Fusarium oxysporum Ste12 Controls Invasive Growth and Virulence Downstream of the Fmk1 MAPK Cascade

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A conserved mitogen-activated protein kinase (MAPK) cascade homologous to the yeast Fus3/Kss1 mating/filamentation pathway regulates virulence in fungal plant pathogens. In the soilborne fungus *Fusarium oxysporum*, the MAPK Fmk1 is required for infection and development of vascular wilt disease on tomato plants. Knockout mutants lacking Fmk1 are deficient in multiple virulence-related functions, including root adhesion and penetration, invasive growth, secretion of pectinolytic enzymes, and vegetative hyphal fusion. The transcription factors mediating these different outputs downstream of the MAPK cascade are currently unknown. In this study, we have analyzed the role of *ste12* which encodes an orthologue of the yeast homeodomain transcription factor Ste12p. *F. oxysporum* mutants lacking the *ste12* gene were impaired in invasive growth on tomato and apple fruit tissue and in penetration of cellophane membranes. However, *ste12* was not required for adhesion to tomato roots, secretion of pectinolytic enzymes, and vegetative hyphal fusion, suggesting that these Fmk1-dependent functions are mediated by other downstream MAPK targets. The $\Delta ste12$ strains displayed dramatically reduced virulence on tomato plants, similar to the $\Delta fmk1$ mutant. These results indicate that invasive growth is the major virulence function controlled by the Fmk1 MAPK cascade and depends critically on the transcription factor Ste12.

Plant-pathogenic fungi have evolved mechanisms to invade their hosts, overcome their defenses, and colonize the host tissue, thereby causing disease. One of these mechanisms involves a highly conserved mitogen-activated protein kinase (MAPK) homologous to the yeast mating/filamentation MAPKs Fus3/Kss1 (Qi and Elion 2005). The essential role of this so-called pathogenicity MAPK (PMK) during infection was first reported in the rice blast fungus *Magnaporthe grisea*. Fungal mutants lacking the *pmk1* gene were not only defective in formation of appressoria but also unable to colonize host plant tissue when inoculated through wound sites, a process known as invasive or infectious growth (Xu and Hamer 1996). PMK orthologues were subsequently found to be required for virulence in a wide array of biologically and taxonomically diverse plant pathogens, sug-

gesting an ancient evolutionary role of this MAPK cascade in fungal pathogenicity on plants (Di Pietro et al. 2001; Lev et al. 1999; Muller et al. 1999; Takano et al. 2000). Although PMK are known to control a number of virulence-related functions, the mechanisms through which they regulate plant infection have so far remained elusive (Zhao et al. 2007).

The PMK orthologue Fmk1 is required for plant infection in the vascular wilt fungus *Fusarium oxysporum*, a soilborne pathogen attacking a wide range of agriculturally important crops (Di Pietro et al. 2003). Mutants lacking the *fmk1* gene are deficient in several virulence-related functions, including adhesion to tomato roots, penetration, invasive growth on plant tissue, and secretion of pectinolytic enzymes (Delgado-Jarana et al. 2005; Di Pietro et al. 2001). Fmk1 is also required for vegetative hyphal fusion, an ubiquitous process in filamentous fungi whose biological function is poorly understood (Prados Rosales and Di Pietro 2008). The downstream transcription factors mediating all these different MAPK outputs are currently unknown. Moreover, the relative importance of each of these Fmk1-controlled functions during the infection process remains to be determined.

In *Saccharomyces cerevisiae*, the homeodomain protein Ste12p is a key transcription factor downstream of the MAPKs Fus3 and Kss1 (Qi and Elion 2005). Ste12p binds to pheromone response elements in the promoters of its target genes and, in cooperation with the TEA/ATTS family transcription factor Tec1, to filamentation response elements to regulate genes involved in invasive growth (Madhani and Fink 1997). Ste12p orthologues have also been identified in filamentous fungi, including human and plant pathogens. *M. grisea* mutants lacking the *MST12* gene were impaired in pathogenicity and invasive growth on host tissue but were still able to form melanized appressoria on the leaf surface (Park et al. 2002). These appressoria developed normal turgor pressure, but failed to form penetration pegs (Park et al. 2004). Similarly, disruption of *ste12* orthologues in *Colletotrichum lagenarium* and *C. lindemuthianum* resulted in dramatically reduced virulence, indicating an essential role of this transcription factor during the infection process (Hoi et al. 2007; Tsuji et al. 2003).

Here, we investigated the role of *ste12* in *F. oxysporum*. Our study addressed two major questions: i) does Ste12 control virulence-related functions downstream of the MAPK Fmk1 and ii) which of these functions are essential for pathogenicity? We found that knockout mutants lacking the *ste12* gene were impaired in invasive growth but not in adhesion to tomato roots, secretion of pectinolytic enzymes, or vegetative hyphal fusion. The $\Delta ste12$ strains had dramatically reduced virulence on tomato plants, suggesting that invasive growth is the main virulence function regulated by the Fmk1 MAPK cascade and depends crucially on the transcription factor Ste12.

