Functional Analyses of Laccase Genes from *Fusarium oxysporum*

D. Cordoba Cañero and M. I. G. Roncero

Departamento de Genética, Universidad de Córdoba, Campus Universitario de Rabanales Edif. Gregor Mendel, E-14071 Córdoba, Spain.
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ABSTRACT

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Six laccase genes, *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5*, and *lcc9*, of the vascular wilt fungus *Fusarium oxysporum* were isolated and characterized. All genes have the characteristic conserved domains for copper binding of phenol oxidase enzymes. Targeted inactivation of *lcc1*, *lcc3*, and *lcc5* resulted in a significant decrease of extracellular laccase activity. Reverse transcription-polymerase chain reaction showed that *lcc1*, *lcc2*, and *lcc9*

were constitutively expressed in culture, whereas *lcc3* and *lcc5* appeared down and up-regulated, respectively, by PacC. Oxidative stress conditions and phenolic compounds altered the growth rate of the $\Delta lcc3$ mutant compared with the wild-type. *lcc1*, *lcc3*, and *lcc9* were expressed in roots and stems during the infection process. However, inactivation of *lcc1*, *lcc3*, and *lcc5* had no detectable effects on virulence on tomato plants.

Additional keywords: copper enzymes, menadione, phenoloxidases, plant pathogens.

Laccases are widely distributed copper-containing phenol oxidases (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) that belong to a small group of enzymes known as blue copper proteins or blue copper oxidases (49), with characteristic copper atoms in their catalytic center. Members of this group are the mammalian plasma protein ceruloplasmin and ascorbate oxidases from plants. Laccases catalyze the oxidation of phenolic compounds with a concomitant reduction of molecular oxygen to water (31). Classification of laccases by means of their reducing substrate is not feasible due to their very broad substrate range, coincident with different enzymes, such as the monophenol monooxygenase tyrosinase (EC 1.14.18.1) (3). Laccases have received much attention in the last decades due to their ability to oxidize lignin-related compounds, as well as highly recalcitrant environmental pollutants, making them useful in biotechnological processes (40).

Laccases are typically found in plants and fungi. Plant laccases participate in radical-based mechanisms involved in lignin polymer formation (6,18,29,37,47), whereas various functions have been reported for fungal laccases. *Aspergillus nidulans* and *A. fumigatus* laccases are required for pigment biosynthesis during conidial development and maturation (51). Laccases from wood-rotting basidiomycetes, such as *Trametes versicolor*, *Lentinula edodes*, and *Pleurotus ostreatus* are involved in lignin degradation (3,25). There is evidence for their different roles in fungal pathogenesis. In the animal pathogen *Cryptococcus neoformans* laccases are involved in melanin synthesis (55) and thus considered as important virulence factors (56). Significant reduction in both intracellular and extracellular laccase activities has been associated with hypovirulence in virus-infected strains of the chestnut blight fungus *Cryphonectria parasitica* (38,39).

Plant pathogenic fungi produce a number of laccase isozymes encoded by multiple genes. In *Rhizoctonia solani*, a soilborne plant-pathogenic fungus infecting a wide range of crop plants,

four laccase genes have been identified (54). In *Gaeumannomyces graminis* var. *tritici*, an important root pathogen of cereals that causes take-all disease, three laccase genes have been cloned and characterized (28). In *Botrytis cinerea*, a broad-host-range necrotrophic pathogen, two laccase genes have been isolated (44). Three genes have been characterized in *Fusarium proliferatum*, an opportunistic pathogen isolated from wheat (24). During the different stages of the fungal life cycle, gene regulation is subject to different environmental conditions and therefore differential expression of laccases implies alternative functions for these enzymes.

F. oxysporum is a vascular wilt pathogen causing disease among a variety of important crop plants (5,11). The species has also been reported as an emerging opportunistic human pathogen in immuno-compromised patients (53). In the present work we have examined the role of laccases in pathogenesis of *F. oxysporum* f. sp. *lycopersici* in tomato. We report the molecular and functional characterization of putative laccase genes, *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5*, and *lcc9*. As shown by reverse transcription-polymerase chain reaction (RT-PCR) *lcc1*, *lcc3*, and *lcc9* are expressed in roots and stems of *Fusarium*-infected plants. Nevertheless, targeted inactivation of *lcc1*, *lcc3*, and *lcc5* had no detectable effect on virulence toward tomato plants.

MATERIALS AND METHODS

Strains and culture conditions. *F. oxysporum* f. sp. *lycopersici* 4287 (race 2) was originally obtained from J. Tello, Universidad de Almería, Spain. The pathotype of the strain was periodically confirmed by plant infection assays. Mutant strains altered in the PacC transcription factor were originally isolated and described by Caracuel et al. (7). Fungal strains were stored at -80°C with 30% glycerol as a microconidial suspension (10). For extraction of DNA and microconidia production, cultures were grown in potato dextrose broth (PDB, Difco, Detroit, MI) at 28°C with shaking at 170 rpm as described previously (10).

