, Brice RE, Corina DL (1970) A colorimetric determination of inositol monophosphates as an assay for D-glucose 6-phosphate-1L-*myo*-inositol 1-phosphate cyclase. Biochem J 119:183–186

Bohnert HJ, Sheveleva E (1998) Plant stress adaptations making metabolism move. Curr Opin Plant Biol 1:267–274

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bray EA, Bailey-Serres J, Weretilnyk E (2000) Response to abiotic stress. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and molecular biology of plants. American Society of Plant Physiologists, pp 1158–1203

Chang SJC, Doubler TW, Kilo V, Suttner V, Schmidt ME, Gibson PT, Lightfoot DA (1997) Association of field resistance to soybean sudden death syndrome (SDS) and cyst nematode (SCN). Crop Sci 37:965–971

Cho Y, Njiti VN, Chen X, Lightfoot DA, Wood AJ (2002) Analysis of trigonelline (Nicotinic acid betaine) in *Glycine max*. I. Alteration of trigonelline concentration in field-grown plants under drought and irrigated conditions. Biol Plant 45 (in press)

Connell JP, Pami S, Iqbal MJ, Huizinga T, Reddy AS (1998) A high through-put procedure for capturing microsatellites from complex plant genomes. Plant Mol Biol Rep 16:341–349

Dixon RA, Harrison MJ, Lamb CJ (1994) Early events in the activation of plant defense responses. Annu Rev Phytopathol 32:479–501

Feys BJ, Parker JE (2000) Interplay of signaling pathways in plant disease resistance. Trends Genet 16:449–455

Flowers TJ, Koyama ML, Flowers SA, Sudhakar C, Singh KP, Yeo AR (2000) QTLs: their place in engineering tolerance of rice to salinity. J Exp Bot 51:99–106

Francs CCD, Thiellement H, Vienne DD (1985) Analysis of leaf proteins by two-dimensional gel electrophoresis. Plant Physiol 78:178–182

Geri C, Cecchini E, Giannakou ME, Covey SN, Milner JJ (1999) Altered pattern of gene expression in Arabidopsis elicited by cauliflower mosaic virus (CaMV) infection and by a CaMV gene VI transgene. Mol Plant-Microbe Interact 12:377–184

Hegeman CE, Good LL, Grabau EA (2001) Expression of D-*myo*-Inositol-3-phosphate synthase in soybean. Implications for phytic acid biosynthesis. Plant Phys 125:1941–1948

Hnetkovsky N, Chang SC, Doubler TW, Gibson PT, Lightfoot DA (1996) Genetic mapping of loci underlying field resistance to sudden-death syndrome. Crop Sci 36:392–400

Iqbal MJ, Meksem K, Njiti VN, Kassem MyA, Lightfoot DA (2001) Microsatellite markers identify three additional quantative trait loci for resistance to soybean sudden-death syndrome (SDS) in Essex × Forrest RILs. Theor Appl Genet 102:187–192

Johnson MD (1994) The *Arabidopsis thaliana myo*-inositol-1-phosphate synthase (EC 5.5.1.4). Plant Physiol 105:1023–1024

Johnson MD, Burk D (1995) Isozymes of 1-L-*myo*-inositol-1-phosphate synthase from *Arabidopsis* (accession no. U30250; PGR95-067). Plant Physiol 109:721–723

Johnson MD, Henry SA (1989) Biosynthesis of inositol in yeast: primary structure of *myo*-inositol 1-phosphate synthase (EC 5.5.1.4) and functional analysis of its structural gene, the *INO1* locus. J Biol Chem 264:1274–1283

Johnson MD, Sussex IM (1995) 1-L-*myo*-inositol 1-phosphate synthase from *Arabidopsis thaliana*. Plant Physiol 107:613–619

Kelly JD, Afanador L, Haley SD (1995) Pyramiding genes for resistance to bean common mosaic virus. Euphytica 82:207–212

Kloppers FJ, Pretorius ZA (1997) Effects of combinations amongst genes LR13, LR34 and LR37 on components of resistance in wheat to leaf rust. Plant Pathol 46:737–750

Kuc' J (1995) Phytoalexins, stress metabolism, and disease resistance in plants. Annu Rev Phytopathol 33:275–297

Larson SR, Raboy V (1999) Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: correspondence with a *low phytic acid* mutation. Theor Appl Genet 99:27–36