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The sequence of the *F. oxysporum ste12* gene has been deposited in GenBank under accession number FJ609797.

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RESULTS

Cloning and targeted knockout of the *F. oxysporum* *ste12* gene.

The complete *ste12* gene of *F. oxysporum* f. sp. *lycopersici* strain 4287 was cloned from a λ EMBL3 genomic library, using as a probe a polymerase chain reaction (PCR) fragment amplified from genomic DNA with primers Ste12-1 and Ste12-2, derived from conserved regions of *ste12* genes. Sequencing of a hybridizing λ genomic clone identified an open reading frame of 2,298 bp, encoding a putative 696-amino-acid protein with a predicted molecular mass of 76.6 kDa and a pI of 6.70 containing a characteristic Ste homeodomain and a double zinc-finger domain (Fig. 1A). The sequence of the *F. oxysporum* *ste12* gene has been deposited in GenBank under accession number FJ609797. Alignment of the nucleotide sequences of the

ste12 cDNA, obtained by reverse-transcriptase (RT)-PCR with gene-specific primers, and the genomic clone revealed the presence of four introns. Southern analysis of total genomic DNA treated with different restriction enzymes produced a banding pattern indicative of *ste12* being present as a single copy in the *F. oxysporum* genome (data not shown). In agreement with this result, a BLASTP search of the complete genome database of *F. oxysporum* detected only one significant match with an identical predicted gene product (FOXG_02103.2). Alignment of the amino acid sequence of *F. oxysporum* Ste12 with sequences from the databases revealed significant identity with Ste12 proteins from other fungi (Fig. 1B and C). Based on these data, we conclude that *F. oxysporum* *ste12* is the structural orthologue of *S. cerevisiae* STE12.

To explore the biological role of Ste12 in *F. oxysporum*, a Δ Ste12 null allele was generated through replacement of the

Fig. 1. Ste12 protein structure. **A**, Scaled cartoon of the domain structure of *Fusarium oxysporum* Ste12 and fungal Ste12 orthologues: *Magnaporthe grisea* Mst12, *Colletotrichum lindemuthianum* ClSte12, *Neurospora crassa* SteA, *Aspergillus nidulans* SteA, *Candida albicans* Cph1, and *Saccharomyces cerevisiae* Ste12p. **B**, Amino acid sequence alignment of the Ste homeodomain of fungal Ste12 orthologues. Regions predicted to form the three α -helices are indicated by black lines. **C**, Amino acid sequence alignment of the C₂H₂ zinc-finger region of fungal Ste12 orthologues. The two C₂H₂ zinc-finger domains are indicated by black lines. Absolutely conserved residues are shaded black, residues conserved in at least 80% are shaded in dark gray, and residues conserved in at least 60% are shaded in light gray.

ste12 open reading frame with the hygromycin resistance cassette, using the split-marker method (Supplementary Fig. 1A) (details below). The knockout constructs were introduced into both the wild-type strain and the $\Delta fmk1$ mutant to study possible epistatic relationships between the two genes. In all, 6 and 19 hygromycin-resistant transformants, respectively, were analyzed by PCR with different combinations of gene-specific primers. Of these, 3 and 10 transformants, respectively, produced amplification products indicative of homologous integration-mediated gene replacement (data not shown). Southern blot analysis of these transformants confirmed the replacement of an 8-kb *EcoRI* fragment corresponding to the wild-type *ste12* allele by a fragment of 3.1 kb, demonstrating that these transformants, which were named $\Delta ste12$ and $\Delta fmk1\Delta ste12$, respectively, lack the *ste12* gene.

To confirm that the phenotype of the $\Delta ste12$ mutants was indeed caused by loss of *ste12* function, a 5.4-kb DNA fragment encompassing the complete *F. oxysporum ste12* gene was introduced into the $\Delta ste12-1$ strain by cotransformation with

the phleomycin resistance marker. Five phleomycin-resistant transformants were analyzed for presence of a functional *ste12* allele by PCR with gene-specific primers Ste12-9 and Ste12-10. A 2.8-kb amplification product identical to that obtained from the wild type was detected in four phleomycin-resistant transformants but not in the $\Delta ste12-1$ strain. We concluded that these cotransformants, denominated $\Delta ste12+ste12$, have an intact copy of the *ste12* gene ectopically integrated into the genome.

Ste12 is not required for vegetative hyphal growth and fusion.