For analysis of gene expression, freshly obtained microconidia from wild-type and mutant strains were inoculated in PDB medium at a final concentration of $5 \times 10^6/\text{ml}$ and germinated for 12 to 14 h at 28°C and 170 rpm. Germlings were washed twice in sterile water and transferred to low carbon (LC) medium con-

Corresponding author: M. I. G. Roncero; E-mail address: ge1gorom@uco.es

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a figure showing the amino acid sequence alignment of *Fusarium oxysporum* deduced laccases with laccases from pathogenic fungi.

taining 11 mM KH₂PO₄, 2 mM MgSO₄·7H₂O, 17 mM NaNO₃, 0.08 mM FeSO₄, 0.06 mM ZnSO₄, 0.06 mM MnSO₄, and 2.5 g of sucrose per liter and incubated 3 days at 28°C with shaking at 170 rpm. Media were buffered with 100 mM Na₂HPO₄ at pH 4, 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, and 50 mM NaCl at pH 6; and 100 mM NaH₂PO₄ and 100 mM NaCl at pH 8.

Nucleic acid manipulations and cloning of the laccase genes.

Total RNA and genomic DNA were extracted following previously reported protocols (9,36). Southern blot analysis and probe labeling were carried out as described (10) using the nonisotopic digoxigenin labeling kit (Roche Diagnostics SL, Barcelona, Spain). The expression analysis was performed by RT-PCR with Superscript II reverse transcriptase (Gibco-BRL, UK). The primer pairs used to amplify the laccase gene transcripts depending on

pH were lcc1-11 and lcc1-12, lcc2-1 and lcc2-12, lcc3-3 and lcc3-4, lcc4-3 and lcc4-4, lcc5-8 and lcc5-9, and lcc9-5 and lcc9-8 (Table 1). PCR conditions were: 40 cycles with denaturation at 94°C for 35 s, annealing at 60°C for 35 s and extension at 72°C for 60 s. An initial denaturation step of 5 min at 94°C and a final elongation step at 72°C for 7 min were performed. The primer pairs used to amplify the laccase gene transcripts during plant infection were lcc1-5 and lcc1-21, lcc2-13 and lcc2-28, lcc3-3 and lcc3-4, lcc4-14 and lcc4-19, lcc5-3 and lcc5-23, and lcc9-5 and lcc9-8 (Table 1). PCR conditions were: 35 cycles with denaturation at 94°C for 35 s, annealing at 62°C for 35 s and extension at 72°C for 40 s. An initial denaturation step of 5 min at 94°C and a final elongation step at 72°C for 7 min were performed. Amplification of the actin gene was used as control, with the primer pair act-1 and act-2, or act-2 and act-Q6. Other routine DNA procedures were performed as described in standard protocols (43).

For cloning of genes *lcc1* and *lcc2*, genomic DNA from *F. oxysporum* isolate 4287 was PCR amplified using the degenerate primers lccFw and lccRv (Table 1) derived from conserved domains of related laccase sequences (GenBank). Sequences of primers lccFw and lccRv are based on the consensus peptides from the copper-binding regions WYHSHYS (II) and WPFHCHI (IV), respectively. Cloning of *lcc3*, *lcc4*, *lcc5*, and *lcc9* genes was achieved using the primers, lcc3-1 and lcc3-2, lcc4-1 and lcc4-2, lcc5-1 and lcc5-2, lcc9-2 and lcc9-3 (Table 1), respectively, derived from the genome sequence of *F. graminearum*, after a BLAST search with the *F. oxysporum* *lcc1* sequence.

The amplified DNA fragments were cloned into the pGEM-T vector (Promega, Corp. Madison, WI) and used to screen a λEMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 4287 (2). Library screening, subcloning, and other routine procedures were performed as described in standard protocols (43). The cDNA clone of *lcc1* gene was obtained from a lambda ZAP cDNA library of *F. oxysporum* strain 4287 (41). Sequencing of both DNA strands from the positive clones was performed using the Dye-deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 Genetic Analyzer apparatus (Applied Biosystems). DNA and protein sequence databases were searched using the BLAST algorithm (1) at the National Center for Biotechnology Information (NCBI) (Bethesda, MD).

Constructions of plasmids vectors and fungal transformation. Gene replacement vectors pDlcc1::hyg, pDlcc3::hyg, and pDlcc5::hyg were constructed by following the general strategy of inserting the hygromycin-resistance (Hyg^R) cassette from plasmid pH1B (52) and thus interrupting the open reading frame (ORF) of the corresponding *lcc* gene. In the case of *lcc3*, the DNA product obtained with primers lcc3-11 and lcc3-15 was cloned into pGEM-T, digested with *Bam*HI to insert the 1.8-kb *Bam*HI fragment containing the hygromycin B resistance cassette from plasmid pH1B, and thus interrupting the ORF. In the *lcc1* and *lcc5* genes, the Hyg^R cassette was inserted into a *Bam*HI sequence, newly created by using the overlapping method (17) with the primers lcc1-14*Bam*HI and lcc1-15*Bam*HI, lcc5-14*Bam*HI, and lcc5-15*Bam*HI, and the amplified final products were cloned into pGEM-T. These constructs were digested with *Bam*HI and the 1.8-kb *Bam*HI fragment containing the Hyg^R cassette was cloned into it, thus disrupting the corresponding *lcc* coding region.

Linear DNA fragments containing the different interrupted *lcc* alleles were generated by amplification of the entire constructs with primer pairs lcc1-7 and lcc1-9, lcc3-11 and lcc3-15, lcc5-6, and lcc5-13 (Table 1), and used to transform protoplasts from *F. oxysporum* f. sp. *lycopersici* strain 4287 following a protocol previously described (10). Hyg^R transformants were isolated as described previously (10).

