The Biotrophic, Non-Appressorium-Forming Grass Pathogen *Claviceps purpurea* Needs a *Fus3/Pmk1* Homologous Mitogen-Activated Protein Kinase for Colonization of Rye Ovarian Tissue

G. Mey,¹ B. Oeser,¹ M. H. Lebrun,² and P. Tudzynski¹

¹Institut für Botanik, