A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis

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**Summary**

The soil-borne vascular wilt fungus *Fusarium oxysporum* infects a wide variety of plant species by directly penetrating roots, invading the cortex and colonizing the vascular tissue. We have identified *fmk1*, encoding a mitogen-activated protein kinase (MAPK) of *F. oxysporum* that belongs to the yeast and fungal extracellular signal-regulated kinase (YERK1) subfamily. Targeted mutants of *F. oxysporum* f. sp. *lycopersici* carrying an inactivated copy of *fmk1* have lost pathogenicity on tomato plants but show normal vegetative growth and conidiation in culture. Colonies of the *fmk1* mutants are easily wettable, and hyphae are impaired in breaching the liquid–air interface, suggesting defects in surface hydrophobicity. *Fmk1* mutants also show reduced invasive growth on tomato fruit tissue and drastically reduced transcript levels of *pl1* encoding the cell wall-degrading enzyme pectate lyase. Conidia of the mutants germinating in the tomato rhizosphere fail to differentiate penetration hyphae, resulting in greatly impaired root attachment. The orthologous MAPK gene *Pmk1* from the rice leaf pathogen *Magnaporthe grisea* complements invasive growth and partially restores surface hydrophobicity, root attachment and pathogenicity in an *fmk1* mutant. These results demonstrate that FMK1 controls several key steps in the pathogenesis of *F. oxysporum* and suggest a fundamentally conserved role for the corresponding MAPK pathway in soil-borne and foliar plant pathogens.

**Introduction**

Fungal plant pathogens have evolved strategies to recognize suitable hosts, penetrate and invade plant tissue, overcome host defences and optimize growth in the plant. To perform these tasks correctly, the fungus must perceive chemical and physical signals from the host and respond with the appropriate metabolic and morpho-genetic changes required for pathogenic development. Such changes include directed hyphal growth, adhesion to the plant surface, differentiation of specialized infection structures and secretion of lytic enzymes and phytotoxins (Knoege, 1996). Many of these responses require the synthesis of specific gene products and depend on conserved signal transduction pathways involving the activation of G proteins (Bölker, 1998), cAMP signalling (Lee and Dean, 1993; Mitchell and Dean, 1995) and mitogen-activated protein kinase (MAPK) cascades (Xu and Hamer, 1996; Xu *et al*., 1998).

Among the MAPKs, the yeast and fungal extracellular signal-regulated kinase (YERK1) subfamily (Kültz, 1998) plays a key role in infection-related morphogenesis and pathogenicity. The best-studied members of this subfamily are Fus3 and Kss1 from the budding yeast *Saccharomyces cerevisiae* that function in different, albeit interconnected, pathways. Fus3 controls the transduction of extracellular mating pheromone signals (Elion *et al*., 1990), whereas Kss1 mediates nitrogen starvation-induced filamentation (Madhani *et al*., 1997). Remarkably, both pathways share a number of components, including the p65PAK kinase Ste20, the MAPK kinase kinase Ste11 and the MAPK kinase Ste7 (Madhani and Fink, 1998). YERK1 subfamily MAPKs have been shown to play key roles in infection structure formation and in invasive growth of phytopathogenic fungi attacking the aerial parts of the plant, including the cereal leaf pathogens *Magnaporthe grisea* and *Cochliobolus heterostrophus* (Xu and Hamer, 1996; Lev *et al*., 1999), the cucumber leaf pathogen *Colletotrichum lagenarium* (Takano *et al*., 2000), the maize pathogen *Ustilago maydis* (Mayorga and Gold, 1999; Muller *et al*., 1999) and the broad-host-range necrotroph *Botrytis cinerea* (Zheng *et al*., 2000).

Little is known about the role of signal transduction in soil-borne fungal plant pathogens. In contrast to many foliar pathogens, soil-borne fungi tend to penetrate the host roots directly without producing fully differentiated infection structures (Mendgen *et al*., 1996). In this study, we have identified *fmk1* encoding a YERK1 family MAPK of the vascular wilt fungus *Fusarium oxysporum*, a
soil-borne facultative parasite that causes economically important losses on a wide variety of crops. The fungus enters the roots directly through penetration hyphae and colonizes the cortex by intra- and intercellular growth (Rodríguez-Galvez and Mendgen, 1995). Once it reaches the vascular tissue, *F. oxysporum* spreads rapidly upwards through the xylem vessels provoking the characteristic wilt symptoms (Beckman, 1987).

Mutants lacking a functional copy of the *fmk1* gene are deficient in pathogenicity but grow and sporulate normally on artificial media. We show that disruption of *fmk1* results in the impairment of a number of putative pathogenicity functions, such as hyphal growth at the liquid–air interface or efficient colonization of living plant tissue. *fmk1* mutants also show greatly reduced transcript levels of a gene encoding the cell wall-degrading enzyme pectate lyase. Using fluorescence microscopy, we establish that spores of a *fmk1* mutant germinating in the tomato rhizosphere are affected in the production of penetration hyphae and that this results in greatly reduced root attachment. We also find that the orthologous MAPK gene *Pmk1* from *M. grisea* complements some, but not all, of the *fmk1* mutant phenotypes. Our results demonstrate that *FMK1* controls several key steps in the pathogenicity process of *F. oxysporum* and suggest a fundamentally conserved role of YERK1 family MAPKs among soil-borne and foliar plant pathogens.

