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The two-component histidine kinase Fhk1 controls stress adaptation and virulence of *Fusarium oxysporum*

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SUMMARY

Fungal histidine kinases (HKs) have been implicated in different processes, such as the osmostress response, hyphal development, sensitivity to fungicides and virulence. Members of HK class III are known to signal through the HOG mitogen-activated protein kinase (MAPK), but possible interactions with other MAPKs have not been explored. In this study, we have characterized *fhk1*, encoding a putative class III HK from the soil-borne vascular wilt pathogen *Fusarium oxysporum*. Inactivation of *fhk1* resulted in resistance to phenylpyrrole and dicarboximide fungicides, as well as increased sensitivity to hyperosmotic stress and menadione-induced oxidative stress. The osmosensitivity of Δ*fhk1* mutants was associated with a striking and previously unreported change in colony morphology. The Δ*fhk1* strains showed a significant decrease in virulence on tomato plants. Epistatic analysis between Fhk1 and the Fmk1 MAPK cascade indicated that Fhk1 does not function upstream of Fmk1, but that the two pathways may interact to control the response to menadione-induced oxidative stress.

INTRODUCTION

Two-component histidine kinase (HK) phosphorelay protein complexes are important components of the signal-sensing machinery of bacteria, plants and fungi, allowing them to sense and adapt to their environment (Catlett *et al*., 2003; West and Stock, 2001). In prokaryotes, this signalling complex is generally composed of two distinct proteins, HK and a response regulator (RR). In response to environmental stimuli, HK autophosphorylates a conserved histidine residue and transfers the phosphoryl group to an aspartic acid residue in the RR protein, leading to changes in gene expression and cell response (West and Stock, 2001). Most eukaryotic two-component systems are composed of hybrid HKs in which the HK and RR domains are present in a single protein. In this case, the phosphate group on the aspartate residue of the HK RR domain is transferred to a histidine phosphotransfer (HPT) protein which, in turn, phosphorylates an aspartate residue of the receiver domain on a RR protein (West and Stock, 2001). Although bacterial HK signalling systems control the cell response through direct activation of gene transcription by the RR protein, eukaryotic HKs are generally found at the head of intracellular signalling pathways that recruit more conventional downstream signalling modules, such as mitogen-activated protein kinase (MAPK) cascades (West and Stock, 2001).

Two-component systems have been well studied in the yeast *Saccharomyces cerevisiae*. The *S. cerevisiae* genome contains one HK, Sln1, one HPT protein, Ypd1, and two RRs, Ssk1 and Skn7. Sln1 acts as a sensor HK, transmitting the high-osmolarity response signal via the Ypd1–Ssk1 phosphorelay to the Ssk2 (Ssk22)/Pbs2/Hog1 MAPK module. Under hyperosmotic conditions, Sln1 is in an unphosphorylated state that is conducive to the activation of the downstream Hog1 MAPK (Hohmann, 2002; Saito and Tatebayashi, 2004). Sln1 also mediates the phosphorylation of Skn7, which acts as a transcription factor during hyperosmotic and oxidative stress and interacts with the cell wall integrity (CWI) MAPK and the calcium/calcineurin pathways (Levin, 2005; Li *et al*., 1998; Morgan *et al*., 1997; Williams and Cyert, 2001). In addition to Sln1, the Hog1 MAPK cascade can be activated by an alternative osmosensing branch via Sho1, Ste11 and Pbs2 (Posas and Saito, 1997).

In filamentous fungi, orthologues of the elements of the yeast Hog1 pathways have been identified by functional and *in silico* analysis (Dixon *et al*., 1999; Fujimura *et al*., 2003; Han and Prade, 2002; Izumitsu *et al*., 2007; Rispail *et al*., 2009; Segmüller *et al*., 2007; Yamashita *et al*., 2008; Zhang *et al*., 2002). For example, *Neurospora crassa* Os-2 is orthologous to yeast Hog1 (Zhang *et al*., 2002) and is activated via the MAPKKK Os-4 and the MAPKK Os-5 (Fujimura *et al*., 2003). Orthologues of Sln1, Sho1, Ypd1, Ssk1, Skn7 and Ste11 are also present in *N. crassa* (Jones *et al*., 2007; Rispail *et al*., 2009). However, the *N. crassa* HK responsible for the high-osmolarity response, Nik-1/Os-1, is not the orthologue of yeast Sln1 (Catlett *et al*., 2003).

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Filamentous fungi contain multiple HKs that have been classified into 11 groups on the basis of the protein sequence (Catlett *et al*., 2003). In filamentous ascomycetes, so far, only members of HK class III have been associated with the high-osmolarity response, including Nik-1/Os-1 in *N. crassa* (Ochiai *et al*., 2001), NikA in *Aspergillus nidulans* (Hagiwara *et al*., 2007), Daf1/Bos1 in *Botrytis cinerea* (Cui *et al*., 2002; Viaud *et al*., 2006), Nik1 in *Cochliobolus heterostrophus* and *Alternaria brassicicola* (Avenot *et al*., 2005; Yoshimi *et al*., 2004) and Hik1 in *Magnaporthe oryzae* (Motoyama *et al*., 2005a). In addition to osmosensitivity, mutations in class III HKs also confer resistance to certain classes of fungicides, and often result in morphological defects (Hagiwara *et al*., 2007; Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004).

Class III HKs have been implicated in the dimorphism and virulence of several human pathogens, such as *Blastomyces dermatiditis* (Nemecek *et al*., 2006), *Candida albicans* (Yamada-Okabe *et al*., 1999), *Histoplasma capsulatum* (Nemecek *et al*., 2006), *Aspergillus fumigatus* (Clemons *et al*., 2002) and *Cryptococcus neoformans* (Bahn *et al*., 2006). The role of class III HKs in the virulence of plant pathogenic fungi is less clear, and depends not only on the fungal species but also on the type of mutation. Thus, the deletion of class III HKs strongly reduced the virulence in *B. cinerea* and *A. brassicicola* (Cho *et al*., 2009; Liu *et al*., 2008; Viaud *et al*., 2006), but had no effect in *M. oryzae* (Motoyama *et al*., 2005a). Similarly, point mutations in the class III HK *Mf-os1* decreased the virulence of *Monilinia fructicola* (Ma *et al*., 2006), but had no such effect in naturally occurring fungicide-resistant field isolates of *B. cinerea* or *A. brassicicola* (Avenot *et al*., 2005; Cui *et al*., 2002).