Transformation vectors harboring the green fluorescent protein (GFP) gene were constructed as follows: the DNA fragment con-

TABLE 1. Oligonucleotides used in this study

Name	Sequence ^a
lccFw	TGGTA(T/C)CA(T/C)AGTCATTATTC
lccRv	ATGTG(A/G)CA(A/G)TG(A/G)AA(C/G)(A/G)GCCA
lcc1-5	AATGGTCATCTTGTAATACAGTT
lcc1-7	AAGTTGCTGCTGGTTTATTTTCGG
lcc1-9	TTATTGACGAGAGTGACGAAG
lcc1-11	TATACCTTGAATCAGACTGGT
lcc1-12	ATGAAGCTCCCTTTACCTCCA
lcc1-14 <i>Bam</i> HI	CAGCAAAATGGATCCGGCTCCTTA
lcc1-15 <i>Bam</i> HI	TAAGGAGCCGGATCCATTTTGTCTG
lcc1-20	GCTTCGCCTTCTACATAC
lcc1-21	GTGTCTAGCTGTACCTGAC
lcc1-21 <i>Xba</i> I	<u>TCTAGAC</u> AGTCCACTGTGATCTGG
lcc2-1	CAAGAGCCAGGTTGTGTCGG
lcc2-12	CCGTCGGAGCAACTCGCT
lcc2-13	GGACACAGGAAAGGTCAATCT
lcc2-27	TTTGATAGATGGAAGTTGTGT
lcc2-28	GTCCTAGTAGCCATAGATT
lcc2-28 <i>Eco</i> RI	<u>GAATTC</u> GATACCCGAGTCATCTGA
lcc3-1	TAAGGTCTGGGGGACAGGACATCAA
lcc3-2	CCTCTACATGAATGGAAGCAGCAT
lcc3-3	GTTTCGGGCTTGACAGACATT
lcc3-4	CGGTGTCACTCAATGTCCAA
lcc3-11	GAAGCGACTGACTATGCGG
lcc3-15	TACTTACTACCTACTTTTCTGG
lcc3-20	GAGGAAGAGGTTACGATTGATA
lcc3-21 <i>Sac</i> II	<u>CCGCGG</u> GTGACTTCTCGCATCACCG
lcc4-1	GCAGTTGACACGCATTGGAAGTTC
lcc4-2	GATTGTCTGTTTCGTGTCACAGT
lcc4-3	TCGCAGCAGACCTTGATCC
lcc4-4	GTCAGTGTAGTCATAGGCAT
lcc4-14	TAATTATGCCTCCAGTTTCAGTC
lcc4-19	CTCTACCAGTGCCCCATC
lcc5-1	GAAATGGGATATGACTCTTGCTGCT
lcc5-2	CTGGGAGGAGGGCTGATTTCGTT
lcc5-3	ATGCTATGATTCAGTCCCTTTT
lcc5-6	GGCAATCAAACCCGCTTATCT
lcc5-8	CCTCTTGTCGTAACCGAAG
lcc5-9	GTTTCGTAATGGGCAGTCTC
lcc5-13	GATGGTGTCAGTTTGTGTCAGC
lcc5-14 <i>Bam</i> HI	TGCTATGATTGGATCCCTTTAATGG
lcc5-15 <i>Bam</i> HI	CCATTAAGAGGATCCCAATCATAGCA
lcc5-23	GCCGTTGACAAAGTTGACTGC
lcc9-2	GAGATCCATGTCGTCGTAAGGGTC
lcc9-3	TTACCTATCTGTTGGTCAACGATGG
lcc9-5	TGATGCAACCAAGGACGAC
lcc9-8	TGAGGATTCGTCGCTTGCTTT
gfp-3 <i>Eco</i> RI	<u>GAATTC</u> GTGAGCAAGGGCGAGGAGC
gfp-7 <i>Xba</i> I	<u>TCTAGAC</u> TGAGCAAGGGCGAGGAGC
gfp-11 <i>Sac</i> II	<u>CCGCGG</u> GTGAGCAAGGGCGAGGAGC
trp-2 <i>Kpn</i> I	GATTACCTCTAAACAAGGGTACC
act-1	ATGACCCAGATCGTCTTCGAG
act-2	GAGGGACCGCCTCGTCGT
act-Q6	GGAGATCCAGACTGCCGCTCAG
λ-int	CGCAACTCGTGAAAGGTA
λ-git	AAGTCCAACCCAGATAACGAT

^a Restriction sites introduced in the original sequences are underlined. Nucleotides in parentheses represent degeneracy for the same position.

taining the GFP ORF without start codon and with the *trpC* terminator, from plasmid ppgdAsGFP (14), was amplified using the primers GFP-3EcoRI, GFP-7XbaI or GFP-11SacII, and TRP-2KpnI, introducing an EcoRI, XbaI, or SacII site at the 3' end, respectively. The resulting fragments were cloned into pGEM-T in such a manner that the new restriction sites were located adjacent to the *ApaI* site of pGEM-T, thus enabling the release by digestion with the appropriate enzyme EcoRI, XbaI or SacII, and *NotI*.

