2009

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

M Javed Iqbal

R Ahsan

Jawad Afzal

A Jamai

K Meksem

See next page for additional authors

Published in *Current Issues in Molecular Biology*, (2009) 11 (Suppl. 1): i11-19

Recommended Citation

Iqbal, M Javed, Ahsan, R, Afzal, Jawad, Jamai, A, Meksem, K, El- Shemy, Hany A. and Lightfoot, David A. "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." (Jan 2009).

Authors

M Javed Iqbal, R Ahsan, Jawad Afzal, A Jamai, K Meksem, Hany A. El- Shemy, and David A. Lightfoot

Curr. Issues Mol. Biol. 11 (Suppl. 1) i11–19. Online journal at www.cimb.org

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

MJ Iqbal[1,2], R Ahsan[1,2], AJ Afzal[2], A Jamai[2,3,4], K Meksem [2,3], H A El- Shemy[2,5], and DA Lightfoot[2]*

[1]Present address: Institute for Sustainable and Renewable Resources (ISRR), Institute for Advanced Learning and Research (IALR), Danville, VA 24540 and Departments of Horticulture and Forestry, Virginia Polytechnic Institute and State University Blacksburg, VA 24061, USA
[2]Center of Excellence: The Illinois Soybean Center, Department of Plant, Soil and Agriculture Systems, MC 4415, SIUC, Carbondale, IL 62901, USA
[3]Plants and Microbes Genomics and Genetics lab, Department of Plant, Soil Sciences, and Agriculture System, Southern Illinois University at Carbondale, Carbondale, IL 62901, USA
[4]Present address: Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA
[5]Faculty of Agriculture Research Park (FARP), Biochemistry Department, Faculty of Agriculture, University of Cairo, 12613 Giza, Egypt

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.

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.

---

*For correspondence: ga4082@siu.edu

Horizon Scientific Press. http://www.horizonpress.com

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^{TM} (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^{TM} 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^{TM} cDNA Synthesis Kit (BIORAD, Hercules, CA). Quantitative real time PCR (RT-PCR) amplification was carried out in a iQ^{TM} 5 Multicolor Real Time PCR Detection System (BIORAD, Hercules, CA) using iQ^{TM} 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