Fusarium oxysporum, a ubiquitous soil-borne ascomycete, causes vascular wilt disease on more than 100 plant species, provoking severe losses in important crops, such as banana, cotton, melon and tomato (Gordon and Martyn, 1997). This fungus is also being recognized as an emerging human pathogen which poses a lethal threat to immunocompromised individuals (Nucci and Anaissie, 2007). Its remarkably broad host range and the array of molecular tools available make *F. oxysporum* an attractive model for studying the different aspects of fungal infection (Di Pietro *et al*., 2003; Michielse and Rep, 2009). It has been shown previously that Fmk1, an orthologue of the yeast mating and filamentation MAPKs Fus3/Kss1, and one of its downstream targets, the transcription factor Ste12, are crucial for the virulence of *F. oxysporum* on tomato plants (Di Pietro *et al*., 2001; Rispail and Di Pietro, 2009). Mutants lacking the *fmk1* gene are impaired in multiple virulence-related functions, such as root adhesion, host penetration, invasive growth on living plant tissue and secretion of cell wall-degrading enzymes, as well as in vegetative hyphal fusion, a ubiquitous process in filamentous fungi (Delgado-Jarana *et al*., 2005; Di Pietro *et al*., 2001; Prados Rosales and Di Pietro, 2008). In contrast with the pleiotropic role of Fmk1, the function of *F. oxysporum* Ste12 is restricted to host penetration, invasive growth and virulence (Rispail and Di Pietro, 2009). In addition to Fmk1, *F. oxysporum* has two additional MAPKs orthologous to yeast Mpk1 and Hog1 (Rispail *et al*., 2009), respectively, as well as at least 21 HKs (N. Rispail and A. Di Pietro, unpublished work). The potential role of these additional signalling modules in the virulence of *F. oxysporum* is currently unknown.

In this study, we investigated the role of *fhk1*, encoding a class III HK of *F. oxysporum*. Our study addressed three major questions: (i) is Fhk1 required for correct cellular adaptation to stresses?; (ii) is Fhk1 required for root infection and virulence?; and (iii) does Fhk1 interact with the Fmk1/Ste12 signalling pathway? We found that deletion mutants lacking the *fhk1* gene were resistant to phenylpyrrole and dicarboximide fungicides, showed increased sensitivity to hyperosmotic and oxidative stresses, and displayed reduced virulence on tomato plants. Interestingly, the increased sensitivity of Δ*fhk1* strains to oxidative stress was restored to the wild-type level in a Δ*fmk1*Δ*fhk1* double mutant, providing new genetic evidence for an interaction between the Fmk1 and Fhk1 signalling pathways.

RESULTS

Cloning and targeted knockout of the *F. oxysporum fhk1* gene

A BLASTP search for orthologues of class III HK in the annotated genome of *F. oxysporum* f. sp. *lycopersici* strain 4287, available at the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html), identified a single gene, *FOXG_01684* (subsequently termed *fhk1* for *Fusarium* histidine kinase 1). Comparison with the orthologous gene of the related species *F. verticillioides* (*FVEG_08048*; AY456038) suggested that the predicted open reading frame of *FOXG_01684* lacked the N-terminal part of the protein as a result of sequencing errors. A 6.8-kb genomic region encompassing the complete *fhk1* gene, including the promoter, coding region and terminator, was sequenced manually, revealing an open reading frame of 3882 bp interrupted by five putative introns, according to the Fgenesh 2.6 prediction server (http://linux1.softberry.com/berry.phtml?topic=fgenesh & group=programs&subgroup=gfind). The *fhk1* gene encodes a putative 1293-amino-acid polypeptide with a predicted molecular mass of 141.83 kDa and a p*I* value of 5.37. The sequence of the *F. oxysporum fhk1* gene has been deposited in GENBANK under accession number GQ871928. Analysis of the Fhk1 sequence with the InterProScan prediction server (http://www.ebi.ac.uk/Tools/InterProScan/) detected all the characteristic domains of class III HKs, including five HAMP repeats (IPR003660) (Aravind and Ponting, 1999), the HK A signal transducer domain (HK;

IPR003661), the HK-like ATPase domain (HATPase; IPR003594) and the RR domain (REC; IPR001789), as well as the highly conserved H-, N-, G1-, G2- and D-boxes of the HK and REC catalytic site (West and Stock, 2001) (Fig. 1A). Alignment of *F. oxysporum* Fhk1 with sequences from the databases revealed significant identity with class III HKs from other fungi, including *F. verticillioides* GmNik1 (98.9% identity), *N. crassa* Os-1 (80.0%), *M. oryzae* Hik1 (77.6%), *C. heterostrophus* Nik1 (61.4%) and *Candida albicans* Nik1 (47.3%) (Fig. 1B,C). On the basis of these data, we conclude that *F. oxysporum fhk1* is the orthologue of *N. crassa os-1*.

To explore the biological role of Fhk1 in *F. oxysporum*, a Δ*fhk1* null allele was generated by replacing most of the *fhk1* open reading frame with the hygromycin resistance cassette, using a polymerase chain reaction (PCR) fusion method (Fig. S1A, see Supporting Information; see Experimental procedures for details). The knockout construct was introduced into both the wild-type strain and the Δ*fmk1* mutant to study possible epistatic relationships between the two genes. Hygromycin-resistant transformants were analysed by PCR with different combinations of gene-specific primers, identifying six and one transformants, respectively, which produced amplification products indicative of homologous integration-mediated gene replacement (data not shown). Southern blot analysis of these transformants confirmed the replacement of a 6-kb *Eco*RI fragment, corresponding to the wild-type *fhk1* allele, by a fragment of 2.8 kb (Fig. S1B, see Supporting Information), demonstrating that these transformants, named Δ*fhk1* and Δ*fmk1*Δ*fhk1*, respectively, lacked a functional *fhk1* gene. By contrast, transformant *efhk1-1* still showed the wild-type fragment, together with another hybridizing fragment, suggesting that it contained an ectopic insertion of the knockout construct (Fig. S1B, see Supporting Information).

To confirm that the phenotype of the Δ*fhk1* mutants was indeed caused by a loss of *fhk1* function, a 9-kb DNA fragment encompassing the complete *F. oxysporum fhk1* gene was introduced into the Δ*fhk1-2* strain by cotransformation with the phleomycin resistance marker. PCR with gene-specific primers Fhk1-1 and Fhk1-2 produced a 6-kb amplification product, identical to that obtained from the wild-type strain, in two phleomycin-resistant transformants, but not in the Δ*fhk1-2* mutant (Fig. S1C, see Supporting Information). We conclude that these cotransformants, denominated Δ*fhk1*+*fhk1*, had integrated an intact copy of the *fhk1* gene into the genome.

Fhk1 is not required for vegetative hyphal growth

To test whether *fhk1* was required for vegetative hyphal growth, the colony growth rate was determined on synthetic medium (SM) adjusted to pH 4.5 or pH 6.5. As reported previously (Prados Rosales and Di Pietro, 2008), the Δ*fmk1* strain showed a significant reduction in growth rate compared with the wild-type at both pH values (Fig. S2, see Supporting Information). By contrast, no significant difference was observed between the wild-type and Δ*fhk1* mutants, or between the Δ*fmk1* single mutant and the Δ*fmk1*Δ*fhk1* double mutant (Fig. S2, see Supporting Information). This suggests that *fhk1* is not required for normal vegetative growth on artificial medium as reported for other class III HKs (Cho *et al*., 2009; Motoyama *et al*., 2005a).

Fhk1 mediates the sensitivity to phenylpyrrole and dicarboximide fungicides

Fungal class III HKs have been shown to confer sensitivity to different classes of fungicide (Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006). We thus tested whether the deletion of *fhk1* in *F. oxysporum* affected the sensitivity to phenylpyrrole and dicarboximide fungicides. Both the wild-type and Δ*fmk1* strains showed a dramatic decrease in growth rate on SM supplemented with 10 μg/mL of fludioxonil or iprodione, compared with SM without fungicide (Fig. 2). By contrast, growth rates of Δ*fhk1* and Δ*fmk1*Δ*fhk1* mutants were not affected by the presence of either type of fungicide. Complementation of Δ*fhk1* with the native *fhk1* gene restored fungicide sensitivity to the wild-type level (Fig. 2). We conclude that Fhk1 mediates the sensitivity of *F. oxysporum* to phenylpyrrole and dicarboximide fungicides.

Fhk1 is required for cellular adaptation to hyperosmotic stress

In addition to fungicide sensitivity, fungal class III HKs have been associated with the response to osmotic stress by acting at the head of the Hog1 MAPK pathway (Liu *et al*., 2008; Yoshimi *et al*., 2005). To determine whether Fhk1 plays a role in osmoregulation, we determined colony growth on SM containing 0.8 M sodium chloride, 0.8 M potassium chloride or 1.2 M glycerol. All three types of osmolyte produced moderate growth inhibition (30%) in the wild-type and the complemented Δ*fhk1*+*fhk1* strains, but only slight growth inhibition in the Δ*fmk1* mutant (15%) (Fig. 3A).

Under salt stress (NaCl and KCl), the Δ*fhk1* mutants showed a similar reduction in radial growth to the wild-type strain, whereas polyol-induced osmotic stress led to a more pronounced growth inhibition, indicating that Δ*fhk1* mutants are more sensitive than the wild-type to high concentrations of glycerol (Fig. 3A). Under all three conditions of hyperosmotic stress, the colony morphology of the Δ*fhk1* mutants differed drastically from that of the wild-type. In contrast with the wild-type or Δ*fmk1* strain, whose colony morphology was normal, albeit with a slower growth rate, colonies of the Δ*fhk1* mutants were very compact with extremely short extending hypha

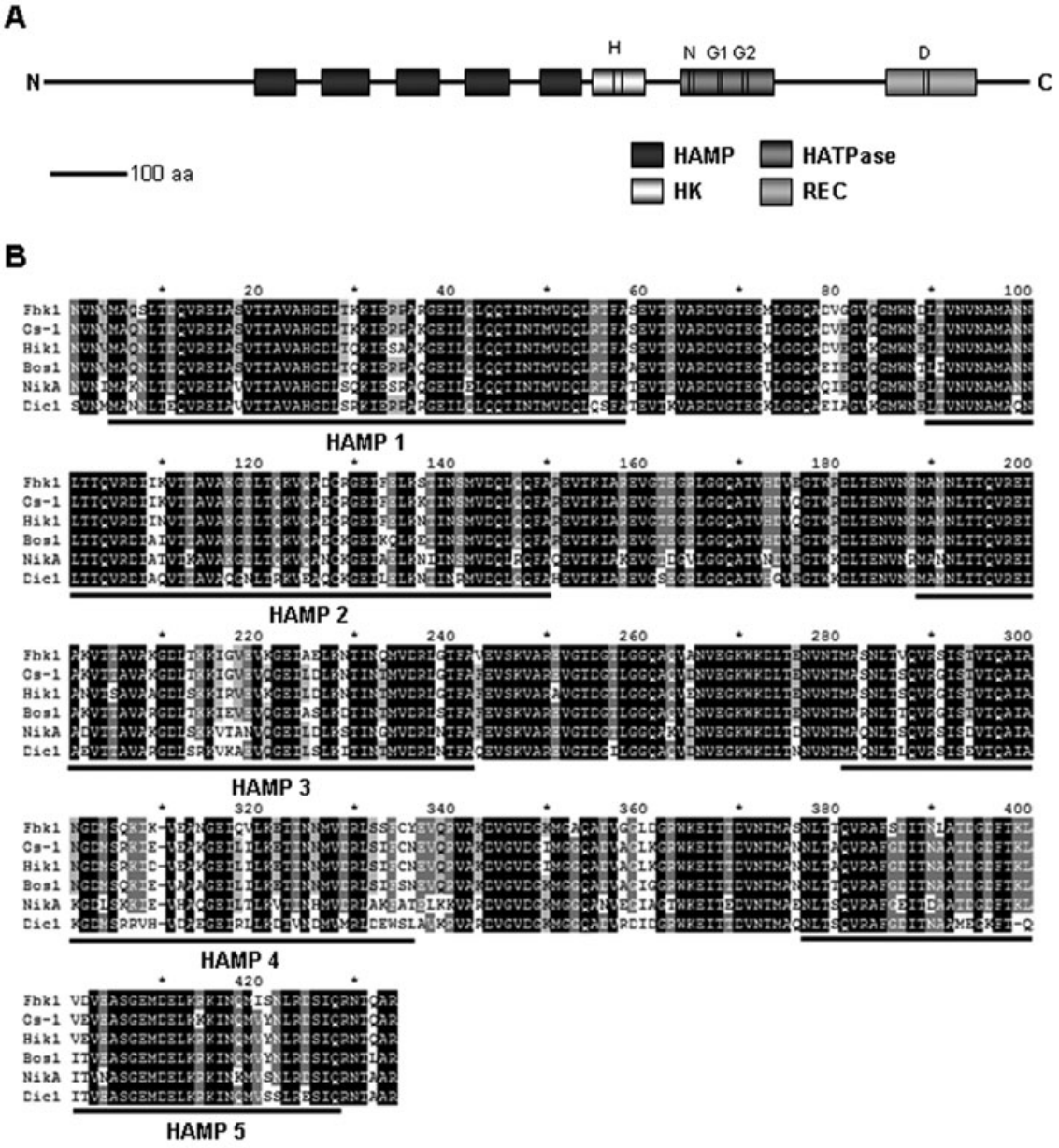

Fig. 1 Fhk1 protein structure. (A) Scaled diagram of the domain structure of *Fusarium oxysporum* Fhk1. The positions of the conserved H-, N-, G1-, G2- and D-boxes are shown. (B) Amino acid alignment of the HAMP repeat region of Fhk1 with *Neurospora crassa* Os1 (AAB01979), *Magnaporthe grisea* Hik1 (BAB40947), *Botrytis cinerea* Bos1 (AAL30826), *Aspergillus nidulans* NikA (AN4479.3) and *Cochliobolus heterostrophus* Dic1 (BAC78679). HAMP domain repeat regions are indicated by black lines. Absolutely conserved residues are shaded in black, residues conserved in at least 80% are shaded in dark grey and residues conserved in at least 60% are shaded in light grey. (C) Cladogram generated by the maximum likelihood method after alignment of *F. oxysporum* Fhk1 with *F. verticillioides* GmNik1 (AAR30126), *F. solani* f. sp. *pisi* Fik (AAD09491), *Neurospora crassa* Os1 (AAB01979), *Magnaporthe grisea* Hik1 (BAB40947), *Botrytis cinerea* Bos1 (AAL30826), *Aspergillus nidulans* NikA (AN4479.3), *Cochliobolus heterostrophus* Dic1 (BAC78679), *Alternaria brassicicola* AbNik1 (AAU10313), *Monilinia fructicola* Mfos1 (ABF60145), *Candida albicans* CaNik1 (BAA24952), *Ajellomyces dermatiditis* Drk1 (ABF13477) and *Cryptococcus neoformans* Tco1 (ABD49452). *Saccharomyces cerevisiae* Sln1 (NP_012119) was included as outgroup.

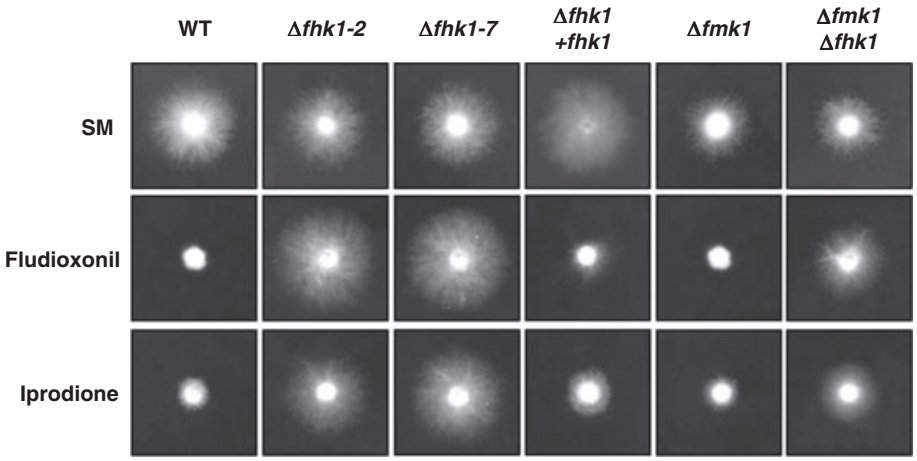

Fig. 2 Fhk1 mediates sensitivity to phenylpyrrole and dicarboximide fungicides. Conidia of the indicated strains were spotted onto plates containing synthetic medium (SM), or SM with 10 μg/mL fludioxonil or 10 μg/mL iprodione, and grown at 28 °C for 3 days.

(Fig. 3A,B). The Δ*fmk1*Δ*fhk1* double mutant showed the same morphological defects as Δ*fhk1*, and a more pronounced decrease in growth rate compared with the Δ*fmk1* strain. Collectively, these results indicate that: (i) Fhk1 is required for correct adaptation to hyperosmotic stress; (ii) Fmk1 acts as a negative regulator of osmotic stress adaptation; and (iii) Fmk1 and Fhk1 act in independent and additive pathways, as the double mutation leads to a more drastic phenotype.

Fhk1 contributes to the oxidative stress response

The oxidative stress response in fungi has been shown to depend on both the Hog1 MAPK and CWI pathways, depending on the nature of the stress-inducing compound (Levin, 2005). As class III HKs have been suggested to act at the top of the Hog1 pathway in filamentous fungi (Liu *et al*., 2008; Yoshimi *et al*., 2005), we tested the effect of compounds inducing either oxidative (menadione, hydrogen peroxide) or nitrosative (sodium nitrite) stress on *F. oxysporum* wild-type and mutant strains. The addition of 10 μg/mL menadione induced only moderate growth inhibition in the wild-type and Δ*fmk1* strains, but had a more drastic effect in Δ*fhk1* mutants (Fig. 4). Interestingly, the double Δ*fmk1*Δ*fhk1* mutant only had a mild phenotype, similar to the wild-type and the Δ*fmk1* strains. No significant differences in growth inhibition between the different strains were observed in response to hydrogen peroxide (Fig. 4). Similarly, no differences were observed between the wild-type and Δ*fhk1* strains in response to 1 mM sodium nitrite (Fig. 4). However, nitrosative stress led to a significant decrease in growth rate in the Δ*fmk1* and Δ*fmk1fhk1* mutants (Fig. 4). We conclude that: (i) Fhk1 contributes to the tolerance to menadione-induced oxidative stress; (ii) Fmk1 negatively regulates the response to menadione-induced oxidative stress, acting either downstream or competing with Fhk1; and (iii) Fmk1 but not Fhk1 is required for nitrosative stress tolerance.

Fhk1 is required for full virulence on tomato plants

The Fmk1 MAPK controls invasive growth functions in *F. oxysporum*, such as the ability to penetrate cellophane sheets (Prados Rosales and Di Pietro, 2008) or to invade and colonize living fruit tissue (Di Pietro *et al*., 2001). We explored whether these invasive growth functions are also mediated by Fhk1. In contrast with Δ*fmk1* and the Δ*fmk1*Δ*fhk1* double mutant, Δ*fhk1* mutants penetrated cellophane sheets and colonized apple slices as efficiently as the wild-type strain (Fig. S3, see Supporting Information).

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. 5).

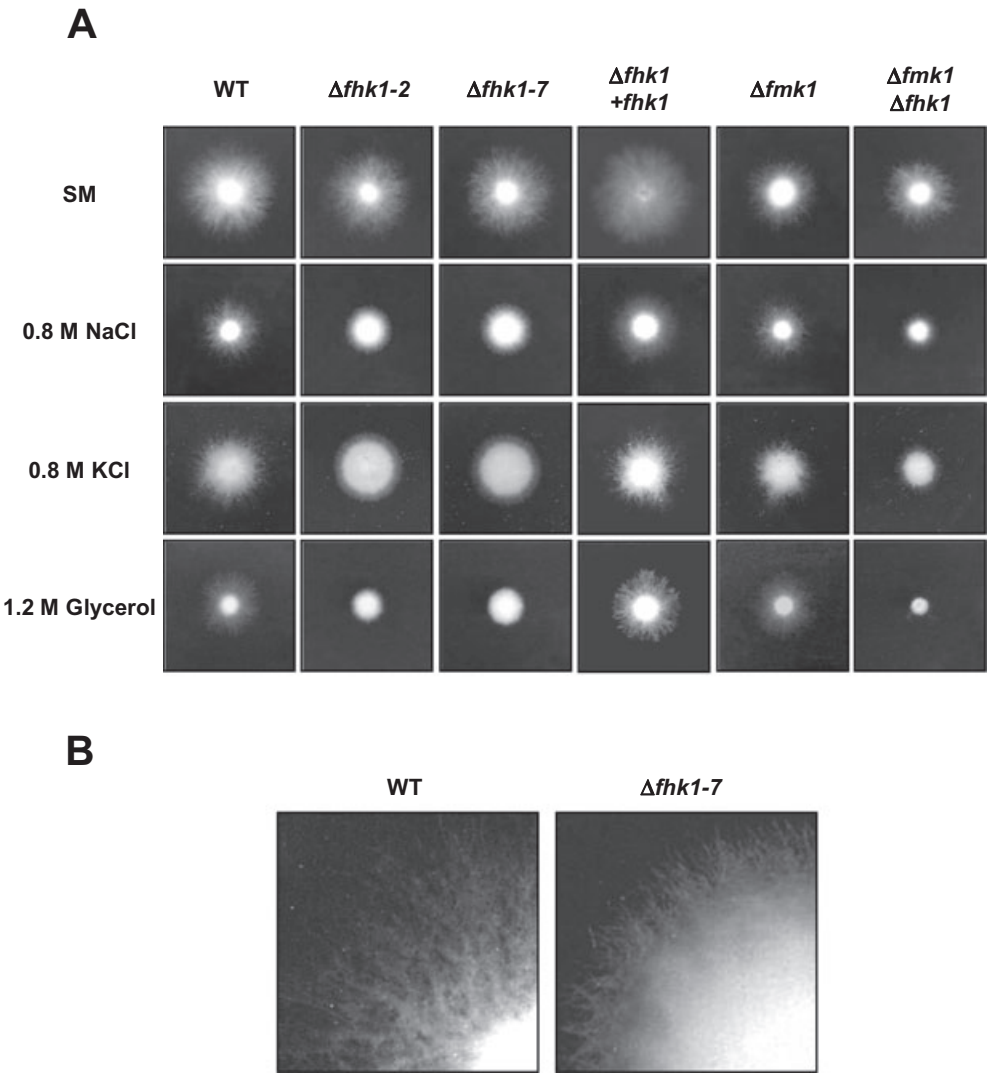

Fig. 3 Fhk1 is required for adaptation to osmotic stress. (A) Conidia of the indicated strains were spotted onto plates containing synthetic medium (SM), or SM with 0.8 M NaCl, 0.8 M KCl or 1.2 M glycerol, and grown at 28 °C for 3 days. (B) Detailed view of the colony edge after salt treatment (0.8 M NaCl) for wild-type and *Δfhk1* mutant strain.

As reported previously (Di Pietro *et al*., 2001), plants inoculated with the Δ*fmk1* mutant had extremely low disease ratings. Two independent Δ*fhk1* mutants, Δ*fhk1-2* and Δ*fhk1-4*, showed significantly reduced virulence compared with the wild-type, with a delay of approximately 10 days in symptom development. The Δ*fmk1*Δ*fhk1* double mutants behaved similarly to the Δ*fmk1* single mutant. We conclude that Fhk1 contributes to the virulence of *F. oxysporum* on tomato plants independent of Fmk1, although it does not mediate invasive growth functions.

DISCUSSION

Fungal HKs are involved in essential cellular processes, such as osmosensing, the oxidative stress response, cell cycle control and virulence (Bahn, 2008). In particular, class III HKs have been shown to mediate the cellular responses to osmotic and oxidative stresses (Motoyama *et al*., 2005a; Viaud *et al*., 2006), to control conidiation and asexual morphology (Liu *et al*., 2008; Viaud *et al*., 2006), to regulate the mould-to-yeast transition of dimorphic fungi (Nemecek *et al*., 2006) and to mediate pathogenicity (Liu *et al*., 2008; Nemecek *et al*., 2006; Viaud *et al*., 2006; Yamada-Okabe *et al*., 1999). However, the role of class III HKs in these different processes differs greatly according to the type of mutation and the fungal species. In this study, we characterized Fhk1, a class III HK from the plant and human pathogen *F. oxysporum*, and studied its role in the mediation of cellular adaptation to different stresses and virulence. In addition, we explored the potential interaction between this HK and

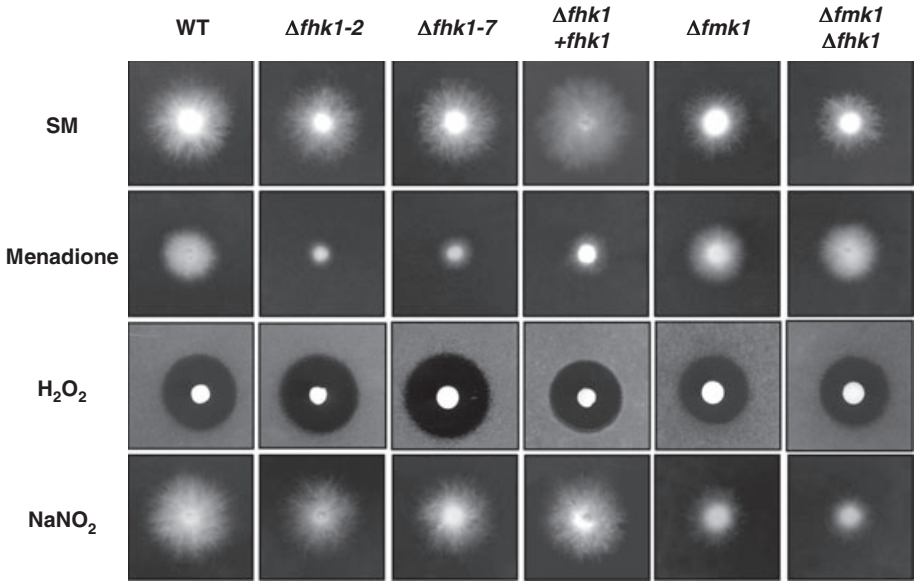

Fig. 4 Fhk1 and Fmk1 play opposing roles in the menadione-induced oxidative stress response. Conidia of the indicated strains were spotted onto plates containing synthetic medium (SM), or SM with 10 μg/mL menadione or 1 mM $NaNO_2$, and grown at 28 °C for 3 days. For H_2O_2 sensitivity assays, conidia were spread onto SM plates and grown for 1 day at 28 °C before placing a filter disc saturated with 3% H_2O_2 at the centre of the plates, followed by growth for two additional days at 28 °C. Growth inhibition was assessed by measuring the clear halo surrounding the filter disc.

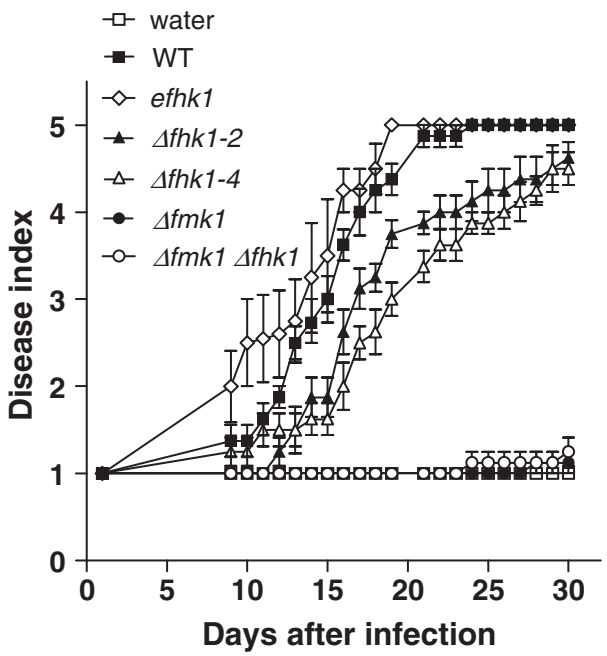

Fig. 5 Fhk1 is required for full virulence of *Fusarium oxysporum* on tomato plants. The graph shows the incidence of *Fusarium* wilt on tomato plants (cultivar Monica) inoculated with the indicated strains. The severity of disease symptoms was recorded at different times after inoculation, using an index ranging from one (healthy plant) to five (dead plant). Error bars represent standard errors calculated from 10 plants.

the pathogenicity MAPK Fmk1. As reported in other filamentous fungi, the predicted *F. oxysporum* Fhk1 protein contains all the characteristic domains of hybrid HKs, as well as the five HAMP repeats specific to class III fungal HKs (Catlett *et al*., 2003). Although the HK, HATPase and REC domains are strictly required for HK activation and its signal transduction function (Catlett *et al*., 2003; Motoyama *et al*., 2005a; West and Stock, 2001), the role of the HAMP domains remains unknown, although mutations in the HAMP domains of *os-1*, *dic1*, *abnik1* and *bos1* are responsible for the increased osmosensitivity and fungicide resistance of *N. crassa*, *C. heterostrophus*, *A. brassicicola* and *B. cinerea*, respectively (Avenot *et al*., 2005; Cui *et al*., 2002; Ochiai *et al*., 2001; Yoshimi *et al*., 2004). These phenotypes, together with the high sequence conservation of the HAMP region in filamentous fungi (Avenot *et al*., 2005) (Fig. 1B), strongly suggest that the HAMP region plays a pivotal role in Fhk1 function. The elucidation of this role will be an important challenge for future studies.

Function of Fhk1 in the stress response

In this study, we have demonstrated that Fhk1 is required for normal sensitivity of *F. oxysporum* to phenylpyrrole and dicarboximide fungicides (Fig. 2). This result confirms the involvement of class III HKs in the sensitivity to phenylpyrrole, dicarboximide and aromatic hydrocarbon fungicides reported for *N. crassa*, *M. grisea*, *C. heterostrophus*, *B. cinerea*, among others (Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004). Although the exact mode of action of these fungicides is still unclear, the finding that the heterologous expression of *M. oryzae hik1* in the yeast *S. cerevisiae* confers susceptibility to this otherwise resistant organism suggests that class III HKs are direct targets of these classes of fungicide (Motoyama *et al*., 2005b). Several lines of evidence indicate that fungicide-activated class III HKs recruit the conserved stress-activated Hog1 MAPK pathway, leading to the aberrant accumulation of glycerol and other stress-related responses (Hagiwara *et al*.,

2009; Kojima *et al*., 2004; Motoyama *et al*., 2005b; Yamashita *et al*., 2008; Yoshimi *et al*., 2005). For instance, the application of fungicide or osmotic stress has been shown to induce the hyperphosphorylation of Hog1, and mutants in Hog1 orthologues are resistant to fungicides (Kojima *et al*., 2004; Noguchi *et al*., 2007; Yoshimi *et al*., 2005). In addition, a recent microarray analysis has shown that the transcriptional response to the phenylpyrrole fungicide fludioxonil in *Aspergillus nidulans* is dependent on Hog1 orthologues and partly overlaps with the transcriptional response to hyperosmotic stress (Hagiwara *et al*., 2009). The fact that *fhk1*-deficient mutants of *F. oxysporum* show a higher sensitivity to hyperosmotic and menadione-induced oxidative stresses suggests that recruitment of the Hog1 pathway on activation of Fhk1 also occurs in *F. oxysporum*.

Higher sensitivity to osmotic stress, including salt and polyols, and to menadione has been reported for most mutants lacking class III HKs, although the exact effect of a given HK mutation on osmotic stress adaptation depends on the fungal species studied (Alex *et al*., 1996; Hagiwara *et al*., 2007; Motoyama *et al*., 2005a; Ochiai *et al*., 2001; Viaud *et al*., 2006; Yoshimi *et al*., 2004). Thus, mutants of *N. crassa*, *C. heterostrophus* and *B. cinerea* show a higher sensitivity to salt (NaCl or KCl) than to polyols (glycerol, sorbitol) (Alex *et al*., 1996; Viaud *et al*., 2006; Yoshimi *et al*., 2004), whereas *M. oryzae Δhik1* mutants show a strong growth reduction on sorbitol but not on salt (Motoyama *et al*., 2005a). In *F. oxysporum*, we detected a similar trend, as Δ*fhk1* mutants were more sensitive to glycerol than to salt stress. Strikingly, the growth of Δ*fhk1* mutants under both types of hyperosmotic stress was associated with a clear morphological phenotype: fungal colonies showed a dense and compact colony morphology with very short hyphae and more aerial mycelium than the wild-type strain (see Fig. 3B). To our knowledge, such a morphological switch in response to hyperosmotic stress has not been reported previously in filamentous fungi. Our finding indicates that the Hog1-dependent stress response may be more complex than previously thought, or that Fhk1 may recruit other signalling pathways in addition to Hog1. The fact that knockout mutants in *sak1*, the *B. cinerea* Hog1 orthologue, are still sensitive to fungicides supports this second hypothesis and suggests that other signalling pathways could also be recruited by these HKs (Liu *et al*., 2008).

