Multigeneic QTL: The Laccase Encoded within the Soybean Rfs2/rhg1 Locus Inferred to Underlie Part of the Dual Resistance to Cyst Nematode and Sudden Death Syndrome

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Received 4 September 2008
Revised 5 October 2008
Accepted 12 October 2008

Abstract

Multigeneic QTL present significant problems to analysis. Resistance to soybean (*Glycine max* (L) Merr.) sudden death syndrome (SDS) caused by *Fusarium virguliforme* was partly underlain by *QRfs2* that was clustered with, or pleiotropic to, the multigeneic *rhg1* locus providing resistance to soybean cyst nematode (SCN; *Heterodera glycines*). A group of five genes were found between the two markers that delimited the *Rfs2/rhg1* locus. One of the five genes was predicted to encode an unusual diphenol oxidase (laccase; EC 1.10.3.2). The aim of this study was to characterize this member of the soybean laccase gene-family and explore its involvement in SDS resistance. A genomic clone and a full length cDNA was isolated from resistant cultivar 'Forrest' that were different among susceptible cultivars 'Asgrow 3244' and 'Williams 82' at four residues R/H168, I/M271, R/H330, E/K470. Additional differences were found in six of the seven introns and the promoter region. Transcript abundance (TA) among genotypes that varied for resistance to SDS or SCN did not differ significantly. Therefore the protein activity was inferred to underlie resistance. Protein expressed in yeast pYES2/NTB had weak enzyme activity with common substrates but good activity with root phenolics. The Forrest isoform may underlie both *QRfs*2 and *rhg1*.
Key words: soybean, laccase, SDS resistance, yeast expression.

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Introduction

Genetic studies have mapped a number of quantitative trait loci (QTL) conferring resistance to sudden death syndrome (SDS; Q*Rfs*, Q*Rsfs1*, Q*Rfs2*, Q*Rfs3*) and soybean cyst nematode (SCN; *rhg1*) on soybean linkage group G (LG G; chromosome 18; Iqbal *et al*., 2001; Triwitayakorn *et al*., 2005). The four QTL for resistance to SDS on LG G range from 0.0 to 30 ± 2.5 cM from *rhg*1, a major gene for resistance to SCN (Meksem *et al*., 1999, 2001). Q*Rfs*2 is about 0.0–0.2 cM from *rhg*1 and may be pleiotropic effect of a single gene (Triwitayakorn *et al*., 2005; Ruben *et al*., 2006), Q*Rfs2* reduces the leaf-scorch index, a measure of foliar symptoms that result from the toxins produced by *Fusarium virguliforme*, the causative agent of SDS, but not the root infection severity (percentage of roots infected with *F. virguliforme*). The *Rfs2*/*rhg*1 region on LG G encompassed between SIUC-Sac13 (Ruben *et al*., 2006) and AFLP marker ATG4 (Meksem *et al*., 2001) was shown to contain five genes; a candidate receptor like kinase gene potentially involved in extracellular signal reception and intracellular signal transduction; and four genes encoding enzymes that might be involved in metabolism. Each or all of these genes might underlie resistance to *F. virguliforme* and or SCN (Triwitayakorn *et al*., 2005; Ruben *et al*., 2006).

Extensive soybean genome sequence from the susceptible cultivar 'Asgrow 3244' has been released to GenBank encompassing *Rfs2/rhg1* (Hague *et al*., 2001). In addition significant sequence resources for this region are available from resistant cultivar 'Forrest' (Ruben *et al*., 2006; Shultz *et al*., 2006a) and susceptible culivar 'Williams 82' (J. Schmutz personal communication 2006). Extensive sequencing of the receptor like kinase in 32 cultivars and 112 plant introductions (PIs) showed there were 9 alleles encoding 5 different proteins (Ruben *et al*., 2006). Allele 1 was perfectly associated with resistance to SCN HgType 0 (race 3). Allele 1 has also been shown to be associated with SDS resistance across a very wide collection of germplasm whereas allele 2 was more associated with susceptibility to SDS (Gibson, 1994; Njiti *et al*., 1997; 2002). The allelic diversity of the neighboring genes has not been well characterized to date. However, since linkage disequilibrium in soybean is often large (97–536 kbp; Hyten *et al*., 2007) significant numbers of alleles among the linked genes are expected.