Laxalt AM, ter Riet B, Verdonk JC, Parigi L, Tameling WI, Vossen J, Harring M, Musgrave A, Munnik T (2001) Characterization of five tomato phospholipase D cDNAs: rapid and specific expression of LePLDbeta1 on elicitation with xylanase. Plant J 26:237–247

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS (1993) Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J Biol Chem 268:24,559–24,563

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–971

Liang P, Pardee AB (1995) Recent advances in differential display. Curr Opin Immunol 7:274–280

Loewus FA (1990) Inositol metabolism in plants, In: Boss WF, Morre DJ, Loewus FA (eds) Wiley-Liss, Inc, New York, vol 9, pp 13–19

Loewus FA, Murthy PPN (2000) *Myo*-inositol metabolism in plants. Plant Sci 150:130–139

Majumder AL, Johnson MD, Henry SA (1997) 1L-*myo*-inositol-1-phosphate synthase. Biochem Biophys Acta 1348:245–256

Meksem K, Doubler TW, Chancharoenchai K, Njiti VN, Chang SJC, Rao-Arelli AP, Cregan PE, Gray LE, Gibson PT, Lightfoot DA (1999) Clustering among loci underlying soybean resistance to *Fusarium solani*, SDS and SCN in a near-isogenic line. Theor Appl Genet 99:1131–1142

Morre' DJ, Boss WF, Loewus FA (1990) Inositol metabolism in plants. Wiley-Liss, New York, USA

Munnik T (2001) Phosphatidic acid: an emerging plant lipid second messenger. Trends Plant Sci 6:227–233

Newman KD, Van-Toai TT (1991) Developmental regulation and organ-specific expression of soybean alcohol dehydrogenase. Crop Sci 3:1253–1257

Njiti VN, Doubler TW, Suttner RJ, Grey LE, Gibson PT, Lightfoot DA (1998) Resistance to soybean sudden death syndrome and root colonization by *Fusarium solani* f. sp. *glycine* in near-isogenic lines. Crop Sci 38:472–477

Njiti VN, Johnson JE, Torto TA, Grey LE, Lightfoot DA (2001) Inoculum rate influences selection for field resistance to soybean sudden death syndrome in the greenhouse. Crop Sci 41: 1726–1731

Porter DR, Burd JD, Shufran KA, Webster JA (2000) Efficacy of pyramiding greenbug (Homoptera: Aphididae) resistance genes in wheat. J Econ Entomol 93:1315–1318

Prabhu RR, Njiti VN, Bell-Johnson B, Johnson JE, Schmidt ME, Klein JH, Lightfoot D-A (1999) Selecting soybean cultivars for dual resistance to soybean cyst nematode and sudden death syndrome using two DNA markers. Crop Sci 39:982–987

Roy KW (1997) *Fusarium solani* on soybean roots: Nomenclature of the causal agent of sudden death syndrome and identity and relevance of *F. solani* form B. Plant Dis 81:259–266

Seehaus K, Tenhaken R (1998) Cloning of genes by mRNA differential display induced during the hypersensitive reaction of soybean after inoculation with *Pseudomonas syringae* pv *glycinea*. Plant Mol Biol 38:1225–1234

Shigaki T, Bhattacharyya MK (2000) Decreased inositol 1,4,5-triphosphate content in pathogen-challenged soybean cells. Mol Plant-Microbe Interact 13:563–567

Stuber CW, Polacco M, Senior ML (1999) Synergy of empirical breeding, marker-assisted selection, and genomics to increase crop yield potential. Crop Sci 39:1571–1583

Truesdell GM, Dickman MB (1997) Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. Plant Mol Biol 33:737–743

Walton TJ, Cooke CJ, Newton RP, Smith CJ (1993) Evidence that generation of inositol 1,4,5-triphosphate and hydrolysis of phosphatidylinositol 4,5-bisphosphate are rapid responses following the addition of fungal elicitor which induces phytoalexin synthesis in lucerne (*Medicago sativa*) suspension culture cells. Cell Sig 5:345–356

Web DM, Baltazar BM, Rao-Arelli AP, Schupp J, Clayton K, Keim P, Beavis WD (1995) Genetic mapping of soybean cyst-nematode race-3 resistance loci in soybean PI 437.654. Theor Appl Genet 91:574–581

Zobrist KF (2000) A partial integrated, genetic and physical map of the soybean genome. MS thesis, Southern Illinois University at Carbondale, Illinois, USA