***PTK1*, a Mitogen-Activated-Protein Kinase Gene, Is Required for Conidiation, Appressorium Formation, and Pathogenicity of *Pyrenophora teres* on Barley**

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Mitogen-activated protein kinases (MAPKs) are a group of protein kinases that execute a wide variety of roles in cellular signal transduction pathways such as osmoregulation, cell wall biosynthesis, growth, and differentiation. A polymerase chain reaction (PCR) with degenerate primers based on conserved regions of known MAPKs was used to clone the MAPK gene *PTK1* from the leaf pathogen *Pyrenophora teres* (anamorph *Drechslera teres*), the causal agent of net blotch of barley (*Hordeum vulgare* L.). The predicted amino acid sequence shows high homology with MAPKs from other phytopathogenic fungi. The gene is present in the genome as a single copy. *PTK1* is expressed during in vitro growth on complete medium, under conidiation-inducing conditions and during infection of barley leaves, as shown by reverse transcription-PCR studies. In order to assess the role of *PTK1* in the life cycle of *P. teres*, targeted gene disruption was conducted. Mutants carrying an interrupted copy of the gene were deficient in conidiation, did not form appressoria on glass surfaces or on barley leaves, lost their ability to infect barley leaves, and could not colonize host tissues following artificial wounding.

Net blotch caused by the leaf pathogen *Pyrenophora teres* Drechs. (anamorph *Drechslera teres*) is one of the most economically devastating diseases of cultivated barley (*Hordeum vulgare* L.) (Weiland et al. 1999). Barley lines range from being highly resistant to highly susceptible to this pathogen, which is a highly variable organism that includes several virulence types (Douiyyssi et al. 1998; Harrabi and Kamel 1990; Robinson and Jalli 1996; Steffenson and Webster 1992a). Nevertheless, little is known about the fungal genes that condition pathogenicity or cause the various observed pathotypes. We initiated molecular genetic studies of this pathogen to define genes responsible for attachment, penetration, and pathogenic growth.

P. teres produces asexual spores, called conidia, which are dispersed in moist air and attach tightly to the barley leaf sur-

face, thus starting the infection cycle. Under high-moisture conditions, a conidium produces a germ tube that grows and differentiates a specialized infection structure called appressorium. After hyphal penetration of an epidermal cell, the fungus develops intracellular infection vesicles that may act like haustoria. After breaching the epidermal cell layer, the fungus grows exclusively in the apoplast of the mesophyll tissue (Keon and Hargreaves 1983). Eventually a lesion develops and, under conditions of high humidity, *P. teres* sporulates and releases more conidia to reinitiate the infection cycle.

In many fungal pathogens, development of penetration structures is triggered by the perception of physical and/or chemical signals from the plant surface (Gilbert and Dean 1996; Hoch et al. 1987; Lee and Dean 1994; Podila et al. 1993; Uchiyama and Okuyama 1990). Thus, signal transduction pathways in filamentous fungal cells are likely to be critical to the establishment of disease. Molecular genetic analysis of several fungal pathogens has demonstrated that mitogen-activated protein kinase (MAPK) signal transduction pathways play important roles in pathogenesis (Lev et al. 1999; Mayorga and Gold 1999; Müller et al. 1999; Takano et al. 2000; Xu and Hamer 1996; Xu et al. 1998; Zheng et al. 2000). MAPKs are a group of protein kinases that execute a wide variety of roles in other cellular signal transduction pathways such as osmoregulation, cell wall biosynthesis, growth, and differentiation (Brewster et al. 1993; Courchesne et al. 1989; Elion et al. 1990; Krisak et al. 1994). The functions of the various cloned MAPK genes in fungal life cycles seem to differ from one pathogen to the other (Table 1). Therefore, we assessed the impact of MAPK pathways from *P. teres* on its life cycle.

RESULTS

Isolation of the MAPK gene *PTK1*.

A nested-primer strategy was used to amplify wild-type genomic DNA. Four degenerate oligonucleotides were designed from conserved amino acid regions of MAPKs in other organisms (see below). After a first round of polymerase chain reaction (PCR) amplification with primers 2 and 4 (located on domains I and VIII, respectively), a nested PCR amplification was performed with primers 1 and 3 (located on domains VIII and II, respectively). Out of the two major PCR products generated (478 and 100 bp), the 478-bp product matched the size

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expected for a segment between regions II and VIII of known protein kinases (Hanks and Quinn 1991; data not shown). Sequence analysis of this PCR fragment revealed a significant similarity to known MAPK genes. The 100-bp PCR product was not considered because of its small size.