Diphenol oxidase laccase (hereafter called as laccase) was considered a strong candidate for Q*Rfs*2 (Triwitayakorn, 2005) and may be part of *rhg1* (Ruben *et al*., 2006). The laccase enzyme (p-diphenol:O2 oxidoreductase; EC 1.10.3.2) is a blue copper-containing oxidase found in plants, fungi, bacteria (Diamantidis *et al*., 2000) and arthropods (Thomas *et al*., 1989; Cardenas and Dankert, 2000). Laccases in plants are present as large multigene families (18–20 members) that can be classified into 6 major sub-groups some of which predate the monocot, dicot split (McCaig *et al*., 2005). Phenotypes of four mutants in Arabidopsis include seed coat color, root development, flowering time though the majority (8) showed no phenotype (Cai *et al*., 2006). Many members are expressed in roots. Together this data suggests it is unlikely most laccases participate directly in cell wall lignifications although a few may (Ranocha *et al*., 2002). Other roles include the hydroxlation of flavonoids, formation of proanthocyanidin or tannin and polymerization of phenolic compounds which protect plants from pathogen and insect attack. A variant laccase might detoxify phenolic fungal toxins, like monorden (Baker and Nemec, 1994) or reduce the frequency of SCN feeding site development. Alternately a laccase expressed in the roots to increase cell wall lignification (Lozovaya *et al*., 2004) might protect against fungal ingress or spread and therefore decrease the amount of toxin produced or translocated (Lozovaya *et al*., 2005).

Laccase genes have been found in all major seed plants and have been well characterized in *Arabidopsis thaliana* (Cai *et al*., 2006), ryegrass (*L. perenne*; Gavnholt *et al*., 2002), maple (*Acer pseudoplatanus*; La Fayette *et al*., 1999), tobacco (*Nicotiana tabacum*; Kiefer-Myer *et al*., 1996), poplar (*Populus trichocarpa*; Ranocha *et al*., 1999) and yellow poplar (*Liriodendron tulipifera*; Fayette *et al*., 1999). Several Poplar laccase mRNAs were expressed in stems, but not in leaves and roots (Ranocha *et al*., 1999). Ryegrass laccase mRNA was differentially expressed in stem and meristem (Gavnholt *et al*., 2002). However, laccase gene family sizes are large (18–20 members) in most species and expression of at least some family member is found in all organs (Cai *et al*., 2006).

Based on the presence of laccase within the region encompassing the *Rfs2/rhg1* resistant locus and the multifunctional role of the enzyme class, it was hypothesized that the soybean laccase might be involved in resistance to SDS and SCN. This paper reports the characterization of the laccase enzyme; identification of laccase alleles; analysis of transcript abundances in roots of several soybean varieties varying in partial resistance to SDS and SCN; and associations with the alleles at the receptor like kinase within the *rhg1* region The possibility that laccase is a candidate for one of the genes underlying both the *rhg1* and the Q*Rfs*2 locus is discussed.

Materials and methods

Plant material

Soybean varieties and genotypes used in this study are listed in Table 1. Alleles for *rhg1* are as listed in Ruben *et al*. (2006) from receptor like kinase sequences. Recombinant inbred line (RIL) ExF23 contains the favorable alleles of the 6 SDS QTL and considered as partially resistant. RIL ExF85 contains the susceptible alleles of the 6 SDS QTL and was considered as susceptible (Iqbal *et al*., 2001; Njiti *et al*., 2001). Forrest contains 4 beneficial QTL and Essex two. The complement of segregating QTL were not completely equivalent to Essex and Forrest in 'Flyer', 'Hartwig', 'Ripley' and 'Spencer' (Farias-Neto *et al*., 2007; Kazi *et al*., 2008) or were not yet characterized ('Hamilton', 'Jack').

Table 1. Soybean varieties and recombinant inbred lines (RIL) studied for the TA of laccase diphenol oxidase like sequence (AY113187) in roots inoculated with SDS pathogen at 7 days.

			Change in TA			
	Name	SDS[a]	PR	S	*rhg1* allele[b]	Seed source
1	RIL23	PR	1.48		1	Southern Illinois University
2	RIL85	S		2.195	6	Southern Illinois University
3	Jack	PR	0.546		2	USDA NSRC, Urbana, IL
4	Flyer	S		0.56	4	USDA NSRC, Urbana, IL
5	Ripley	PR	0.928		5	USDA NSRC, Urbana, IL
6	Hamilton	PR	1.094		1	USDA NSRC, Urbana, IL
7	Spencer	S		1.000	4	USDA NSRC, Urbana, IL
8	Hartwig	PR	0.57		1	USDA NSRC, Urbana, IL
9	Essex	S		nd	6	Southern Illinois University
10	Forrest	PR	nd		1	Southern Illinois University
11	Williams82	S		nd	6	USDA NSRC, Urbana, IL
12	A3244	S		nd	4	USDA NSRC, Urbana, IL
Mean± SEM[c]			0.92±0.17	1.25±0.42		

[a] The resistance (R) and susceptibility (S) is based on Njiti *et al*. (2001).
[b] The *rhg1* allele is after Ruben *et al*., (2006) based on the receptor like kinase haplotype.
[c] The two means are not significantly different (p >0.05).