To test the role of Ste12 in vegetative hyphal growth, colony diameter was determined on rich (yeast-peptone-glucose [YPG]) or poor (minimal medium [MM]) growth medium. No significant differences were detected between wild-type and $\Delta ste12$ mutants (Supplementary Fig. 2A). By contrast, $\Delta fmk1$ and $\Delta fmk1\Delta ste12$ strains had a significantly reduced growth rate, particularly on MM. These results argue that the growth

Fig. 2. Ste12 is not required for hyphal agglutination and vegetative hyphal fusion. Strains were grown overnight in potato dextrose broth diluted 1:50 with water and supplemented with 20 mM glutamic acid. **A**, Fungal culture was transferred to a microwell and observed in a Leica binocular microscope. Note the presence of mycelial aggregates in the wild-type (wt) and $\Delta ste12$ strains. **B**, Fungal cultures were observed in a Leica DMR microscope using the Nomarsky technique. Note the presence of vegetative fusions between hyphae and microconidial germ tubes of the wild-type and $\Delta ste12$ strains. Bar = 10 μ m.

reduction observed in the $\Delta fmk1$ mutants is not mediated by Ste12. Colonies of $\Delta fmk1$ and $\Delta fmk1\Delta ste12$ but not those of $\Delta ste12$ strains also developed less aerial hyphae than those of the wild type (results not shown).

The number of microconidia produced by $\Delta fmk1$ and $\Delta fmk1\Delta ste12$ strains was 3 to 4 times higher than that of the wild type on YPG, and 10 to 20 times higher on MM. The $\Delta ste12$ strain showed a three- to fourfold increase on both types of media, suggesting that Ste12 has only a minor role in controlling conidiation and that the dramatic increase in conidiation on MM observed in the $\Delta fmk1$ mutant is not mediated by Ste12.

To test whether Ste12 plays a role in the stress response of *F. oxysporum*, we compared hyphal growth of the fungal strains on MM under conditions of osmotic (0.8 M NaCl), oxidative (menadione at 10 $\mu\text{g ml}^{-1}$), or cell wall stress (Congo Red at 20 $\mu\text{g ml}^{-1}$ or Calcofluor White at 20 $\mu\text{g ml}^{-1}$). No differences in growth rates were detected between wild-type, $\Delta ste12$, $\Delta fmk1$, and $\Delta fmk1\Delta ste12$ strains (data not shown).

The Fmk1 MAPK is required for efficient vegetative hyphal fusion of *F. oxysporum* (Prados Rosales and Di Pietro 2008). In liquid culture, hyphal fusion leads to the production of networks that are macroscopically visible as hyphal aggregates in the wild-type strain (Fig. 2A). By contrast, hyphae of the $\Delta fmk1$ strain do not undergo fusion and fail to produce aggregates. We found that $\Delta ste12$ strains produced aggregates similar to those of the wild-type strain, indicating the presence of vegetative hyphal fusion. This was further confirmed by microscopic examination of the germlings of different $\Delta ste12$ strains. Hyphal

fusion bridges and germ tube fusion events were detected at frequencies similar to those of the wild-type strain (Fig. 2B). By contrast, no hyphal fusions were observed in the $\Delta fmk1$ and $\Delta fmk1\Delta ste12$ mutants after extensive microscopic analysis, even though the hyphae frequently made physical contact with each other. We conclude that the essential role of the MAPK Fmk1 in vegetative hyphal fusion is not mediated by the Ste12 transcription factor.

Ste12 is not required for adhesion to tomato roots.

Among the virulence-related phenotypes reported in the $\Delta fmk1$ mutants is the inability to adhere to and colonize tomato roots (Di Pietro et al. 2001). We tested whether Ste12 is required for efficient root adhesion. Roots of tomato seedlings were immersed for 24 h in microconidial suspensions of the different strains, then washed by vigorous shaking in water and observed in a stereomicroscope. The wild-type strain had efficiently adhered to the lateral roots and formed a dense hyphal network covering most of the root surface (Fig. 3). As previously reported, the $\Delta fmk1$ strain failed to adhere to and colonize the roots. By contrast, the adhesion efficiency of the $\Delta ste12$ strain but not that of the $\Delta fmk1\Delta ste12$ double mutant was similar to that of the wild-type strain. Microscopic observation of the colonized root surface revealed the presence of hyphal fusion events between germ tubes and hyphae of the wild type or the $\Delta ste12$ strains (results not shown). We conclude that the essential role of the MAPK Fmk1 in adhesion of *F. oxysporum* to tomato roots is not mediated by the Ste12 transcription factor.

Fig. 3. Ste12 is not required for hyphal adhesion to tomato roots. Roots of tomato seedlings were immersed for 24 h in microconidial suspensions of the indicated strains, washed by vigorous shaking in water, and observed in a Leica binocular microscope; wt = wild type. Hyphae adhered to the plant surface are visible as a white mass covering the roots.

