Influence of the chloride channel of *Fusarium oxysporum* on extracellular laccase activity and virulence on tomato plants

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CLC-type voltage-gated chloride channels are a family of proteins which mediate chloride transport across the plasma and intracellular membranes. A *clc1* gene from the vascular wilt fungus *Fusarium oxysporum* was characterized and disrupted. The predicted Clc1 protein contained highly conserved transmembrane and CBS domains of this protein family and showed significant identities to the *Saccharomyces cerevisiae* GEF1 and the *Cryptococcus neoformans* CLC-A chloride channels. Inactivation of *clc1* caused a deficiency in laccase activity which was more severe than that found in any of the structural laccase mutants previously described. The addition of copper sulphate to the growth medium resulted in total recovery of extracellular laccase activity in $\Delta clc1$ mutants, although it did not activate transcription of any laccase genes. The pleiotropic phenotype displayed by the *Fusarium* chloride channel-deficient mutants included a significant delay in the development of disease on tomato plants, with a higher sensitivity to oxidative stress compounds as well as a significant decrease in laccase activity, thus suggesting a possible connection between virulence and the two processes. Nevertheless, we cannot rule out that additional phenotypes present in the $\Delta clc1$ mutants could play an essential role in the full virulence of *Fusarium*.

Received 26 November 2007

Revised 14 February 2008

Accepted 15 February 2008

INTRODUCTION

Chloride channels belonging to several protein families are widely distributed among prokaryotic and eukaryotic organisms (Jentsch *et al.*, 1999, 2002). Their physiological functions in higher organisms include the regulation of cell volume, the control of electrical excitability and transepithelial transport (Schmidt-Rose & Jentsch, 1997). Voltage-gated chloride channels belonging to the CLC family have been highly conserved during evolution and are widely represented in organisms ranging from bacteria and yeasts to plants and animals. CLC proteins are structurally unrelated to other classes of ion channels. Topological models that are mainly based on hydropathy analysis suggest the presence of about 12–13 transmembrane domains, labelled D1 to D13 (Jentsch *et al.*, 1990).

The mammalian CLC family contains nine genes originally identified by the functional expression of the *Torpedo* chloride channel CLC-0 in *Xenopus* oocytes (Jentsch *et al.*, 1990). Although the biological role of many CLC channels

remains unknown, others have been well characterized. For example, mutations in some CLC channels are associated with genetic diseases such as myotonia for CLC-1, Dent disease for CLC-5 or osteoporosis for CLC-7 (Jentsch *et al.*, 1999). Their expression is tissue-specific as in the case of CLC-1 and CLC-5, or broadly represented as in CLC-2. These channels are present in the plasma membrane (CLC-1), in intracellular organelles (CLC-5, CLC-7), or they have more than one localization (CLC-2) (Jentsch *et al.*, 1999, 2005).

GEF1 is the only gene encoding a CLC chloride channel that has been identified in yeast (Greene *et al.*, 1993). The deduced protein, which is localized in the medial Golgi compartment (Schwappach *et al.*, 1998), plays a role in vesicular cation homeostasis (Gaxiola *et al.*, 1998). *gef1* mutants exhibit a defect in iron metabolism with a phenotype of poor growth on low-iron media in the presence of a non-fermentable carbon source.

Yeasts have both low-affinity and high-affinity iron-uptake systems. Fet3p oxidase, a key component of the high-affinity uptake system, is a multicopper-containing glycoprotein that requires the post-translational insertion of four copper ions for its activity (Taylor *et al.*, 2005), thus closely relating copper and iron homeostasis. Copper loading on Fet3p occurs in post-Golgi vesicles mediated

Abbreviation: BCS, bathocuproinedisulphonic acid.

The GenBank/EMBL/DDBJ accession number for the gene sequence *clc1* is EU030436.

A supplementary sequence alignment is available with the online version of this paper.

by the action of a P-type ATPase, Ccc2p. Gef1p is localized in the same Golgi compartment as Ccc2p (Gaxiola *et al.*, 1998). Gaxiola *et al.* (1998) reported that impaired Fet3p activity in the *gef1* mutants was due to a defect in vesicular copper homeostasis. In the absence of a counter-ion such as Cl^- , cation transport results in an unfavourable electrochemical potential that may affect the copper loading of apoFet3p by limiting the H^+ or Cu^+ transport.

In *Cryptococcus neoformans*, an opportunistic fungal pathogen for immunocompromised individuals, laccase is an important cell-wall-associated virulence factor (Zhu *et al.*, 2001; Williamson, 1994). A cryptococcal mutant defective in *vph1*, a gene encoding a vacuolar proton pump H^+ -ATPase that maintains the low pH in this organelle, was affected in both laccase activity and virulence, although laccase transcription appeared to be preserved (Zhu *et al.*, 2003). The defect in vesicular acidification led to ineffective metalation of the laccase enzyme that could be restored by the addition of exogenous copper. Nevertheless cryptococcal $\Delta\text{clc-a}$ mutants were defective in laccase activity and laccase mRNA was undetectable (Zhu & Williamson, 2003). Again, the addition of copper to the media restored both laccase transcription and activity.

Fusarium oxysporum is a soilborne fungus that causes large economic losses in a wide variety of crops and has also been reported as an opportunistic human pathogen (Di Pietro *et al.*, 2003). In order to understand the interaction between *F. oxysporum* f. sp. *lycopersici* and the tomato plant, the potential role of laccase production by this plant pathogen was examined (Cordoba Cañero & Roncero, 2008). In the present paper, we report the isolation and molecular characterization of the *clc1* gene encoding a chloride channel in *F. oxysporum*. Mutants lacking a functional copy of the gene showed a significant decrease in virulence, as well as a defect in laccase activity, which was restored by the addition of copper. In contrast to the corresponding cryptococcal mutants, the transcription of laccase genes was unaffected in Δclc1 mutants of *F. oxysporum*.

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. Fungal strains were stored at -80°C with 30 % glycerol as a microconidial suspension (Di Pietro & Roncero, 1998). For the extraction of DNA and microconidia production, cultures were grown in potato dextrose broth (PDB, Difco) at 28°C with shaking at 170 r.p.m. as described previously (Di Pietro & Roncero, 1998).

The *F. oxysporum* Δlcc strains were obtained by targeted inactivation mediated by DNA transformation of the wild-type strain with gene replacement vectors containing the hygromycin resistance (Hyg^R) cassette and interrupting the ORF of the corresponding *lcc* gene. The deficient mutant strains were described previously (Cordoba Cañero & Roncero, 2008).

To analyse gene expression, freshly obtained microconidia from the wild-type and mutant strains were inoculated into PDB medium at a

final concentration of 5×10^6 microconidia ml^{-1} and germinated for 12–14 h at 28°C and 170 r.p.m. The germings were then washed twice in sterile water and transferred to a synthetic liquid medium containing 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.9 mM KH_2PO_4 , 2.7 mM KCl, 12.5 mM NH_4NO_3 , 0.08 mM FeSO_4 , 0.06 mM ZnSO_4 , 0.06 mM MnSO_4 and 10 g sucrose l^{-1} as the carbon source. The media were buffered with 100 mM Na_2HPO_4 at pH 4; 50 mM Na_2HPO_4 , 50 mM NaH_2PO_4 and 50 mM NaCl at pH 6; and 100 mM NaH_2PO_4 and 100 mM NaCl at pH 8.

Nucleic acid manipulations and cloning of the *clc1* gene. Total RNA and genomic DNA were extracted following previously reported protocols (Chomczynski & Sacchi, 1987; Raeder & Broda, 1985). Southern blot analysis and probe labelling were carried out as described by Di Pietro & Roncero (1998) using the non-isotopic digoxigenin labelling kit (Roche Diagnostics). Other routine DNA procedures were performed according to standard protocols (Sambrook & Russell, 2001). Cloning of the *clc1* gene was achieved using the primers *clc1*-1 and *clc1*-2 (Table 1) derived from the genome sequence of *Fusarium graminearum*, available at the website http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html, after a BLAST search with the *C. neoformans* *CLC-A* gene.

Library screening, subcloning and other routine procedures were performed as described in standard protocols (Sambrook & Russell, 2001). Both the DNA strands of the positive clones were sequenced using the Dyedexy Terminator Cycle Sequencing kit (Applied Biosystems) on an ABI Prism 377 Genetic Analyzer apparatus (Applied Biosystems). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990, 1997) at the National Center for Biotechnology Information (NCBI).

Construction of plasmid vectors and fungal transformation.

The gene replacement vector pDcl1::hyg was constructed by following the general strategy of inserting the Hyg^R cassette from

Table 1. Oligonucleotides used in this study

Name	Sequence* (5'–3')
clc1-7	aagagtgtgatgagtcgcca
clc1-8	aggaggagattgccgagatt
clc1-10	aatcccgaacaccagcgat
clc1-12BamHI	tatctatggatccaaggttccg
clc1-13BamHI	cgggacctggatccataggata
lcc1-1	tataccttgaatcagactggt
lcc1-12	atgaagctccctttacctcca
lcc2-1	caagagccagggtgtcgg
lcc2-12	ccgtcggagcaactcgct
lcc3-3	gttccgggcttgacagacatt
lcc3-4	cgggtgcagtcaatgtccaa
lcc4-5	tgctccgataacgactccac
lcc4-14	taattatgccttcagttcagtc
lcc5-4	ggcgatggcattaatagcag
lcc5-12	cgacatactggaacaacac
lcc9-5	tgatagcaaccaaggacgac
lcc9-8	tgaggattcgctgctgtttt
act-1	atgaccagatcgcttcgag
act-2	gagggaccgcctcgctgt
λ -int	cgcaactcgtgaaaggta
λ -git	aagtccaaccagataacgat

*Restriction sites introduced by targeted mutation are underlined.

plasmid pH1B (Turgeon *et al.*, 1987) and interrupting the ORF of the *clc1* gene. The Hyg^R cassette was inserted into a *Bam*HI sequence that was newly created by the overlapping method (Ho *et al.*, 1989) with the *clc1*-12*Bam*HI and *clc1*-13*Bam*HI primers. The final products were then cloned into pGEM-T. This construct was 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 coding region (Fig. 1).

The linear DNA fragment containing the interrupted *clc1* allele was amplified with the *clc1*-8 and *clc1*-10 primers (Table 1) and used to transform protoplasts of the *F. oxysporum* wild-type strain following a previously described protocol (Di Pietro & Roncero, 1998). Hyg^R transformants were isolated as described previously (Di Pietro & Roncero, 1998).

RT-PCR. To determine *clc1*, *lcc1*, *lcc3*, *lcc4*, *lcc5* and *lcc9* gene expression, total RNA from samples obtained after 3 days growth on CuSO_4 -containing medium, or in the presence of the copper-chelator bathocuproinedisulphonic acid (BCS) (Fluka), was used as a template for semiquantitative reverse transcription-polymerase chain reactions (RT-PCR). Briefly, total RNA was treated with RNase-free DNase (Roche Diagnostics) and reverse transcribed into cDNA with M-MLV reverse transcriptase (Invitrogen) using a poly-dT antisense primer. The cDNA was then used for PCR amplification with the following sense and antisense primers: *lcc1*-1 and *lcc1*-12, *lcc2*-1 and *lcc2*-12, *lcc3*-3 and *lcc3*-4, *lcc4*-5 and *lcc4*-14, *lcc5*-4 and *lcc5*-12, *lcc9*-5 and *lcc9*-8, *clc1*-7 and *clc1*-8 (Table 1). PCR conditions included 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. A different number of amplification rounds (10, 20 and 30) were used as indicated in Results. The actin gene was used as the internal control using act-1 and act-2 primers (Table 1).

Enzymic assays. Culture supernatants were harvested by centrifugation at 10 000 r.p.m. for 10 min and then by filtration through a Monodur filter (15 µm pore size). The laccase activity in the extracellular fluids was determined spectrophotometrically by monitoring the product formation at 30 °C, using syringaldazine 1 mM (dissolved in methanol) ($\epsilon_{525}=65\,000\text{ M}^{-1}\text{ cm}^{-1}$), in 0.1 M sodium phosphate buffer pH 5.5 as substrate. One unit of laccase oxidizes 1 µmol syringaldazine per minute.

Laccase activity was detected by non-dissociating SDS-PAGE (Laemmli, 1970) using acrylamide concentrations of 7.5 % for the separating gel and 4 % for the stacking gel. The running buffer was Tris/glycine (pH 8.3) containing 0.1 % SDS. Samples were mixed at a 4:1 ratio (v/v) with sample buffer [0.5 M Tris/HCl (pH 6.8), 8 % glycerol (v/v), 0.1 % bromophenol blue and 1.6 % SDS (w/v)]. To perform specific activity staining, gels were equilibrated by 5 min immersion in 0.2 M sodium phosphate buffer at pH 5.5 and were then stained by incubating at room temperature in 0.2 M sodium phosphate buffer (pH 5.5) containing 0.2 % *p*-phenylenediamine as the substrate.

Sensitivity to oxidative stress. To determine menadione sensitivity, a 5 µl drop containing from 5×10^3 to 2×10^4 freshly obtained microconidia was transferred to 15 g l^{-1} synthetic medium (SM) agar plates (Di Pietro & Roncero, 1998). The plates contained 10 g sucrose l^{-1} as the carbon source and 0.1 % NH_4NO_3 as the nitrogen source and were supplemented with 10 µg menadione ml^{-1} (Sigma) dissolved in absolute ethanol after autoclaving. They were then incubated at 28 °C for 2 days as described previously (Di Pietro & Roncero, 1998).

Virulence assays. Tomato seeds, which were sterilized as previously described (Huertas-Gonzalez *et al.*, 1999), were used for the plant inoculation assays (Di Pietro & Roncero, 1998). Two-week-old tomato seedlings (cultivar Vemar) were inoculated with *F. oxysporum* f. sp. *lycopersici* strains by immersing the roots in a microconidial suspension. The seedlings were then planted in vermiculite and kept in a growth chamber at 25 °C with a 14 h photoperiod. Plants immersed in sterile water were used as controls. After inoculation, the severity of disease symptoms was recorded at different times using an index ranging from 1 (healthy plant) to 5 (dead plant). Twenty plants were used for each treatment, and the experiment was repeated at least three times.

Fig. 1. Targeted replacement of *F. oxysporum clc1*. (a) Physical map and gene replacement vector for the *clc1* gene. The coding region shown in grey indicates the orientation of the ORF and the introns. Small arrows represent the positions of the primers used for amplification of the gene-replacement cassette. (b) Southern analysis of the *clc1* transformants and the wild-type strain. Genomic DNA digested with *Bam*HI was hybridized with the indicated probe. Size bands are shown in kb.

RESULTS

Isolation and characterization of the *clc1* gene

A DNA fragment encoding a putative voltage-gated chloride channel of *F. oxysporum* f. sp. *lycopersici* was isolated by PCR amplification using the *clc1*-1 and *clc1*-2 primers (Table 1) derived from the sequence of the *F. graminearum* gene orthologous to *CLC-A* of *C. neoformans*. After confirming its identity by sequencing, the single amplified fragment obtained was cloned and used to probe a *F. oxysporum* λ -EMBL3 genomic library (Anaya & Roncero, 1995). The three positive clones obtained were amplified by PCR using the flanking λ -EMBL3 primers λ -int and λ -git (Table 1), thus amplifying the entire DNA

inserts from the genomic clones. These were sequenced by DNA-walking with specific synthetic oligonucleotides. The sequences were analysed using the BLAST algorithm (Altschul *et al.*, 1990) available at NCBI. The gene sequence, named *clc1*, was deposited in the EMBL GenBank under the accession number EU030436.

The *clc1* gene revealed a 2475 nt ORF, interrupted by three introns of 115, 50 and 55 nt, identified by comparison with related proteins. The gene encodes a predicted protein of 825 aa with a calculated mass of 90.9 kDa and a pI of 7.3.