In the dimorphic human pathogen *B. dermatiditis*, a class III HK has been shown to control the yeast-to-hyphal transition, a process related to pseudohyphal growth in *S. cerevisiae* which is mediated by the conserved MAPK Kss1 (Nemecek *et al*., 2006). In plant pathogenic fungi, Kss1 orthologues, such as *F. oxysporum* Fmk1, play a key role in plant pathogenicity (Di Pietro *et al*., 2001). As the upstream elements of Fmk1 are still unknown, we reasoned that Fhk1 could function as an upstream element of the Fmk1 pathway. As the Δ*fmk1* mutant had the same sensitivity to fungicides and to osmotic stress as the wild-type (see Figs 2 and 3), we conclude that Fmk1 is probably not a direct downstream element of Fhk1. However, we found evidence suggesting that the Fmk1 and Fhk1 pathways may interact to control the response to menadione-induced oxidative stress. Thus, the increased sensitivity to menadione of the Δ*fhk1* mutant was rescued to wild-type levels in the Δ*fmk1*Δ*fhk1* double mutant (see Fig. 4), indicating that Fmk1 may be a negative regulator of the oxidative stress response, and that one function of Fhk1 may be to inhibit Fmk1 during the response to oxidative stress. The role of Fmk1 in stress responses may be even more complex, as we observed that the Δ*fmk1* mutant is slightly more sensitive to nitrosative stress, which is generally thought to be under the control of the CWI pathway (Brown *et al*., 2009). Collectively, our results suggest that the pathogenicity MAPK Fmk1 may participate, at least partially, in controlling the responses to different types of stress. In *C. heterostrophus*, a recent report has shown that the pathogenicity MAPK Chk1, but not Hog1, controls the fungal response to oxidative stress after activation of its upstream element ChSte11 (Izumitsu *et al*., 2009). However, the phenotype described for the *C. heterostrophus chk1*-deficient mutant in response to oxidative stress is somewhat different from that of *F. oxysporum*, highlighting the complexity of stress signalling in filamentous fungi. The clarification of the connection between HKs, Fmk1, Hog1 and the CWI MAPK will be an important challenge to better understand how filamentous fungi adapt to changes in the environment.

Role of Fhk1 in the virulence of *F. oxysporum*

In addition to the important role of Fhk1 in fungicide sensitivity and the stress response, we found that *fhk1* deletion decreased significantly the virulence of *F. oxysporum* in tomato plants. HKs have been reported as pathogenicity determinants of human pathogens, such as *Candida albicans*, *B. dermatiditis*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (see Kruppa and Calderone, 2006 and references therein). In plant pathogens, the involvement of class III HKs in virulence has so far only been studied in air-borne pathogens, leading to conflicting results, with mutant phenotypes ranging from highly virulent to completely avirulent depending on the type of mutation, genetic background and fungal species studied. HKs are required for full virulence in the necrotrophic fungi *B. cinerea* and *A. brassicicola*, but not in the hemibiotroph *M. oryzae* (Cho *et al*., 2009; Liu *et al*., 2008; Motoyama *et al*., 2005a; Viaud *et al*., 2006). Thus, there is an important need to address the role of class III HKs in a soil-borne pathogen, such as *F. oxysporum*, which exists in a completely different lifestyle and environment. In this study, we have shown that, although Δ*fhk1* mutants retain their capacity to colonize fruit tissue and

penetrate cellophane membranes, they show a clear reduction in disease symptoms in tomato plants. The fact that Δ*fhk1* mutants are still able to cross cellophane and to colonize living fruit tissue suggests that the role of Fhk1 in virulence is mainly restricted to post-penetration events. Similarly, Δ*abnik1* and Δ*bos1* mutants of *A. brassicicola* and *B. cinerea*, respectively, were still able to form appressoria and to penetrate, but were defective in colonizing healthy plant tissue (Cho *et al*., 2009; Viaud *et al*., 2006). Two hypotheses could explain the observed attenuated virulence phenotype of Δ*fhk1* mutants. On the one hand, the higher sensitivity to different types of stress decreases the likelihood of the mutants surviving within the adverse environment of the host. The plant defence response, for example, is well known to involve the generation of different oxidative agents, such as hydrogen peroxide and superoxide (Huckelhoven and Kogel, 2003; Scott and Eaton, 2008). On the other hand, Fhk1 functions upstream of a signalling pathway that partly contributes to virulence. In *F. oxysporum* and other plant pathogenic fungi, virulence is under the control of the conserved pathogenicity MAPK cascade and its main downstream target, the transcription factor Ste12 (Di Pietro *et al*., 2001; Rispail and Di Pietro, 2009; Rispail *et al*., 2009). Our failure to detect any epistatic effects between Fhk1 and Fmk1 [see Fig. 5 and Fig. S3 (Supporting Information)] suggests that the role of Fhk1 in virulence is likely to be independent of the pathogenicity MAPK. In *B. cinerea*, mutation of the Hog1 orthologue Sak1 led to a similar pathogenicity defect as deletion of the HK Bos1 (Liu *et al*., 2008; Segmüller *et al*., 2007). Thus, it is possible that Fhk1 also recruits the Hog1 MAPK in *F. oxysporum*. The functional link between Fhk1 and the Hog1 pathway, as well as their potential roles in the stress response and virulence of *F. oxysporum*, will be the subject of future investigations.

EXPERIMENTAL PROCEDURES

Fungal isolates and culture conditions

Fusarium oxysporum f. sp. *lycopersici* race 2 wild-type strain 4287 (FGSC 9935) was used in all experiments. The generation and molecular characterization of the *F. oxysporum* Δ*fmk1* mutant have been described previously (Di Pietro *et al*., 2001). All fungal strains were stored as microconidial suspensions at −80 °C with 30% glycerol. For the extraction of genomic DNA and for microconidia production, cultures were grown in potato dextrose broth (PDB; Difco, Detroit, MI, USA) at 28 °C with shaking at 170 rpm (Di Pietro and Roncero, 1998). Cellophane invasion assay was performed as described previously (Prados Rosales and Di Pietro, 2008). For phenotypic analysis of colony growth, aliquots of 2×10^5 microconidia were spotted onto SM containing 1% glucose as unique carbon source and 0.2% $NaNO_3$ as nitrogen source, and buffered at pH 6.5 with 50 mM phosphate buffer (Di Pietro and Roncero, 1998). When needed, SM was supplemented with sodium chloride, potassium chloride, glycerol, menadione or sodium nitrite at the indicated concentrations. For fungicide assay, 1 mg/mL stock solutions of fludioxonil and iprodione, prepared in dimethylsulphoxide (DMSO) or ethanol, respectively, were used to complement SM at the indicated concentration. Fungal growth on fungicide-supplemented SM was compared with that on SM complemented with 0.01% DMSO or ethanol, respectively. All chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Química SA, Madrid, Spain). To determine growth at pH 4.5 or pH 6.5, media were buffered with 50 mM phosphate buffer. After inoculation, plates were incubated for 3 days at 28 °C. To determine H_2O_2 sensitivity, 1×10^6 microconidia were evenly spread onto the surface of SM and grown overnight at 28 °C before adding a filter paper disc imbibed with 3% H_2O_2 (v/v) solution at the centre of the plate. Growth inhibition induced by H_2O_2 was determined after 3 days of growth at 28 °C by assessing the size of the clear halo surrounding the filter paper disc.

