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The identification and characterization of four laccases from the plant pathogenic fungus *Rhizoctonia solani*

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Abstract Four distinct laccase genes, *lcc1*, *lcc2*, *lcc3* and *lcc4*, have been identified in the fungus *Rhizoctonia solani*. Both cDNA and genomic copies of these genes were isolated and characterized. Hybridization analyses indicate that each of the four laccase genes is present in a single copy in the genome. The *R. solani* laccases can be divided into two groups based on their protein size, intron/exon organization, and transcriptional regulation. Three of these enzymes have been expressed in the fungus *Aspergillus oryzae*. Two of the recombinant laccases, r-lcc1 and r-lcc4, as well as the native lcc4 enzyme were purified and characterized. The purified proteins are homodimeric, comprised of two subunits of approximately 66 kDa for lcc4 and 50–100 kDa for the recombinant lcc1 protein. These laccases have spectral properties that are consistent with other blue copper proteins. With syringaldazine as a substrate, lcc4 has optimal activity at pH 7, whereas lcc1 has optimal activity at pH 6.

Key words Laccas · Phenol-oxidas · *Rhizoctonia solani* · Fungi

Introduction

Laccases are copper-containing enzymes that catalyze the oxidation of phenolic compounds and the four-electron reduction of dioxygen to water (Messerschmidt and Huber 1990). Such enzyme activity has been found in both plants and filamentous fungi, although it is important to note that the laccases from these two sources differ in their active copper centers, redox potentials, and substrate specificity (Mayer 1987). The enzyme may be involved in various biosyntheses and cellular de-toxification in which the oxidation of phenolic compounds occurs, but its precise role is unknown. Mature laccases have a primary structure of approximately 500 amino acids that are predicted to fold into a three-domain barrel capable of binding four copper atoms (Mayer 1987; Messerschmidt and Huber 1990; Solomon et al. 1992).

Biochemical analyses of fungal culture media have shown that individual species may express several laccase isozymes which can differ with regard to pH optimum, substrate specificity, molecular weight, and cellular location (Blaich and Esser 1975; Leonowicz et al. 1978; Bollag and Leonowicz 1984; Mayer 1987). In *Botrytis cinerea* three different laccases can be distinguished by their differential expression under induction by various substrates, as well as by their different isoelectric points (pI), pH optima, and molecular weights (Marbach et al. 1983, 1984). *Armillaria mellea* produces two laccases that do not cross-react immunologically and have different pI (Rehman and Thurston 1992). Whether these isozymes are the products of separate laccase genes or originate from differential post-translational modification of a single gene product is unknown. Of the seven fungi *Neurospora crassa* (Germann and Lerch 1986; Germann et al. 1988), *Coriolus hirsutus* (Kojima et al. 1990), *Cryphonectria* parasitica (Choi et al. 1992), *Agaricus bisporus* (Perry et al. 1993), *Aspergillus nidulans* (Aramayo and Timberlake, 1990), *Phlebia radiata* (Saloheimo et al. 1991) and the basidiomycete PM1(Coll et al. 1993), from which laccase genes have been isolated, characterized and reported, only *A. bisporus* has been shown to encode multiple (two) laccases, and they share 93% amino-acid identity.

Interest in laccases as industrial enzymes has increased recently due to their potential to degrade both highly toxic phenolics and lignin. However, most

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characterized fungal laccases function optimally at acidic pH which may not be compatible with potential industrial applications. The fungus *Rhizoctonia praticola* has been shown to produce a laccase that functions at neutral pH (pH 6.5–7.5), and whose production can be increased by a factor of 30 by exposure of the fungus to *p*-anisidine (Bollag et al. 1979; Shuttleworth et al. 1986). We report here the isolation and molecular characterization of four distinct laccase genes from a related phytopathogenic species *Rhizoctonia solani*.

Materials and methods

Materials. Chemicals used as buffers and substrates were commercial products of at least reagent grade. Chromatography was performed on either Pharmacia FPLC or conventional low-pressure open systems. Spectroscopic assays were conducted on either a spectrophotometer (Shimadzu UV 160 U) with a 1-cm quartz cuvette or a microplate reader (Molecular Devices, Menlo Park, Calif). N-terminal sequencing was performed on an ABI 476A sequencer (Applied Biosystems, Foster City, Calif.). Triplicate quantitative amino-acid analyses with internal standards were performed on a HP AminoQuant instrument (Hewlett-Packard). SDS-PAGE and native IEF analysis were performed respectively on Mini Protean II and Model 111 Mini IEF cells (Bio-Rad, Hercules, Calif.). Western-blot analyses were done on a Mini Transblot cell (Bio-Rad) with an alkaline phosphatase kit (Bio-Rad). Molecular-weight determination by size exclusion gel filtration was carried out on a Sephacryl S-300 column (1×112 cm) by using Blue Dextran (2000 kDa), bovine IgG (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and horse heart myoglobin (17 kDa) to calibrate the column. All oligonucleotides were synthesized using an Applied Biosystems 394 DNA/RNA synthesizer. Restriction digests, plasmid DNA isolations, DNA cloning, and DNA ligations were performed using standard molecular biology protocols (Sambrook et al. 1989). Reagents were purchased from Boehringer Mannheim and Gibco/BRL. Nucleotide sequences were determined using an Applied Biosystems automatic DNA sequencer, Model 363A, version 1.2.0 . *Saccharomyces cerevisiae* and *Escherichia coli* strains used for molecular biology experiments were all commercial strains. *R. solani* RS22 belongs to anastomosis grouping 6 (AG-6) and can be obtained as sample #358730 from the International Mycological Institute, Genetic Resource Reference Collection, Bakeham Lane, Egham, Surrey TW20 9TY, England. An *Aspergillus oryzae* amylase-deficient variant was kindly provided by Dr. Howard Brody of Novo Nordisk Biotech, Davis, Calif.