Three positive clones were isolated following screening of a λ EMBL3 library of genomic DNA of *P. teres* strain 15A with the 478-bp PCR fragment as a probe. The three clones corresponded to the same genomic region, as deduced from restriction analysis. A 5.5-kb *Hind*III fragment that hybridized to the probe was subcloned into pBlueScript II KS+ (Stratagene, La Jolla, CA, U.S.A.). The resulting plasmid was named pPTK1-5.5H. The nucleotide sequence of a 3.2-kb region was determined. It contained an open reading frame with 1,265 bp coding a 352 amino acid polypeptide. Four introns of 55, 53, 51, and 48 bp, respectively, were identified on the basis of the alignment with other MAPKs and the presence of conserved splice-site consensus sequences that are characteristic of filamentous fungi. The calculated molecular weight and pI of the encoded protein were 40.8 and 6.56 kDa, respectively. Comparisons with database entries demonstrated high similarities to other fungal MAPKs such as Chk1 of *Cochliobolus heterostrophus* with 98.9% identity (Lev et al. 1999); Bmp1 of *Botrytis cinerea*, 94.6% identity (Zheng et al. 2000); Cmk1 of *Colletotrichum lagenarium*, 92.9% identity (Takano et al. 1999); Fsmapk of *Nectria haematococca*, 92.9% identity (Li et al. 1997); Pmk1 of *Magnaporthe grisea*, 91.8% identity (Xu and Hamer 1996); or Kpp2-Ubc3 of *Ustilago maydis*, 73.3% identity (Mayorga and Gold 1999; Müller et al. 1999) (Fig. 1). We designated the corresponding *P. teres* gene *PTK1*. The deduced amino acid sequence of *PTK1* contains all 11 conserved protein kinase domains (Hanks and Quinn 1991) in addition to the TEY sequence, a site for threonine-tyrosine phosphorylation required for kinase activation (Kültz 1998).

Southern blot analysis with several restriction enzymes indicated that the *PTK1* gene is present as a single copy in the genome of *P. teres* (data not shown).

Expression of *PTK1*.

PTK1 expression was studied by reverse transcription (RT)-PCR during in vitro growth and during infection of barley leaves. Control reactions were performed with total RNA from uninfected leaves. Genomic DNA was used as template to compare sizes of the amplified fragments with or without introns (416 and 363 bp, respectively). The 363-bp amplification product of *PTK1* was obtained from RNA extracted from mycelium grown in complete medium (CM) or from conidiophores and conidia produced on peanut leaves-oatmeal agar (POA) plates as well as during infection of barley leaves at all time points studied (Fig. 2). This amplified fragment was absent in the uninfected barley leaves. Cloning and sequencing of the bands confirmed that their sequences completely matched those of the genomic *PTK1* clone, except for the absence of the intron.

PTK1 is essential for pathogenicity and invasive growth in barley leaves.

To determine the function of the *PTK1* gene in the life cycle of *P. teres*, a gene disruption strategy was conducted. A disruption vector, pKO_{ptk1}, was constructed containing a hygromycin B resistance gene cassette next to an internal 416-bp fragment of the *PTK1* gene (Fig. 3A). pKO_{ptk1} was linearized and introduced into the wild-type strain 15A by a single cross-over at the homologous locus, and transformants were selected on medium containing hygromycin B. Among 43 hygromycin-resistant transformants, nine contained an interrupted copy of the *PTK1* gene, as shown by Southern blot analysis (Fig. 3B). Three of these *ptk1* mutant strains con-

Table 1. The function of different mitogen-activated protein kinases (MAPKs) in fungal life cycles