SDS assays
Seeds were germinated in sterilized sand:soil (1:1) mix in a growth chamber and inoculated with *F. virguliforme* spores as described earlier (Iqbal *et al*., 2002, 2005). Root samples from control and inoculated plants were collected at seventh day after inoculation.

RNA isolation and cDNA synthesis
Total RNA from frozen roots or leaves was extracted using plant RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Contaminating DNAs were removed by DNAase treatment during the RNA isolation process. For the isolation of full length laccase, cDNA was synthesized using SMART™ RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA) according to manufacturer's instructions. The cDNA was amplified using laccase gene (AY113187) specific primers (Forward 5′ATGGAGCCTGCCAAAACCATTCAC3′; Reverse 5′CTAACAAAGAGGAAGATCCACAGGA3′). The PCR product was cloned in to pGEM-T vector (Promega, Madison, WI, USA) and transformed according to the manufacturer's instructions.

Expression of laccase in yeast
The cloned cDNA was removed from vector by *Eco*R1 digestion; gel purified and sub-cloned into *Eco*R1-digested pYES2/NTB (Invitrogen, Carlsbad, CA) fusion vector according to the manufacturer instructions. The presence of inserts was confirmed by restriction analysis followed by gel electrophoresis. The orientation of the insert was determined by DNA sequencing using ABI 377 automated DNA sequencer.

Total RNA was extracted from control INVSc1 yeast cells, non-induced and induced samples using plant RNeasy Mini Kit (QIAGEN, Valencia, CA) and the expression of cloned laccase was confirmed by Northen hybridization using NorthernMax™ kit (Ambion, Austin, TX) according to their instructions.

Total proteins from induced, non-induced and control yeast cells and the recombinant expressed protein were detected by Western hybridization using Anti-Xpress™ (Cat # R910–25, Invitrogen, Carlsbad, CA) as primary antibody and antimouse-peroxidase conjugate (Cat # NA931VS, Amersham, Piscataway, NJ) as secondary antibody. A second Western was probed solely with a mouse monoclonal IgG antibody Anti-Xpress™ HRP (Cat # R911–25, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Enzyme assays
Laccase activity was calculated from the rate of oxidation of 5 mM of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 100 mM sodium acetate (pH 5.0) at 420 nm after Hoopes and Dean (2001) and the oxidation of O-phenylenediamine (OPDA) after Zuyun *et al*., (1998). In gel staining following electrophoresis was in 50 mM sodium acetate (pH 5.0) containing 1% (v/v) dimethyl sulfoxide and 2 mM 1,8-diaminonaphthalene and incubated at 30ºC until activity bands developed (Hoopes and Dean, 2001).

Analysis of laccase transcript abundance in response to F. virguliforme *inoculation*
Initial laccase gene-family transcript abundance (TA) measurements used reverse Northern hybridization (Iqbal *et al*., 2005) in *F. virguliforme* inoculated and non-inoculated roots of RIL 23 and 'Essex'. An EST representing *G. max* laccase (AI495260) was spotted on a membrane. Probe synthesis, hybridization and post-hybridization treatments and data analysis were carried out as described in Iqbal *et al*. (2002, 2005).

For RT-PCR, total RNA was isolated from inoculated and control roots. cDNA was synthesized from 1 µg using iScript™ cDNA Synthesis Kit (BIORAD, Hercules, CA). Quantitative real time PCR (RT-PCR) amplification was carried out in a iQ™ 5 Multicolor Real Time PCR Detection System (BIORAD, Hercules, CA) using iQ™ SYBR® Green Supermix (BIORAD, Hercules, CA) and 10 pmoles of forward and reverse primers in 20 µl reaction volume. The transcript abundances (TA) of two different laccases were estimated. Primers selective for AY113187, the laccase at the *Rfs2/rhg1* locus, (Scaffold 121, LgG, near Satt309) were made. The forward primer was 5′GTCCATCTTGCAGGCTCACCAC3′ and the reverse primer was 5′TTGGGTCGAAGTTCCC GAAACC3′). Primers selective for the EST AI495260 (forward primer 5′ ATGCATTGCCACTTTGATGTCC 3′ and reverse primer 5′ AGTAGGCAAACCAAAATTCCGG 3′) was measured. This laccase is potentially encoded at two loci in the genome, both on LG H, Scaffold_112 near Satt568 and Scaffold 137, near Satt247. The reactions were carried out in triplicate. Soybean β-tubulin (forward primer 5′ CGCACCCTTAAGCTCACCACC 3′, reverse primer 5′ TTTCCACGGACATCGCAGAAGC 3′) was amplified in duplicates from the same cDNA as a control in separate reactions to normalize the data. Data normalization and fold changes in TA between treated and non-treated samples were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Absolute values for TA among cultivars were calculated by reference to the fresh weight of roots from which RNA was extracted and compared to the 18S rRNA band fluorescence and the tubulin amplicon abundance (Iqbal *et al*., 2005).