Mycelial growth. *R. solani* mycelia were grown at room temperature at 150 rpm in 500-ml Ehrlenmeyer flasks containing 100 ml of Czapek Dox media [3.0 g $NaNO_3$, 1.0 g Na_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_20$, 0.01 g $FeSO_4 \cdot 7H_20$, 2.5 g L-asparagine, 0.025 g $CaCl_2$–$2H_2O$, 0.0015 g $CuSO_4$, 1.0 ml trace metals (22.0 g $ZnSO_4 \cdot 7H_2O$, 11 g H_3BO_3, 5.0 g $MnCl_2 \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.6 g $CoCl_2 \cdot 5H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, 1.1 g $(NH_4)_6MO_7O_{24} \cdot 4H_2O$, 50 g Na_2EDTA in 1 l) in 900 ml of water, pH 6.8; 100 ml of a 30% sterile sucrose solution was added after autoclaving]. For induction experiments, 0.246 g of *p*-anisidine was dissolved in 1 ml of ethanol, diluted to 5 ml with sterile water and then added to a 3-day-old, 100-ml culture. Mycelia was harvested from all cultures after 4 days of growth and flash-frozen in liquid Nitrogen before storing at − 80 °C.

Genomic DNA isolation. Frozen mycelia were ground with dry ice to a fine powder in an electric coffee grinder. The powdered mycelia were dissolved in 20 ml of lysis buffer (100 mM EDTA, 10 mM Tris-Cl, 1% Triton X-100, 500 mM guanidine-HCl, 200 mM NaCl; pH 8.0) per 2 g of mycelium, and DNase-free RNase A was added to a final concentration of 20 μg/ml followed by an incubation for 30 min at 37 °C. Proteinase K was then added to a final concentration of 0.8 mg/ml and the mixture was incubated for 2 h at 50 °C with gentle agitation. Insoluble debris was removed by centrifugation for 20 min at 12–15 000 g. The cleared supernatant was applied to a Qiagen tip-500 column (QIAGEN Inc., Los Angeles), washed and eluted according to the manufacturer's instructions.

RNA preparation. Two grams of frozen mycelia were ground with dry ice to a fine powder in an electric coffee grinder. The powdered mycelia were dissolved in 20 ml of buffer R1 (4 M guanidine thiocyanate; 100 mM Tris-Cl, 25 mM $MgCl_2$, 25 mM EDTA; pH 7.5) to which 1.6 ml of 25% Triton X-100 and 40 μl of 2-mercaptoethanol were added. Following a 15-min incubation on ice, 20 ml of 3 M NaAc, pH 6.0, was added and the mixture was again incubated for 15 min on ice. The lysate was cleared by centrifugation at 15 000 g for 30 min at 4 °C. RNA was precipitated from the supernatant by the addition of 0.8 vol of isopropanol, followed by a 5-min. incubation on ice and centrifugation at 15 000 g for 30 min at 4 °C. After dissolving the RNA pellet in 16 ml buffer R2 (20 mM Tris-Cl, 1 mM EDTA; pH 8.0), and removing insoluble particles by centrifugation at 15,000 g for 15 min. at 4 °C, 4 ml of buffer R3 (2 M NaCl, 250 mM MOPS; pH 7.0) was added. The solution was loaded onto a QIAGEN-tip 500 and the RNA eluted according to the manufacturer's instructions. Poly-A^+ RNA was selected on an oligo-dT column using standard conditions (Sambrook et al. 1989).

Polymerase chain reaction (PCR). To identify the laccase gene sequences from *R. solani*, a 220-nt fragment of genomic DNA was amplified using PCR with degenerate oligonucleotide primers based upon two amino-acid sequences conserved among all characterized laccases [IHWHGFFQ and TFWYHSH; amino acids 84–91 and 126–132, respectively, of the *Coriolus hirsutus* mature laccase protein (Kojima et al. 1990)]. Genomic DNA (0.5 μg) was mixed with primers using standard PCR conditions and the reactions were first incubated for 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 50–60 °C, 1 min at 72 °C, and one cycle of 5 min at 72 °C.

Genomic DNA library preparation. *R. solani* genomic DNA (30 μg) was partially digested with the restriction enzyme *Sau*3A. Fragments between 6 kb and 15 kb were isolated by preparative gel electrophoresis, further purified with Beta-Agarase (New England Biolabs, Beverly, Mass.) according to manufacturer's instructions, and subsequently ligated to Lambda (λ) phage EMBL4 arms with *Bam*HI ends. The resulting molecules were packaged in vitro using Gigapack II packaging extracts (Stratagene, Los Angeles, Calif.) to create a library of 170 000 phages that were used to infect *E. coli* K802 and amplified 100-fold for future use.

Library screening. Approximately 25 000 plaques from an amplified genomic library were screened using as a probe the 220-nt laccase PCR fragment that had been random labeled with α-dCTP-^{32}P. Conditions were the same as for high-stringency Southern hybridizations.

cDNA library cloning. Double-stranded cDNA was synthesized as described previously (Kofod et al. 1994). Gel-fractionation was used to isolate cDNAs between 1.7 and 3.5 kb, and one-half of this preparation was ligated to non-palindromic *Bst*XI adaptors (Invitrogen), while the second-half was ligated to hemi-phosphorylated *Eco*RI/*Not*I adaptors (Gibco BRL). The first fraction was then inserted into the yeast expression vector pYES2.0 (Invitrogen) that had been linearized with *Bst*XI. After amplification in *E. coli*, purified plasmid DNA was electroporated into electro-competent *S. cerevisiae* W3124 cells [*MAT*α *ura3-52 leu2-3, 112 his3-Δ200*

Δpep4-1137 Δprc1::HIS3 prb1::LEU2 (van den Hazel et al. 1992)]. Aliquots were plated on YNB (1.7 g yeast nitrogen base without amino acids, and 5 g $(NH_4)_2SO_4$ per l) with 2% glucose. After 4 days growth at 30 °C, the resulting colonies were replica plated onto YNB with 0.1% glucose, 2% galactose and 1 g/l of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The *Eco*RI-adapted cDNA fraction was ligated with λZiplox, *Eco*RI arms (Gibco BRL), and a library consisting of 4.4×10^7 recombinant phages was created.