Ste12 regulates expression of cell-wall-degrading enzymes.

Another virulence-related phenotype of the $\Delta fmk1$ mutant is a significant reduction in pectinolytic activity secreted during growth on plates containing polygalacturonic acid (PGA) (Di Pietro et al. 2001). We tested whether Ste12 mediates Fmk1-dependent regulation of pectinolytic activity on PGA in the presence of 2% glucose. As reported previously, the $\Delta fmk1$ mutant had strongly reduced clear halo production compared with the wild-type strain (Fig. 4). By contrast, $\Delta ste12$ strains produced clear halos of size similar to those of the wild-type strain. Unexpectedly, $\Delta fmk1\Delta ste12$ double mutants consistently produced larger halos than the $\Delta fmk1$ single mutant. These results suggest that Ste12 may function in repression rather than activation of pectinolytic genes under the growth conditions tested.

We also determined the ability of the different strains to produce clear halos on carboxymethyl cellulose (cellulase activity) or starch (amylase activity). On both substrates, the $\Delta fmk1$ strain showed no significant difference in lytic activity compared with the wild type (Fig. 4). However, $\Delta ste12$ and $\Delta fmk1\Delta ste12$ mutants were clearly reduced in halo production on starch, and also showed a small but consistent reduction on carboxymethyl cellulose. Thus, Ste12 and Fmk1 appear to have distinct functions in the regulation of secreted lytic enzymes.

Ste12 is required for invasive growth and pathogenicity on plants.

The Fmk1 MAPK controls invasive growth functions in *F. oxysporum*, such as the ability to penetrate cellophane membranes (Prados Rosales and Di Pietro 2008) or to invade and colonize living fruit tissue (Di Pietro et al. 2001). Here, we

explored whether these invasive growth functions are mediated by Ste12. Similar to $\Delta fmk1$ and in contrast to the wild-type strain, $\Delta ste12$ and $\Delta fmk1\Delta ste12$ mutants were unable to penetrate cellophane membranes (Fig. 5A). The $\Delta ste12$ strains also had significantly reduced invasive growth on tomato fruit (Fig. 5B) and on apple slices (Fig. 5C). Both cellophane penetration and fruit tissue colonization were restored in the $\Delta ste12+ste12$ complemented strains.

Plant infection was performed by inoculating the roots of tomato seedlings with microconidial suspensions of the different strains. Disease symptoms in plants inoculated with the wild-type strain increased steadily throughout the experiment, and most of the plants were dead 20 days after inoculation (Fig. 6). As reported previously (Di Pietro et al. 2001), plants inoculated with the $\Delta fmk1$ mutant had extremely low disease ratings. Three independent $\Delta ste12$ mutants were all strongly impaired in virulence but disease ratings were slightly higher than those of the $\Delta fmk1$ strain (one representative mutant is shown in Figure 6). Complementation of the $\Delta ste12-1$ mutant with the wild-type *ste12* allele restored virulence. The $\Delta fmk1\Delta ste12$ double mutants behaved similarly to the $\Delta fmk1$ single mutant. We conclude that, similar to Fmk1, Ste12 controls functions important for invasive growth and is essential for pathogenicity of *F. oxysporum* on tomato plants.

DISCUSSION

A conserved MAPK cascade orthologous to the yeast mating and filamentation pathway controls plant infection in a wide array of biologically and taxonomically diverse fungi (Zhao et al. 2007). Mutants lacking this PMK grow well, in general, on artificial substrates but are unable to infect living plant tissue. PMK thus plays a specific role in plant infection, making it one of the most evolutionarily conserved fungal virulence factors known to date.

How exactly this MAPK cascade regulates fungal virulence is still unclear. In leaf pathogens, PMK is required for differentiation of the appressorium, a highly specialized infection structure that develops extreme turgor pressure and is essential for host penetration (Takano et al. 2000; Xu and Hamer 1996). However, PMK mutants also fail to produce disease symptoms when inoculated through wound sites, a situation in which appressoria are dispensable for infection (Xu and Hamer 1996), suggesting that PMK must control additional virulence functions. This conclusion is further supported by the finding that fungal pathogens which do not develop classical appressoria, such as *Botrytis cinerea*, *F. oxysporum*, *Claviceps purpurea*, or *Cryphonectria parasitica*, also require the orthologous MAPK for plant infection (Choi et al. 2005; Di Pietro et al. 2001; Mey et al. 2002; Zheng et al. 2000). Although PMK have been shown to control a number of virulence functions, the cellular mechanisms through which they regulate these functions remain largely unknown (Zhao et al. 2007).