The *lcc1*, *lcc2*, and *lcc3* promoter regions and ORFs without stop codons were amplified from genomic DNA using the primer pairs *lcc1*-20 and *lcc1*-21XbaI, *lcc2*-27 and *lcc2*-28EcoRI, and *lcc3*-20 and *lcc3*-21SacII, respectively, introducing the indicated restriction sites at the 5' ends of the corresponding genes. Each amplified DNA fragment was cloned into pGEM-T so that the new restriction sites were adjacent to the *NotI* site present in pGEM-T. These vectors were linearized by digestion with EcoRI, XbaI or SacII, and *ApaI*, and ligated with the appropriated fragments from the above mentioned GFP constructs.

Plate assay for extracellular laccase production. Freshly obtained microconidia (10^4 /ml) were inoculated into agar plates of synthetic medium (SM) (10) containing 5 or 0.5 g of sucrose per liter, glycerol, polygalacturonic acid, or pectin from citrus fruits (both from Sigma) as the carbon source, and incubated at 28°C for 2 to 3 days as described previously (10). After this period, colonies were transferred, by using the broad-end of a 1-ml pipette tip, to wells made into plates supplemented with 2 mM 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma), and incubated at 28°C for 2 to 3 days, by a modification of the previously described procedure (46). The development of an intense bluish green color surrounding the colonies was considered a positive result.

Determination of the sensitivity of laccase mutants to oxidative stress. For determination of menadione sensitivity, a 5- μ l drop containing from 5×10^3 to 5×10^4 freshly obtained microconidia was transferred to 15 g/liter SM agar plates (10) containing 10 g of sucrose per liter as the carbon source and 12.5 mM NH_4NO_3 as the nitrogen source, and supplemented with 10 μ g/ml of menadione (Sigma), dissolved in absolute ethanol, after autoclaving. Plates were incubated at 28°C for 2 days as described previously (10).

Determination of the sensitivity of mutants to phenolic compounds. For determination of phenolic compound sensitivity in wild-type and mutants strains, freshly obtained microconidia (10^4 /ml) were inoculated into 96-well plates containing PDB supplemented with different phenolic compounds. The appropriate phenolic compound concentrations, initially determined by using a concentration range from 0 to 100 mM, was that resulting in about 50% survival with the wild-type strain, monitoring the growth over 3 days. Five phenolic compounds were assayed: chlorogenic acid (5 mM), vanillic acid (0.25 mM), *p*-coumaric acid (2.5 mM), caffeic acid (5 mM), and L-tyrosine (50 mM). Plates were incubated at 28°C and growth density was determined during 2 to 3 days at 2 to 12 h intervals by absorbance at 600 nm using a SpectroFluor Plus (Tecan Trading Ag, Switzerland).

Virulence assays and RT-PCR from infected tomato plant tissues. Tomato seeds, sterilized as previously described (19),

were used for plant inoculation assays (10). Two-week-old tomato seedlings (cv. Vemar) were inoculated with *F. oxysporum* f. sp. *lycopersici* strains by immersing the roots in a microconidial suspension, planted in vermiculite and maintained in a growth chamber. At different times after inoculation, severity of disease symptoms was recorded using an index ranging from 1 (healthy plant) to 5 (dead plant). Twenty plants were used for each treatment. Assays for invasive growth on tomato fruits (cv. Daniela) were carried out as described (10).

RT-PCR experiments were performed essentially as described (10). Fifteen tomato plants from each treatment were removed at 2 and 5 days after inoculation, and five at 8 and 12 days. Total RNA was isolated from roots and stems and subject to RT-PCR with gene-specific primers flanking introns. The primers used to amplify the transcript of the actin gene were act2 and actQ6, and those for laccase genes were *lcc1*-5, *lcc1*-21, *lcc2*-13, *lcc2*-28, *lcc3*-3, *lcc3*-4, *lcc4*-14, *lcc4*-19, *lcc5*-3, *lcc5*-23, *lcc9*-5, and *lcc9*-8 (Table 1).

RESULTS

Isolation and characterization of laccase encoding genes. Genomic DNA from the wild-type strain 4287 of *F. oxysporum* f. sp. *lycopersici* was used for PCR amplification using primers based on conserved fungal laccase domains, as described in previously. The PCR amplified fragments obtained with each degenerate primer pair were cloned into pGEM-T and the identity of the cloned inserts was confirmed by sequencing, before being used to probe a λ EMBL3 genomic library of *F. oxysporum* isolate 4287 (2). The three positive clones obtained, one for *lcc1* and two for *lcc2*, were subject to PCR amplification using the flanking λ EMBL3 primers, λ -int and λ -git (Table 1), thus amplifying the entire inserts from the genomic clones. These *F. oxysporum* inserts were sequenced using the strategy of DNA-walking with specific synthetic oligonucleotides. The gene sequences, named *lcc1* and *lcc2*, have been deposited as GenBank accession nos. EF990894 and EF990895, respectively.

Isolation of additional laccase genes, designated *lcc3*, *lcc4*, *lcc5*, and *lcc9* accordingly to their isolation order, was accomplished by amplification of *F. oxysporum* genomic DNA with specific primer pairs (*lcc3*-1 and *lcc3*-2, *lcc4*-1 and *lcc4*-2, *lcc5*-1 and *lcc5*-2, and *lcc9*-2 and *lcc9*-3) based on the coding sequence of *F. graminearum* genes with identity to fungal laccases. The PCR products obtained were cloned and, after confirming their identity by sequencing, used to probe an *F. oxysporum* genomic library (2). The positive clones were amplified and sequenced using specific synthetic oligonucleotides. The gene sequences have been deposited as GenBank accession nos. EF990899, EF990896, EF990897, and EF990898, respectively.