Southern and Northern hybridizations. Hybridizations were carried out using standard techniques on Zeta Probe Blotting membrane (BioRad) or Hybond N membrane (Amersham, Arlington Heights, Ill.) with 5×10^5 cpm/ml of probe. For Southern hybridizations the following fragments were used as probes: for *lcc1* and *lcc3*, a genomic fragment from nt 717 to nt 1933 of *lcc1* encompassing aa 130–441; for *lcc2*, a genomic fragment from nt 150 to nt 1992 encompassing aa 1–341; and for *lcc4*, the entire cDNA clone. The entire cDNA clones for *lcc2*, *lcc3* and *lcc4* were used as probes for Northern hybridizations.

Vector construction for heterologous expression. The coding sequences of *lcc1*, *lcc2* and *lcc4* were cloned into the expression vector pMWR1 at unique SfiI (5′ end) and *Nsi*I (3′ end) sites such that gene expression was controlled by the *A. oryzae* TAKA-amylase transcription and translation signals (Christensen et al. 1988). The *lcc4* cDNA was cloned into another expression vector, pMWR3, at unique SwaI (5′ end) and NsiI (3′ end) sites. pMWR3 differs from pMWR1 only in that it lacks the TAKA amylase signal peptide; therefore secretion and processing rely on the native laccase signals.

Fungal transformation. Protoplasts of Aspergillus oryzae were prepared using Novozyme 234 and a sucrose step gradient, and subsequently co-transformed with the laccase expression plasmids and a plasmid containing the *A. nidulans amdS* gene as described in Yelton et al. (1984). The transformation mix was plated onto minimal medium plates containing 10 mM acetamide as the sole nitrogen source (Gomi et al. 1992). ABTS activity was scored on minimal plates with 10 mM acetamide and 1 g/l of ABTS.

Purification of laccase from p-anisidine-induced R. solani culture broth. The culture broth (400 ml, pH 4.6, 1.0 mS) whose gelatinous, pinkish material was initially removed by a DEAE-Sephadex A-25 (Sigma Chemical, St. Louis, Mo.; 70-ml) column under 10 mM KAcetate, pH 5, was applied on a SP-Sepharose (Parmacia Biotech, Piscataway, N.J.; 10-ml) column, pre-equilibrated with 20 mM of NaAcetate, pH 4.5, and eluted with a linear gradient of 0–0.5 M NaCl. After chromatography on a Mono-S column (Pharmacia, 1-ml) under identical buffer conditions, the laccase was further purified by gel filtration on a BioGel P-60 column (Bio-Rad, 200-ml) under 20 mM of KAcetate, 0.5 M KCl, pH 4.5, to yield a pure laccase preparation, as judged by SDS-PAGE. An overall 14-fold purification and a 1% recovery were achieved.

Purification of r-lcc4 from A. oryzae culture broth. The concentrated broth (360 ml, 1.1 mS, pH 7) was applied on a Q-Sepharose (120- ml) column pre-equilibrated with 10 mM Tris-Cl, pH 7.5. The active fractions that passed through the column during loading and washing were pooled, the pH adjusted to 5.3, and applied onto a SP-Sepharose column (60-ml) pre-equilibrated with 10 mM MES, pH 5.3. The laccase was then eluted with a 0–1 M NaCl gradient and was pure as judged by SDS-PAGE. A purification of 78-fold and a recovery of 12% were achieved.

Purification of r-lcc1 from A. oryzae culture broth. The concentrated broth (280 ml, 0.6 mS, pH 7.3) was applied to a Q-Sepharose column (70 ml) pre-equilibrated with 10 mM Tris-Cl, pH 7.5, and eluted with a gradient of 0-2 M NaCl. The pooled active fractions were pH adjusted to 5.3 and applied onto a SP-Sepharose column (60-ml) pre-equilibrated with 10 mM MES, pH 5.3, and eluted with a 0–1 M NaCl gradient. The pooled active fractions were made 1.7 M in $(NH_4)_2SO_4$, pH 6.4, and applied to a Phenyl-Superose column (Pharmacia, 1-ml) pre-equilibrated with 100 mM Na-phosphates, 1.7 M $(NH_4)_2SO_4$, pH 6.4. The laccase was eluted late in a gradient of 0–100% of 10 mM Na-phosphate, pH 6.4, to give a pure preparation as judged on SDS-PAGE. A purification of 130-fold and a recovery of 5% were achieved.

Enzymatic assay. Syringaldazine oxidation was carried out with 20-μ M of substrate in Britton and Robinson buffers (B and R: 0.1 M boric acid, 0.1 M acetic acid, 0.1 M phosphoric acid mixed with 0.5 M NaOH to desired pH) at 20 °C by monitoring absorbance change at 530 nm at which the extinction coefficient was 65 $mM^{-1}cm^{-1}$ (Bauer and Rupe 1971). ABTS oxidation was carried out with 1 mM ABTS in B and R buffers at 20 °C by monitoring absorbance change at 405 nm at which the extinction coefficient was 35 $mM^{-1}cm^{-1}$ (Childs and Bardsley 1975). The overlay ABTS oxidase activity assay was carried out by pouring cooled ABTS-agarose (0.1 g ABTS, 1 g agarose, 50 ml H_2O) over a native IEF gel and incubating at 20 °C.

De-glycosylation. De-glycosylation by Endo/N-glycosidase F was carried out in accordance with the instruction of the manufacturer (Boehringer-Mannheim) (3.6 units of glycosidase for 2–7 μg of laccase). The extent of de-glycosylation was estimated from the mobility on SDS-PAGE.

Protein sequencing and copper detection. Partial protein digestions were carried out with Lys-C and Glu-C proteases (from *Achromobacter* and *Bacillus licheniformes*, respectively), the resulting peptides were purified by RF-HPLC (under a linear gradient of 80% 2-propanol, plus 0.08% aqueous TFA in 0.1% aqueous TFA), and their N-termini were sequenced. The Cu content of the laccases was determined by 2,2′-biquinoline titration (Felsenfeld 1959). Protein concentrations were measured based on the extinction coefficients determined by amino-acid analysis and MWt was deduced from the DNA sequence.