The homeodomain transcription factor Ste12p functions downstream of the Fus3 and Kss1 pathways in yeast (Qi and Elion 2005). Here, we have characterized the Ste12 orthologue from *F. oxysporum* and studied its role in mediating distinct virulence-related functions controlled by the Fmk1 MAPK cascade (Fig. 7). As reported in other filamentous fungi, *F. oxysporum* Ste12 contains a characteristic Ste-like homeodomain in the N-terminal region of the protein, as well as two C-terminal C₂H₂ zinc-finger motifs which are absent in Ste12 orthologues from the *Saccharomycotina* (Fig. 1). The Ste12 homeodomain was previously shown to be required for DNA binding in *Cryptococcus neoformans* (Chang et al. 2004) and to mediate binding of *Colletotrichum lindemuthianum* Ste12 to the putative target sequence TGAAACA (Hoi et al. 2007). By

Fig. 4. Ste12 plays differential roles in regulation of lytic enzymes. Production of extracellular polygalacturonase, cellulase, or amylase activity was determined on plates containing polygalacturonic acid, carboxymethyl cellulose, or starch, respectively. Enzyme activity is visualized as a contrasting halo underneath the fungal colony.

Fig. 5. Ste12 is required for invasive growth. Microconidial suspensions of the indicated strains were spot inoculated on different substrates for invasive growth assays; wt = wild type. **A**, Penetration of cellophane membranes. Colonies were grown for 4 days on a plate with minimal medium covered by a cellophane membrane (before); then, the cellophane with the colony was removed and plates were incubated for an additional day (after). Invasive growth on **B**, tomato and **C**, apple fruit inoculated with microconidia and incubated at 28°C for 5 or 4 days, respectively.

Fig. 6. Ste12 is required for virulence of *Fusarium oxysporum* on tomato plants. Incidence of Fusarium wilt on tomato plants (cv. Monica) inoculated with the indicated strains. Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Error bars represent standard errors calculated from 10 plants.

contrast, the role of the double zinc-finger region is currently unknown. The zinc-finger domain was dispensable for DNA binding in *Cryptococcus neoformans* (Chang et al. 2004). However, both homeodomain and zinc-finger domain of *M. grisea* Mst12 were required for virulence on rice plants (Park et al. 2004). The high sequence conservation of the zinc-finger region, which includes two introns that were shown to undergo differential splicing in *Colletotrichum lindemuthianum* (Hoi et al. 2007), strongly suggests that this domain plays a pivotal role in Ste12 function. Elucidating this role will be an important challenge for future studies.

Ste12 regulates only a subset of the Fmk1 MAPK cascade outputs.

To test whether Ste12 mediates signaling outputs downstream of Fmk1, we systematically compared the phenotypes of $\Delta fmk1$, $\Delta ste12$, and $\Delta fmk1\Delta ste12$ mutants. The $\Delta fmk1$ and $\Delta ste12$ strains shared a number of characteristic phenotypes, indicating that Ste12 could be located in the same pathway as Fmk1; however, we also identified several functions that were affected in the $\Delta fmk1$ strain but intact in $\Delta ste12$ mutants. Such Ste12-independent functions include vegetative hyphal growth and development on artificial substrates. As previously reported, the $\Delta fmk1$ strain showed reduced hyphal growth on poor medium (MM) (Prados Rosales and Di Pietro 2008). By contrast, hyphal growth of the $\Delta ste12$ mutants was similar to that of the wild type. Deletion of *ste12* also had no detectable effect on hyphal growth of *M. grisea* and *Cryphonectria parasitica* (Deng et al. 2007; Park et al. 2002) but resulted in significantly reduced growth in *Neurospora crassa* (Li et al. 2005). As previously noted in *Cryphonectria parasitica* (Deng et al. 2007), *F. oxysporum* $\Delta ste12$ mutants displayed a significant increase

in asexual sporulation. However, this increase was much less pronounced than in the $\Delta fmk1$ strain, supporting the idea that Ste12 plays only a minor role in regulating vegetative growth and development of *F. oxysporum*.

Another function requiring Fmk1 but not Ste12 is vegetative hyphal fusion. Deletion of *fmk1* was previously shown to result in impaired hyphal fusion (Prados Rosales and Di Pietro 2008). By contrast, the $\Delta ste12$ mutants produce hyphal fusions at frequencies similar to those of the wild-type strain. Similar to the wild type, hyphal fusion events in $\Delta ste12$ strains were observed both at the interior of mature colonies and between germinating conidia, and were preceded by positive tropic responses such as reorientation of the hyphal growth axis toward the neighboring hyphae or germ tubes.

Vegetative hyphal fusion has been suggested to play a role in the formation of hyphal networks during colonization of tomato roots. *F. oxysporum* mutants lacking the WW domain protein Fso1, which controls vegetative hyphal fusion independently of the Fmk1 MAPK cascade, are also impaired in formation of hyphal aggregates and root adhesion (Prados Rosales and Di Pietro 2008). The results from the present study provide further support for this hypothesis. The $\Delta ste12$ strains, which were as efficient as the wild type in hyphal fusion, also behaved similarly to the wild type in production of hyphal networks and root adhesion. As expected, these phenotypes were impaired in the $\Delta fmk1$ and the $\Delta fmk1\Delta ste12$ strains which lack hyphal fusion. Thus, vegetative hyphal fusion, formation of hyphal aggregates, and tomato root adhesion are controlled by the Fmk1 cascade via transcriptional regulators other than Ste12, whose identity remain to be determined.

Mutants lacking Fmk1 have reduced extracellular pectinolytic activity when grown in the presence of polygalactu-

Fig. 7. Model for the role of Ste12 in *Fusarium oxysporum*. Ste12 regulates invasive growth downstream of the Fmk1 mitogen-activated protein kinase, whereas the remaining Fmk1-controlled functions are mediated by other, unknown transcriptional regulators. Additional pathways may also regulate Ste12 activity. The invasive growth function mediated by Ste12 plays a key role in virulence on plants.

ronic acid and glucose (Delgado-Jarana et al. 2005; Di Pietro et al. 2001). Recently, Hoi and associates (2007) reported a decrease of pectinase activity in $\Delta ste12$ mutants of *Colletotrichum lindemuthianum*. In the present study, *F. oxysporum* $\Delta ste12$ mutants showed wild-type levels of pectinolytic activity, suggesting that Ste12 is not required for activation of pectinase genes under the conditions studied. Unexpectedly, $\Delta fmk1\Delta ste12$ double mutants had consistently higher pectinase levels than the $\Delta fmk1$ single mutant, suggesting that Ste12 may act as a negative regulator of pectinase gene expression. To our knowledge, this is the first evidence for a repressor role of Ste12 in filamentous fungi, although Ste12p was recently shown to function as a repressor of the *PRY3* gene in yeast (Bickel and Morris 2006). In contrast to pectinase activity, extracellular amylase and cellulase activities were positively regulated by Ste12, suggesting that Ste12 can play both positive and negative roles in the regulation of gene expression. The mechanism of this dual mode of action is currently unknown but may be related to post-transcriptional or post-translational modifications. In a recent study, a differentially spliced version of the *CLSTE12* gene had a dominant negative effect on yeast invasive growth and *Colletotrichum lagenarium* pathogenicity (Hoi et al. 2007).

Ste12 mediates invasive growth, the main virulence function controlled by the Fmk1 MAPK cascade in *F. oxysporum*.

Among different *fmk1* mutant phenotypes tested in the present study, only two were shared by the $\Delta ste12$ mutants. The first phenotype is invasive growth. Similar to the $\Delta fmk1$ strain, $\Delta ste12$ mutants were dramatically impaired in invasive growth functions, including cellophane penetration and colonization of living fruit tissue. The second phenotype is virulence. Tomato plants infected with the $\Delta ste12$ mutants showed dramatically reduced disease symptoms that were nearly as low as those of the $\Delta fmk1$ strain.

These results allow two main conclusions regarding the role of Ste12 in Fmk1-mediated signaling and pathogenesis. First, Ste12 is a key factor for virulence of the root pathogen *F. oxysporum*, as previously reported for the orthologous Ste12 proteins in the appressorium-forming leaf pathogens *M. grisea* and *Colletotrichum* spp. (Hoi et al. 2007; Park et al. 2002; Tsuji et al. 2003) and in the wound-infecting stem canker pathogen *Cryphonectria parasitica* (Deng et al. 2007). Recently, an *ste12* orthologue of the mycorrhizal fungus *Glomus intraradicis* was found to be upregulated during root penetration of the host plant *Medicago truncatula*, and its heterologous expression fully restored virulence of the *Colletotrichum lindemuthianum* $\Delta clste12$ mutant (Tollot et al. 2009). Thus, the role of Ste12 in plant infection appears to be broadly conserved among fungal pathogens, and even symbionts with diverse infection strategies and host species. This is strongly reminiscent of the role of the PMK MAPK, which is also required for virulence in a wide array of plant-pathogenic fungi (Zhao et al. 2007).

Second, $\Delta ste12$ and $\Delta fmk1$ mutants of *F. oxysporum* only share phenotypes related to invasive growth but none of the other Fmk1-regulated functions such as pectinolytic activity, vegetative hyphal fusion, or root adhesion. Yet, both classes of mutants have dramatically reduced virulence. This result strongly suggests that invasive growth is the main virulence function controlled by the Fmk1 MAPK in *F. oxysporum*. Root adhesion was previously shown to contribute only marginally to virulence on tomato plants, because $\Delta fso1$ mutants that are impaired in hyphal fusion, formation of hyphal aggregates, and root adhesion, exhibited only a slight reduction in virulence levels (Prados Rosales and Di Pietro 2008).

In *Magnaporthe grisea*, Mst12 was also shown to mediate a subset of functions controlled by the Pmk1 MAPK. Although *pmk1* mutants failed to form appressoria and were also unable to grow invasively from wound sites (Xu and Hamer 1996), *mst12* mutants produced appressoria developing normal turgor pressure but unable to form penetration pegs (Park et al. 2004). Interestingly, the *mst12* mutant was defective in microtubule reorganization associated with penetration peg formation, suggesting a function of Mst12 in establishment of cell polarity (Park et al. 2004). How exactly Ste12 mediates polarity establishment in filamentous fungi and how this translates into invasive growth during plant infection remains to be determined.

In *S. cerevisiae*, Ste12p is a direct target of the Fus3 and Kss1 MAPKs (Elion et al. 1993). A similar role in the orthologous MAPK signaling pathway has been suggested in filamentous fungi, including plant pathogens (Li et al. 2005; Park et al. 2004). The present study adds circumstantial evidence to the hypothesis that *F. oxysporum* Ste12 functions in an invasive growth-specific branch downstream of the Fmk1 MAPK cascade (Fig. 7). This evidence is based on the shared invasion phenotypes of the $\Delta ste12$ and $\Delta fmk1$ mutants, and on the observation that $\Delta fmk1\Delta ste12$ double mutants display a phenotype similar to that of the $\Delta fmk1$ single mutant. However, because direct phosphorylation of Ste12 by the MAPK has not yet been demonstrated in filamentous fungi, this hypothesis remains to be confirmed. Mutation of a putatively conserved MAPK phosphorylation site did not modify the function of Mst12 in *M. grisea* (Park et al. 2004), suggesting that the molecular mechanisms regulating Ste12 activity in filamentous fungi could be different from those in yeast. Finally, Ste12 may also be regulated by additional pathways distinct from the Fmk1 cascade. This may explain our unexpected finding of reduced amylase activity in $\Delta ste12$ and $\Delta fmk1\Delta ste12$ but not $\Delta fmk1$ strains, suggesting that the activating role of Ste12 is independent of the MAPK pathway. Interestingly, Ste12p was shown to interact with the transcriptional regulators Flo8p and Mss11p, which are regulated by the cAMP-PKA pathway, to activate expression of the glucoamylase gene *STA1* in *Saccharomyces* spp. (Kim et al. 2004). Thus, it is feasible that, in fungal pathogens, multiple signaling pathways may converge on the Ste12 transcription factor to regulate invasive growth and infection of plant hosts.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

F. oxysporum f. sp. *lycopersici* race 2 wild-type strain 4287 (FGSC 9935) was used in all experiments. Generation and molecular characterization of the *F. oxysporum* $\Delta fmk1$ mutant was described previously (Di Pietro et al. 2001). All fungal strains were stored as microconidial suspensions at -80°C with 30% glycerol. For extraction of genomic DNA and for microconidia production, cultures were grown in potato dextrose broth (PDB; Difco Laboratories, Detroit) at 28°C with shaking at 170 rpm (Di Pietro and Roncero 1998). Cellophane invasion assays were carried out as described (Prados Rosales and Di Pietro 2008). For macro- and microscopic analysis of hyphal fusion, fungal strains were grown overnight in PDB diluted 1:50 with water and supplemented with 20 mM glutamic acid, and observed in a Leica binocular microscope or a Leica DMR microscope using the Nomarsky technique. Photographs were recorded with a Leica DC 300F digital camera. For determination of colony growth, aliquots of 2×10^5 microconidia were spotted onto YPG (1% yeast extract, 1% peptone, 7% glucose, and 1.5% agar) or MM plates, and colony diameter was measured after 8 days at 28°C . Conidiation was quantified on the same plates by transferring a 20-mm² area excised from the

center of the colony to an Eppendorf tube containing 500 ml of water, vortexing vigorously for 30 s, and counting 10- μ l aliquots under a Leica DMR microscope. Experiments included four replicates and were performed at least twice with similar results.

Nucleic acid manipulations.

Genomic DNA was extracted from *F. oxysporum* mycelium following previously reported protocols (Chomczynski and Sacchi 1987; Raeder and Broda 1985). Southern analysis and probe labeling were carried out as described (Di Pietro and Roncero 1998) using the nonisotopic digoxigenin-labeling kit (Roche Diagnostics SL, Barcelona, Spain). Other routine DNA procedures were performed as described in standard protocols (Sambrook and Russell 2001).