The positions of introns in the laccase genes were initially determined by comparison with other related laccase polypeptides. In the *lcc1* gene, intron position, as well as the transcriptional 5' terminus and the poly (A) signal site, were confirmed by sequencing of the cDNA clone isolated from an *F. oxysporum* cDNA library (41). Table 2 summarizes the structural features of the laccase genes isolated in this work and their deduced products

TABLE 2. Structures of laccase gene open reading frames (ORF) and analysis of the predicted laccase proteins from *Fusarium oxysporum*

	Gene		Deduced protein			
	ORF (bp)	Introns (bp)	Number of amino acids	kDa	pI	Location ^a
<i>lcc1</i>	1,755	51, 60, 92	585	64.8	6.8	I
<i>lcc2</i>	1,920	53, 50, 59, 51, 48, 53	640	72.5	6.4	I
<i>lcc3</i>	1,869	53, 52, 53, 56	623	68.6	5.0	C
<i>lcc4</i>	1,698	49, 54	566	62.4	4.0	E
<i>lcc5</i>	1,890	32, 100	630	69.8	5.6	E
<i>lcc9</i>	1,638	42, 43, 40	546	61.2	4.8	E

^a I, intracellular; C, cell or membrane bound; E, extracellular.

analyzed by using the Lasergene Navigator and the BLAST algorithm (1) at the NCBI. The subcellular locations of predicted proteins were determined by the algorithms PSORT, available at wolfsort.org, and SOSUI, available online by Mitaku Group, Department of Applied Physics, Nagoya University.

F. oxysporum laccase genes encoded predicted proteins ranging from 547 to 639 amino acids, with a calculated molecular mass from 65 to 70 kDa, and pIs from 4.0 to 7.0 (Table 2). All deduced gene products contained the four conserved putative Cu-binding sites, characteristic of these proteins. The degree of identity among deduced laccases from *F. oxysporum* ranged from 61.2% (Lcc3 and Lcc9) to 16% (Lcc5 and Lcc9). Lcc4 had the highest identities (40 to 50%) with laccases from the tomato pathogen *B. cinerea* (44) and the cereal pathogen *G. graminis* (28).

Promoter regions were scanned manually and by using the MatInspector algorithm for putative transcription factor binding sites indicated by the position of different regulatory motifs (Table 3). These include metal-responsive elements (MRE) (13), stress-responsive elements (STRE) (45), CreA-binding elements (CreA) (22), and xenobiotic-responsive elements (XRE) (27,42).

Expression of laccase genes depends on pH. Gene expression by pH is regulated in fungi via a conserved signaling cascade whose terminal component is the zinc finger transcription factor PacC/Rimp1p (7,26,35,50). In *F. oxysporum*, transcription of *pacC* increases in alkaline conditions but is almost undetectable at acidic growth conditions (7). Two types of *pacC* mutants were previously isolated and characterized by Caracul et al. (7); the $\Delta pacC$ loss of function mutant displayed an acidity-mimicking phenotype resulting in poor growth at alkaline pH and high transcript levels for acid-expressed genes. Conversely, the *pacC^C* mutant, carrying a dominant activating *pacC^C* allele, showed increased *pacC* transcription and protein levels and displayed an alkalinity-mimicking phenotype with constitutive expression of alkaline-expressed genes.

In the present work, an RT-PCR approach was used to characterize pH-dependent transcription of six laccase genes from *F. oxysporum*. Total RNA was purified from wild-type and *pacC* mutant strains grown for 3 days in low carbon (2.5 g of sucrose per liter) minimal medium at different pH values. For PCR amplification of each transcript, a pair of specific primers flanking an intron was used (described above). As shown in Figure 1, expression of *lcc4* was never detected and no differences were observed in transcript accumulation of *lcc1*, *lcc2*, and *lcc9* between the *pacC* mutants and the wild-type strain. However, transcript accumulation of *lcc3* increased at pH 4 and 6 in the wild-type and in the $\Delta pacC$ mutant, whereas no expression was detected in the

pacC^C mutant. In contrast, transcript accumulation of *lcc5* was induced in the wild-type and the *pacC^C* mutant strains at pH 6, but was absent in the $\Delta pacC$ mutant. Taken together these results indicate that the *PacC* transcription factor down-regulates *lcc3* gene expression, and up-regulates *lcc5* gene expression.

In vivo analysis of gene expression during plant infection.

To test whether laccase genes are expressed during infection of tomato plants by *F. oxysporum* f. sp. *lycopersici*, RT-PCR with gene-specific primers flanking an intron was used to detect the presence of laccase transcripts in roots and stems of tomato plants at different time points after inoculation (Fig. 2). Gene-specific transcripts were never observed in the noninoculated control plants. Transcription of the *F. oxysporum* actin gene was detected in roots and stems after inoculation (Fig. 2). Expression of *lcc2*, *lcc4*, and *lcc5* gene was never observed during infection, whereas *lcc1*, *lcc3*, and *lcc9* transcripts were detected in roots and stems of infected tomato plants. In the case of the *lcc1* gene, expression was observed in roots and stems 5 to 12 days after infection. Transcription of *lcc3* and *lcc9* was detected in roots and stems 8 and 12 days after infection, indicating a differential expression pattern of laccase genes during the infection process and independent of the plant tissue.