Organism	Gene	Function	Reference
<i>Aspergillus nidulans</i>	<i>MPKA</i>	Germination of conidia and polarized growth	Bussink and Osmani 1999
<i>Botrytis cinerea</i>	<i>BMP1</i>	Growth and pathogenicity on different plant species	Zheng et al. 2000
<i>Candida albicans</i>	<i>HOG1</i>	Glycerol accumulation and virulence	San Jose et al. 1996; Alonso-Monge et al. 1999
	<i>MKC1</i>	Growth and morphogenesis under stress conditions	Navarro-Garcia et al. 1995
<i>Cochliobolus heterostrophus</i>	<i>CHK1</i>	Hyphal integrity, conidiation, appressorium formation and pathogenicity on corn	Lev et al. 1999
<i>Colletotrichum lagenarium</i>	<i>CMK1</i>	Pathogenicity, germination of conidia, appressorium formation and invasive growth in cucumber	Takano et al. 2000
<i>Magnaporthe grisea</i>	<i>PMK1</i>	Appressorium formation and pathogenicity on rice	Xu and Hamer 1996
	<i>MPS1</i>	Penetration peg formation, aerial hyphal growth and conidiation	Xu et al. 1998
	<i>OSM1</i>	Response to hyperosmotic stress and cellular turgor control	Dixon et al. 1999
<i>Nectria haematococca</i>	<i>FsMAPK</i>	Unknown	Li et al. 1997
<i>Ustilago maydis</i>	<i>KPP2-UBC3</i>	Mating (pheromone signal) and pathogenic development on corn	Müller et al. 1999; Mayorga and Gold 1999
<i>Saccharomyces cerevisiae</i>	<i>SMK1</i>	Sporulation	Krisak et al. 1994
	<i>HOG1</i>	Osmolyte synthesis in response to high osmolarity	Brewster et al. 1993
	<i>FUS3</i>	Mating in response to pheromone signaling	Elion et al. 1990
	<i>KSS1</i>	Filamentous growth	Courchesne et al. 1989
	<i>MPK1</i>	Cell wall remodeling in response to hypotonic shock	Torres et al. 1991
<i>Schizosaccharomyces pombe</i>	<i>PMK1</i>	Cell integrity	Toda et al. 1996
	<i>SPK1</i>	Conjugation and meiosis in response to mating pheromones	Gotoh et al. 1993
<i>Zygosaccharomyces rouxii</i>	<i>ZrHOG1</i>	Both involved in osmolyte synthesis in response to high osmolarity	Iwaki et al. 1999
<i>Pyrenophora teres</i>	<i>PTK1</i>	Conidiation, appressorium formation, and pathogenicity on barley	This work

tained two copies of the vector integrated at the homologous locus, whereas the other six *ptk1* mutants contained a single copy of the disruption vector at the same position. Transcript analysis of the *ptk1* mutant strains was carried out by RT-PCR during in vitro growth on CM with the F7 and R8 *PTK1* specific primers located at the 5' and 3' coding region of the gene, respectively. Control reactions were performed with total RNA from the wild-type strain. Genomic DNA from the wild-type strain was used as template to compare sizes of the amplified fragments, with or without introns (903 and 744 bp, respectively). The 744-bp amplification product of *PTK1* was obtained from RNA from the wild-type strain, but it was absent when RNA from the *ptk1* mutant strains was used as template (data not shown), confirming that the gene was no longer active.

To determine whether *PTK1* is essential for pathogenicity on barley, we inoculated barley leaves with mycelial sus-

pensions of all *ptk1* mutant strains and the wild type and one ectopic integration mutant as controls. Mycelium was used as inoculum instead of spores for proper comparisons between wild-type and *ptk1* mutant strains because mutants were not able to produce conidia, as shown below. Typical net blotch lesions were formed upon inoculation with wild-type mycelia as well as with the ectopic integration mutant (Fig. 4). No lesions were detected after inoculation with any of the *ptk1* mutant strains, indicating that they had lost their pathogenicity. In order to test the ability of the *ptk1* mutants to colonize plant tissues, barley leaves were wounded by abrading the surface with sand before inoculation. Again, typical net blotch lesions were formed upon inoculation with wild-type or with the ectopic integration mutant, but no signs of disease were detected with any of the *ptk1* mutants (data not shown), indicating that they were not able to colonize the host tissue.

Fig. 1. Alignment of the predicted amino acid sequence encoded by the *PTK1* gene to several mitogen-activated protein kinase (MAPKs). Alignment was performed with the CLUSTAL W 1.8 program (Thompson et al. 1994). Identical amino acids are indicated as white letters on black background. Similar residues are shown on a gray background. Gaps introduced for the alignment are indicated by hyphens. Protein kinase domains are indicated by Roman numerals (Hanks and Quinn 1991). TEY sequence required for kinase activation is marked by asterisks (Kültz 1998). GenBank accession nos. for *P. teres* Ptk1, *C. heterostrophus* Chk1, *B. cinerea* Bmp1, *C. lagenarium* Cmk1, *N. haematococca* Fsmapk, *M. grisea* Pmk1, and *U. maydis* Kpp2–Ubc3 are AF272831, AF178977, AF205375, AF174649, U52963, U70134, and AF193614, respectively.

PTK1 is required for conidiation and appressorium formation.