### Results

#### Isolation of the *fmk1* gene encoding a YERK1 family MAPK from *F. oxysporum*

A DNA fragment containing the complete coding region of an *F. oxysporum* MAPK gene was isolated by polymerase chain reaction (PCR) amplification with primers oligFMK1 and oligFMK2 derived from the *Fusarium solani* MAPK cDNA clone (Li et al., 1997). Part of the gene was then used to probe a *F. oxysporum* lambda EMBL3 genomic library and isolate a clone containing the entire MAPK gene with the 5′- and 3′-flanking sequences. Sequencing revealed the presence of an open reading frame (ORF) of 1068 nucleotides encoding a putative protein of 355 amino acids. Three potential introns were identified within the ORF by comparing the sequences of the *F. oxysporum* genomic clone and the *F. solani* cDNA clone. Figure 1 shows an alignment of the predicted amino acid sequence with that of other YERK1 MAPKs. The *F. oxysporum* MAPK has between 99% and 93% identity with the MAPK of *F. solani*, CMK1 of *C. lagenarium*, Pmk1 of *M. grisea*, Bmp1 of *B. cinerea* and Chk1 of *C. heterostrophus*. Identities with Fus3 and Kss1 of *S. cerevisiae* are 59% and 58% respectively. The gene was named *fmk1* for *Fusarium* MAP kinase 1.

Southern analysis of *F. oxysporum* genomic DNA digested with different restriction enzymes was performed...
using two different probes: fmk1p1 encompasses the complete fmk1 coding region, whereas fmk1p2 is a PCR fragment corresponding to the first 160 nucleotides of the gene. The hybridizing banding pattern indicated that at least three different genes hybridized to probe fmk1p1, whereas fmk1p2 only hybridized to a single gene, the fmk1 gene (data not shown). We conclude that fmk1 is a single-copy gene and that the F. oxysporum genome contains additional, structurally related MAPK genes.

**FMK1 is essential for vascular colonization and pathogenicity**

Mutants carrying a disrupted copy of the fmk1 gene were generated using a one-step gene replacement. Gene replacement vector pDFmk1 was constructed by inserting the phleomycin resistance cassette (ble) into a KpnI site located in the fmk1 coding region immediately downstream of the threonine and tyrosine residues representing putative phosphorylation sites for MAPK kinase (Johnson et al., 1996). A linear PCR fragment containing the disrupted fmk1 gene was used to transform F. oxysporum f. sp. lycopersici strain 4287, which is highly virulent on tomato plants. Transformants were selected on medium containing phleomycin, and genomic DNA was subjected to PCR with primers oliFmk1 and oliFmk2, which amplify the entire fmk1 coding region. The wild-type strain 4287 and two putative transformants, ND1 and ND2, gave the expected 1.2 kb fragment, whereas two other transformants, D1 and D2, produced a larger 3.8 kb fragment whose size was consistent with the disrupted fmk1 allele (results not shown). The gene replacement event in these two transformants was confirmed by Southern analysis with probe fmk1p2: strain 4287 and transformants ND1 and ND2 contained a 5.8 kb HincIII fragment corresponding to the wild-type fmk1 allele, whereas in D1 and D2, this fragment was replaced by a larger 8.4 kb fragment. These results indicate that transformants D1 and D2 contain a single copy of the replacement vector that has been integrated by double homologous recombination, thus generating a disrupted copy of fmk1. Consistent with gene replacement, the same single fragment hybridized with a ble probe (results not shown). Transformants ND1 and ND2 each contained an additional hybridizing fragment of larger size that was indicative of ectopic insertion of the replacement vector.

To determine expression of the fmk1 gene in the different strains, total RNA from mycelia grown in synthetic medium supplemented with either pectin or polygalacturonic acid sodium salt as the carbon source was either hybridized to probe fmk1p2 or used as a template for reverse transcriptase (RT)–PCR with primers oliFmk1 and oliFmk2. No transcript could be detected by Northern analysis even after prolonged exposure times, suggesting that fmk1 is expressed at extremely low levels in F. oxysporum. However, a fragment of about 1 kb corresponding to the expected size of the fmk1 cDNA was amplified in the wild-type strain and in both ectopic insertion transformants but was absent in the two transformants D1 and D2 (results not shown). Cloning and sequencing of the fragment confirmed that it was identical to the fmk1 genomic DNA except for the absence of the three introns.

Infection assays on tomato plants were performed to determine the effect of fmk1 inactivation on pathogenicity. Two-week-old tomato plants were inoculated by immersing the roots in a microconidial suspension of the fmk1 mutants and the control strains. After planting them in vermiculite, plants were scored for disease symptoms at different time intervals. The development of disease symptoms is shown in Fig. 2: plants inoculated with the wild-type strain and the ectopic insertion transformants showed characteristic wilt symptoms starting 11 days after inoculation. The severity of disease increased steadily throughout the experiment, and most of the plants were dead 20 days after inoculation. In contrast, plants inoculated with the fmk1 mutants failed to show any visible disease symptoms during this period of time. To determine colonization of the plant vascular tissue by the fungus, plants were removed at different times after

![Graph showing disease index over days after inoculation](image)

**Fig. 2.** Incidence of Fusarium wilt caused by different F. oxysporum strains on tomato plants (cv. Vemar). Severity of disease symptoms was recorded at different times after inoculation, using an index ranging from 1 (healthy plant) to 5 (dead plant). Symbols refer to plants inoculated with the wild-type strain 4287 (filled circles), fmk1 mutant D1 (open squares), ectopic integration transformant ND1 (open inverted triangles) and the uninoculated control (filled diamonds). Error bars indicate the standard deviations from 20 plants for each treatment.

inoculation, roots and stems were cut into 2 cm fragments, surface sterilized and transferred to potato dextrose agar plates with or without phleomycin. F. oxysporum was readily isolated from all plants tested that had been inoculated with the wild-type strain or the ectopic insertion transformants, but was only recovered in one out of 10 samples taken from plants inoculated with fmk1 mutants (results not shown). The identity of the isolated strains, i.e. presence of the functional or the disrupted fmk1 allele, was confirmed by PCR analysis of the genomic DNA. We conclude that FMK1 is required for full virulence and for efficient vascular colonization of tomato plants.