Targeted disruption of laccase genes. Loss-of-function mutants carrying a disrupted laccase allele were generated as described above. The hygromycin resistance cassette was inserted into an existing *Bam*HI site of the coding region of the *lcc3* gene (Fig. 3B), or into a newly created *Bam*HI site in the coding region of *lcc1* and *lcc5* genes (Fig. 3A and B). The knockout constructs were introduced into the wild-type strain by DNA transformation and *Hyg^R* selection allowed the isolation of transformants harboring the disrupted *lcc1*, *lcc3*, or *lcc5* alleles. A number of these *Hyg^R* transformants were analyzed by restriction enzyme diges-

TABLE 3. Summary of relevant response elements present in the promoter regions of *F. oxysporum* laccase genes.

Genes	Position of response elements ^a				
	MRE	XRE	ACE1	STRE	PacC
<i>lcc1</i>	-225 -413 -83	-875	-725 -59	-106 -180	-101 -373
<i>lcc2</i>	-466 -582		-300	-400	
<i>lcc3</i>	-323			-95 -882	-1091 -1100
<i>lcc4</i>	-792 -1199	-1081		-119 -229	-769 -1288
<i>lcc5</i>				-1211	-1365
<i>lcc9</i>	-1077 -1094			-992	-240

^a The location of each element upstream from the start codon is indicated. MRE (TGC(G/A)CNC) metal responsive elements; XRE (TCACGC) xenobiotic responsive elements; ACE1 copper-responsive transcription factor (TCT₄₋₆GCTG); STRE (CCCCT) stress responsive elements; PacC (GCCA(A/G)G) ambient pH response factor PacC.

Fig. 1. Expression analysis of laccase genes depending on ambient pH. Total RNA from wild type and *pacC^C* and $\Delta pacC$ mutants grown at pH 4, 6, or 8 as indicated were reverse transcription-polymerase chain reaction amplified using the specific primers described in text. *Fusarium oxysporum* genomic DNA (gDNA) was used as a template for amplification control. Actin gene amplification, with primer pair act-1 and act-2, was used as RNA quantity control.

tion of genomic DNA and Southern hybridization. One, four, and six transformants with a homologous gene replacement were obtained for *lcc1*, *lcc3*, and *lcc5*, respectively (Fig. 3B, D, and F). RT-PCR analyses confirmed the absence of *lcc1*, *lcc3*, and *lcc5* transcripts in the corresponding null-mutants (data not shown) which were further characterized. In the case of genes *lcc2*, *lcc4*, and *lcc9*, no homologous recombination transformants were identified, although more than 100 *Hyg^R* colonies were obtained from each disruption construct.

Influence of carbon source on laccase activity. The wild-type strain grown on different carbon sources showed the highest laccase activity when grown on a carbon-limited medium. The results of the plate assays indicated that laccase activity increased on low concentrations of sucrose, glycerol, or polygalacturonic acid containing plates (Fig. 4). Nevertheless, the highest levels were detected when *F. oxysporum* was grown on a medium with a high pectin concentration. Laccase gene deficient mutants (*lcc1*, *lcc3*, and *lcc5*) produced halos of considerable less intensity than the wild-type strain, in all assayed conditions, indicating a decrease in laccase activity (data not shown).

Sensitivity of laccase mutants to oxidative stress. The oxidative burst is one of the earliest plant responses to the attack of pathogenic fungi (30). Therefore, the defense mechanism against oxidants produced within the host environment might constitute an important virulence factor for plant-infecting microbes. The intermediary products of the activity of laccases are free radicals, able to interact with a variety of other compounds. In order to determine the effect of laccase gene inactivation on the response to oxidative stress, colony growth of specific laccase knockout mutants, in comparison to the wild-type strain, was assayed on minimal medium plates containing menadione at 10 μ g/ml, a redox-cycling agent (20). The growth of Δ *lcc1* and Δ *lcc3* deficient mutants in the presence of the oxidant was severely impaired, whereas Δ *lcc5* showed no difference in growth rate compared with the wild-type strain (Fig. 5). These results suggest that *lcc1* and *lcc3* proteins may play a role in defense against the oxidants produced by the host plant. On the other hand, *PacC^C* and Δ *PacC* mutants showed a marked reduction in growth rate in the presence of menadione, suggesting an essential role of this transcription factor under stress conditions.

Sensitivity of laccase mutants to plant-related phenolic compounds. Plants produce a diverse array of secondary metabolites with antifungal activity. Some of these are part of the pathogen-induced response (phytoalexins) or are produced under stress conditions, such as phenolic compounds (33,34). The pathway for phenolic acid metabolism comprises the initial steps of general phenylpropanoid metabolism produced from cinnamate, such as *p*-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins (48). The free acids rarely accumulate to high levels within plant cells; usually, they are conjugated to sugars, cell wall carbohydrates, or organic acid compounds.

The defense mechanism of *F. oxysporum* against plant phenolic compounds may be important for survival within the host cells. Thus, the growth of the wild-type strain and different laccase deficient mutants in the presence of phenolic compounds was determined.

As shown in Figure 6, the Δ *pacC* mutant was more sensitive to L-tyrosine, an aromatic amino acid precursor for plant defense compounds (8), whereas the dominant active *pacC^C* was more resistant in comparison to the wild-type strain, suggesting the regulation of tyrosinase activity by the transcription factor *pacC* in *F. oxysporum*. On the other hand, all the laccase mutants showed similar sensitivity to L-tyrosine and to *p*-coumaric acid, another non-nitrogenated monophenol, as the wild-type strain.