With the aim to determine at which infection step the *ptk1* mutants are affected, we studied their ability to produce conidia and to form appressoria in comparison to the wild-type and the ectopic integration mutant. Mycelium from the wild-type strain, the ectopic integration mutant, and the *ptk1* mutants were inoculated on POA plates, and conidium formation was checked 1 week later. Microscopic observation demonstrated that hyphae of the wild-type strain and ectopic integration mutant differentiated into conidiophores bearing conidia (Fig. 5A and C). In contrast, all *ptk1* mutants formed abnormally elongated conidiophores, three to four times longer than those produced by the wild type, and no conidia were detected at their tips (Fig. 5B and D). Hyphal tips of the wild-type strain and the ectopic integration mutant responded to the presence of a glass surface by swelling to form small appressoria (Fig. 5E). When mycelium from the *ptk1* mutants was inoculated on glass slides, the hyphae continued to grow and did not form any appressorium (Fig. 5F). The appressorium-defective phenotype was not rescued in the *ptk1* mutants after the addition of exogenous cyclic AMP (cAMP) and no hyphae hooking or swelling was detected (data not shown). The same differences in appressorium formation were observed when barley leaves were inoculated with the wild-type strain and *ptk1* mutants (Fig. 5G to I). Wild-type hyphae responded to the presence of the barley leaf surface by growing toward the regions close to anticlinal cell walls where they formed appressoria and penetrated through the cuticle and the underlying cell wall into the epidermal cell (Fig. 5G and H). In contrast to the wild-type strain, hyphae from the *ptk1* mutants continued to grow on the leaf surface without developing any appressorium and no attempt to penetrate was observed (Fig. 5I). Colonies of *ptk1* strains, however, showed the same morphology as the wild-type strain on minimal medium (MM) or CM plates (data not shown), and no significant difference in growth rate between the wild-type and *ptk1* mutants was found during growth on those media or under osmotic stress conditions (data not shown). These results demonstrate that *PTK1* gene is required for conidiophore differentiation and conidiation as well as for appressorium formation, but it is dispensable for vegetative growth.

DISCUSSION

In order to determine the roles of MAPK pathways in the life cycle of the ascomycete *P. teres*, we identified and characterized a MAPK gene of this fungus, *PTK1*, which is highly homologous to other known fungal MAPKs. It is an extracellular, signal-regulated kinase belonging to the YERK1 subfamily of the MAPK family, whose main function in other eukaryotes is the transduction of extracellular signals (Kültz 1998).

Our results demonstrate that *PTK1* is dispensable for vegetative growth on MM or CM plates as well as under osmotic stress conditions because we did not detect any difference between the wild-type strain and the *ptk1* mutants. In contrast, mutants in the MAPK gene *CHK1* from the closely related fungal pathogen *C. heterostrophus*, which together with *P. teres* belongs to the former *Helminthosporium* spp., showed altered colony morphology with poorly developed aerial hy-

phae and autolysis during vegetative growth (Lev et al. 1999). Moreover, the *PTK1* transcripts were present in fungal mycelium during vegetative growth and in conidiophores and conidia as well as during the infection process, suggesting that the *PTK1* gene may be expressed during all developmental stages tested. This indicates that regulatory mechanisms may occur at the posttranscriptional level, as widely described for protein kinase activities (Hunter 1995).

To discern the function of the *PTK1* gene, we generated *ptk1* mutants and characterized their phenotypes. They form abnormally elongated conidiophores that do not bear any conidia. A defect in conidiation has been observed previously in *C. heterostrophus* *chk1* mutants, where no conidia were formed (Lev et al. 1999), and in *cmk1* mutants of *C. lagenarium* (Takano et al. 2000) or *M. grisea* *mps1* mutants (Xu et al. 1998), where a significant reduction in conidiation has been described. These results are in contrast to those obtained in *pmk1* mutants of *M. grisea* (Xu and Hamer 1996) and *B. cinerea* *bmp1* mutants (Zheng et al. 2000), where conidiation was unaffected. An abnormal elongation of conidiophores in MAPK mutants has not been described.

ptk1 mutants are unable to produce appressoria on glass surfaces or barley leaves. The involvement of MAPKs in appressorium formation has been described previously in the *PMK1* gene of *M. grisea* (Xu and Hamer 1996) and its homologs, the *CHK1* gene of *C. heterostrophus* (Lev et al. 1999) and the *CMK1* gene of *C. lagenarium* (Takano et al. 2000). In all of these cases, the inactivation of the corresponding MAPK gene led to an inability to produce appressoria. In *M. grisea*, it was proposed that the Pmk1 MAPK pathway works cooperatively with a cAMP pathway for appressorium formation because the addition of cAMP induced the early steps of appressorium formation in *pmk1* mutants, even though they failed to form mature appressoria (Xu and Hamer 1996). When exogenous cAMP was added to *ptk1* mutants, however, it was not possible to induce the initiation of appressorium formation, suggesting that the *PTK1* gene acts independently of a cAMP signaling pathway for appressorium formation.

ptk1 mutants also lost their pathogenicity in barley leaves and were unable to grow invasively in barley plants, similar to *pmk1* mutants of *M. grisea* (Xu and Hamer 1996), *chk1* mutants of *C. heterostrophus* (Lev et al. 1999), *cmk1* mutants of *C. lagenarium* (Takano et al. 2000), and *bmp1* mutants of *B. cinerea* (Zheng et al. 2000). At present, little is known about

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) products showing expression of *PTK1*. First-strand cDNAs generated from total RNA isolated from mycelium grown on complete medium (lane 1), conidiophores and conidia formed on peanut leaves–oatmeal agar (lane 2), infected leaves 3 and 5 days postinoculation (lanes 3 and 4, respectively), and uninoculated leaves (lanes 5 and 6) were used as templates for PCR with the gene-specific primers R5 and F6. Lane M, 100-bp ladder marker. Lane C, PCR with genomic DNA as template was performed as a control.

invasive growth inside plants in fungal pathogens. Thus, studies of fungal genes expressed differentially during this development stage could give clues about the involvement of MAPKs in pathogenicity.

The multiple phenotype shown by *ptk1* mutants (deficiency in conidiophore differentiation and conidiation, appressorium formation, and pathogenicity and invasive growth in barley leaves) may indicate that the gene is involved in different pathways, each responsive to different extracellular signals. In

this case, specific responses could be achieved by additional related components or pathways. Various sets of phenotypes were found in fungi carrying mutations on different MAPK genes (Table 1). The findings in several phytopathogenic fungi emphasize the different functions of similar signaling MAPK pathways in closely related ascomycete foliar pathogens, even though all of them play crucial roles in fungal pathogenesis.

Appressoria are thought to be required by fungi to invade plants (Emmett and Parbery 1975), although their function

Fig. 3. Disruption of *Pyrenophora teres* *PTK1* gene. **A**, Disruption vector pKOptk1 and the *PTK1* locus. Vector pKOptk1 was constructed by cloning an internal 416-bp fragment of the *PTK1* gene (white bar) into the pAN7-1 vector (Punt et al. 1987) next to the hygromycin B resistance gene *HPH* (black arrow). The vector was linearized at the unique *Bss*HII restriction site located in the *PTK1* internal fragment. By homologous recombination through a single crossover, the gene was interrupted by a single-copy insertion event (A1; *ptk1* mutants 2, 3, 4, 6, 7, and 8) or by tandem insertion of two copies (A2; *ptk1* mutants 1, 5, and 9). Restriction enzyme sites: B = *Bam*HI; Bs = *Bss*HII; E = *Eco*RV. Methionine and stop codons are indicated by inverted black triangles. The position of a *Eco*RV restriction site that is 9 kb away from the *Eco*RV restriction site located in the coding region of the gene is indicated by a broken line. **B**, Southern blot analysis of genomic DNA from the wild-type strain (WT), *ptk1* mutants (1 to 9), and one ectopic integration transformant (E). Disruption vector pKOptk1 was included as control (V). DNA was digested with *Eco*RV (upper panel) or with *Eco*RV and *Bam*HI (lower panel). Blot was probed with the 416-bp internal fragment of the *PTK1* gene contained in the disruption vector (white bar; upper panel) or the complete pKOptk1 vector (lower panel).

may vary among fungi. *M. grisea* and *C. lagenarium* produce large and heavily melanized appressoria that enable them to generate a high, turgor-based pressure and penetrate into their hosts (Chida and Sisler 1987; Chumley and Valent 1990; Howard and Valent 1996; Kawamura et al. 1997; Kubo et al. 1991; Money and Howard 1996; Perpetua et al. 1996). In both fungi, mutants affected in melanin production or defective in appressorium formation are nonpathogenic as a result of their inability to penetrate the plant epidermis (Takano et al. 2000; Xu and Hamer 1996). Appressoria from *Cochliobolus* and *Alternaria* species are smaller, often swelling structures and nonmelanized. In both cases, albino mutants are fully virulent (Guillen et al. 1994; Kubo et al. 1989; Tanabe et al. 1995), as in the case of the albino mutant of *P. teres* (Löscher and Schäfer, unpublished results). In addition, α -deficient mutants of *C. heterostrophus* that have a severe reduction in the ability to form appressoria, are fully virulent (Horwitz et al. 1999). It has been suggested that small nonmelanized appressoria cannot generate mechanical force sufficient for infection and may not be critical for infection by *C. heterostrophus*. Fungi producing this type of appressoria also may depend on other mechanisms, e.g., enzymatic action, to gain entry into their hosts (Horwitz et al. 1999). Our data seem to be in contrast with those obtained in *C. heterostrophus* because *ptk1* mutants cannot produce the small nonmelanized appressoria made by the wild-type strain and are no longer pathogenic. It is possible that appressorium formation in *P. teres* is necessary as a developmental step before colonization of the host tissue, and if this step does not take place, colonization will not occur. This would explain why wounded barley leaves are not infected by the *ptk1* mutants. It is also possible that *PTK1* regulates genes that are directly necessary for appressorium formation and a different set of genes necessary for colonization of the host tissue. Because the role of the small nonmelanized appressoria during the infection process is not clear, the question remains whether the loss of pathogenicity in the *ptk1* mutants is a result of their inability to form appressoria. The MAPK mutants of *P. teres* should enable us to identify and study downstream components of the Ptk1 MAPK cascade in order to understand the relationship between the fungal signaling pathway, its involvement in conidiation and appressorium formation, and its pathogenicity on barley.

