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

Dolores Cordoba Cañero and M. Isabel G. Roncero

Deptoamento de Genetica, Universidad de Cordoba, Campus de Rabanales, Edif. Gregor Mendel, E-14071 Cordoba, Spain

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 D*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 D*clc1* mutants could play an essential role in the full virulence of *Fusarium*.

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

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**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 vacular 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 metaliation of the laccase enzyme that could be restored by the addition of exogenous copper. Nevertheless cryptococcal Δclc-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 Δclc1 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 x 10\(^6\) microconidia ml\(^{-1}\) and germinated for 12–14 h at 28 °C and 170 r.p.m. The germlings were then washed twice in sterile water and transferred to a synthetic liquid medium containing 0.8 mM MgSO_4\(_\cdot\)7H\(_2\)O, 2.9 mM KH\(_2\)PO\(_4\), 2.7 mM KCl, 12.5 mM NH\(_4\)NO\(_3\), 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 medium were buffered with 100 mM Na\(_2\)HPO\(_4\) at pH 4; 50 mM Na\(_2\)HPO\(_4\), 50 mM NaH\(_2\)PO\(_4\) and 50 mM NaCl at pH 6; and 100 mM Na\(_2\)HPO\(_4\) 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 DyeDeoxy 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 pDclc1: :hyg was constructed by following the general strategy of inserting the Hyg\(^R\) cassette from

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence* (5’–3’*)</th>
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</tr>
<tr>
<td>clc1-8</td>
<td>aggaggaggtagtggcagatt</td>
</tr>
<tr>
<td>clc1-10</td>
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<tr>
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<td>clc1-13BamHI</td>
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</tr>
<tr>
<td>lcc1-1</td>
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*Restriction sites introduced by targeted mutation are underlined.
plasmid pH1B (Turgeon et al., 1987) and interrupting the ORF of the clc1 gene. The HygR cassette was inserted into a BamHI sequence that was newly created by the overlapping method (Ho et al., 1989) with the clc1-12BamHI and clc1-13BamHI primers. The final products were then cloned into pGEM-T. This construct was digested with BamHI and the 1.8 kb BamHI fragment containing the HygR 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). HygR 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 CuSO4-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) (ε525=65 000 M⁻¹ 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 × 10⁻⁷ to 2 × 10⁴ 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 1⁻¹ as the carbon source and 0.1 % NH₄NO₃ 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.

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://wolfpsort.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 β1-x1-β2-x2-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). HygR 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 Δclc1 mutants were grown for 5 days on minimal medium containing sucrose, with or without 250 μM 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 Δclc1 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 (Δlcc5) (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 minimal medium containing sucrose, with or without 250 μM 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 Δclc1 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 (Δlcc5) (Cordoba Cañero & Roncero, 2008). Activity was restored in both instances by adding copper sulphate (Fig. 2).

![Fig. 2. Extracellular laccase activity from wild-type (wt), laccase-deficient (lcc) and Δlcc1 mutants.](http://mic.sgmjournals.org)
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 Δclc1 mutants to oxidative stress**

To investigate whether the decrease in laccase activity shown by Δclc mutants affects oxidative stress sensitivity, colony growth on plates containing 10 μg ml⁻¹ of the redox-cycling agent menadione was determined (Hwang et al., 2002). The growth of the Δclc 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 Δclc mutants, using liquid and solid media. However, the simultaneous presence of both compounds seemed to be highly toxic for *F. 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 Δclc1 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 Δclc1 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
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 Δclc1 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 Δclc1 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 Δclc1 strains with impaired laccase activity appear to be even more sensitive than the individual laccase mutants to oxidative stress.

Although virulence analyses of Δclc1 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 Δclc1 mutants indicates that copper loading into proteins occurs to some extent in F. oxysporum, probably due to the functionality of additional chloride channels.

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