For isolation of the *ste12* gene, genomic DNA of *F. oxysporum* was used for PCR amplification with primers Ste12-1 and Ste12-2, deduced from conserved regions of the Ste12 zinc-finger domain. The amplified DNA fragment was cloned into the pGEM-T vector (Promega Corp., Madison, WI, U.S.A.) and used to screen a λ EMBL3 genomic library of *F. oxysporum* f. sp. *lycopersici* isolate 4287. Library screening, subcloning, and other routine procedures were performed as described in standard protocols (Sambrook and Russell 2001). Sequencing of both DNA strands of the hybridizing clones was performed at the Servicio de Secuenciación Automática de DNA, SCAI (University of Córdoba, Spain) using the Dyedexy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) 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).

Construction of Δ ste12 null allele and fungal transformation.

Replacement of the *F. oxysporum ste12* gene was performed by the split-marker method (Catlett et al. 2003). PCR reactions were routinely performed with the High Fidelity Template PCR system (Roche Diagnostics SL) using a Perkin Elmer GeneAmp System 2400. First, 1.2- and 1.5-kb DNA segments flanking the *ste12* coding region were amplified from genomic DNA of *F. oxysporum* strain 4287 with primer pairs Ste12-For/Ste12-KO1 and ste12-KO2/Ste12-Rev1, respectively (Supplementary Table 1). The 5' regions of Ste12-KO1 and Ste12-KO2 contained the complementary sequence of the M13 reverse and M13 forward primers, respectively, which were used to amplify the cassette containing the hygromycin B resistance gene under control of the *Aspergillus nidulans gpdA* promoter (Punt et al. 1987). In the second reaction, 100 ng of each flanking region was mixed with 100 ng of the hygromycin B resistance cassette and used as templates to fuse each flanking region to partially overlapping truncated versions of the hygromycin B resistance gene, using primer combinations Ste12-For/Hyg-Y and Ste12-Rev1/Hyg-G, respectively. The obtained split-marker fragments were used to cotransform protoplasts of the *F. oxysporum* wild-type strain or the Δ *fmk1* mutant to hygromycin resistance, and transformants were purified by monoconidial isolation as described (Di Pietro and Roncero 1998). Transformants showing homologous insertion of the construct were detected by PCR of genomic DNA with primers Ste12-13 and M13 reverse, and confirmed by Southern analysis of genomic DNA treated with *Eco*RI and hybridized with a labeled probe obtained by PCR amplification with primers Ste12-KO2 and Ste12-Rev1. For complementation experiments, a 5.4-kb DNA fragment encompassing the entire *ste12* gene was amplified by PCR from *F. oxysporum* genomic DNA using primers Ste12-13 and Ste12-Rev2 and introduced

into protoplasts of the Δ *ste12* mutant by cotransformation with the phleomycin resistance cassette amplified from plasmid pAN8-1 (Mattern et al. 1988). Phleomycin-resistant transformants were selected as described (Di Pietro et al. 2001), and the presence of the wild-type *ste12* allele in the complemented transformants was detected by PCR of genomic DNA with primers Ste12-13 and Ste12-Rev2 and by Southern analysis with the *ste12* probe.

Cell-wall-degrading enzyme activity assays.

Plate assays for polygalacturonase, cellulase, and amylase activities were performed by determining the clear halo surrounding fungal colonies after precipitation or staining of the substrates PGA, carboxymethyl cellulose (CMC), or starch, respectively (all from Sigma, Madrid). Aliquots of 2×10^5 microconidia were spotted onto solid synthetic medium (2% glucose, 0.2% ammonium sulfate, and 1.5% agar) adjusted to pH 7 with 0.1 M phosphate buffer and supplemented with 0.5% (wt/vol) of the appropriate substrate. Colonies were grown for 3 days at 28°C. PGA plates were precipitated for 5 min with 0.4 N HCl and washed thoroughly with water. CMC plates were stained for 30 min with 0.1% Congo Red (Sigma), followed by 30 min destaining with 0.1 M NaCl. Starch plates were precipitated with 100% ethanol, washed thoroughly with water, and kept for 24 h at 4°C. All experiments included four replicates and were performed twice with similar results.

Virulence and root adhesion assays.

Tomato plant inoculation assays were performed in a growth chamber as described (Di Pietro and Roncero 1998). At different times after inoculation, severity of disease symptoms was recorded using an index ranging from 1 (healthy plant) to 5 (dead plant). Ten plants were used for each treatment. Invasive growth assays on tomato fruit (cv. Daniela) and apple slices (cv. Golden Delicious) were carried out as described previously (Di Pietro et al. 2001; Sánchez López-Berges et al. 2009), using three replicates. Root adhesion assays were performed as described (Di Pietro et al. 2001) using three replicates. All virulence and root adhesion experiments were performed three times with similar results.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Broad Institute *Fusarium* comparative database homepage:
www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html