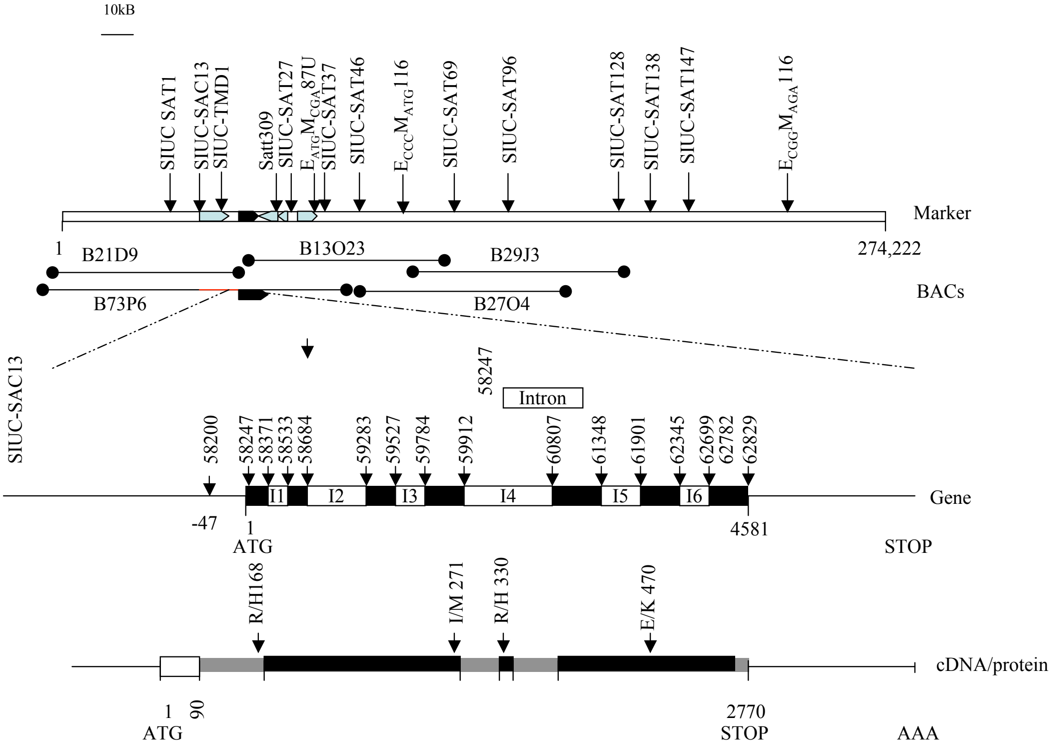

Fig. 1. The physical map of the laccase gene found at the *rhg1* locus. Shown by the open white bar are marker positions in relation to the first 274, 222 kbp of AX196295. An interval spanning 10 Kbp encoding the putative *laccase* gene is shown by a black arrow. In the middle panel the introns (white 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.

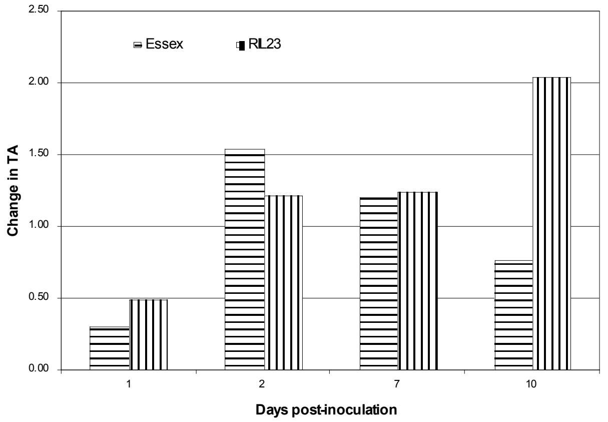

Fig. 2. Change in transcript abundance (TA) of AY113187, the laccase at the *Rfs2/rhg1* locus, measured by reverse Northern hybridization in roots of RIL23 and Essex at 1–10 dai. The hybridization intensity measured from the inoculated samples was divided by that of non-inoculated samples. Scale of 1 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

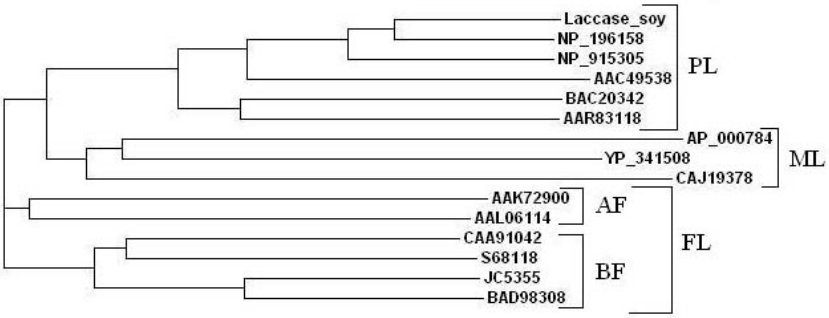

Fig. 3. Amino acid similarity between plant (PL), microbial (ML) and fungal (FL) laccases. The plant sequences included were of soybean (Laccase_soy; AY113187, the laccase at the *Rfs2/rhg1* locus), *Arabidopsis thaliana* (NP_196158), *Oryza sativa* (NP_915305), Tobacco (AAC49538), *Rhus vernicifera* (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.

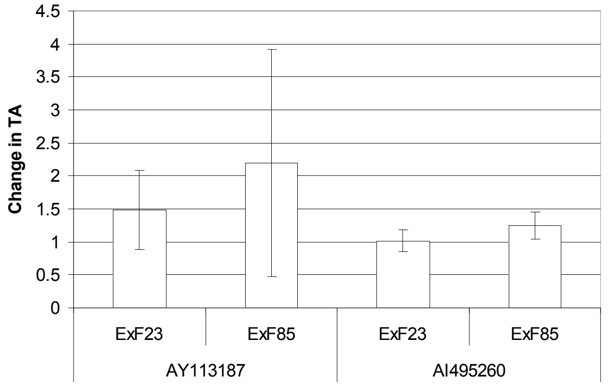

Fig. 4. Change in TA of two ESTs (AY113187 the laccase at the *Rfs2/rhg1* locus and AI495260 a duplicated laccase on LgH and) representing two soybean laccases in RIL23 and RIL85 contrasting for the presence of SDS QTL at 7 dai. The results are mean of 3 independent experiments. In each experiment, roots 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).