MATERIALS AND METHODS

Fungal strains and culture conditions.

P. teres strain 15A causing the net-type symptoms typical of the net blotch of barley (Weiland et al. 1999) was stored as dried mycelium at room temperature or in 30% glycerol at -70°C . The pathotype of the isolate was periodically confirmed by plant infection assays in a growth chamber. For extraction of DNA and RNA, mycelium was obtained from cultures grown in CM (Leach et al. 1982) in Erlenmeyer flasks on a rotary shaker at 150 rpm and 28°C . Five-day-old mycelium was harvested and blended with a commercial blender. The resulting homogenates were washed with sterile water, centrifuged, and resuspended in 4 vol of sterile water (wt/vol) and an aliquot transferred to fresh CM and incubated for 14 h. For induction of conidiation, an aliquot of the fungal homogenate was transferred to POA (Speakman and Pommer 1986) and incubated at 18°C under near-UV light (TLD 36

W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips) for a 12-h photoperiod for 1 week. The growth rate of the *P. teres* wild-type strain and *ptk1* mutants was tested on MM (CM agar without yeast extract and casein hydrolysate), CM agar, and under osmotic stress on CM agar supplemented with varying concentrations of NaCl (0.2 to 1 M). In these experiments, plates were inoculated with a 6-mm diameter plug of mycelium and allowed to grow for 12 days at 24°C . The diameter of the colony was then measured.

Isolation, cloning, and sequencing of PTK1.

Four degenerate primers were designed on the basis of conserved amino acid regions among different MAPKs: primer 1 (5'-CCAICKIGTNGCIACRTAYTC-3') and primer 4 (5'-AYYTCIGGIGCICKRTAVYA-3') were designed on the conserved region of domain VIII; primer 2 (5'-GCITAYGGIRTNGTNTG-3') on the conserved region of domain I; and primer 3 (5'-GTIGCNATRAARAARAT-3') on the conserved region of domain II (Hanks and Quinn 1991).

P. teres genomic DNA from strain 15A was first amplified with primers 2 and 4. The 50- μl PCR reaction contained 1.5 mM MgCl_2 , 0.2 mM dNTP (each), 150 pmol of each primer, 100 ng of genomic DNA, 1 U of *Taq* polymerase, and $1\times$ PCR buffer (Gibco BRL, Paisley, U.K.). The PCR was initiated by denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 40°C for 1 min, and 72°C for 1 min. PCR cycling included a final extension step at 72°C for 10 min and a cooling step to 4°C .

After amplification with primers 2 and 4, the PCR reaction was diluted to 1:50 and 1 μl was used to perform a nested PCR amplification with primers 1 and 3 under the same conditions as described above. A 478-bp PCR fragment was obtained, cloned into pCR 2.1-TOPO vector (Invitrogen, Leek, The Netherlands), and used as a probe for the screening of a genomic IEMBL3 library of *P. teres* strain 15A. Library screening under high-stringency conditions, subcloning, and other routine procedures were performed by standard protocols (Sambrook et al. 1989). Sequencing was performed on

Fig. 4. Pathogenicity assay on barley leaf segments. Intact barley leaf segments were inoculated with the wild-type strain (WT) and nine mutants carrying an interrupted copy of the *PTK1* gene (1 to 9). Controls consisted of leaf segments inoculated with water (C) as well as leaf segments infected with an ectopic integration transformant (E). Picture was taken 5 days postinoculation.