Results

Purification and characterization of the major extracellular laccase from *R. solani* cultures

R. solani culture supernatant was subjected to isoelectric-focusing (IEF), and oxidase activity was detected by overlaying the gel with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)-containing agarose. A prominent phenoloxidase with a pI of approximately 8.0 was detected. This enzyme was purified by ion-exchange chromatography. The purified protein is blue in color and has a UV-visible spectrum with maxima near 280 and 600 nm, similar to those reported for other plant and fungal laccases (Nakamura 1958; Mosbach 1962). The amino-terminal sequence of the mature protein was examined and determined to be: AVRNYKFDIKNVNVAPDGFQRPIVSVNGLVPGTL. This sequence can be aligned with the amino-termini of laccases from *C. hirsutus* and *P. radiata* (Kojima et al. 1990; Saloheimo et al. 1991).

Isolation of laccase genes by polymerase chain reaction (PCR) amplification

A 220-nt PCR fragment was amplified from *R. solani* genomic DNA using oligonucleotide primers based on two regions of conserved amino-acid sequences among laccases. A single, cloned PCR fragment was sequenced revealing an open reading frame with 60% homology to the corresponding region of a laccase gene from *C. hirsutus* (Kojima et al. 1990) . Sequencing of 15 additional, independent, PCR clones showed that at least three distinct laccase genes were present, assigned as *lcc1*, *lcc2* and *lcc3*. Using the PCR amplicon as a probe, nine potential genomic clones were isolated from a *R. solani* library. Further characterization of the clones by restriction and hybridization analyses localized the three genes to different *Eco*RI fragments common to eight of the phage clones, suggesting gene linkage. A restriction map generated from these results, as diagrammed in Fig. 1, places *lcc1* 1.7 kb from *lcc3*, and *lcc3* 1.3 kb from *lcc2*.

The DNA sequences of *lcc1*, *lcc2* and *lcc3* were determined from the genomic clones (Fig. 2). A comparison between the DNA sequence of the three genes with the restriction map shown in Fig. 1 shows that all three genes share the same transcription orientation. cDNA clones were also isolated for *lcc2* and *lcc3*, and their DNA sequences were determined. The cDNA sequence of *lcc2* matches 100% the predicted coding portions of the genomic sequence, while the *lcc3* cDNA and genomic sequence differ at 26 nt which introduces amino-acid changes at four residues as indicated in Fig. 2. These differences can be explained by the presence of either multiple copies or alleles of the *lcc3* gene.

Lcc1, *lcc2* and *lcc3* encode proteins of 576, 599 and 572 amino-acids (aa) respectively. *Lcc1* and *lcc3* share 87% identity at the amino acid level, and *lcc*2 shares 64% identity to the other two proteins (Fig. 2). Each gene product begins with a 19 or 20 amino-acid sequence characteristic of fungal signal peptides (Perlman and Halvorson 1983; von Heijne 1986). Comparison between the three genes shows that their structure is identical since each contains 13 introns that are located at homologous positions; interestingly, all three genes contain an exon (exon 5) composed of 3 nt. The N-terminal sequence of each protein was aligned with the amino-acid sequence obtained from the purified laccase. Although homology was high enough to align the sequences, *lcc1* matched only 13 out of the 34 amino acids, while *lcc2* and *lcc3* matched 18 and 14 out of 34, suggesting that none of these were the laccase that we had originally purified and characterized.

To identify the gene that encodes the major extracellular laccase purified from *R. solani*, cDNA was prepared from poly(A)$^+$ RNA obtained from mycelia induced with *p*-anisidine and cloned into the yeast

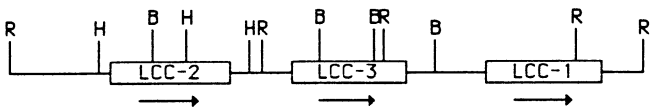

Fig. 1 Organization of *lcc1*, *lcc2* and *lcc3* within the *R. solani* genome as determined by restriction-enzyme mapping and DNA-sequence analysis. Restriction-enzyme sites are labeled as followed: B = *BamHI; H* = *Hin*dIII, and *R* = *Eco*RI. The direction of transcription is indicated by an *arrow* below each gene.

lcc1

MARTIFLVSV SLFVSAVLAR TVEYGLKISD GEIAPDGVKR **NA**TLVNGGYP 50
GPLIFANKGD TLKVKVQNKL TNPEMYRTTS IHWHGLLQHR NADDDGPSFV 100
TQCPIVPRES YTYTIPLDDQ TGTYWYHSHL SSQYVDGLRG PLVIYDPKDP 150
HRRLYDVDDE KTVLIIGDWY HESSKAILAS **GNI**TRQRPVS ATINGKGRFD 200
PDNTPANPDT LYTLKVKRGK RYRLRVI**NSS** EIASFRFSVE GHKVTVIAAD 250
GVSTKPYQVD AFDILAGQRI DCVVEANQEP DTYWINAPLT NVP**NKT**AQAL 300
LVYEEDRRPY HPPKGPYRKW SVSEAIIKYW NHKHKHGRGL LSGHGGLKAR 350
MIEGSHHLHS RSVVKRQ**NET** TTVVMDESKL VPLEYPGAAC GSKPADLVLD 400
LTFGLNFATG HWMINGIPYE SPKIPTLLKI LTDEDGVTES DFTKEEHTVI 450
LPKNKCIEFN IKGNSGIPIT HPVHLHGHTW DVVQFGNNPP NYVNPPRRDV 500
VGSTDAGVRI QFKTDNPGPW FLHCHIDWHL EEGFAMVFAE APEAVKGGPK 550
SVAVDSQWEG LCGKYDNWLK SNPGQL• 576