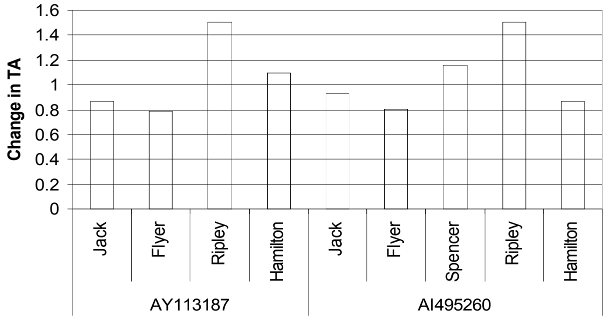

Fig. 5. Change in TA of two ESTs AY113187 (at *Rfs2/rhg1* on LgG) and AI495260 (on LgH) representing two soybean laccases in different soybean varieties at 7 dai. The roots of 3–5 plants were pooled together and TA was measured by quantitative real time-PCR. Each experiment was triplicated (three technical 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. 6 B; lane 5).

Western hybridization showed that the Anti-Xpress™ antibody reacted with the induced fusion protein containing the Xpress™ epitope (Fig. 6 c, 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

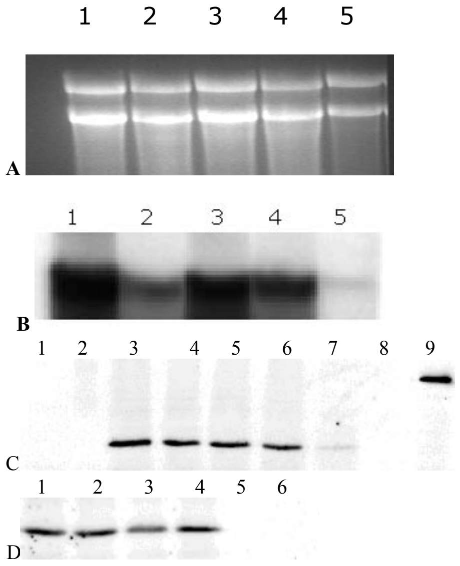

Fig. 6. Expression of diphenol oxidase laccase from AY113187, the laccase at the *Rfs2/rhg1* locus, in induced and non-induced yeast samples by Northern hybridization and Western hybridization. Panel A: Total RNA. Panel B: Northern hybridization. In each lanes 1 and 3 were induced, lanes 2 and 4 were 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.

References

Afzal, A.J., Natarajan, A., Saini, N., Iqbal, M.J., Geisler, M., El Shemy, H., Mungur, R., Wilmitzer, L., and Lightfoot, D.A. (2009) The nematode resistance allele at the *rhg1* locus alters the proteome and metabolome of soybean roots. Plant Physiol. (in press).

Ashfield, T., Bocian, A., Held, D., Henk, A., Marek, L., Danesh, D., Penuela, S., Meksem, K., and Lightfoot, D.A., Young, N., Shoemaker, R., and Innes, R. (2003) Genetic and physical mapping of the soybean *Rpg1-b* disease resistance gene reveals a complex locus containing several tightly linked families of NBS/LRR genes. Mol. Plant Microbe Interact. *16*, 817–826.

Baker, R.A., and Nemec, S. (1994) Soybean sudden death syndrome, Isolation and identification of a new phytotoxin from cultures of the causal agent, *Fusarium solani* (abstract) Phytopathol. *84*, 1144.

Cai, X., Davis, E.J., Ballif, J., Liang, M., Bushman, E., Haroldsen, V., Torabinejad, J., and Wu, Y. (2006) Mutant identification and characterization of the laccase gene family in Arabidopsis. J. Exp. Bot. *57*, 2563–2569.

Cardenas W, and Dankert, J.R. (2000) Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish. Fish Shellfish Immunol. *10*, 33–46.

Davin, L.B., Bedgar, D.L., Katayama, T., and Lewis, N.G. (1992) On the stereoselective synthesis of (+)-pinoresinol in *Forsythia suspense* from its achiral precursor, coniferyl alcohol Phytochemistry *31*, 3869–3874.