Determination of laccase copy number in the soybean genome
A minimum tiling path (MTP, build 2) of soybean bacterial artificial chromosome library (BAC) developed at Southern Illinois University, Carbondale, IL (Shultz *et al*., 2006b) contained 8, 064 clones representing ~1-fold coverage of the soybean genome. In order to determine laccase homologous sequences, a α^{32}P dCTP labeled laccase cDNA probe was hybridized to this set of MTP clones as described earlier (Shopinski *et al*., 2006) and number of hybridizing BACs were counted to estimate the number of paralogs in the genome.

Results and discussion

Sequence analysis of alleles of the laccase at Rfs2/rhg1.
Alignment of the 1, 770 bp protein coding portion of the cDNA (AY113187) with 4, 615 bp from the equivalent genomic sequence (AF527604) of the Forrest allele of the laccase at *Rfs2/rhg1* showed the gene was encoded

by seven exons (Fig. 1; 47–172, 286–437, 1036–1280, 1537–1665, 2560–3101, 3654–4098, 4452–4582 bp). The protein predicted to be encoded had a theoretical MW of 64.934 MDa and a pI of 9.23 from 590 amino acids. Two of the introns (I2 and I4) were unusually large at 599 and 895 bp.

Comparison of the Forrest to the Asgrow 3244 allele of the laccase at *Rfs2/rhg1* showed 4 conservative changes in amino acid sequence; R/H168, I/M 271, R/H 330, E/K 470 (Fig. 1). Non-conservative changes from residues 112 to 116 and S/A 356 were frame shift errors in the Forrest allele sequences posted in 2002. Comparing genomic sequences showed no SNPs in the second or third introns but a 70–80 bp region of divergence in the first intron of the Forrest allele; a 159 bp region in the fourth intron; a 35 bp region in the fifth intron; and a 92 bp region in the sixth intron.

Comparison of the Williams 82 allele of the laccase at *Rfs2/rhg1* to Forrest and Asgrow 3244 showed equally significant similarity with multiple SNPs (9–24) in every intron that distinguished the 3 alleles. In the first intron Williams 82 was very different from both Forrest and A3244 showing just 85 % identical sequence due to the 70–80 bp insertion (Supplemental Fig. 1).

Comparing promoter regions showed significant differences among the three alleles that may have effects on transcript abundance. The promoter of Forrest shared just 89 % identity in the first 300 bp that encompassed the core promoter with the Williams 82 and A3244 alleles that did not differ from one another in that region. Differences encompassed but did not disrupt the potential TATA box regions at −40, −32 and −11. From −300 bp to −2, 000 bp distal to the transcription start site alleles were 100 % identical between Forrest, Williams 82 and Asgrow 3244. In summary, the sufficient differences found among the three alleles suggested that transcript abundance might differ significantly between cultivars with different alleles, particularly the Forrest allele.

Effect of F. virguliforme *infestation on laccase mRNA abundance*

Reverse Northern hybridizations indicated some correlation between laccase TA and resistance (Fig. 2) at later stages (day 10) of root-pathogen interaction. The initial decrease in TA at day 1 after inoculation looks most likely the result of stress caused by the process of inoculation and transfer of roots into new media. The TA at day 2 and 7 after inoculation does not indicate any significant change in the resistant and susceptible inoculated roots. Days 1–7 are considered as early to middle phases of the *F. virguliforme* and soybean root interaction (Iqbal *et al*., 2005). However the probe used was not strictly gene specific and may have measured the TA of a set of related laccase genes in the soybean genome (Fig. 3). Further the day 10 effect may not be specific because nearly all transcript abundances are reduced by this stage in a susceptible cultivar (Iqbal *et al*., 2005).

The inoculation and TA analysis experiment were conducted multiple times in ExF23 and ExF85 RILs contrasting for the SDS QTL concentrating on day 7 after inoculation when many gene transcript differ