On the other hand, *pacC^C* and Δ *lcc3* were more sensitive to chlorogenic acid and its precursor, caffeic acid, compared with Δ *pacC* and the wild-type strain. Chlorogenic acid is the major soluble phenolic in Solanaceous species, such as potato and

tomato (32), and may act directly as a defense compound, or alternatively serves as a precursor in the synthesis of lignin, suberin, and other polyphenolic barriers. Vanillic acid, a precursor of lignin synthesis, showed a growth-inhibitory effect in all laccase or *pacC* mutants, in comparison to the wild-type strain.

Pathogenicity of laccase mutant strains. The effect of laccase gene mutations on virulence of *F. oxysporum* was determined by root infection assays of tomato plants. Two-week-old plants were inoculated by immersing the roots in a microconidial suspension of the wild-type strain, Δ *lcc1*, Δ *lcc3*, or Δ *lcc5* mutants. Plants were scored for vascular wilt symptoms at different time intervals. Plants inoculated with the wild-type strain produced characteristic wilt symptoms starting 7 days after inoculation. Disease severity increased steadily throughout the experiment, and most of the plants were dead 15 days after inoculation. Plants inoculated with laccase deficient mutants showed similar symptoms to those inoculated with the wild-type strain. Virulence experiments were performed three times with similar results.

Intracellular localization of GFP-tagged laccases from *F. oxysporum*. Analysis of the predicted amino acid sequence of laccase proteins suggested an intracellular location for Lcc1 and Lcc2, whereas Lcc3 was predicted to be a transmembrane (TM) protein with one TM domain, a large (>500 amino acids) N-terminal intracellular domain and a short (50 amino acids) extracellular C-terminal domain. In contrast, Lcc4, Lcc5, and Lcc9 showed predicted extracellular locations.

Fig. 2. Expression analysis of laccase genes during plant infection. Total RNA from roots and stems of infected plants at the indicated days after inoculation was reverse transcription-polymerase chain reaction (PCR) amplified using the specific primers described in text. PCR amplification of *Fusarium oxysporum* genomic DNA (gDNA) was used as an amplification control. Actin gene amplification, with act-2 and act-Q6, was used for quantity control.

In order to demonstrate these cellular locations, translational fusions of Lcc1, Lcc2, and Lcc3 laccases with GFP were constructed. The GFP coding region was fused in frame to the 3' end of the coding regions of each laccase gene. The constructs included an untranslated 5' region in order to maintain gene regulation under the respective laccase gene promoters (20). Lcc1-GFP, Lcc2-GFP, and Lcc3-GFP constructs were used for co-transformation of wild-type strain protoplasts with plasmid pAN8-1 which confers phleomycin resistance. Figure 7 shows fluorescence microscopy images of three representative transformants, harboring single integration events for each of the constructs. Confirming its previously predicted location, Lcc1-GFP

fusions appeared cytosolic, whereas Lcc2-GFP seemed to accumulate in small cytoplasmic vesicles and Lcc3-GFP was observed in cell walls and septa.

DISCUSSION

Although the biological functions of several fungal laccases have been studied, the functions of the majority of laccases remain uncharacterized. Laccase can protect fungal pathogens from toxic phytoalexins and tannins existing in the host environment (30). In the human pathogen *C. neoformans*, laccase and the related product melanin are as essential virulence factors (55). In

Fig. 3. Targeted replacement of *Fusarium oxysporum* laccase genes. Physical maps of genes **A**, *lcc1*, **C**, *lcc3*, and **E**, *lcc5* and their replacement vectors **A**, pDlcc1, **C**, pDlcc3, and **E**, pDlcc5. The coding regions are shown in gray indicating the orientation of the ORFs and the introns. Small arrows represent the position of the primers used for amplification of the gene replacement cassettes. Southern analysis of disrupted transformants **B**, *Δlcc1*, **D**, *Δlcc3*, and **F**, *Δlcc5* and the wild-type strain. Genomic DNAs were digested with *EcoRI*, *SacI*, or *HindIII*, as indicated, and hybridized with the gene probe indicated. Band sizes are in kilobases.

B. cinerea, laccase secretion protects the fungus from toxic metabolites produced by cucumber fruits, and reduces lignification activities by the host. On the other hand, it has been shown that resveratrol, a phytoalexin produced by grapevine, acts as a fungicide that is converted by *B. cinerea* laccase into a compound that is toxic to the fungus (44).

Six laccase genes from the plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici* were identified and characterized in this work. Their deduced protein sequences show high identities between *lcc3* and *lcc9* (64%) and lower identities (22 to 33.5%) between the rest. One explanation for redundancy in laccase genes would be their different roles and regulatory mechanisms depending on environmental conditions, pH, biotic and abiotic stresses, nutrition, and developmental phase. The promoters of the six laccase genes contained putative metal responsive elements like those found in the promoters of metallothionein genes (21) induced by a range of heavy metals. On the other hand, many identified fungal laccase genes are induced by metals, especially by copper (4,16).