lcc2

MARSITSLFA LSLVASAFAR VVDYGFDVAN GAVAPDGVTR NAVLVNGRFP 50
GPLITANKGD TLKITVRNKL SDPTMRRSTT IHWHGLLQHR TAEEDGPAFV 100
TQCPIPPQES YTYTMPLGEQ TGTYWYHSHL SSQYVDGLRG PIVIYDPHDP 150
YRNYYDVDDE RTVFTLADWY HTPSEAIIAT HDVLKTIPDS GTINGKGKYD 200
PASANT**NNTT** LENLYTLKVK RGKRYRLRII **NAS**AIASFRF GVQGHKCTII 250
EADGVLTKPI EVDAFDILAG QRYSCILKAD QDPDSYWINA PITNVLNTNV 300
QALLVYEDDK RPTHYPWKPF LTWKISNEII QYWQHKHGSH GHKGKGHHHK 350
VRAIGGVSGL SSRVKSRASD LSKKAVELAA ALVAGEAELD KRQNED**NST**I 400
VLDETKLIPL VQPGAPGGSR PADVVVPLDF GLNFANGLWT IN**NVS**YSPPD 450
VPTLLKILTD KDKVDASDFT ADEHTYILPK NQVVELHIKG QALGIVHPLH 500
LHGHAFDVVQ FGDNAPNYVN PPRRDVVGVT DAGVRIQFRT DNPGPWFLHC 550
HIDWHLEEGF AMVFAEAPED IKKGSQSVKP DGQWKKLCEK YEKLPEALQ• 599

Fig. 2 Sequence of the *R.solani* laccase proteins as determined by DNA sequencing. The DNA sequence of both strands of all four genes, as well as the cDNA sequence for *lcc2*, *lcc3* and *lcc4*, were determined and have been submitted to the EMBL data library under the accession numbers Z54275, Z54276, Z54215 and Z54277. Amino-acid numbers are indicated at the right of the figure. Amino-acid changes resulting from differences between the cDNA and genomic sequences, are indicated below the affected residues. Putative signal sequences are *underlined*. Amino-acid sequences of lcc4, as determined by amino-terminal sequencing, are *overlined*. Tentative N-linked glycosylation sites (NXS/T) are in *bold letters*

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lcc3
MARTIFLVSV SLFVSAVLAR TVEYNLKISN GKIAPDGVER DATLVNGGYP  50
GPLIFANKGD TLKVKVQNKL TNPDMYRTTS IHWHGLLQHR NADDDGPAFV 100
TQCPIVPQAS YTYTMPLGDQ TGTYWYHSHL SSQYVDGLRG PLVIYDPKDP 150
HRRLYDIDDE KTVLIIGDWY HTSSKAILAT GNITLQQPDS ATINGKGRFD 200
PDNTPANPNT LYTLKVKRGK RYRLRVINSS AIASFRMSIQ GHKMTVIAAD 250
GVSTKPYQVD SFDILAGQRI DAVVEANQEP DTYWINAPLT NVANKTAQAL 300
LIYEDDRRPY HPPKGPYRKW SVSEAIIKYW KHKHGRGLLS GHGGLKARMM 350
EGSLHLHGRR DIVKRQNETT TVVMDETKLV PLEHPGAACG SKPADLVIDL 400
TFGVNFTTGH WMINGIPHKS PDMPTLLKIL TDTDGVTESD FTQPEHTIIL 450
PKNKCVEFNI KGNSGLGIVH PIHLHGHTFD VVQFGNNPPN YVNPPRRDVV 500
GATDEGVRFQ FKTDNPGPWF LHCHIDWHLE EGFAMVFAEA PEAIKGGPKS 550
VPVDRQWKDL CRKYGSLPAG FL*                               572

lcc4
MLSSITLLPL LAAVSIPAEAA VRNYKFDIK NVNVAPDGFQ RPIVSVNGLVP 50
GTLITANKG  DTLRINVTNQ LTDPSMRRAT TIHWHGLFQA TTADEDGPAF 100
VTQCPIAQNL SYTYEIPLHG QTGTMWYHAH LASQYVDGLR GPLVIYDPND 150
PHKSRYDVDD ASTVVMLEDW YHTPAPVLEK QMFSTNNTAL LSPVPDSGLI 200
NGKGRYVGGP AVPRSVINVK RGKRYRLRVI NASAIGSFTF SIEGHRLTVI 250
EADGIPHQPL PVDSFQIYAG QRYSVIVEAN QTAANYWIRA PMTVAGAGTN 300
ANLDPTNVFA VLHYEGAPNA EPTTEQGSAI GTALVEENLH ALINPGAPGG 350
SAPADVSLNL AIGRSTVDGI LRFTFNNIKY EAPSLPTLLK ILANNASNDA 400
DFTPNEHTIV LPHNKVIELN ITGGADHPIH LHGHVFDIVK SLGGTPNYVN 450
PPRRDVVRVG GTGVVLRFKT DNPGPWFVHC HIDWHLEAGL ALVFAEAPSQ 500
IRQGVQSVQP NNAWNQLCPK YAALPPDLQ*                       529
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Fig. 2 (continued)

expression vector pYES2.0 (in this vector, transcription is induced by galactose and repressed by glucose). Approximately 3000 yeast transformants from this library were cultured initially on agar plates containing 2% glucose, and 4-days later replica-plated to agar plates containing 2% galactose ABTS. After 24 h of growth on galactose plus ABTS, a single colony displayed a light-green halo which gradually turned to dark purple. DNA sequencing of the insert from this colony established the existence of a fourth laccase gene, *lcc4*.

As shown in Fig. 2, *lcc4* encodes a protein of 530 amino acids, and shares 48%, 50% and 49% identity with *lcc1*, *lcc2* and *lcc3*, respectively. The predicted protein sequence of this laccase can be aligned with 34 of the 34 amino acids obtained from the amino-terminus sequencing of the purified mature protein. The open reading frame of the cDNA encodes an additional 22 amino acids preceding the amino-terminus of the mature protein comprising what appears to be a signal peptide that is processed at the carboxyl side of Ala-22. Subsequent cloning and sequencing of the genomic clone for *lcc4* revealed that this gene contains 19 introns. The genomic clone and the cDNA differed at 29

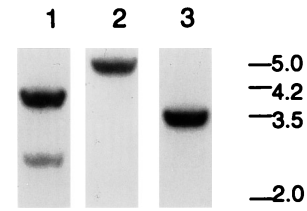

Fig. 3 Southern-blot analysis of the laccase genes. Each lane contains 5 µg of *R. solani* genomic DNA that was digested with the restriction enzyme *EcoR*I, and hybridized with ^{32}P-labeled DNA from *lcc1* (*lane 1*), *lcc2* (*lane 2*), and *lcc4* (*lane 3*). Molecular-weight standards are represented by dashed lines and their sizes are indicated

nucleotides. Of these, 24 changes fall at the third nt of a codon and thus do not alter the amino-acid sequence of the protein; the remaining five base changes result in amino-acid replacements as indicated in Fig. 2.