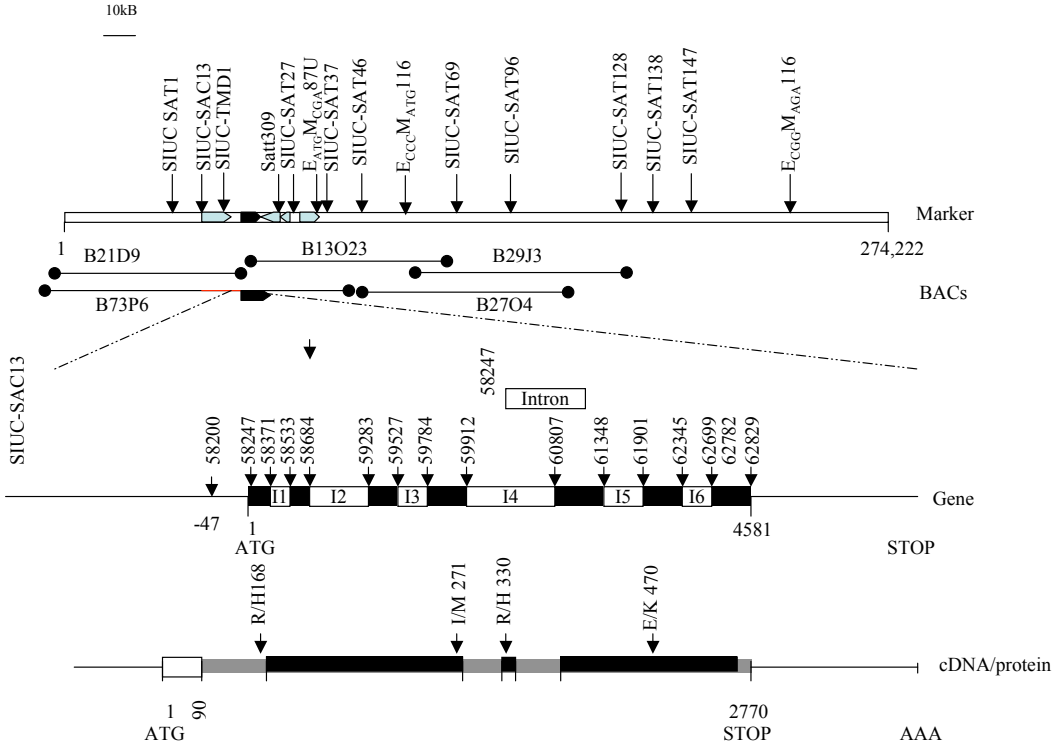

Fig. 1. The physical map of the laccase gene found at the *rhg1* locus. Shown by the open white bar are marker positions in relation to the first 274, 222 kbp of AX196295. An interval spanning 10 Kbp encoding the putative *laccase* gene is shown by a black arrow. In the middle panel the introns (white boxes) and exons (black boxes) are shown. In the lower panel the positions of markers and amino acid substitutions in the cDNA among the alleles of Forrest, Asgrow 3244 and Williams 82 are shown. Copper binding domains are indicated by the black boxes.

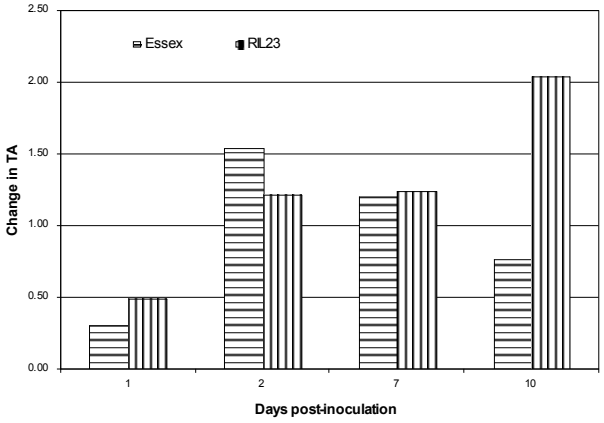

Fig. 2. Change in transcript abundance (TA) of AY113187, the laccase at the *Rfs2/rhg1* locus, measured by reverse Northern hybridization in roots of RIL23 and Essex at 1–10 dai. The hybridization intensity measured from the inoculated samples was divided by that of non-inoculated samples. Scale of 1 on Y-axis represents no change in TA.

between resistant and susceptible genotypes but not because transcription has ceased in the susceptible cultivars (Iqbal *et al*., 2005). The results indicated some significant differences among cultivars. However, there was no significant correlation between change in TA of laccase and the SDS response. In contrast, the number of favorable alleles of SDS QTL (Table 1; Fig. 4) and the allele at *Rfs2/rhg1* predicted RLK were associated with SDS response. Together the results indicate that there is no direct correlation between inoculation induced changes in laccase transcript abundance at 7 days after inoculation (dai) and the heritable resistance of a genotype to SDS.

In fact the laccase at *Rfs2/rhg1* was increased in TA in roots at day 10 after inoculation in RIL23 (Figs 4 & 5). However, the roots of the susceptible genotypes were dead to the extent that there were not many intact transcripts. The laccase TA increased after inoculation only in RIL23 but not Hartwig or Jack that also show root resistance. However, it is possible the assay used had an effect because the correlation between field resistance to SDS and the seedling assay used here is not perfect (Njiti *et al*., 2001). The expression of laccase could be increased at later developmental stages of the soybean roots (Njiti, 1997; Luo *et al*., 1999). Taken together though the data indicate it is the steady state amount of laccase mRNA and/or the isoform expressed from it that are most likely underlying the effects on resistance to SDS among different cultivars.