Dean, J.F.D., and Eriksson, K.-E.L. (1994) Laccase and deposition of lignin in vascular plants. Holzforschung *48*, 21–33.

Diammantidis, G., Effosse, A., Potier, P., and Bally, R. (2000) Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirllum lipoferum.* Soil Biol. Biochem. *342*, 919–927.

Farias-Neto, A.F., Hashmi, R., Schmidt, M.E., Carlson, S.R., Hartman, G.L., Li, S., Nelson, R.L., and Diers, B.W. (2007) Mapping and confirmation of a sudden death syndrome resistance QTL on linkage group D2 from the soybean genotypes ‘PI 567374’ and ‘Ripley’. Mol. Breed. *20*, 53–62.

Gao, H., Narayanan, N.N., Ellison, L., and Bhattacharyya, M.K. (2005) Two classes of highly similar coiled coil-nucleotide binding-leucine rich repeat genes isolated from the Rps1-k locus encode Phytophthora resistance in soybean. Mol. Plant Microbe Interact. *18*, 1035–1045.

Gavnholt, B., Larsen, K., and Rasmussen, S.K. (2002) Isolation and characterization of laccase cDNAs from meristematic and stem tissue of ryegrass (*Lolium perenne*) Plant Sci. *162*, 581–590.

Gibson, P.T., Shenaut, M.A., Njiti, V.N., Suttner, R.J., and Myers Jr, O. (1994) Soybean varietal response to sudden death syndrome. In, D. Wilkinson (ed), Proc Twenty-fourth Soybean Seed Res. Conf., Chicago, IL 6–7 Dec, 1994 (Washington DC: Am Seed Trade Assoc), pp 436–446.

Hauge, B.M., Wang, M.L., Parsons, J.D., and Parnell, L.D. (2001) Nucleic acid molecules and other molecules associated with soybean cyst nematode resistance. WO 01/51627 PCT/US01/00552 Patent # 20030005491.

Hobbs, D.H., Flintham, J.E., and Hills, M.J. (2004) Genetic control of storage oil synthesis in seeds of Arabidopsis. Plant Physiol. *136*, 3341–3349.

Hoopes, J.T., and Dean, J.F.D. (2001) Staining electrophoretic gels for laccase and peroxidase activity using 1,8-diaminonaphthalene. Anal. Biochem. *293*, 96–101.

Hyten, D.L., Choi, I.Y., Song, Q., Shoemaker, R.C., Nelson, R.L., Costa, J.M., Specht, J.E., and Cregan, P.B. (2007) Highly variable patterns of linkage disequilibrium in multiple soybean populations. Genetics *175*, 1937–1944.

Iqbal, M.J., Meksem, K., Njiti, V.N., Kassem, M.A., and Lightfoot, D.A. (2001) Microsatellite markers identify three additional quantative trait loci for resistance to soybean sudden-death syndrome (SDS) in Essex x Forrest RILs. Theor. Appl. Genet. *102*, 187–192.

Iqbal, M.J., Afzal, A.J., Yaegashi, S., Ruben, E., Triwitayakorn, K., Nijiti, V.N., Ahsan, R., Wood, A.J., and Lightfoot, D.A. (2002) A pyramid of loci for partial resistance to *Fusarium solani* f. sp. *glycines* maintains *Myo*-inositol 1-phosphate synthase expression in soybean roots. Theor. Appl. Genet. *105*, 1115–1123.

Iqbal, M.J., Yaegashi, S., Ahsan, R., Shopinski, K., and Lightfoot, D.A. (2005) Root response to *F. solani* f. sp. *glycines*, Temporal accumulation of transcripts in partially resistant and susceptible soybean. Theor. Appl. Genet. *110*, 1429–1438.

Kazi, S., Shultz, J.L., Afzal, A.J., Njiti, V.N., and Lightfoot, D.A. (2007) Different loci underlie resistance to soybean SDS root infection and leaf scorch in RILs from ‘Flyer’ by ‘Hartwig’. Theor. Appl. Genet. *116*, 967–977.

Kiefer-Myer, M.-C., Gomord, V., O’Connell, A., Halpin, C., and Faye, L. (1996) Cloning and sequence analysis of laccase-encoding cDNA clones from tobacco. Gene *178*, 205–207.