In plant pathogens, ambient pH may affect the expression of putative virulence factors. *PacC* is a key component acting both as a transcriptional activator of alkaline-expressed genes and repressing acid-induced genes (7,12,50). Caracul et al. (7) propose that *PacC* functions as a negative regulator of virulence genes in *F. oxysporum*, most probably by preventing the appropriate expression of acidic genes important for infection because

the pH of the tomato plant is in the acidic range (5.5 to 6.0). The promoter regions of *F. oxysporum lcc3* and *lcc5* genes contain three *PacC* binding consensus sites, whereas the rest of laccase genes contain two, one, or none. Indeed, transcription of *lcc3* and *lcc5* was pH dependent and regulated by *PacC*. Expression of *lcc3* occurs in acidic conditions and is down regulated by *PacC*, while expression of *lcc5* occurs only at pH 6 and is up regulated by *PacC*. Our results suggest that *lcc5* expression at neutral pH is mediated by *PacC* together with other signaling mechanisms that suppress expression at acidic and alkaline pH. Conversely, *lcc1*, *lcc2*, and *lcc9* were constitutively transcribed in all the in vitro conditions tested, whereas the *lcc4* transcript was never detected.

Lcc3 may be considered as a potential virulence factor negatively regulated by *PacC* and expressed in roots and stems during infection of tomato plants. Transcription of genes *lcc2*, *lcc4*, and *lcc5* was not detected in the in planta assays, indicating a non-essential role during the pathogenic process. Both *lcc1* and *lcc9* were expressed during infection of tomato plants, although expression seemed independent of pH.

In order to assess their role in *F. oxysporum* pathogenicity we disrupted some of the laccase encoding genes. $\Delta lcc1$, $\Delta lcc3$, and $\Delta lcc5$ mutants exhibited the same degree of virulence as the wild-type strain, suggesting that these laccases are not essential for pathogenicity. Since *F. oxysporum* f. sp. *lycopersici* produces a number of laccases, it cannot be excluded that the loss of one particular isozyme could be compensated by others, even if they displayed different modes of action. Moreover, BLAST analysis of the genome database of *F. oxysporum* with laccase protein sequences deduced in this work and laccase proteins from related organisms identified 15 different potential laccases. Six of the sequences matched with the cloned genes *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5*, and *lcc9*, characterized in this work. The deduced amino acid sequences of these putative laccases have the characteristic fungal laccase signature sequences L1 to L4 (23) with identities ranging from 23 to 33% with *lcc1*, and 37 to 54% with *lcc2*.

The highest laccase induction was observed in liquid cultures containing increasing pectin concentrations, indicating that pectin, a major component of plant cell walls, and its derived break-

Fig. 4. Influence of carbon source and concentration on laccase production. Three-day-old colonies of wild-type *Fusarium oxysporum* f. sp. *lycopersici* grown on minimal medium plates containing the indicated carbon source and concentration were transferred to identical plates but supplemented with 2 mM 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS). An intense green color surrounding the colonies developed after incubation at 28°C for 2 days, indicating laccase activity. PGA: polygalacturonic acid.

Fig. 5. Effect of menadione on colony growth of laccase mutants and the wild-type strain. Serial dilutions of microconidial suspensions of the indicated strains were spotted onto minimal medium agar plates containing 10 g of sucrose per liter, with or without menadione at 10 μg/ml and incubated for 3 days at 28°C.

Fig. 6. Sensitivity of laccase mutants to phenolic compounds. Growth was determined by measuring the OD₆₀₀ value at indicated time points. (□) wild type strain, (▲) $\Delta lcc3$ mutant, (+) $\Delta pacC$ mutant, (○) $pacC^C$ mutant. Error bars indicate standard deviations calculated from three independent experiments.

down products are inducers of laccase production in the tomato pathogen *F. oxysporum*.

A large number of plant antifungal secondary metabolites are phenolic compounds whose detoxification facilitates host colonization by fungi. The increased sensitivity to plant phenolic compounds shown by some laccase mutants suggests a role for these enzymes during the infection process. Lcc3 protein appeared to be localized in the cell wall, while the other laccases tested appeared to be cytoplasmic or extracellular. This difference may account for the higher sensitivity of $\Delta lcc3$ mutants to phenolic

compounds and to menadione, an oxidative stress-inducer, due to a higher accessibility of the cell wall-localized enzyme.

During host colonization, fungal pathogens usually encounter the plant defense response involving the generation of active oxygen species and the death of host cells surrounding the infection site (30). In order to protect themselves and to exploit this hostile environment, fungi produce a variety of enzymes useful for infection of hosts. Intermediary reaction products of laccase activity are free radicals, which may easily interact with other generated radicals (15). As previously mentioned, $\Delta lcc3$ and

Fig. 7. Localization of laccases by translational fusion with the green fluorescent protein (GFP) gene. Fluorescence microscopy observation of germings from the indicated *Fusarium oxysporum* transformants grown for 14 h in potato dextrose broth. Arrows indicate the fluorescence accumulation in septa from the *Lcc3::GFP* transformant.

Δlcc1 mutants were more sensitive to oxidative stress than the wild-type strain, suggesting that these enzymes may play a protective role against active oxygen species.

These results, together with the location of laccase proteins, *Lcc1* in the cytoplasm and *Lcc3* in the membrane or cell wall bound, suggest that these two proteins are more exposed to oxidative stress and toxic compounds, and therefore they are presumed to be involved in fungal protection. In contrast, *Lcc5* is a predicted extracellular protein presumably less implicated in protection against plant defense mechanisms.

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