Identification of diphenol oxidase (laccase) sequences in soybean genome

The lack of correlation between the inherited resistance and the laccase expression raised questions as to the effect of the gene on overall laccase gene family TA. Therefore, the copy number of nearly identical laccase paralogs present in the soybean genome was investigated by high stringency hybridizations to a set of BAC clones covering a minimum tiling path (MTP) of the soybean genome (Shultz *et al*., 2006b). There were 19 BAC clones that hybridized consistently on two filters; B37O05, B38G01, B40D11, B44J09, B10A18, B48I14, B44I16, H38F23, H25M14, H42H20, H57E15, H52G24, H07F23, H64G11, H72M11, H62O22, H71F11, H69G07 and H76L13. The BAC clones B44J09 and B48I14 were present on soybean LG A1 (from Build 4 and 3), H62O22 was identified on soybean LG A2 (Build 3), H64G11 and H69G07 on soybean LG D1BW (Build 3) and H07F23 was identified on soybean LG O. BAC clone B44J09, positive for laccase was identified on soybean LG J. in the version 4 of the soybean physical map. The rest of the BAC clones could not yet be assigned to specific linkage groups but were from different contigs. The soybean BAC clones B21D9 and B73P6 were not present on the membrane because they were not fingerprinted (Wu *et al*., 2004). The presence of at least 20 copies (19 plus the one at the *Rfs2/rhg1* locus) of laccase-like sequences in the soybean genome was not surprising. In ryegrass (*Lolium perenne*), Gavnholt *et al*. (2002) reported the presence of 25 laccase genes. The Arabidopsis database search identified 12 predicted laccase genes (Cai *et al*., 2006). The difference in the number of laccase genes in *Arabidopsis* and ryegrass was attributed to the differences in their genome size. If that is true, then the presence of 20 laccase genes in soybean can be attributed to its genome size and the polyploid nature of parts of its genome (Shultz *et al*., 2006a). However, whether all the

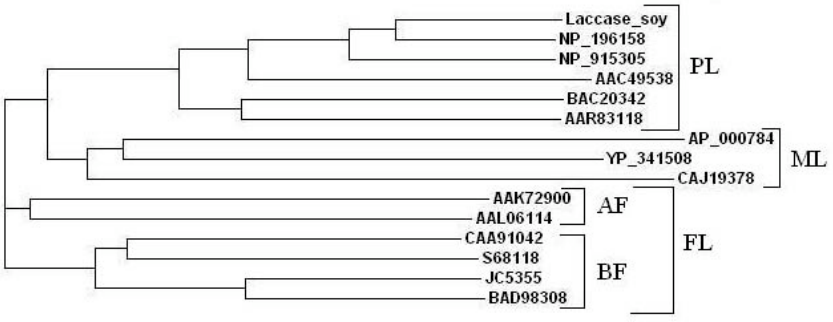

Fig. 3. Amino acid similarity between plant (PL), microbial (ML) and fungal (FL) laccases. The plant sequences included were of soybean (Laccase_soy; AY113187, the laccase at the *Rfs2/rhg1* locus), *Arabidopsis thaliana* (NP_196158), *Oryza sativa* (NP_915305), Tobacco (AAC49538), *Rhus vernicifera* (BAC20342) and *Gossypium arboreum* (AAR83118). The microbial laccases include *Escherichia coli* (AP_000784), *Pseudoalteromonas haloplanktis* (YP_341508) and *Pedomicrobium* sp. (CAJ19378). The fungal laccases include two ascomycetes fungi (AF) *Fusarium proliferatum* (AAK72900) *Botryotinia fuckeliana* (AAL06114) and four basidiomycetes fungi (BF) *Thanatephorus cucumeris* (CAA91042), *Rhizoctonia solani* (S68118), *Trametes villosa* (JC5355) and *Trametes versicolor* (BAD98308. The tree is based on Multiple Alignments using ClustalW v1.8.

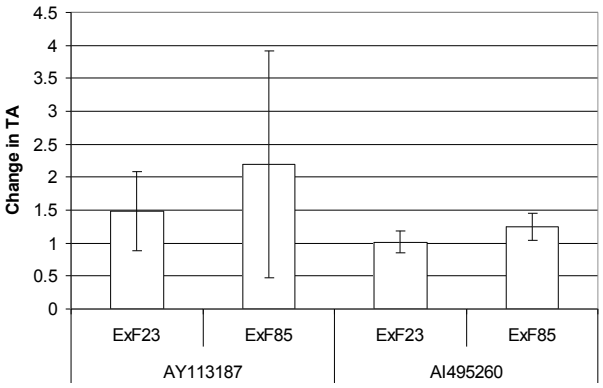

Fig. 4. Change in TA of two ESTs (AY113187 the laccase at the *Rfs2/rhg1* locus and AI495260 a duplicated laccase on LgH and) representing two soybean laccases in RIL23 and RIL85 contrasting for the presence of SDS QTL at 7 dai. The results are mean of 3 independent experiments. In each experiment, roots of 3–5 plants were pooled together and TA was measured by quantitative real time-PCR. Bars indicated standard deviation.

soybean laccase like sequences identified are functional genes has not been determined.