The existence of 11 transmembrane domains, predicted by the PSORT (<http://wolfsort.org>) and SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui>) algorithms, suggests a membrane-associated location of the *F. oxysporum* Clc1 protein. Like the majority of CLC proteins, *F. oxysporum* Clc1 has a long carboxy-terminal cytoplasmic region (262 aa) comprising two copies of a CBS domain (from cystathionine- β -synthase). These structural domains, which contain a typical $\beta 1$ - $\alpha 1$ - $\beta 2$ - $\beta 3$ - $\alpha 2$ fold (Bateman, 1997; Ponting, 1997), have been found in pairs among unrelated proteins from different phyla and are believed to have a diversity of functions (Estevez & Jentsch, 2002), although no clear role has emerged so far.

An *in silico* search for Clc1 orthologues in the available *Fusarium* group genomic databases (http://www.broad.mit.edu/annotation/genome/fusarium_group) revealed the existence of four putative chloride channels in each of the species *F. oxysporum*, *F. graminearum* and *Fusarium verticillioides*. Identity with both *F. graminearum* and *F. verticillioides* orthologues was about 95 %. On the other hand, alignment with putative chloride channels from filamentous fungi showed the highest homology to *Aspergillus nidulans* and *Aspergillus fumigatus*, 64 and 65 %, respectively (see Supplementary Fig. S1, available with the online version of this paper). However, identity with CLC-A from *C. neoformans* was 50 % and nearly 40 % with Gef1p from *Saccharomyces cerevisiae* (Flis *et al.*, 2002; Zhu & Williamson, 2003).

Targeted disruption of the *clc1* gene

Loss-of-function mutants carrying a disrupted *clc1* allele were generated using the one-step gene replacement strategy (Ho *et al.*, 1989). Hyg^R selection allowed the isolation of eight transformants that were analysed by restriction enzyme digestion and Southern hybridization of genomic DNA (Fig. 1). Six transformants with a homologous gene replacement were obtained harbouring a disrupted *clc1* allele. RT-PCR analyses confirmed the absence of *clc1* transcripts in the null mutants that were further characterized.

To investigate the relationships between laccases and the Clc1 chloride channel, laccase activity was determined and the proteins were analysed by non-dissociating SDS-PAGE from *F. oxysporum* culture supernatants. The wild-type strain and the $\Delta clc1$ mutants were grown for 5 days on

minimal medium containing sucrose, with or without 250 μ M copper sulphate (Sigma). The culture supernatants were then harvested to determine laccase activity and analyse the isoenzymes by non-dissociating SDS-PAGE.

In comparison to the wild-type strain, the $\Delta clc1$ mutants showed a significant decrease in laccase activity in the absence of copper sulphate (Fig. 2). This deficiency was comparable to that observed in the most affected individual laccase mutant ($\Delta lcc5$) (Cordoba Cañero & Roncero, 2008). Activity was restored in both instances by adding copper sulphate (Fig. 2).

Laccase gene expression is not regulated by copper in *F. oxysporum*

Several authors have reported that copper induces laccase expression in different fungi (Collins & Dobson, 1997; Galhaup *et al.*, 2002; Litvintseva & Henson, 2002; Soden & Dobson, 2001). To investigate the influence of copper on laccase gene expression mediated by the chloride channel in *F. oxysporum*, semiquantitative RT-PCR analysis was used to assess *lcc* transcripts from the wild-type strain, three independent mutants defective in the *clc1* gene and one ectopic transformant grown on copper or in the presence of the copper-chelator BCS. An identical expression pattern was observed for the *lcc1*, *lcc2*, *lcc3*, *lcc4*, *lcc5* and *lcc9* genes in all the strains analysed (Fig. 3). Transcripts were not detected for any of the genes after

Fig. 2. Extracellular laccase activity from wild-type (wt), laccase-deficient (*lcc*) and $\Delta clc1$ mutants. (a) Crude enzyme obtained from culture supernatants of strains grown for 5 days on minimal medium with or without copper sulphate. Error bars indicate standard deviations calculated from three independent experiments. (b) Analysis of isoenzymes in a zymogram.