**FMK1 is dispensable for growth and conidiation in culture**

Inactivation of fmk1 did not cause any measurable growth defect in standard liquid media such as potato dextrose broth or synthetic medium supplemented with different carbon sources. Both hyphal morphology and the extent of microconidia production were indistinguishable from the wild type (data not shown). On complete solid medium, growth and colony morphology of fmk1 mutants was very similar to that of the wild-type strain, although a slight reduction in growth rate was consistently observed (Fig. 3A). We conclude that FMK1 does not play an essential role in vegetative growth and conidiation in culture.

**FMK1 contributes to maintaining surface hydrophobicity of aerial hyphae**

To determine whether the fmk1 mutants are affected in surface hydrophobicity, water droplets were placed on the surface of fungal colonies grown on complete medium. After 4 h, the water drops were still suspended on the hydrophobic surface of the wild-type strain but had soaked into the surface of the mutant strains (results not shown). To determine the ability of hyphae to breach the liquid–air interface, 100 μl drops of a microconidial suspension were placed in a plastic Petri dish and left at 100% humidity. After 24 h, close to 100% of the conidia had germinated in both the wild-type strain and the fmk1 mutant. After 72 h, both strains had produced filamentous mycelium that grew vigorously within the drop of liquid (not shown). After 96 h, as shown in Fig. 4B, the hyphae of the wild-type strain grew extensively on the surface of the drop, whereas those of the fmk1 mutant failed to emerge from the liquid (Fig. 3B). We conclude that FMK1 is required for maintaining full hydrophobicity of aerial hyphae.

**FMK1 is required for efficient invasive growth on living plant tissue**

To determine whether FMK1 is required for invasive growth of F. oxysporum on living host plant tissue, tomato fruits were inoculated by puncturing the epidermis with a sterile pipette tip and injecting a microconidial suspension into the fruit tissue. Figure 3C shows the fruit after 7 days’ incubation at 100% humidity: the wild-type strain and the ectopic insertion transformants had colonized and rotted the fruit tissue surrounding the site of inoculation, forming a dense mycelial mat on the surface of the fruit. Sequential microscopic observation showed that this mycelial mat was produced by colonization hyphae that emerged from the site of inoculation and extended radially over the hydrophobic fruit surface, forming an increasingly dense hyphal network. Within the rotten fruit tissue, invasive fungal hyphae were observed that spread vigorously into the adjacent parts of the tissue. In contrast, the fmk1 mutants had a strongly reduced capacity to macerate the fruit tissue and failed to produce aerial mycelium on the fruit surface. Microscopic observation revealed that, although most of the mutant microconidia had germinated within the fruit tissue, subsequent hyphal growth was blocked or greatly reduced compared with the wild-type strain. We conclude that FMK1 plays a key role in invasive growth and maceration of living plant tissue.

**Expression of genes encoding pectinolytic enzymes is differentially affected in fmk1 mutants**

F. oxysporum secretes an array of cell wall-degrading enzymes, including those depolymerizing pectin such as endo- and exopolypgalacturonases and pectate lyases (Di Pietro and Roncero, 1996a, b). Expression of the encoding genes can be detected during infection in planta and in liquid culture on synthetic medium supplemented with pectin, polygalacturonic acid sodium salt or tomato vascular tissue (Di Pietro and Roncero, 1998; Huertas et al., 1999; Garcia-Maceira et al., 2000). To determine whether the strongly impaired capacity of the fmk1 mutants to colonize and macerate living plant tissue resulted partly from a defect in the activation of genes encoding pectinolytic enzymes, conidia from the fmk1 mutants and the control strains were germinated overnight in potato dextrose broth, washed in water and transferred to synthetic medium containing either citrus pectin or polygalacturonic acid sodium salt as the carbon source. After 12 h, RNA was extracted from mycelia and subjected to gel blot and Northern hybridization analysis. Figure 4 shows hybridization with probes encoding different pectinolytic enzyme-encoding genes. No or only slight differences in transcript levels were detected between the fmk1 mutants and the control strains in

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genes encoding endopolygalacturonases PG1 (Di Pietro and Roncero, 1998) and PG5 (GenBank accession no. AF078156), as well as exopolygalacturonase PGX4 (García-Maceira et al., 2000), in mycelia grown on both pectin (Fig. 4A–C) and polygalacturonic acid sodium salt (results not shown). In contrast, mRNA levels of a gene encoding the endopeptate lyase PL1 (Di Pietro and Roncero, 1996b; Huertas-González et al., 1999) were greatly reduced in the mycelia of both fmk1 mutants grown on polygalacturonic acid sodium salt, compared with the wild-type and the ectopic control strains (see Fig. 4D). Equal loading of lanes was checked using the \( F. oxysporum \) nitrate reductase gene nit1 that is inducible by nitrate (García-Pedrajas and Roncero, 1996) as a positive control (Fig. 4E) and by staining RNA directly on the filter (Fig. 4F). We conclude that FMK1 is required for correct expression of the pl1 gene under the conditions used in this study.

**FMK1 is essential for differentiation of infection hyphae, attachment to tomato roots and penetration**

Infection-related morphogenesis of \( F. oxysporum \) involves
germination of spores in response to root exudates and differentiation of penetration hyphae able to enter the root (Rodríguez-Gálvez and Mendgen, 1995). To determine whether FMK1 plays a role in the initial stages of infection, roots of 3-week-old tomato plants were immersed in Erlenmeyer flasks with synthetic medium lacking any carbon or nitrogen source and containing microconidia of wild-type strain 4287 or fmk1 mutant D1. After 24 h incubation under gentle shaking, >90% of the microconidia had germinated in both the wild-type strain and the mutant. However, whereas the wild-type germlings became visibly attached to the root, no such attachment was observed in the mutant. The difference became increasingly clear after 72 h incubation, as shown in Fig. 5. Most of the wild-type germlings had attached to the root and initiated hyphal growth on the root surface, whereas the propagules of the mutant remained in suspension.