Orthologs of the laccase at Rfs2/rhg1

Plant laccases are closely related. The deduced amino acid sequence clusters the plant laccases form are distinct (Fig. 3) from microbial and fungal laccases. That separation reflects genetic similarities, evolutionary distances and similar but distinct physiological roles in each species. There is no evolutionary distinction between monocot, dicot and angiosperm laccases (Gavnholt *et al*., 2002). However, a high rate of divergence among dicot laccases has been reported (Gavnholt *et al*., 2002). The differences in amino acid sequence between the plant laccases, fungal laccases and ascorbate oxidases are less pronounced (Ranocha *et al*., 1999), but enough to form separate groups. Within plants, the soybean laccase paralog analyzed here showed a ~75% identity with an *Arabidopsis thaliana* laccase (AtLAC12; Cai *et al*., 2006), 67% identity with an *Oryza sativa* (cv. japonica) putative laccase and 63% identity with a *Pinus taeda* laccase. Mutants in AtLAC 12 did not show any phenotypes (Cai *et al*., 2006).

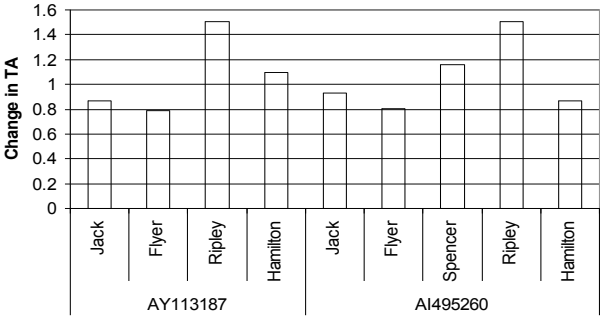

Fig. 5. Change in TA of two ESTs AY113187 (at *Rfs2/rhg1* on LgG) and AI495260 (on LgH) representing two soybean laccases in different soybean varieties at 7 dai. The roots of 3–5 plants were pooled together and TA was measured by quantitative real time-PCR. Each experiment was triplicated (three technical replicates) and means are plotted.

Characterization of the soybean laccase enzyme

The soybean laccase cDNA representing the protein coding portion of the Forrest allele was placed in a yeast expression vector. The expression vector clone was re-sequenced from both ends and no changes were found. The expression of the laccase in yeast was confirmed first by Northern hybridization (Fig. 6, A and B). There was a strong hybridization signal in the lanes containing the RNA samples from galactose-induced yeast cells. However, there was a weak signal in the RNA samples that were isolated from cells that were not induced indicating low constitutive transcription abundance. Yeast cells that were not transformed by the recombinant INVSc1 carrying the laccase gene did not show any expression (Fig. 6B; lane 5).

Western hybridization showed that the Anti-Xpress™ antibody reacted with the induced fusion protein containing the Xpress™ epitope (Fig. 6c, Lane 3–7). No signal was detected from non-induced cells (Lane 8). A positive control, lacZ containing Xpress™ epitope for the detection of lacZ was also used (Lane 9). The lacZ gene encoding β-galactosidase was expressed in yeast cell under the control of the GAL1 promoter. The N-terminal encoding Xpress™ epitope and polyhistidine tag will add ~3.4 kDa to the 67 kDa of recombinant protein. The expressed protein was found to be 70.3 kDa. Therefore the actual size of the diphenol oxidase laccase protein was 67 kDa, confirming the size of the protein as identified from the