Fig. 3. Effect of a functional chloride channel and the presence of copper on laccase gene expression. Semiquantitative RT-PCR of total RNAs, from *F. oxysporum* wild-type (wt), ectopic transformant (*Ect*) and three $\Delta clc1$ mutants, grown with CuSO₄ (250 μ M) or with the copper-chelator BCS (1 mM) amplified after 20 or 30 cycles, as indicated. Genomic DNA (gDNA) and the actin gene were used as controls.

10 RT-PCR cycles (data not shown). Moreover, the transcription profile appeared to be copper-independent. This suggests that laccase activity deficiency and its recovery by the addition of copper may be due to the defective transport of anions via Clc1, which promotes electro-neutrality and allows the loading of copper into the laccase apoproteins, rather than an effect on the transcriptional regulation of the laccase structural genes.

Sensitivity of $\Delta clc1$ mutants to oxidative stress

To investigate whether the decrease in laccase activity shown by $\Delta clc1$ mutants affects oxidative stress sensitivity, colony growth on plates containing 10 μ g ml⁻¹ of the redox-cycling agent menadione was determined (Hwang *et al.*, 2002). The growth of the $\Delta clc1$ mutants in the presence of menadione was severely impaired (Fig. 4), indicating that the loss of the chloride channel produces a marked sensitivity to oxidative stress conditions. An attempt was made to determine the capacity of copper to alleviate menadione resistance in the $\Delta clc1$ mutants, using liquid and solid media. However, the simultaneous presence of both compounds seemed to be highly toxic for *F.*

Fig. 4. Sensitivity of wild-type (wt) strain, ectopic (*Ect*) and $\Delta clc1$ mutants to menadione. Serial dilutions of microconidial suspensions from the indicated strains were spotted on minimal medium agar plates containing 1% sucrose, with or without menadione (10 μ g ml⁻¹), and incubated for 3 days at 28 °C.

oxysporum since no growth was detected in any strains assayed (data not shown).

Effect of *clc1* deletion on virulence

To determine the importance of the *clc1* gene on virulence of *F. oxysporum*, root infection assays with tomato plants were performed. Two-week-old plants were inoculated by immersing the roots in a microconidial suspension of the wild-type strain, three targeted $\Delta clc1$ mutants and an ectopic transformant. Plants were scored for vascular wilt symptoms at different time intervals. The development of disease is shown in Fig. 5. Plants inoculated with the wild-type strain or the ectopic transformant began to show characteristic wilt symptoms 10 days after inoculation. Disease severity increased steadily throughout the experiment and most of the plants were dead 15 days after inoculation. Plants inoculated with the $\Delta clc1$ mutants showed a significant delay in the progression of disease, although most of these plants were dead 25 days after inoculation.

DISCUSSION

F. oxysporum laccase-deficient mutants were obtained and characterized in a previous study (Cordoba Cañero & Roncero, 2008). The disruption of three individual laccase genes had no effect on fungal pathogenicity. This is most likely due to the fact that the loss of a particular laccase isoenzyme is compensated for by redundant enzymes. This redundancy, together with the difficulty of simultaneous inactivation of multiple laccase genes, makes a precise

Fig. 5. Virulence of *F. oxysporum* f. sp. *lycopersici* $\Delta clc1$ mutant on tomato plants. The severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain 4287 (■), $\Delta clc1\#3$ (●), $\Delta clc1\#5$ (□), $\Delta clc1\#10$ (△), ectopic $clc1\#12$ (▲) and the uninoculated control (◆). Bars indicate the standard error from 20 plants for each treatment.

assessment of the requirement of laccases for pathogenicity of this filamentous fungus a difficult task.

To investigate the mechanisms controlling the coordinated expression of laccases in *F. oxysporum*, the *clc1* gene was characterized in the present study. The predicted Clc1 gene product shows 11 putative transmembrane domains and high identities with other CLC chloride channels from *C. neoformans* (CLC-A) (Williamson, 1994) and *S. cerevisiae* (Gef1p) (Greene *et al.*, 1993), respectively.