Gene copy number

Southern hybridizations were carried out to determine the copy number of each of the laccase genes within *R. solani* (Fig. 3). Three filters that had been cross-linked to *R. solani* genomic DNA were separately hybridized to probes made from fragments of *lcc1* (Fig. 3, lane 1), *lcc2* (Fig. 3, lane 2) and *lcc4* (Fig. 3, lane 3). The *lcc1* probe hybridized to two DNA fragments that were 4.0 and 2.6 kb in size, representing *lcc1* and *lcc3*, respectively. The *lcc2* probe hybridized to a single band of 5.3 kb, and the *lcc4* to one band of 3.5 kb. For each gene, the hybridizing DNA fragment is large enough to encode only one complete copy of the gene suggesting that each of the laccase genes is present in a single copy. This result also suggests that the differences found between the cDNA and genomic sequences of *lcc3* and *lcc4* are due to allelic differences.

Transcriptional regulation

The cDNA clones of *lcc2* and *lcc4* were hybridized separately to poly(A)$^+$ RNA isolated from mycelia that had been grown in the presence (Fig. 4, lanes 1, 3 and 5) or the absence (Fig. 4, lanes 2 , 4 and 6) of *p*-anisidine. Hybridization of the *lcc2* probe occurred at conditions that allow cross-hybridization between *lcc1*, *lcc2* and *lcc3*. A single transcript of approximately 2.0 kb can be seen only in the uninduced RNA lane (Fig. 4, lane 2). A faint transcript of the same size can be detected in the induced RNA lane after a long exposure (Fig. 4, lane 3). Identical results were obtained when the *lcc2* and *lcc3* cDNAs were hybridized separately to RNA at high-stringency conditions that prevent cross-hybridization between the two genes (data not shown). The *lcc4* cDNA hybridizes to a single band of about 1.8 kb in size in both the induced and the uninduced samples

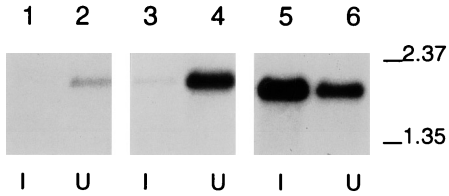

Fig. 4 Northern-blot analysis of laccase transcripts. Each lane contains1 mg of poly-A RNA that was isolated from *R. solani* mycelia, denatured, and electrophoresed through a 1.5% agarose/5.4% formaldehyde gel. After transferring to filters, laccase mRNA was detected by hybridization with ^{32}P-labeled cDNA of *lcc2* (*lanes 1–4*) and *lcc4* (*lanes 5 and 6*). *Lanes 1, 3 and 5* contain RNA isolated from cultures induced with *p-anisidine* (*I*) ; *lanes 2, 4 and 6* contain RNA from uninduced cultures (*U*). The autoradiograms shown in *lanes 1, 2, 5 and 6* were developed after 16 h exposure. *Lanes 3 and 4* were exposed for 5 days. The molecular weights of fragments were estimated by comparison to MWt standards as indicated

(Fig. 4, lanes 5 and 6, respectively). The signal for the induced culture is slightly stronger than that seen in cultures grown without *p*-anisidine, corroborating that the expression of this laccase can be induced by this compound. In addition, the mRNA for *lcc4* appears to be present at a higher level than that of the other three laccases in non-induced mycelia (Fig. 4, lanes 2 and 6).

Heterologous expression of *R. solani* laccase genes in *A. oryzae*

Expression vectors were constructed for three of the laccase genes (*lcc1*, *lcc2* and *lcc4*) by ligating the TAKA amylase promoter and terminator sequences at the 5′ and 3' ends, respectively, of each gene as described in Materials and methods. The resulting constructs were used to transform an *A. oryzae* host strain, and transformant colonies were screened for their ability to oxidize ABTS on agar plates. Further analysis of these transformants in shake-flask cultures showed that levels of recombinant (r-)lcc1 and r-lcc4 increased steadily in most transformants from day 2 to day 6. In contrast, r-lcc2 levels only increased to a smaller extent after 4 days of growth, followed by a decrease to background levels suggesting that this enzyme was unstable in *A. oryzae*. The low expression level of r-lcc2 was also confirmed by SDS-PAGE.

Purification and characterization of recombinant laccases from *A. oryzae* culture broth

We chose to focus our protein characterization work on r-lcc1 and r-lcc4, in addition to the native (wt-) lcc4, *R. solani* enzyme. The initial concentration of the culture broth by diafiltration, using a membrane with a molecular-weight (MWt) cut-off of 100 kDa, removed significant amounts of small contaminants and enriched for laccases. Sequential chromatographies allowed the purification of *R. solani* laccases. For the

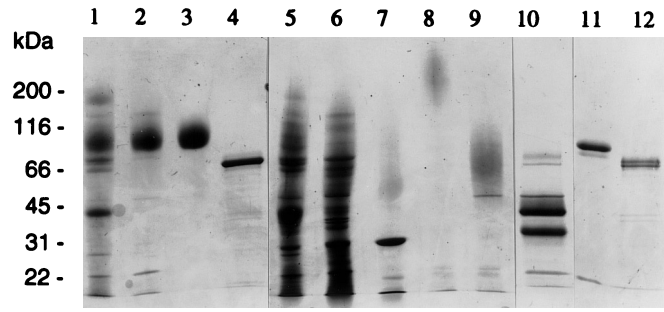

Fig. 5 SDS-PAGE (4–15% gels) for *R. solani* laccases. For r-lcc4: *lane 1*, *A. oryzae* broth in which r-lcc4 is expressed; *lane 2*, pooled r-lcc4 fractions from Q-Sepharose; *lane 3*, purified r-lcc4 from SP-Sepharose; *lane 4*, de-glycosylated r-lcc4. For r-lcc1: *lane 5*, *A. oryzae* broth in which r-lcc1 is expressed; *lane 6*, pooled r-lcc1 fractions from Q-Sepharose; *lane 7*, pooled r-lcc1 fractions from SP-Sepharose; *lane 8*, purified r-lcc1 from Phenyl-Superose (sample loaded without de-naturation in boiling water); *lane 9*, purified r-lcc1 from Phenyl-Superose (sample loaded after de-naturation in boiling water); *lane 10*, de-glycosylated r-lcc1 (sample loaded after de-naturation in boiling water). For wt-lcc4: *lane 11*, purified wt-lcc4; *lane 12*, de-glycosylated wt-lcc4

inducible wt-lcc4, r-lcc4, and r-lcc1, overall purification of 14-, 78-, and 35- fold was achieved, respectively. Figure. 5 shows the SDS-PAGE analysis of the laccase fractions from various stages of the purification.