Fig. 5. Effects of targeted disruption of *PTK1* on conidiation and appressorium formation. Under conidiation-inducing conditions, conidiophore tips of the wild-type strain differentiate and produce conidia (**A and C**). Conidiophores from the *ptk1* mutant strains are approximately three to four times longer than those of the wild-type and do not produce conidia (**B and D**). **A and B**, Light microscopy images taken 5 days postinoculation (dpi) on peanut leaves–oatmeal agar (POA) plates (bar = 50 μ m; 1 = mycelium; 2 = conidiophore; 3 = conidium). **C and D**, Scanning electronic micrographs taken 5 dpi on POA plates (bar = 50 μ m). On a glass surface, wild-type hyphal tips swell to form small appressoria (arrows) (**E**), whereas mutant strains do not produce appressoria (**F**). **E and F**, Fluorescence microscopy images of mycelium stained with calcofluor 24 h postinoculation (bar = 50 μ m). On barley leaves, wild-type hyphae form appressoria and penetrate through the cuticle (**G and H**). Hyphae from the mutant strains grow on the leaf surface without developing appressoria, and penetration attempts are not observed (**I**). **G, H, and I**, Scanning electronic microscopy images of infected barley leaves 24 h postinoculation. **G**, Bar = 10 μ m; **H and I**, Bar = 20 μ m.

double-stranded DNA by MWG Sequencing Service (MWG-Biotech, Ebersberg, Germany) and AGOWA Sequencing Service (AGOWA GmbH, Berlin, Germany). Sequence data analyses were carried out with the Wisconsin Package, Version 8.3 (Genetics Computer Group, Madison, WI, U.S.A.) and Lasergene Programs (DNASTar, Madison, WI, U.S.A.).

Nucleic acid isolation and analysis.

Genomic DNA was extracted from *P. teres* mycelium by a DNA Isolation Kit (Gentra Systems, Minneapolis, MN, U.S.A.). Three micrograms of DNA were digested with appropriate enzymes and subjected to Southern hybridization analysis, as described in standard protocols (Sambrook et al. 1989), with the use of a nonisotopic DIG-dUTP labeling kit (Boehringer Mannheim, Mannheim, Germany), in accordance with the manufacturer's instructions. Total RNA was prepared according to the protocol of Kay et al. (1987).

Expression studies.

Expression of the *PTK1* gene was carried out by RT-PCR, either in vitro or during infection of barley leaves. Total RNA from *P. teres* strain 15A was extracted from mycelium grown on CM liquid medium or from conidia and conidiophores harvested from POA plates and used for in vitro growth studies. Barley leaf segments were infected with *P. teres* strain 15A, and 2 and 5 days postinoculation total RNA was isolated. Leaves inoculated with sterile water were used as control. In order to eliminate possible DNA contaminations, total RNA was treated with DNase I (Life Technologies, Karlsruhe, Germany), according to the manufacturer's instructions. One microgram of total DNA-free RNA was subjected to RT-PCR with the antisense primer R5 5'-ATCCAGAGTTGTCTTCGGTCGA-3' and the sense primer F6 5'-TCCATGTTCTGTCTGAGAACAACACTACG-3', both flanking the second intron of the gene. RT-PCR was performed with the Access RT-PCR System (Promega Corp., Madison, WI, U.S.A.), following the manufacturer's instructions. First-strand cDNA synthesis was performed at 48°C for 45 min followed by a denaturation step at 94°C for 3 min. Second-strand cDNA synthesis and PCR amplification conditions consisted of 40 cycles at 94°C for 1 min, 59°C for 1 min, and 68°C for 1 min. PCR cycling included a final extension step at 68°C for 7 min and a cooling step to 4°C. Aliquots of the PCR products were analyzed on 2.5% agarose gels. The amplified fragments were cloned into the pGEM-T vector (Promega, Mannheim, Germany) and sequenced in order to confirm their identity.

Transcript analysis of the *ptk1* mutant strains was also carried out by RT-PCR with the *PTK1*-specific antisense primer R8 5'-CAGAGATCGGATGTACTCGCG-3' and the sense primer F7 5'-CAATGTCTCCGAGCAGTACGATATC-3', located at the 3' and 5' coding region of the gene, respectively. Total RNA was extracted from 5-day-old mycelium grown on CM liquid medium. Control reactions were performed with total RNA from the wild-type strain grown under the same conditions. The RT-PCR reactions were performed under the same conditions as described above. Aliquots of the PCR products were analyzed on 2.5% agarose gels.

Transformation-mediated gene disruption.