To facilitate microscopic observation of the initial infection steps, we transformed both the wild-type strain and a fmk1 mutant with the GFP gene driven by the constitutive gpdA promoter, using hygromycin resistance as the selectable marker. Five hygromycin-resistant transformants were isolated from each strain, checked in a fluorescence microscope and one strongly fluorescent transformant was selected for each strain. Both the fluorescent wild-type strain and the fmk1 mutant showed the same characteristics as their correspondent non-fluorescent parentalss as regards pathogenicity on tomato plants, colonization of tomato fruits and growth and sporulation in liquid culture (data not shown).

To follow the early stages of infection, roots of tomato plants were inoculated with conidia from the fluorescent wild-type strain or the fmk1 mutant. After planting in vermiculite, plants were removed each day, and the roots were observed under a fluorescence microscope. Figure 6 shows micrographs taken at different times after inoculation. After 1 day, most conidia from both the wild type and the mutant had germinated. However, whereas wild-type conidia differentiated a thin germ tube that elongated and attached to the root surface (Fig. 6A), most conidia from the mutant failed to show germ tube elongation and, instead, became swollen and appeared as egg-shaped structures (Fig. 6B and C). The difference became increasingly clear 2 days after inoculation. By this time, roots inoculated with the wild-type strain were covered with infection hyphae, whereas <5% of the germinated conidia from the fmk1 mutant had produced any infection hyphae (not shown). Four to 7 days after inoculation, infection hyphae growing within the cortex tissue and the xylem vessels were observed in plants inoculated with the wild-type strain (Fig. 6D and E). In contrast, no fungal structures were detected inside roots inoculated with the mutant. Even 7 days after inoculation, the fluorescent, egg-shaped germlings were still present at the root surface, indicating that they remained viable, but hardly any hyphal growth was detected. We conclude that FMK1 is essential for complete differentiation of infection hyphae, attachment to the root surface and penetration.

M. grisea Pmk1 fully restores invasive growth and partially complements hydrophobicity, root attachment and pathogenicity in an fmk1 mutant

To determine whether the orthologous MAPK Pmk1 from the rice blast fungus M. grisea can function in F. oxysporum, a 2423 bp PCR fragment containing the Pmk1 gene with 1003 bp of the 5'-flanking and 79 bp of 3'-flanking sequence was amplified from plasmid pFL1 (Xu and Hamer, 1996) using gene-specific primers. After cloning into pGEM-T, the resulting plasmid was used together with vector pANBlue3 containing the hygromycin resistance cassette to co-transform protoplasts of fmk1 mutant D1. Eleven out of 16 hygromycin-resistant transformants contained one or several copies of Pmk1, as indicated by Southern analysis of genomic DNA digested with different restriction enzymes and hybridized to the Pmk1 probe (results not shown).

To check whether the Pmk1 gene could restore invasive growth in the fmk1 mutant, tomato fruits were injected with conidial suspensions from three transformants, D1-Pmk1-1, D1-Pmk1-2 and D1-Pmk1-3, carrying one, multiple and two copies of the Pmk1 gene respectively. As controls, the wild-type strain 4287 and the fmk1 mutant D1 were also inoculated. Figure 7A shows that the transformants carrying the Pmk1 gene
were as efficient in growing invasively on the tomato fruit tissue as the wild-type strain. Both maceration and production of aerial mycelium on the fruit surface were comparable in these strains, independent of the Pmk1 copy number. The three transformants partially regained the ability to induce pl1 expression on polygalacturonic acid sodium salt, although to a lesser extent than the wild type, as demonstrated by Northern analysis (results not shown). When assayed for their ability to grow on the surface of a drop of liquid, transformants D1-Pmk1-1, D1-Pmk1-2 and D1-Pmk1-3 showed differential responses: multicopy transformant D1-Pmk1-2 produced aerial mycelium as well as the wild type, whereas transformants D1-Pmk1-1 and D1-Pmk1-3 showed very limited hyphal growth on the drop (results not shown). To confirm copy number-dependent complementation of this character, the study was extended to all 16 hygromycin-resistant transformants. Only those carrying multiple copies of Pmk1, but not those carrying none, one or two copies of the heterologous MAPK gene fully regained the ability to grow on the drop of liquid. Consistent with these results, colonies of D1-Pmk1-1 and D1-Pmk1-3 were easily

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**Fig. 6.** Penetration and colonization of tomato roots by *F. oxysporum*. Roots of tomato plants were inoculated with microconidia of transformants of wild-type strain 4287 or fmk1 mutant D1 constitutively expressing green fluorescent protein. Root samples were observed directly under an epifluorescence microscope using a filter set for fluorescein isothiocyanate. Magnifications $\times$ 1000 (A, B, D and E) and $\times$ 1600 (C).

A. Germinated conidia and penetration hyphae of wild-type strain 4287 attaching to the root surface 24 h after inoculation.
B. and C. Germinated conidia of fmk1 mutant D1 on the root surface and in the rhizosphere, respectively, 24 h after inoculation. Germlings are swollen and vacuolated and fail to differentiate penetration hyphae.
D. and E. Infection hyphae of wild-type strain 4287 growing in the root cortex, 5 days after inoculation (D); and in a root xylem vessel, 7 days after inoculation (E).

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**Fig. 7.** The *M. grisea* Pmk1 gene complements invasive growth and pl1 expression in fmk1 mutants. Invasive growth on tomato fruits of the wild-type strain 4287 (top), fmk1 mutant D1 (right) and two transformants of the fmk1 mutant, D1-Pmk1-1 (left) and D1-Pmk1-2 (bottom), carrying one and multiple copies of the *M. grisea* Pmk1 gene respectively. The picture was taken after 5 days’ incubation at 100% humidity.

wettable, whereas those of D1-Pmk1-2 were as water repellent as the wild-type colonies (results not shown).

Complementation of pathogenicity was tested by inoculating roots of tomato plants with microconidia from strains D1-Pmk1-1, D1-Pmk1-2 and D1-Pmk1-3, as well as with the mutant D1 and the wild-type strain. After 10 days, the plants inoculated with the wild-type strain showed initial wilting symptoms, whereas those inoculated with the Pmk1 transformants still appeared completely healthy (results not shown). After 15 days, plants inoculated with the wild-type strain were severely wilted, and those inoculated with the transformants showed slight initial symptoms. After 20 days, all plants inoculated with the wild-type strain were dead. At this time, those inoculated with the transformants showed intermediate wilting symptoms and, after 30 days, most of them were dead. No significant difference in virulence was detected between the three transformants D1-Pmk1-1, D1-Pmk1-2 and D1-Pmk1-3. The original fmk1 mutant did not produce any disease symptoms throughout the whole duration of the experiment. In the root attachment assay, germinating microconidia from the three transformants carrying Pmk1 were able to attach to tomato roots, although to a lesser extent than the wild-type strain (results not shown). We conclude that M. grisea Pmk1 fully complements invasive growth on tomato fruits in an fmk1 mutant and partially restores surface hydrophobicity, root attachment and pathogenicity.

Discussion

Many of the economically important plant pathogenic fungi are soil borne and attack their hosts through the root or the hypocotyl. The early steps involved in fungal root infection have been studied considerably less than the corresponding stages in pathogens attacking the aerial parts of the plant, mainly because of methodological limitations. Generally, the morphogenetic events preceding penetration in root pathogens are viewed as less complex than those occurring in foliar pathogens, because the former usually lack fully differentiated infection structures such as appressoria (Mendgen et al., 1996). We provide evidence that signalling pathways controlling root infection share common features with those reported in leaf pathogens. The MAPK FMK1 from the soil-borne vascular wilt fungus F. oxysporum is part of a signal transduction pathway involved in the formation of infection hyphae, root attachment and penetration, as well as invasive growth on the living plant tissue. Targeted inactivation of the fmk1 gene abolishes pathogenicity of F. oxysporum on tomato plants. This result is not unexpected, as loss of or severe reduction in pathogenicity was also reported in mutants of the leaf pathogens M. grisea, C. lagenarium and C. heterostrophus lacking functional MAPKs, all of which share over 90% identity with Fmk1 (Xu and Hamer, 1996; Lev et al., 1999; Takano et al., 2000). Interestingly, the F. oxysporum fmk1 gene is dispensable for vegetative growth and conidiation in culture. Similar results have been reported for Pmk1 from M. grisea (Xu and Hamer, 1996). In contrast, MAPK mutants of C. heterostrophus and C. lagenarium showed severe defects in conidiation (Lev et al., 1999; Takano et al., 2000), whereas those of B. cinerea were reduced in vegetative growth (Zheng et al., 2000). Our results suggest that FMK1 acts specifically in signalling during the interaction of F. oxysporum with the plant host and is not required under conditions encountered by the fungus grown in culture.

FMK1 controls infection-related morphogenesis and root attachment

To determine how and when FMK1 participates during infection, we compared the ability of the wild-type and mutant strains to accomplish different pathogenicity-related functions. The first steps of infection in soil-borne fungi usually involve spore germination in response to stimuli exuded by host roots and the production of germ tubes that differentiate into infection hyphae (Mendgen et al., 1996). Germination rates in the wild type and the mutant were comparable, indicating that FMK1 is not essential for germination under the conditions tested. MAPK mutants of M. grisea and B. cinerea also showed no defect in conidial germination (Xu and Hamer, 1996; Zheng et al., 2000), whereas reduced germination rates were observed in the C. lagenarium Cmk1 mutants (Takano et al., 2000).

Once conidia from the wild-type strain had germinated, they differentiated thin infection hyphae that became attached to the root surface. Production of penetration hyphae by F. oxysporum and their ability to penetrate directly the root epidermal walls has been reported previously (Rodríguez-Gálvez and Mendgen, 1995). Attachment of F. oxysporum germlings was macroscopically evident: tomato roots immersed in a conidial suspension under gentle shaking became completely covered with the fungal propagules. The germlings remained attached to the roots even after vigorous shaking, indicating that fungal adhesion was highly effective. So far, fungal attachment to the plant surface has mainly been associated with foliar pathogens (Hamer et al., 1988; Mendgen et al., 1996). Adhesion of macroconidia of the root- and hypocotyl-infecting fungus Nectria haematococca (F. solani) to a polystyrene surface has been reported and found to be rapidly induced by plant extract and associated with the presence of a 90 kDa glycoprotein (Kwon and Epstein, 1993).

In contrast to the wild type, strains lacking a functional
copy of fmk1 failed to attach to tomato roots. Fluorescence microscopy analysis suggests that lack of attachment in the mutants results mainly from failure in differentiating infection hyphae in the presence of tomato roots. Instead, germings from the mutant evolved into swollen, egg-shaped structures that became highly vacuolated. The aberrant morphogenesis of the fmk1 germings somewhat resembled that observed during failure in appressorium formation in M. grisea pmk1 mutants (Xu and Hamer, 1996). Interestingly, MAPK mutants of the necrotrophic pathogen B. cinerea also failed to form penetration hyphae, but instead grew as thin mycelium on the plant surface (Zheng et al., 2000). Even in the few cases in which germings of the F. oxysporum fmk1 mutant produced hyphal structures, these were unable to attach efficiently to the root surface and failed to penetrate. As a consequence, no mycelium from the mutant was detected within the inoculated tomato roots. Conversely, fluorescent injection hyphae of the wild-type strain were readily observed in the root cortex and vascular tissue of inoculated plants. Thus, even though F. oxysporum does not produce apparent appressoria, FMK1 controls the differentiation of infection hyphae in a similar manner to that by which the orthologous MAPKs regulate infection-related morphogenesis in foliar pathogens.