On SDS-PAGE, the induced wt-lcc4 appears to have a MWt of about 66 kDa (Fig. 5, lane 11), while r-lcc4 migrates as a smear over 70–85 kDa (Fig. 5, lane 3). When r-lcc1 is denatured by SDS and 2-mercaptoethanol, a smear of MWt 200 kDa is seen on SDS-PAGE (Fig. 5, lane 8). However, when the SDS/2-mercaptoethanol treatment is followed by incubation of the sample in boiling water, r-lcc1 shows a smear of MWt 50–80 kDa and a defined band around 45 kDa (Fig. 5, lane 9). Both the smear and the band have an N-terminal amino-acid sequence identical to the predicted amino-acid sequence of lcc1.

Treatment with endoglycosidases results in a mobility increase for all three laccases. For the wt, the treatment yields a pair of bands migrating at about MWt 60 kDa (Fig. 5, lane 12). For r-lcc4, endoglycosidase treatment yields one band of about MWt 60 kDa (Fig. 5, lane 4). For r-lcc1, the treatment yields a sample which, after being reduced and denatured, shows a minor doublet of bands around 60 kDa and three major bands of MWt approximately 27, 40, and 45 kDa (Fig. 5, lane 10). N-terminal sequencing has established that the bands around MWt 40, 45, and 60 kDa have the N-terminus of r-lcc1, whereas the band at MWt 27 kDa has an N-terminus resulting from a cleavage at the Arg345-carboxyl site.

On native IEF, the induced wt-lcc4 gives two bands near pH 7.5; r-lcc4 shows three bands near pH 7.2, 7.5, and 7.8; and r-lcc1 yields four bands near pH 5. An ABTS overlay assay shows that these bands are all active.

Table 1 Properties of wt-lcc4, r-lcc4 and r-lcc1

Laccase	MWt[a] kDa	pI	Glycosylation[b] w/w, %	λ_{max} (nm) (ε, mM^{-1} cm^{-1})	Cu/ subunit	Optimal pH	
						ABTS[c]	Syringaldizine
wt-lcc4	66	7.5	≈ 10	276 (66), 602 (2.5)	2.1	≤ 2.7	7.0
r-lcc4	70–85	7.5	≈ 20	276 (66), 602 (4.7)	3.4	≤ 2.7	7.0
r-lcc1	50–80	5.0	≈ 10	276 (111), 602 (4.2)	3.6	≤ 2.7	6.0

[a] Estimated from SDS-PAGE. The gel-filtration of wt-lcc4 on S-300 yielded a MWt of 130 kDa. The ultrafiltration of wt-lcc4, r-lcc4, and r-lcc1 on S1Y100 membrane suggested a MWt of > 100 kDa. The SDS-PAGE of r-lcc1 without 2-mercaptoethanol pre-treatment suggested a MWt of 200 kDa
[b] Estimated from the mobility change on SDS-PAGE with or without glycosidase pre-treatment
[c] Lower assaying pH limit

Size-exclusion gel filtration of the induced wt-lcc4 protein gives a native MWt of 130 kDa, indicating the dimeric nature of the protein. Like wt-lcc4, r-lcc1 and r-lcc4 can also be concentrated by a membrane with MWt cut-off of 100 kDa. It appears that r-lcc1 and r-lcc4 may be dimeric as well.

The UV-visible absorption maxima for the three laccases are around 275 and 605 nm (Table 1). The ratio of absorbance at 280 to that at 600 is 26 for wt-lcc4, 14 for r-lcc4, and 22 for r-lcc1. The ratio of absorbance at 330 to that at 600 is 3.7 for the wt-lcc4, 1.0 for r-lcc4, and 3.3 for r-lcc1. Based on absorbance at 280, the photometric titration showed the stoichiometry values of Cu per subunit as 2.1, 3.4, and 3.6 for the wt-lcc4, r-lcc4, and r-lcc1, respectively.

N-terminal sequencing shows that r-lcc4 is processed in *A. oryzae* at the same site as that of the wt-lcc4 in *R. solani* (Fig. 2). Up to the position where sequencing has been carried out, all three proteins match 100% the sequence derived from DNA analysis. Partial amino-acid sequences determined from internal fragments of wt-lcc4 also match that derived from the DNA sequence of lcc4. Protein sequencing results indicate that there is no C-terminal processing of the mature wt-lcc4.

The pH-activity profiles for the three laccases are shown in Fig. 6. The wt-lcc4 and r-lcc4 have pH optima around pH 7 when syringaldazine serves as the substrate, in comparison with a pH of 6 for r-lcc1. With ABTS as the substrate, the optimal pH is ≤ 2.7 for all three laccases. The specific activities at optimal pH are given in Table 1. All three laccases showed poor stability at room temperature over pH 3–9 in acetate-phosphate-borate and Bis-Tris buffers.

The laccases in crude culture broths from r-lcc1 and r-lcc4 transformants showed similar pH-activity profiles as that of the purified enzymes. The broth of r-lcc2 transformants showed optimal activities at pH 6 and ≤ pH 4 with syringaldazine and ABTS as substrate, respectively. While wt-lcc4 and r-lcc4 cross-reacted well with polyclonal rabbit sera antibodies raised against a *Polyporus pinsitus* laccase, r-lcc1 showed very poor cross-reactivity in Western blotting. Table 1 summarizes the properties of the purified laccases.