LaFayette, P.R., Eriksson, K.-E.L., and Dean, J.F.D. (1999) Characterization and heterologous expression of laccase cDNAs from xylem tissue of yellow-poplar (*Liriodendron tulipifera*) Plant Mol. Biol. *40*, 23–35.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods *24*, 402–408.

Lozovaya, V.V., Lygin, A.V., Zernova, O.V., Li, S., Hartman, G.L., and Widholm, J.M. (2004) Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. Plant Physiol. Biochem. *42*, 671–679.

Lozovaya, V.V., Lygin, A.V., Zernova, O.V., Li, S., Widholm, J.M., and Hartman, G.L. (2005) Lignin degradation by *Fusarium solani* f. sp. *glycines.* Plant Dis. *90*, 77–82.

Luo, Y., Myers Jr, O., Lightfoot, D.A., and Schmidt, M.E. (1999) Root colonization of soybean cultivars in the field by *Fusarium solani* f. sp. *glycines.* Plant Dis. *83*, 1155–1159.

McCaig, B.C., Meagher, R.B., and Dean, J.F.D. (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis thaliana*. Planta *221*, 619–636.

Meksem, K., Doubler, T.W., Chancharoenchai, K., Njiti, V.N., Chang, S.J.C., Rao-Arelli, A.P., Cregan, P.E., Gray, L.E., Gibson, P.T., and Lightfoot, D.A. (1999) Clustering among loci underlying soybean resistance to *Fusarium solani*, SDS and SCN in near-isogenic lines. Theor. Appl. Genet. *99*, 1131–1142.

Meksem, K., Pantazopoulos, P., Njiti, V.N., Hyten, D.L., Arelli, P.R., and Lightfoot, D.A. (2001) ‘Forrest’ resistance to the soybean cyst nematode is bigenic, saturation mapping of the *Rhg*1 and *Rhg*4 loci. Theor. Appl. Genet. *103*, 710–717.

Njiti, V.N., Gray, L.E., and Lightfoot, D.A. (1997) Rate reducing resistance to *Fusarium solani* f. sp. *phaseoli* underlies field resistance to soybean Sudden Death Syndrome (SDS) Crop Sci. *37*, 132–138.

Njiti, V.N., Doubler, T.W., Suttner, R.J., Gray, L.E., Gibson, P.T., and Lightfoot, D.A. (1998) Resistance to soybean sudden death syndrome and root colonization by *Fusarium solani*f. sp. *glycines* in near-isogeneic lines. Crop Sci. *38*, 472–477.

Njiti, V.N., Johnson, J.E., Torto, T.A., Gray, L.E., and Lightfoot, D.A. (2001) Inoculums’ rate influences selection for field resistance to soybean sudden death syndrome in the greenhouse. Crop Sci. *41*, 1726–1731.

Njiti, V.N., Meksem, K., Iqbal, M.J., Johnson, J.E., Kassem, M.A., Zobrist, K.F., Kilo, V.Y., and Lightfoot, D.A. (2002) Common loci underlie field resistance to soybean sudden death syndrome in Forrest, Pyramid, Essex, and Douglas. Theor. Appl. Genet. *104*, 294–300.

Prabhu, R.R., Njiti, V.N., Bell-Johnson, B., Johnson, J.E., Schmidt, M.E., Klein, J.H., and 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.

Ranocha, P., Chabannes, M., Chamayou, S., Danoun, S., Jauneau, A., Boudet, A.M., and Goffiner, D. (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. *129*, 145–155.

Ranocha, P., McDougall, G., Hawkins, S., Sterjiades, R., Borderies, G., Stewart, D., Cabanes-Macheteau, M., Boudet, A., and Goffiner, D. (1999) Biochemical characterization, molecular cloning and expression of laccases-a highly divergent gene family in Poplar. Eur. J. Biochem. *259*, 485–495.

Ruben, E., Aziz, J., Afzal, A.J., Njiti, V.N., Triwitayakorn, K., Iqbal, M.J., Yaegashi, S., Arelli, P.R., Town, C.D., Meksem, K., and Lightfoot, D.A. (2006) Genomic analysis of the ‘Peking’ *rhg1* locus, candidate genes that underlie soybean resistance to the cyst nematode Molec. Genet. Genom. *276*, 320–330.

Searle, I.R., Men, A.E., Laniya, T.S., Buzas, D.M., Iturbe-Ormaetxe, I., Carroll, B.J., and Gresshoff, P.M. (2003) Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science *299*, 109–112.

Shopinski, K.L., Iqbal, M.J., Afzal, A.J., Shultz, J.L., Jayaraman, D., and Lightfoot, D.A. (2006) Development of a pooled probe method for locating small gene families in a physical map of soybean using stress related paralogues and a BAC minimum tile path. Plant Methods *2*, 20–28.

Shultz, J.L., Jayaraman, D., Shopinski, K.L., Iqbal, M.J., Kazi, S., Zobrist, K., Bashir, R., Yaegashi, S., Lavu, N., Afzal, A.J., Yesudas, C.R., Kassem, M.A., Wu, C., Zhang, H.B., Town, C.D., Meksem, K., and Lightfoot, D.A. (2006a) The soybean genome database (SoyGD), A browser for display of duplicated, polyploid, regions and sequence tagged sites on the integrated physical and genetic maps of *Glycine max.* Nucleic Acids Res. *34*, D1-D8.

Shultz, J.L., Yesudas, C.R., Yaegashi, S., Afzal, A.J., Kazi, S., and Lightfoot, D.A. (2006b) Three minimum tile paths from bacterial artificial chromosome libraries of the soybean (*Glycine max* cv 'Forrest'), Tools for structural and functional genomics. Plant Methods *2*, 9–18.

Sterjiades, R., Dean, J.F.D., Gamble, G., and Himmelsbach, D.S. (1993) Extracellular laccases and peroxidases from sycamore maple (*Acer pseudopplatanus*) cell suspension cultures Reactions with monolignols and lignin model compounds. Planta *190*, 75–87.

Triwitayakorn, K., Njiti, V.N., Iqbal, M.J., Yaegashi, S., Town, C.D., and Lightfoot, D.A. (2005) Genomic analysis of a region encompassing *QRfs1* and *QRfs2*, genes that underlie soybean resistance to sudden death syndrome. Genome *48*, 125–138.

Wu, C., Sun, S., Nimmakayala, P., Santos FA., Springman, R., Ding, K., Meksem, K., Lightfoot, D.A., and Zhang, H.B. (2004) A BAC and BIBAC-based physical map of the soybean genome. Genome Res. *14*, 319–326.

Zuyun, H., Houping, H., Ruxiu, C., and Yun'e, Z. (1998) Organic solvent enhanced spectrofluorimetric method for determination of laccase activity. Anal. Chim. Acta *374*, 99–103.