The easily wettable phenotype of the mutant and the inability of the hyphae to escape the surface tension of water droplets is strongly reminiscent of phenotypes reported in fungal mutants lacking hydrophobins. Mutants of Schizophyllum commune lacking the hydrophobin Sc3 are unable to breach the water–air surface, whereas hyphal growth in aqueous medium is indistinguishable from that of wild-type strains. These mutants are also impaired in attachment to hydrophobic surfaces (Wösten et al., 1994). Likewise, the hydrophobin Mpg1 from M. grisea, which is strongly expressed during the early stages of pathogenesis, is required for surface attachment and appressorium formation (Talbot et al., 1993). We speculate that failure of the fmk1 mutants to express surface hydrophobin genes may be related, at least in part, to their inability to attach to tomato roots. A possible link between G protein-mediated signalling pathways and hydrophobin gene expression has been suggested previously in the chestnut blight fungus Cryphonectria parasitica. Hypovirus-infected strains of this pathogen showing perturbed G protein-mediated signalling and attenuated virulence (Nuss, 1996) also show greatly reduced accumulation of the surface hydrophobin cryparin (Carpenter et al., 1992).

Besides hydrophobins, additional factors may also be involved in fungal attachment to the plant surface. Mucin-like cell surface proteins have been shown to accumulate in cysts of the oomycete pathogen Phytophthora infestans during infection and have been suggested to play a role in adhesion to the host plant (Görnhardt et al., 2000). Intriguingly, Flo11, a mucin-like cell surface protein that mediates cell-to-cell adhesion and flocculation in S. cerevisiae and is essential for filamentous invasive growth (Lambrechts et al., 1996) is strongly regulated by the Kss1-mediated pathway (Madhani et al., 1999). In F. oxysporum, the presence of mucin-like proteins and their role in pathogenesis has not been studied so far.

**FMK1 is required for invasive growth on living host plant tissue**

An interesting feature of YERK1 family MAPK mutants is that, in addition to being deficient in infection-related morphogenesis and penetration, they are also unable to grow invasively on living plant tissue (Xu and Hamer, 1996; Takano et al., 2000; Zheng et al., 2000). Similarly, we found that F. oxysporum fmk1 mutants have a strongly reduced ability to grow invasively on tomato fruit tissue. The difference from the wild-type strain was apparent in two key parameters, maceration of the fruit tissue surrounding the site of inoculation and production of aerial mycelium on the fruit surface. Failure of the fmk1 mutants to produce aerial hyphae on the hydrophobic surface of tomato fruits may be causally related to the hydrophobicity defect discussed above. On the other hand, the reduced ability to macerate the fruit tissue suggests alterations in the expression profile of cell wall-degrading enzymes. To follow this hypothesis, we determined transcript accumulation for a number of genes encoding pectinolytic enzymes of F. oxysporum under controlled growth conditions on artificial media. Pectinases are among the first cell wall-degrading enzymes secreted by plant pathogenic fungi upon contact with the host tissue (Walton, 1994). We found that the expression of genes encoding two endopolygalacturonases and an endopolygalacturonase was not significantly altered in fmk1 mutants, whereas transcripts of pl1 encoding an endopolygalacturonase were drastically reduced in comparison with the wild-type and control strains. pl1 was previously shown to be expressed during plant infection (Huertas-González et al., 1999), and the enzymatic activity band corresponding to the gene product was detected in extracts of tomato roots infected with F. oxysporum (Di Pietro and Roncero, 1996a). We speculate that the reduced expression of pl1 in the fmk1 mutants may contribute to their incapacity to macerate tomato fruit tissue, although it appears likely that additional cell wall-degrading enzymes participating in maceration of the fruit tissue may be downregulated as well, as a consequence of fmk1 inactivation. Interestingly, expression of a polygalacturonase gene from S. cerevisiae was recently found to be dependent on the Kss1-mediated pathway that
regulates invasive filamentous growth (Madhani et al., 1999). Likewise, expression of a cellobiohydrolase gene from C. parasitica was greatly reduced in strains showing transgenic co-suppression of a G protein alpha-subunit gene (Wang and Nuss, 1995). However, two lines of evidence indicate that 
\[ \text{pl1} \] 
by itself is not essential for invasive growth: first, targeted disruption of \text{pl1} \ does not affect invasive growth of \text{F. oxysporum} \ (A. Di Pietro, M.D. Huertas González and M.I.G. Roncero, unpublished); secondly, the \text{M. grisea Pmk1} \ gene fully complements invasive growth in \text{fmk1} \ mutants but only partially restores \text{pl1} \ expression.

A heterologous MAPK partially complements signalling for pathogenesis-related functions in \text{F. oxysporum}.

The conserved structure among members of the YERK1 family suggests a high degree of functional relatedness. This has been confirmed partly by complementation experiments. The \text{M. grisea Pmk1} \ gene could partially rescue the phenotype of \text{S. cerevisiae fus3 kss1} \ double mutants in mating assays when expressed under the control of a strongly inducible yeast promoter (Xu and Hamer, 1996). Recently, the \text{C. lagenarium Cmk1} \ gene was shown to complement appressorium formation defects in \text{M. grisea pmk1} \ mutants (Takano et al., 2000), indicating that MAPK signalling pathways for infection structure formation are highly conserved in these two foliar pathogens.

In contrast, the lifestyles and infection mechanisms of \text{M. grisea} \ and \text{F. oxysporum} \ are highly divergent. \text{M. grisea} \ infects rice plants in a manner typical of foliar pathogens: conidia attach to the leaf and produce germ tubes that differentiate into dome-shaped melanized appressoria, able to generate sufficient turgor pressure to penetrate the plant cuticular surface (Talbot, 1995). Conversely, and similar to other soil pathogens, \text{F. oxysporum} \ spores germinate in response to root exudates to produce penetration hyphae that attach to the root surface and penetrate it directly (Rodriguez-Gálvez and Mendgen, 1995).

To what extent are signalling components from such diverse plant pathogens able to complement each other? Our data show that Pmk1 from \text{M. grisea}, which differs from FMK1 in only eight amino acid residues (marked by asterisks in Fig. 1), can partially restore pathogenesis-related functions in an \text{fmk1} \ mutant, although to a different extent. Although invasive growth was fully complemented by \text{Pmk1}, independently of the number of copies of the heterologous gene in the transformants, only those strains carrying multiple copies of \text{Pmk1} \ regained surface hydrophobicity to the same extent as the wild type, and those with single or double \text{Pmk1} \ copies showed partial complementation of the mutant phenotype. Similarly, \text{Pmk1} \ partially restored root attachment and pathogenicity in the \text{fmk1} \ mutant. Two conclusions can be drawn from these results: first, complementation of FMK1 functions by the heterologous Pmk1 is incomplete. At present, it is unclear whether the reduced functionality of Pmk1 results from lower levels of enzyme produced under the control of the heterologous promoter, a decrease in MAPK activity caused by the differences in amino acid residues or a combination of both. The copy number-dependent complementation of surface hydrophobicity suggests that MAPK dosage may be important for at least some of the signalling outputs.

A second conclusion is that different pathogenicity-related functions in \text{F. oxysporum} \ require different levels of MAPK activity. It appears that increasing MAPK function is needed for invasive growth and \text{pl1} \ expression, surface hydrophobicity and morphogenetic differentiation of infection hyphae, root attachment and pathogenesis. A similar scenario has been reported for Fus3, which regulates different outputs of the pheromone response pathway in \text{S. cerevisiae} \ (Elion et al., 1990). By comparing a series of partial function \text{fus3} \ point mutants, the authors concluded that increasing Fus3 function is required for transcriptional activation of pheromone-induced genes, cell agglutination, cell cycle arrest in G1 and the formation of mating projections, in this order (Farley et al., 1999). Thus, in both the yeast and the \text{F. oxysporum} \ system, morphogenetic response appears to require higher or more persistent MAPK activation levels than gene transcription. This observation is intriguing, because it has been suggested previously that the formation of mating projections in yeast and the differentiation of infection structures in filamentous fungi may have common evolutionary origins, as both processes involve polarized cell growth (Madhani and Fink, 1998).

In conclusion, the present and other recent studies support the view that, in spite of the diversity of infection mechanisms developed by plant pathogenic fungi, the key steps leading to plant infection and their underlying signalling pathways are essentially the same. Promising directions for future research efforts include exploring the nature of the environmental cues that activate these signalling pathways and identifying the downstream effector genes that function in pathogenesis.

Experimental procedures

Fungal isolates and culture conditions

\text{F. oxysporum} \ f. sp. \ \text{lycopersici} \ strain 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Spain, and stored at \(-80\)°C with glycerol as a microconidial suspension (Di Pietro and Roncero, 1998). The pathotype of the isolate was confirmed periodically by plant infection assays. For the extraction of DNA, mycelium was obtained from cultures.
grown in potato dextrose broth (PDB; Difco) as described previously (Di Pietro and Roncero, 1998). For analysis of gene expression, freshly obtained microconidia were germinated for 14 h in PDB, germings were washed twice in sterile water and transferred to synthetic medium (Di Pietro and Roncero, 1996a) containing 1% (w/v) pectin from citrus fruits or 1% polygalacturonic acid sodium salt (PGA) (both from Sigma) as the carbon source and 0.1% (w/v) sodium nitrate as the nitrogen source.

**Nucleic acid manipulations and PCR cloning of the fmk1 gene**

Total RNA and genomic DNA were extracted from *F. oxysporum* mycelium according to previously reported protocols (Raeder and Broda, 1985; Chomczynski and Sacchi, 1987). Southern and Northern analyses and probe labelling were carried out as described previously (Di Pietro and Roncero, 1998) using the non-isotopic digoxigenin labelling kit (Boehringer Mannheim). Experiments were carried out twice with similar results.

Genomic DNA from *F. oxysporum* isolate 4287 was used for PCR amplification on a Perkin-Elmer GeneAmp System 2400 using the primers oliFmk1 (5'-ATGTCCCGATCGAACC CCCCC-3') and oliFmk2 (5'-CTGTTACCTCATATACTCTCTGG -3') derived from the *F. solani* MAPK cDNA clone (Li et al., 1997). The following PCR conditions were used: 35 cycles with denaturation at 94°C for 35 s, annealing at 58°C for 35 s and extension at 72°C for 90 s. An initial denaturation step of 5 min at 94°C and a final elongation step at 72°C for 7 min were performed. The amplified DNA fragment was cloned into pGEM-T (Promega) and used to screen a lambda 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 et al., 1989). Sequencing of both DNA strands was performed directly on the lambda clone at the Servicio de Secuenciación Automática de DNA, CIB, CSIC, Madrid, Spain, using the dye-deoxy terminator cycle sequencing kit (PE Biosystems) on an ABI Prism 377 genetic analyser apparatus (Applied Biosystems). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (Bethesda, MD, USA).

**Construction of plasmid vectors and fungal transformation**

Gene replacement vector pDFmk1 was constructed as follows: a 2.8 kb fragment containing the phleomycin resistance cassette (ble) under the control of the *Aspergillus nidulans* *gpdA* promoter was amplified from plasmid pAN8-1 (Mattern et al., 1988) by PCR with primers gpd2K (5'-CATGTCAGATACGATAGCCCG-3') and trp2K (5'-GATTACCTCATAACGACGGAATCC-3'), introducing a KpnI site at both ends. The fragment was cloned into pGEM-T, released by digestion with KpnI and ligated into the KpnI site located in the *fmk1* coding region cloned in pGEM-T. A linearized fragment containing the *fmk1* coding region interrupted by the phleomycin resistance cassette was generated by amplifying the entire construct with primers oliFmk1 and oliFmk2 using the Perkin-Elmer Gene Amp XL PCR kit (PE Biosystems) under the following conditions: 10 cycles with denaturation at 94°C for 10 s, annealing at 58°C for 30 s and extension at 68°C for 8 min, followed by 15 cycles with the same conditions except that 20 s were added successively to the 8 min extension in each cycle. Initial denaturation was 75 s at 94°C, and final elongation was 7 min at 68°C. The amplified fragment was used to transform protoplasts of *F. oxysporum* strain 4287 into phleomycin resistance according to a protocol described previously (Di Pietro and Roncero, 1998). Transformants were selected on minimal medium plates adjusted to pH 8.0 containing 3 μg ml⁻¹ phleomycin (Cayla), purified by monodonal isolation and stored as macroconidial suspensions at −80°C. To check for replacement of the *fmk1* gene, PCR on genomic DNA of transformants was performed with primers oliFmk1 and oliFmk2 as described above.

A vector for transformation with the green fluorescent protein (GFP) was constructed as follows: an EcoRI–HindIII fragment from plasmid pgpdAsGFP containing the sGFP gene inserted between the *gpdA* promoter and the *trpC* terminator of *A. nidulans* (Fernández-Ábalos et al., 1998) was subcloned into Bluescript pBKS, excised with NotI–HindIII and inserted into the corresponding sites of pANBlue3 containing the hygromycin resistance cassette (Di Pietro and Roncero, 1998). The resulting vector was used to transform protoplasts of the wild-type strain 4287 and the *fmk1* mutant D1. Hygromycin-resistant transformants were selected as described previously (Di Pietro and Roncero, 1998).

For complementation experiments, a 2.4 kb fragment containing the *M. grisea* *Pmk1* gene was amplified from plasmid pFL1 (Xu and Hamer, 1996) using primers Pmk3 (5'-CATGTCAGATACGATAGCCCG-3') and Pmk4 (5'-ACGTCTAGTTTCCTCGCTGCA-3') and subcloned into pGEM-T. The resulting vector and plasmid pANBlue3 were used in the proportion 3:1 to co-transform protoplasts of strain D1 to hygromycin resistance. The presence and copy number of the *Pmk1* gene in hygromycin-resistant transformants was determined by Southern analysis of genomic DNA digested with BamHI or HindIII and hybridized to the *Pmk1* probe.

**Assays for pathogenicity and root adhesion**

Seeds from tomato cultivar Vemar were kindly provided by Novartis Seeds. Ten-day-old seedlings in the first true leaf stage were inoculated with *F. oxysporum* strains as described previously (Di Pietro and Roncero, 1998). Briefly, roots were immersed in a suspension of *5 × 10^6* microconidia ml⁻¹ for 30 min before planting in vermiculite. Twenty plants were used for each treatment. Plants were maintained in a growth chamber as described previously (Di Pietro and Roncero, 1998). At different times after inoculation, severity of disease symptoms was recorded using an index from 1 (healthy plant) to 5 (dead plant). For the determination of plant colonization by *F. oxysporum*, roots and stems of infected tomato plants harvested at different times after inoculation were cut into 2 cm fragments, surface sterilized by soaking for 20 min in 0.7% sodium hypochlorite and transferred to potato dextrose agar plates (Panreac) containing 3 μg ml⁻¹ phleomycin in the case of the transformants.

For adhesion assays, the roots of tomato seedlings were...
placed in Erlenmeyer flasks containing a suspension of 10^7 microconidia ml^{-1} and incubated at 28°C and 120 r.p.m. Adhesion of germlings to the root surface was observed macroscopically at different times after inoculation. The experiments were performed three times with similar results.

**Fluorescence microscopy**

Roots of tomato plants were inoculated with conidia from the wild-type strain or the fmk1 mutant carrying the GFP gene as described above. After different time periods, plants were removed from the vermiculite, and the roots were observed directly under an epifluorescence microscope (Leica DMRB) using a filter set for fluorescein isothiocyanate (BP450–490 excitation, 510–520 emission). Photographs were taken with a Leica 35 mm camera, and images were transferred to Adobe Photoshop version 4.0. The experiment was performed twice with similar results.

**Assays for hyphal growth under different conditions**

Growth and conidiation of *F. oxysporum* strains in culture was determined in PDB and on complete medium at 28°C. To determine surface hydrophobicity, 100 μl drops of water were placed on the surface of fungal colonies grown for 4 days on complete medium and allowed to stand for 4 h. To determine hyphal growth at a liquid–air interface, 1 were placed directly on the bottom of a plastic Petri dish, incubated at 28°C under conditions of 100% humidity and observed in a binocular microscope. The experiment was performed twice with similar results.

To assay invasive growth of *F. oxysporum* strains, tomato fruits (cultivar Daniela) were washed under running tap water and surface sterilized by immersion in ethanol for 5 min. After air drying, the epidermis was punctured with a sterile pipette tip, and 10 μl of a microconidial suspension (5 x 10^6) was injected into the fruit tissue. Fruits were incubated at 28°C under conditions of 100% humidity. Colonization of the fruit tissue and formation of a mycelial mat on the fruit surface was determined at different times after inoculation. Spore germination and hyphal growth of autofluorescent strains within the fruit tissue was observed under an epifluorescence microscope as described above. Experiments were performed at least three times with similar results.

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García-Pedrajas, M.D., and Roncero, M.I.G. (1996) A molecular microconidia ml^{-1} and incubated at 28°C and 120 r.p.m. Adhesion of germlings to the root surface was observed macroscopically at different times after inoculation. The experiments were performed three times with similar results.

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