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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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Abstract

Multigeneic QTL present significant problems to analysis. Resistance to soybean (Glycine max (L) Mer.) 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 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 QRfs2 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; QRfs, QRfs1, QRfs2, QRfs3) 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 rhg1, a major gene for resistance to SCN (Meksem et al., 1999, 2001). QRfs2 is about 0.0–0.2 cM from rhg1 and may be pleiotropic effect of a single gene (Triwitayakorn et al., 2005; Ruben et al., 2006), QRfs2 reduces the leaf-scourch 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/rhg1 region on LG G encompassed between SIU-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 cultivar ‘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.

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Diphenol oxidase laccase (hereafter called as laccase) was considered a strong candidate for QRfs2 (Tritvitatayakorn, 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 hydroxylation 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-Meyer 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 QRFs2 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.

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<td>12 A3244</td>
<td>S</td>
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*The resistance (R) and susceptibility (S) is based on Njiti et al. (2001).
*The rhg1 allele is after Ruben et al., (2006) based on the receptor like kinase haplotype.
*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 DNase 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′ATGGAGCCTGCAAAAACATTCCAGC3′; Reverse 5′CTAAACAGGGAGGTACACGGAG3′). 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 EcoR1 digestion; gel purified and sub-cloned into EcoR1-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.

**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 done in 50 mM sodium acetate (pH 5.0) containing 1% (v/v) dimethyl sulfoxide and 2 mM 1,8-diaminonaphthalene and incubated at 30°C until activity bands developed (Hoopes and Dean, 2001).

**Analysis of laccase transcript 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 α²-SP 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 kDa 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...
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 O. 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
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).

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-sequence 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
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 fluorimetric 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 resistance 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.

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References


related paralogues and a BAC minimum tile path. Plant Methods 2, 20–28.

Supplementary Material

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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 activity at the Rfs2/rhg1 locus.
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