Supplementary Material

```
Query   1097  TTTAACATATAATTATACTTGGGAAAAATTTACTAGAAATAGTAATAATAGATTCTCTAA   1156
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57768  TTTAACATATAATTATACTTGGGAAAAATTTACTAGAAATAGTAATAATAGATTCTCTAA  57827

Query   1157  CACTTTCTCCTAACATAGTCTATGATTAATTTAAATTTATTGAAAACTATGAAGTTATGA   1216
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57828  CACTTTCTCCTAACATAGTCTATGATTAATTTAAATTTATTGAAAACTATGAAGTTATGA  57887

Query   1217  GAGAATTATTATTTTTGTAATTTTTAAGAAATTTCAACTAGAGAAAATATGTTTAAAAGA   1276
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57888  GAGAATTATTATTTTTGTAATTTTTAAGAAATTTCAACTAGAGAAAATATGTTTAAAAGA  57947

Query   1277  GCCTGCTGTTAAACTTTCTCAAAATTTATTTTCAACCTCTAACCGCAGACTTCTGAAATA   1336
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  57948  GCCTGCTGTTAAACTTTCTCAAAATTTATTTTCAACCTCTAACCGCAGACTTCTGAAATA  58007

Query   1337  AGCATTCATGCACTTTATTAACTAGCGGGTGCAACAACTCCTATGGGTTTGGAACCAAGT   1396
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58008  AGCATTCATGCACTTTATTAACTAGCGGGTGCAACAACTCCTATGGGTTTGGAACCAAGT  58067

Query   1397  TAAGTTTCCCTTTGGGGTCTGACCTCAACTAAATTAACCTAATCTGCCTAACCTCAAAGG   1456
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58068  TAAGTTTCCCTTTGGGGTCTGACCTCAACTAAATTAACCTAATCTGCCTAACCTCAAAGG  58127

Query   1457  ACTTTATCTTTCCCCCACCTCTAATCCACCCTATAAAAGCACCCTCTCCCACTCTTACTT   1516
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58128  ACTTTATCTTTCCCCCACCTCTAATCCACCCTATAAAAGCACCCTCTCCCACTCTTACTT  58187

Query   1517  GCATTGCAACCTTAACCTTCAGCATTCACACTAAGGTGTTCCTTGCTCGCCAAAAGATCA   1576
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58188  GCATTGCAACCTTAACCTTCAGCATTCACACTAAGGTGTTCCTTGCTCGCCAAAAGATCA  58247

Query   1577  TGGAGCCTGCCAAAACCATTCACAACAATGTCAAATACTCCCCCATCTTCTTAGCCATCT   1636
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  58248  TGGAGCCTGCCAAAACCATTCACAACAATGTCAAATACTCCCCCATCTTCTTAGCCATCT  58307

Query   1637  TTGTTCTGATCTTAGCTTCAGCATTGTCTTCAGCAAATGCCAAGATTCACGAGCACGAGT   1696
              ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  58308  TTGTTCTGATCTTAGCTTCAGCATTGTCTTCAGCAAATGCCAAAATTCACGAGCACGAGT  58367

Query   1697  TAGTAGTACAATCTCGCACTCTGCAAGCTCAGAAGGGCTCAGGACGGATGAGACGCAAGT   1756
              | || ||||| |||| |||||| |   |||  ||   ||| ||||  ||   |  |  ||
Sbjct  58368  TTGTTGTACATTCTCTCACTCT-CTTTCTCTTAATTTCTCTGGACTTATTTTATTCTTGT  58426

Query   1757  CGATAAGAACTCAGATCCGCGAGATAATAATGACCTAGCTGAAGATAGTAATGGAATAGG   1816
                 |   |||||   ||||  |||||||||| |||||||||  ||| |||||||||||||
Sbjct  58427  TTTTTTTAACTCTTTTCCGTTAGATAATAATTACCTAGCTGTTGATTGTAATGGAATAGG  58486

Query   1817  AAGAAGCAACTCCAGTGAAGAGGCTGTG-AAAACCCACAACAGCATCACCGTGAATGGAC   1875
                |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||
Sbjct  58487  TTGAAGCAACTCCAGTGAAGAGGCTGTGCAAAACCCACAACAGCATCACCGTGAATGGAC  58546
```

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.

Caister Academic Press

New Books

www.caister.com

Caliciviruses
Molecular and Cellular Virology
Edited by: **G.S. Hansman, J. Jiang, K.Y. Green**
c. 250 pp., April 2010
ISBN: 978-1-904455-63-9 $310 / £159
The most important research findings. Timely and comprehensive reviews. Discussion of past and current research.

Epstein-Barr Virus
Latency and Transformation
Edited by: **Erle S. Robertson**
c. 220 pp., April 2010
ISBN: 978-1-904455-62-2 $310 / £159
Expert virologists comprehensively review this important subject from a genetic, biochemical, immunological, and cell biological perspective. Essential reading.

Anaerobic Parasitic Protozoa
Genomics and Molecular Biology
Edited by: **C.G. Clark, P.J. Johnson, R.D. Adam**
c. 210 pp., March 2010
ISBN: 978-1-904455-61-5 $310 / £159
Internationally acclaimed researchers critically review the most important aspects of research on anaerobic parasitic protozoa.

Lentiviruses and Macrophages
Molecular and Cellular Interactions
Edited by: **Moira Desport**
c. 410 pp., March 2010
ISBN: 978-1-904455-60-8 $310 / £159
Top lentivirus and macrophage specialists comprehensively review cutting-edge topics in the molecular and cellular biology of the lentivirus-macrophage interaction.

Microbial Population Genetics
Edited by: **Jianping Xu**
c. 230 pp., March 2010
ISBN: 978-1-904455-59-2 $310 / £159
Details the major current advances in microbial population genetics and genomics.