A 416-bp internal fragment from the *PTK1* gene was amplified with the gene-specific primers R5-H 5'-CCCAAGCTT-

GGGATCCAGAGTTGTCTTCGGTCGA-3' and F6-H 5'-CCCAAGCTTGGGTCCATGTTCTGTCTGAGAACAACACTACG-3', both containing a *Hind*III restriction site. The 50 µl of PCR reaction contained 1.5 mM MgCl₂, 0.2 mM dNTP (each), 20 pmol of each primer, 20 ng of DNA from pPTK1-5.5H, 1 U of *Taq* polymerase, and 1× PCR buffer (Life Technologies). The PCR was initiated by denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min. PCR cycling included a final extension step at 72°C for 10 min and a cooling step to 4°C. The 416-bp fragment obtained was treated with *Hind*III and cloned into the unique *Hind*III restriction site of vector pAN7-1 (Punt et al. 1987) containing the hygromycin-resistant gene *HPH* as selective marker (Fig. 3A). The resulting plasmid, pKO_{ptk1}, was linearized at the unique *Bss*HII restriction site located in the *PTK1* internal fragment. This linearized construction was used to transform protoplasts of *P. teres* strain 15A.

Protoplast formation was achieved by treating 6 g of fresh mycelium with 4 ml (vol/wt) of 5% driselase enzyme solution per g (InterSpex Products, San Mateo, CA, U.S.A.) in 0.7 M NaCl, with shaking at 100 rpm, at 28°C for 2 to 3 h. Protoplast solution was then transferred to ice to avoid cell wall regeneration and filtered twice, first through a double-sterile gauze and then through a nylon membrane (cutoff 50 µm). In order to remove the remaining enzyme, protoplasts were washed three times with 0.7 M NaCl and centrifuged at 3,000 rpm for 10 min at 4°C. Finally, they were resuspended in 1 ml of solution Sorb-TC (1.2 M Sorbitol; 50 mM CaCl₂; and 10 mM Tris-HCl, pH 7.0). Protoplasts (10⁷) were heat-shocked at 48°C for 5 min before adding 20 µg of transforming DNA in a final volume of 200 µl. After 10 min of incubation on ice, 1 vol of polyethylene glycol (PEG)-TC (60% PEG 4,000; 50 mM CaCl₂; and 10 mM Tris-HCl, pH 7.0) was added. Ice incubation was extended for an additional 15 min, followed by the addition of 8 vol of PEG-TC. These transformed protoplasts were added to 400 ml of CM and divided among 20 petri dishes. After 2 days incubation at 24°C, the regeneration of protoplasts was observed. Petri dishes were then overlaid with 10 ml of hygromycin medium (1.2% wt/vol agar in water containing 225 µg of hygromycin B per ml) (DUCHEFA, Haarlem, The Netherlands). The incubation continued at 24°C until hygromycin-resistant transformants appeared.

Pathogenicity tests.

The barley cultivar Hazera (Steffenson and Webster 1992b) was used for all virulence assays. Plants were grown in 11-cm pots at 16°C with a 16-h photoperiod (7,500× at pot level) and 70% relative humidity. Five leaf segments (7 cm in length) from 4-week-old barley plants were fixed onto filter paper and placed in a 94-mm petri dish with 10 ml of water. Five-day-old mycelium was harvested from CM liquid cultures and blended with a commercial blender. The resulting homogenates were washed with sterile water, centrifuged, and resuspended in 4 vol of sterile water (wt/vol). Each leaf segment was inoculated with a 50-µl drop of the fungal homogenate and incubated at plant culture conditions for 1 week. Full disease symptoms became apparent 4 to 7 days after inoculation. Wounded barley leaves were used to check the ability of the fungus to colonize host tissues. Wounds were produced by abrading the leaf surface with white quartz sand (Sigma-

Aldrich, Deisenhofen, Germany). Each experiment was independently repeated at least four times. Control samples consisted of intact or wounded leaf segments inoculated with a 50- μ l drop of sterile water.

In vitro and in vivo assays for appressorium formation.

In order to assess the ability of the fungus to produce appressoria in vitro, 5-day-old mycelia from the wild-type strain, one ectopic integration mutant, and the *ptk1* mutants were harvested from CM liquid cultures and blended with a commercial blender. The resulting homogenates were washed with sterile water, centrifuged, and resuspended in 4 vol of sterile water (wt/vol). A 100- μ l aliquot of the homogenate was diluted in 400 μ l of fresh CM or in CM supplemented with exogenous 10 mM cAMP and transferred to sterile glass slides. The slides were incubated in petri dishes lined with moist filter paper and covered and sealed with Parafilm in a growth chamber at 16°C with a 16-h photoperiod and 70% relative humidity. Twenty-four hours postinoculation, samples were stained with 10 μ g of calcofluor per ml and appressorium formation was checked by fluorescence microscopy.

Appressorium formation was also tested during the infection process. Barley leaves were inoculated with aliquots of fungal homogenates from the wild-type strain and the *ptk1* mutants with sterile cotton buds that had been immersed in the corresponding homogenate. Twenty-four hours after infection, samples were collected and appressorium formation was checked by scanning electronic microscopy.

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