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 labelling were carried out as described previously (Di Pietro and Roncero, 1998) using the nonisotopic digoxigenin labelling kit (Roche Diagnostics SL, Barcelona, Spain). Other routine DNA procedures were performed as described in standard protocols (Sambrook and Russell, 2001).

For sequencing of the *fhk1* gene, genomic DNA of *F. oxysporum* was used for PCR amplification with primers Fhk1-For/Fhk1-KO1, Fhk1-1/Fhk1-2 and Fhk1-KO2/Fhk1-Rev designed from the conserved regions of *F. graminearum* and *F. verticillioides fhk1* and its flanking genes (Table S1, see Supporting Information). The amplified DNA fragments were cloned into the pGEM-T vector (Promega, Madison, WI, USA). Sequencing was performed at the Servicio de Secuenciación Automática de DNA (SCAI, University of Cordoba, Spain) using the Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 377 Genetic Analyser apparatus (Applied Biosystems) employing the pGEMT vector-specific primers SP6 and T7 as well as gene-specific primers (Table S1, see Supporting Information). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul *et al*., 1990).

Construction of the Δ*fhk1* null allele and fungal transformation

Knockout of the *F. oxysporum fhk1* gene was performed by fusion PCR (Yang *et al*., 2004). PCRs were routinely performed

with the High Fidelity Template PCR system (Roche Diagnostics SL) using a Perkin-Elmer GeneAmp System 2400. First, 1.1- and 1.6-kb DNA segments flanking the *fhk1* coding region were amplified from the genomic DNA of *F. oxysporum* strain 4287 with primer pairs Fhk1-1/Fhk1-KO1 and Fhk1-KO2/Fhk1-2, respectively (Table S1, see Supporting Information; Fig. 2). The 5′ regions of Fhk1-KO1 and Fhk1-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 the control of the *Aspergillus nidulans gpdA* promoter (Punt *et al*., 1987). In the second reaction, the two *fhk1* flanking regions were mixed with the hygromycin B resistance cassette in a molar proportion of 1:1:3 and fused using the external Fhk1-1 and Fhk1-2 primers (Table S1, see Supporting Information; Fig. 2). The construct generated was used to transform 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 previously (Di Pietro and Roncero, 1998). Transformants showing the homologous insertion of the construct were detected by PCR amplification of genomic DNA with primers Fhk1-1 and Fhk1-2, and confirmed by Southern analysis of genomic DNA digested with *PstI* and hybridized with a labelled probe obtained by PCR amplification with primers Fhk1-KO2 and Fhk1-2 (Fig. 2). For complementation experiments, a 9-kb DNA fragment encompassing the entire *fhk1* gene was amplified by PCR from *F. oxysporum* genomic DNA using primers Fhk1-for and Fhk1-2 (Table S1, see Supporting Information), and introduced into protoplasts of the Δ*fhk1* mutant by cotransformation with the phleomycin resistance cassette amplified from plasmid pAN8-1 (Mattern *et al*., 1988). Phleomycin-resistant transformants were selected as described previously (Di Pietro *et al*., 2001), and the presence of the wild-type *fhk1* allele in the complemented transformants was detected by PCR of genomic DNA with primers Fhk1-1 and Fhk1-2.

Plant infection assays

Tomato plant inoculation assays were performed in a growth chamber as described previously (Di Pietro and Roncero, 1998). At different times after inoculation, the severity of disease symptoms was recorded using an index ranging from one (healthy plant) to five (dead plant). Ten plants were used for each treatment, and experiments were performed in triplicate. Invasive growth assays on tomato fruits or apple slices (cultivar Golden Delicious) were carried out as described previously (Di Pietro *et al*., 2001; Lopez-Berges *et al*., 2009) using three replicates.

Phylogenetic analysis

The deduced amino acid sequences of *F. oxysporum* Fhk1 and fungal orthologues were aligned using the CLUSTALW algorithm (Thompson *et al*., 1994) and cleaned by GBlocks v0.91b (Castresana, 2000). The PHYML 3.0 program (Guindon and Gascuel, 2003) was used to perform a 1000 nonparametric bootstrap phylogenetic analysis of the resulting alignment of 911 amino acid characters with the maximum likelihood method after optimization of the settings by the MODELGENERATOR program, version 0.85 (Keane *et al*., 2006). The analysis was performed using the LG substitution model (Le and Gascuel, 2008), with a gamma distribution parameter alpha of 1.18. The phylogenetic relationship between Fhk1 and other class III HK sequences was depicted in a phylogenetic tree constructed using the TREEVIEW 1.6.6 program (Page, 1996) (Fig. 1C).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Targeted disruption of the *Fusarium oxysporum fhk1* gene. (A) Physical maps of the *fhk1* locus and the gene replacement construct obtained by polymerase chain reaction (PCR) fusion (Δ*fhk1* allele). (B) Southern hybridization analysis of wild-type strain 4287 (1) and transformants *efhk1-1* (2), Δ*fhk1-2* (3), Δ*fhk1-4* (4), Δ*fhk1-7* (5), Δ*fmk1* (6) and Δ*fmk1*Δ*fhk1* (7). Genomic DNA treated with *Pst*I was hybridized with the probe indicated in (A). (C) PCR amplification of genomic DNA of the wild-type strain 4287 (1), knockout mutant Δ*fhk1-2* (2) and complemented strains Δ*fhk1+fhk1 1* (3) and Δ*fhk1+fhk1* 2 (4), using primers Fhk1-1 and Fhk1-2 (*fhk1*) or M13 Forward and PHL (*Phleo*).

Fig. S2 Fhk1 is not required for vegetative hyphal growth. Conidia of the indicated strains were spotted onto plates with synthetic medium (SM) buffered at the indicated pH values and grown at 28 °C for 3 days.

Fig. S3 Fhk1 is not required for invasive growth. Microconidial suspensions of the indicated strains were applied for the different invasive growth assays. (A) Invasive growth on apple slices

inoculated with microconidia and incubated at 28 °C for 4 days. (B) Penetration of cellophane sheets. Colonies were grown for 4 days on a plate with minimal medium covered by a cellophane sheet (Before); the cellophane sheet with the colony was removed and the plates were incubated for an additional day (After).

Table S1 Oligonucleotides used in this study.

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