Borrelia
Molecular Biology, Host Interaction and Pathogenesis
Edited by: **D. Scott Samuels and Justin D. Radolf**
c. 630 pp., March 2010
ISBN: 978-1-904455-58-5 $310 / £159
Written by renowned scientists in the field who have made seminal contributions to the field, this book is a comprehensive guide to the pathogenic *Borrelia*.

Influenza
Molecular Virology
Edited by: **Qinghua Wang and Yizhi Jane Tao**
c. 200 pp., February 2010
ISBN: 978-1-904455-57-8 $310 / £159
NS1, hemagglutinin, nucleoprotein, glycoproteins, M2 channel, virulence, polymerase, microarrays, vaccine design.

RNA Interference and Viruses
Current Innovations and Future Trends
Edited by: **Miguel Angel Martínez**
c. 280 pp., February 2010
ISBN: 978-1-904455-56-1 $310 / £159
Expert RNAi specialists from around the world have teamed up to produce a timely and thought-provoking review of the area.

Retroviruses
Molecular Biology, Genomics and Pathogenesis
Edited by: **Reinhard Kurth and Norbert Bannert**
c. 520 pp., January 2010
ISBN: 978-1-904455-55-4 $310 / £159
Genomics, molecular biology and pathogenesis , comprehensively covering all the recent advances.

Metagenomics
Theory, Methods and Applications
Edited by: **Diana Marco**
x + 212 pp., January 2010
ISBN: 978-1-904455-54-7 $310 / £159
Essential reading for all researchers performing metagenomics studies. Highly recommended.

Aspergillus
Molecular Biology and Genomics
Edited by: **M. Machida and K. Gomi**
x + 238 pp., January 2010
ISBN: 978-1-904455-53-0 $310 / £159
Systematics, bioinformatics, systems biology, regulation, genetics, genomics, metabolism, ecology, development.

Environmental Molecular Microbiology
Edited by: **Wen-Tso Liu and Janet K. Jansson**
viii + 232 pp., January 2010
ISBN: 978-1-904455-52-3 $310 / £159
Current technology and applications. Microbial diversity, phylogeny, communities, 16S rRNA, metagenomics, metaproteomics, microarrays, fingerprinting, soil, water, plants, humans, biofilms.

Neisseria
Molecular Mechanisms of Pathogenesis
Edited by: **Caroline Genco and Lee Wetzler**
x + 270 pp., January 2010
ISBN: 978-1-904455-51-6 $310 / £150
Genomics, biofilms, adhesion, invasion, immunity, complement, apoptosis, vaccine, epidemiology, antibiotic resistance.

Frontiers in Dengue Virus Research
Edited by: **K.A. Hanley and S.C. Weaver**
viii + 304 pp., January 2010
ISBN: 978-1-904455-50-9 $310 / £150
Evolution, epidemiology, translation, replication, pathogenesis, host, animal models, mosquito interactions, transmission, vaccine, drugs, immunotherapy.

ABC Transporters in Microorganisms
Edited by: **Alicia Ponte-Sucre**
xii + 260 pp., August 2009
ISBN: 978-1-904455-49-3 $310 / £150

Pili and Flagella
Current Research and Future Trends
Edited by: **Ken Jarrell**
x + 238 pp., August 2009
ISBN: 978-1-904455-48-6 $310 / £150

Lab-on-a-Chip Technology
Edited by: **K. E. Herold and A. Rasooly**
Vol 1: Fabrication and Microfluidics
ISBN: 978-1-904455-46-2 $310 / £150
Vol 2: Biomolecular Separation and Analysis
ISBN: 978-1-904455-47-9 $310 / £150

Bacterial Polysaccharides
Current Research and Future Trends
Edited by: **Matthias Ullrich**
xii + 358 pp., June 2009
ISBN: 978-1-904455-45-5 $310 / £150

Microbial Toxins:
Current Research and Future Trends
Edited by: **Thomas Proft**
viii + 192 pp., May 2009
ISBN: 978-1-904455-44-8 $310 / £150

Acanthamoeba:
Biology and Pathogenesis
Author: **Naveed Khan**
viii + 290 pp., February 2009
ISBN: 978-1-904455-43-1 $310 / £150

Bacterial Secreted Proteins:
Secretory Mechanisms and Role in Pathogenesis
Edited by: **Karl Wooldridge**
xii + 512 pp., April 2009
ISBN: 978-1-904455-42-4 $310 / £150

www.caister.com