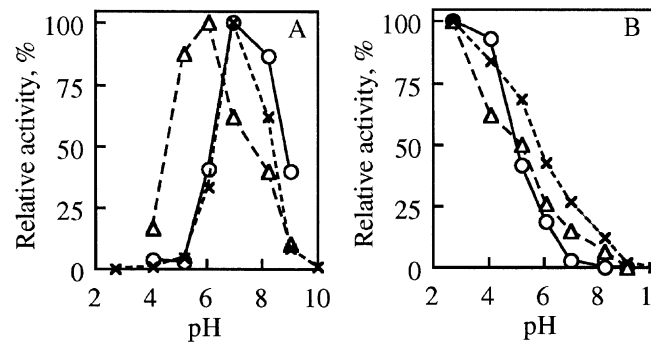

Fig. 6A, B Comparison of the pH-activity profiles of the wt-lcc4 (○), r-lcc4 (×), and r-lcc1 (△). **A** syringaldazine oxidation; **B** ABTS oxidation

Discussion

We report here on the identification and characterization of four laccase genes from the plant pathogenic fungus *R. solani*.These proteins show 50–89% identity and can be grouped into two classes by their homology, gene structure, and transcriptional regulation. The first class of laccases, comprising *lcc1*, 2 and *3*, are linked within a 12-kb fragment of the genome, share 67–89% similarity and contain 11 introns each of which occur at identical positions. Transcription of at least two of these genes occurs at a low constitutive level that can be further suppressed by exposure of the fungus to *p*-anisidine. In contrast, the gene for lcc-4 contains 19 introns, and shares only 48–50% amino-acid identity with the remaining three genes. The mRNA for this enzyme appears to be present at higher basal levels in the cell, and its transcription can be further increased in response to *p*-anisidine.

Under the induction conditions used in our study, only one laccase with relatively high abundance has

been purified from *R. solani* culture broth, wt-lcc4. Based on SDS-PAGE, the laccase subunit of 66 kDa is somewhat smaller than that reported for the *R. praticola* laccase (78 kDa) (Bollag et al. 1979). The ratio of the absorbance at 280 nm to that at 600 nm equals 26 which is larger than the value of 14 found for that of r-lcc4. This is presumably due to Cu loss during the purification, since only two, instead of four, Cu components per subunit are detected. The low recovery yields for wt-lcc4 (1%) and r-lcc1 (5%) are attributed to their low stability, as well as to difficulty in the separation of other impurities.

More-thorough characterization of *R. solani* laccases has been carried out with the recombinant laccase, r-lcc4. Its UV-visible spectral properties are similar to those of *Polyporus versicola* and *Rhus* laccases (Reinhammar 1972). The photometric titration using biquinoline shows that r-lcc4 has four Cu per subunit. The pI of r-lcc4 is similar to that of wt-lcc4. As judged by SDS-PAGE, it appears that lcc4 is more extensively glycosylated in *A. oryzae* than in *R. solani* . The protein does contain seven Asn-X-Ser/Thr motifs which serve as the recognition sites for the attachment of N-linked carbohydrates (Kobata 1992), as well as numerous serine and threonine residues scattered throughout the protein to which O-linked glycans may bind (see Fig. 2).

Recombinant lcc1 exhibits severe smearing on SDS-PAGE, presumably due to extensive heterologous glycosylation. Since the corresponding native form has not been isolated, it is unclear whether this glycosylation pattern is related to the expression host. However, this protein also contains many potential sites for both N-linked and O-linked glycosylation (see Fig. 2). It appears that purified r-lcc1 is quite unstable. Simple heat treatment in preparing the sample for SDS-PAGE can result in significant modification (Fig. 5, lanes 8 and 9). Treatment with commercial endo/N-glycosidase F apparently results in a cleavage at Arg345. According to the DNA sequence, such cleavage yields two polypeptide fragments of 39 and 23 kDa, which correlates to the two bands observed on SDS-PAGE (40 and 27 kDa, Fig. 5, lane 10, respectively). It is unclear whether the cleavage is proteolytic due to minute contamination from either glycosidases or r-lcc1 preparations.

Like *R. praticola* laccase, the wt-lcc4 and r-lcc4 preparations have optimal activities around pH 7 for methoxyphenol substrates (Bollag et al. 1979; Bollag and Leonowicz 1984). The optimal activity of r-lcc1 is more acidic. The wt-lcc4 preparation shows a lower specific activity than that of r-lcc4, possibly due to the partial loss of Cu during protein purification. With the non-phenolic substrate ABTS, wt-lcc4, r-lcc4 and r-lcc1 all show optimal activity at the lowest pH examined (pH 2.7). The substrate-specific differences observed between the pH profiles of the two laccases could be explained by the nature of the substrates. Syringaldizine is a phenol whose oxidation involves the release of two protons, while ABTS is a non-phenolic benzothiazoline whose oxidation involves no proton gain or loss. The pH-induced changes in laccase activity might only affect the pH optima relative to substrates whose oxidation requires the gain or loss of protons (Xu et al. 1995). By photometric titration in pHs ranging from 1.5 to 11, both ABTS and syringaldazine show reversible transformation with a pKa of 2.2 and 8.2, respectively. Whether such pH-induced transformation of substrates plays an important role in the pH activity profiles of the different laccases is not clear.

Although biochemical evidence has suggested that many fungi produce multiple laccases with varying characteristics, the only report to-date of a laccase gene family describes two genes from the mushroom *A. bisporus* that share 93% homology. The *A. bisporus* genes reside within 6 kb of each other on the same chromosome, similar to what is seen with *lcc1*, *lcc2* and *lcc3* (communication personal, Christopher F. Thurston). In contrast, the *R. solani* laccase family comprises at least four genes sharing 48–89% similarity, differing by their pH optima and transcriptional regulation. Why a fungus would require more than one laccase is unknown, but one explanation is that different laccases function under conditions of changing environmental influences or that they function at specific developmental phases. Until a role for this enzyme in fungal cells has been clearly defined any explanations will remain purely speculative.

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