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fost12, the *Fusarium oxysporum* homolog of the transcription factor Ste12, is upregulated during plant infection and required for virulence ☆

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ABSTRACT

We have identified a *Fusarium oxysporum* homolog of the Ste12 transcription factor that regulates mating and filamentation in *Saccharomyces cerevisiae*. The corresponding gene, *fost12*, from a highly virulent strain of *F. oxysporum* f. sp. *phaseoli*, was confirmed to share a high level of similarity and possessed the STE and C2H2 domains characteristic of the fungal Ste12 transcription factor family of proteins. Disruption of *fost12* resulted in no visible alterations of colony morphology or *in vitro* growth characteristics. However, the disruption mutants showed a substantial reduction in virulence when inoculated in common bean seedlings. *In planta* transcription of *fost12* is drastically increased between 12 and 24 h after inoculation, as detected by real-time RT-PCR. The results of the transcriptional analyses carried out in several *F. oxysporum* strains during axenic growth suggest that the *fost12* gene product is a virulence factor required to deal with the nutritional stress confronted by the pathogen during host plant colonization.

1. Introduction

Fusarium oxysporum is an ubiquitous fungus able to display different lifestyles and modes of nutrition to survive as a saprophyte or infect plants and cause vascular disease. An unanswered question regarding the adaptation to these different roles is how the fungus detects and responds to environmental changes.

In eukaryotic cells, the family of serine/threonine protein kinases known as mitogen-activated protein (MAP) kinases is involved in the transduction of a variety of extracellular signals and the regulation of several developmental processes. The *F. oxysporum fmk1* gene encodes a MAP kinase of the YERK1 subfamily, and mutants of *F. oxysporum* f. sp. *lycopersici* carrying an inactivated copy of *fmk1* are defective in pathogenicity on tomato plants (Di Pietro et al., 2001). These results demonstrated that a MAP kinase pathway must be involved in pathogenicity and/or virulence.

In *Saccharomyces cerevisiae* the transcription factor Ste12 is activated by the Fus3 and Kss1 MAPKs for regulating the mating and filamentation processes, respectively. Homologs of Ste12 identified in several pathogenic fungi have been shown to be involved in pathogenicity. In *Magnaporthe oryzae* the appressoria formed by mutants defective in *MST12* have normal appressorium turgor but fail to develop penetration pegs, thus indicating that the transcription factor Ste12 is essential for pathogenesis (Park et al., 2004). In contrast, the *Cst1* product is essential for penetration and infectious growth but dispensable for appressorium formation in *Colletotrichum lagenarium* (Tsuji et al., 2003). Mutants of *Cryphonectria parasitica* defective in *CpSTE12* show reduced virulence and are female sterile (Deng et al., 2007); in addition many genes affected by hypovirus are down-regulated in these mutants. *Cph1*, the homolog of STE12 in the human pathogen *Candida albicans*, is required for hyphal morphogenesis, invasive hyphal growth and virulence while the Ste12alpha and Ste12a gene products of *Cryptoccocus neoformans* are important for virulence in a serotype specific manner (Yue et al., 1999; Chang et al., 2000), but are largely dispensable for mating.

F. oxysporum is a soil-borne vascular pathogen which shows an infectious strategy different to the ones exhibited by the plant pathogens above mentioned. From this point of view it would be interesting to determine whether a STE12 homolog is also expressed in this fungus and, if so, what is the role of this transcription factor during the infection process. In previous reports we have described highly virulent strains of *F. oxsyporum* f. sp. *phaseoli*

☆ Nucleotide sequence accession number: nucleotide sequence data for the *Fusarium oxysporum* f.sp. *phaseoli* fost12 gene is available in the GenBank database under Accesion No. EF450109.

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that are able to kill the host (*Phaseolus vulgaris* L.) in about 2 weeks and weakly virulent strains that cause a lower degree of damage (Alves-Santos et al., 2002b). These differences in the amount of damage caused to the host suggests a differential expression of genes involved in virulence. Our recent findings in the expression of the transcription factor ftf1 (Ramos et al., 2007) not only support this hypothesis, but also indicate that different degrees of virulence may correlate with the presence or absence of certain genes.

The objectives of the present work were to confirm that *F. oxysporum* has a functional homolog of Ste12, to analyze its expression pattern both during *in vitro* culture and *in planta* infection and to shed light on its role in virulence towards the host plant.

2. Materials and methods

2.1. Fungal strains and culture conditions

The *F. oxysporum* strains used in this study were FOP-SP1, FOP-SP2, FOP-SP4, FOP-SP8 and AB82. All were collected in El Barco de Avila (Spain). FOP-SP1 and FOP-SP2 are highly virulent pathogens (*F. oxysporum* Schlechtend:Fr f.sp. *phaseoli* J.B. Kendrick and W.C. Kendrick) that belong to the same VCG (VCG 167), race (race 6), and IGS polymorphism group (IGS A); FOP-SP4 and FOP-SP8 are weakly virulent pathogens (*F. oxysporum* Schlechtend:Fr f.sp. *phaseoli* J. B. Kendrick and W. C. Kendrick), that belong to the same VCG (VCG 166), race (race 6), and IGS polymorphism group (IGS A), while AB82 is a non-pathogenic strain (VCG 167 and IGS polymorphism A) (Alves-Santos et al., 1999, 2002a). Fungal cultures were established from colonies resulting from the subculture of a single conidium stored on 25% glycerol v/v at −80 °C (Alves-Santos et al., 1999). All strains were grown in potato dextrose broth (PDB; Difco Laboratories, Detroit) or potato dextrose agar (PDA; Difco Laboratories) as previously described (Alves-Santos et al., 1999). Cultures were incubated at 22–28 °C with continuous light for 5 days (liquid cultures) or up to 1 week (solid media). For the analysis of gene expression, freshly obtained microconidia were germinated for 4 days in YPSS medium (Talbot et al., 1993) at 150 rpm and 28 °C. Mycelia were washed twice in sterile water and incubated for 12 h in synthetic liquid medium (Di Pietro and Roncero, 1996) containing 1% (w/v) glucose as the carbon source and 0.1% (w/v) NH_4NO_3 as the nitrogen source (SM), synthetic medium lacking the carbon source (SM-C) and synthetic medium lacking the nitrogen source (SM-N). Media were buffered to pH 4 with 100 mM NaH_2PO_4 and 100 mM NaCl, to pH 6 with 50 mM Na_2HPO_4 and 50 mM NaH_2PO_4, and to pH 8 with 100 mM Na_2HPO_4. Mycelia were collected by filtration through two layers of cheesecloth.

2.2. Pathogenicity tests

Inoculations of *P. vulgaris* L. cv. Blanca Riñón with conidia from different strains of *F. oxysporum* f. sp. *phaseoli* were made as previously described (Alves-Santos et al., 1999, 2002a). All the strains were simultaneously tested in each inoculation experiment to reduce the variability in the cultivar response due to environmental conditions. Each inoculation was repeated three times and 20 replica plants were included per treatment. Disease progression rate (DPR) was measured using the formula $DPR = \Sigma_i X_i/n$, where X_i = CIAT disease index and n = days after inoculation.

2.3. Nucleic acid manipulations

Genomic DNA was extracted from *F. oxysporum* mycelium according to the procedures previously described (Alves-Santos et al., 1999, 2002b; Ramos et al., 2007). DNA fragments were purified from gels using the GeneClean Kit (Bio 101, Calsbad, CA) and cloned into pGEM-T-Easy vector (Promega, Madison, WI) or pBluescript KS+ vector (Stratagene, La Jolla, CA). GeneClean Turbo Kit (Bio 101, Calsbad, CA) was used to purify DNA fragments obtained following PCR. Restriction enzyme digestions, agarose gel fractionation, ligations and transformations of *Escherichia coli* strain DH5α, were performed following standard protocols (Sambrook et al., 1989). Plasmid DNA preparations were purified using the High Pure Isolation kit (Roche Diagnostics, Mannheim, Germany). Total RNA was isolated from frozen mycelium by using the guanidine hydrochloride protocol (Logemann et al., 1987). Southern and Northern analyses were carried out as described previously (Ramos et al., 2007). DNA probes were labeled with Digoxigenin-dUTP by the PCR method using the Biotools DNA polymerase (B&M Labs, SA) and genomic DNA or cDNA from strain FOP-SP1 as template. Prehybridization, hybridization, washings and detection were performed using a chemiluminescent detection procedure with CDP-Star (Roche Diagnostics) according to the manufacturer's recommendations. Primer sequences used to obtain the PCR amplicons employed as probes for the Southern or Northern blots are listed in Table 1.

Table 1
Oligonucleotides used in this study.

Oligonucleotides	Oligonucleotides sequence and orientation[a]	Amplicon size (bp)
STE12Deg1	(+) GTY GAY TCR CAR CCY GAY CA	378
STE12Deg2	(−) GAA GAA RAC YTT YTG YTT YTT YTG	
12.11B	(−) GCCGTTACATCATACCGCTGG	2369
12.13A	(+) GCCATCGACTCGACTGACCAA	
12.22	(−) AGAGACTGGTAAGAAGAAGGGGCG	2801
12.13A	(+) GCCATCGACTCGACTGACCAA	
12*Hind*IIID	(+) AGAAGCTTCCAATCCCAGCCTAAT	1077
12*Xba*I	(−) TTGTCTAGACTTGCTGCAGGGC	
12*Eco*RIB	(−) GGGGGCAGAATTCTACGCTTGAG	1094
12SacIE	(+) TCACGAGCTCTTGCCCCATTCCC	
HphA	(−) CACAGCCATCGGTCCAGACG	636
HphB	(+) GCCTGAAACCGAACTGCCCG	
FOST12-1589F	(+) CCACTGCAGTAAAGCATTCTCAAGA	82
FOST12-1589R	(−) ACCATCTCCGCCATCTTCAC	
FOST12-1589M1	(+) FAM-ACCTGGCGCAACACAA-MGB	
BTUBFOX-187F	(+) CGAGCGCATGAGTGTCTACTTC	87
BTUBFOX-187R	(−) TGGTACCAGGCTCAAGATCGA	
BTUBFOX-187 M	(+) FAM-TTGCCAGAGGCCTCGTT-MGB	

The underlined regions are added restriction sites according to the name of each primer.

[a] Sequence and orientation of the oligonucleotides relative to the C-terminal (+) or the N-terminal (−) region of the deduced protein.