Chloride is the most abundant extracellular anion in nature. Its different specific channels allow chloride transport across the cell membrane to reduce the electrochemical gradient. In *S. cerevisiae*, Gef1 is required for cation homeostasis and copper metalation of Fet3, a related multicopper oxidase involved in iron uptake (Gaxiola *et al.*, 1998). On the other hand, the *C. neoformans* $\Delta clc-a$ mutant, affected in a chloride channel, is deficient in laccase activity due to a failure in copper metabolism (Zhu & Williamson, 2003). The cryptococcal laccase gene product is involved in the synthesis of melanin-like pigments that protect the fungus against oxidative attack by the host cells. In fact, *C. neoformans* mutants affected in the laccase-encoding gene, *CNLAC1*, have an avirulent phenotype (Salas *et al.*, 1996).

The *F. oxysporum* *clc1*-targeted mutants show a marked decrease in laccase activity comparable to that shown by the previously described $\Delta lcc5$ mutant (Cordoba Cañero & Roncero, 2008). Nevertheless, laccase activity was restored in both types of mutants by the addition of copper sulphate to the growth medium. Studies on the relationship between GEF1 and the multicopper iron transporter Fet3 in *S. cerevisiae* provide insight into the functions of the cryptococcal chloride channel and copper protein laccases.

Both laccases and Fet3 are members of the multicopper oxidase (MCO) protein family, with similar substrate specificities including diphenol oxidase and ferroxidase activities (Davis-Kaplan *et al.*, 1998; Gaxiola *et al.*, 1998; Li & Kaplan, 1998). Additional research has indicated that the chloride ion is an allosteric effector of the enzyme metalation required for effective copper loading on the Fet3 iron transporter, and that GEF1 is involved in chloride anion transport to the subcellular compartments where copper addition takes place (Davis-Kaplan *et al.*, 1998).

On the other hand, the disruption of *clc-a* in *C. neoformans* affects the expression of laccase and again this effect appears to be reversed by the addition of exogenous copper. However, the present paper demonstrates that copper sulphate induces laccase activity in *F. oxysporum*, but does not activate the investigated laccase-encoding genes at the transcriptional level.

In addition, the *Fusarium* $\Delta clc1$ mutants show similar growth rates to the wild-type strain in iron-limited media (data not shown). This contrasts with the behaviour of the *Saccharomyces* *gef1*-defective mutants, suggesting that iron metabolism remains normal in $\Delta clc1$ strains. One hypothesis is that additional chloride channels supply sufficient metalation of the *F. oxysporum* Fet3 orthologue, although not at the required level for the laccase enzymes to function in a complete manner. Similar results have been described for *C. neoformans* (Zhu *et al.*, 2003).

The plant oxidative burst is one of the earliest responses to attacks by pathogenic fungi (Mayer *et al.*, 2001). Therefore, counter-defence mechanisms against oxidants produced within the host environment might constitute important virulence factors for plant-infecting microbes. The intermediate products of laccase activity are free radicals, which are able to interact with a variety of other radicals. Accordingly, *F. oxysporum* null mutants in single laccase genes show increased sensitivity to oxidative stress in comparison to the wild-type strain (Cordoba Cañero & Roncero, 2008), whereas the $\Delta clc1$ strains with impaired laccase activity appear to be even more sensitive than the individual laccase mutants to oxidative stress.

Although virulence analyses of $\Delta clc1$ on tomato plants demonstrated a significant delay in the disease index, all plants died after 25 days. This delay might be due to some defect in laccase production displayed by the mutants, suggesting the implication of laccases for the development of full virulence of *F. oxysporum*, although the individual genes have been shown not to be essential. On the other hand, the residual laccase activity observed in $\Delta clc1$ mutants indicates that copper loading into proteins occurs to some extent in *F. oxysporum*, probably due to the functionality of additional chloride channels.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Antonio Di Pietro (UCO) for his helpful discussions and Esther Martinez for her skilful technical

assistance. This research was supported by grant BIO2004-00276 from the Spanish Ministerio de Educacion y Ciencia and CVI-138 from Junta de Andalucia. D.C.C. had a PhD fellowship from the Ministerio de Educacion y Ciencia.

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Edited by: J.-R. Xu