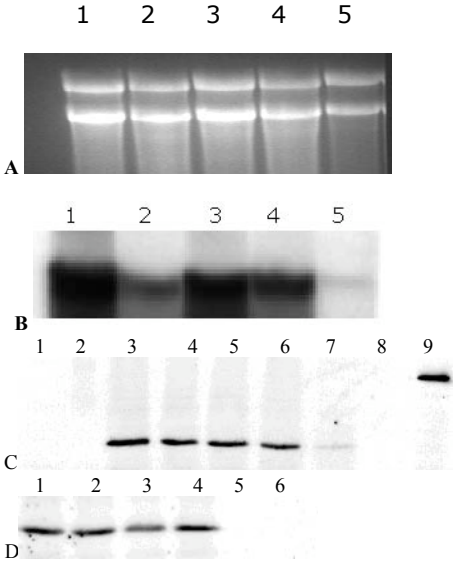

Fig. 6. Expression of diphenol oxidase laccase from AY113187, the laccase at the *Rfs2/rhg1* locus, in induced and non-induced yeast samples by Northern hybridization and Western hybridization. Panel A: Total RNA. Panel B: Northern hybridization. In each lanes 1 and 3 were induced, lanes 2 and 4 were non-induced, lane 5 contained RNA isolated from INSc1 yeast cells. PanelC shows Western hybridization for the detection of expression of the recombinant fusion protein using Anti-Xpress™ antibody. Lanes 1 and 8 contained proteins from non-induced cells; lane 2 was a protein marker, lanes 3 to 7 contained proteins from induced cells; and lane 9 contained tagged lacZ protein. Panel D shows Western hybridization with the Anti-Xpress™-HRP (horseradish peroxidase-conjugated) antibody that react with induced fusion protein containing Xpress™ epitope. Lanes 1 to 4 contained proteins from recombinant yeast cells induced for the expression of diphenol oxidase laccase; lanes 5 and 6 contained proteins from non-induced cells.

deduced amino acid sequence. A number of common substrates were used to determine the laccase activity of the expressed protein. However, very low level of activity was observed in the gel assay (results not shown). The level of activity was very low and could not be quantified by either flourimetric assay. Phenol oxidases usually have specificity toward particular electron acceptors but be able to use a wide variety of electron donors (Zuyun *et al*., 1998; Hoopes and Dean, 2001). The soybean laccase described here appeared to be specific for both. Alternately the protein produced in yeast was not properly folded or lacked an essential cofactor in yeast.

Conclusions

Map based cloning is an important tool in soybean gene identification (Searle *et al*., 2003; Ashfield *et al*., 2003; Gao *et al*., 2005). However, the association of metabolic genes present in the QTL regions with traits is problematic (Hobbs *et al*., 2004). The clustering of genes and iterations among enzyme encoding genes might each only have small effects on the trait. SDS resistance has two components, resistance to root infection and resistance to the leaf scorch caused by the toxin (Njiti *et al*., 1997; 1998). The increases in laccase TA after 10 dai in *F. virguliforme* infested roots in a partially resistant RIL23 line may be due to the ability of the other components of resistance in that genotype to increase the abundance of defense related transcripts. Laccase TA might have only a supporting role in partial resistance. Based on the TA abundance changes in the inoculated roots, it is not likely that increases in the expression of laccase contributes to the SDS QTL named *Rfs2* or the cyst nematode resitance locus *rhg1* (Afzal, 2007; Afzal *et al*., 2009). Rather the higher expression found in resistant cultivars and the amino acid changes in the enzyme isoform found in resistant cultivars were inferred to underlie resistance. Further analysis will require analysis of this locus on linkage group G and the syntenic locus on what appears to be linkage group O BAC clone H07F23 with gene specific probes, transgenic plants and allelic mutant series.

Acknowledgements

This research was funded over the past 11 years in part by grants from the NSF 9872635, and USB 2228–6228. The integrated genetic and physical map was based upon work supported by the National Science Foundation under Grant No. 9872635. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation. The past and continuing support of SIUC, Office of the Vice Chancellor for Research to MJI, RA, JA and DAL is appreciated. The authors thank Dr. P. Gibson, O Myers Jr., and M. Schmidt for assistance with germplasm development and maintenance from 1991–2000 and Chris Town (TIGR) for assistance with DNA sequence analysis and interpretation. We thank the “Soybean Genome Project, at DoE Joint Genome Institute” for release of the WGS reads and scaffolds.

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Supplementary Material

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Query    1097  TTTAACATATAATTATACTTGGGAAAAATTTACTAGAAATAGTAATAATAGATTCTCTAA    1156
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Query    1157  CACTTTCTCCTAACATAGTCTATGATTAATTTAAATTTATTGAAAACTATGAAGTTATGA    1216
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query    1637  TTGTTCTGATCTTAGCTTCAGCATTGTCTTCAGCAAATGCCAAGATTCACGAGCACGAGT    1696
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Query    1697  TAGTAGTACAATCTCGCACTCTGCAAGCTCAGAAGGGCTCAGGACGGATGAGACGCAAGT    1756
               | || ||||| |||| |||||| |   |||  ||   ||| ||||  ||   |  |  ||
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Sbjct  58487   TTGAAGCAACTCCAGTGAAGAGGCTGTGCAAAACCCACAACAGCATCACCGTGAATGGAC  58546
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Supplemental Fig. 1. Alignment of the DNA sequence of Williams 82 and A3244 in the promoter region, first exon and first intron of the laccase at the *Rfs2/rhg1* locus.

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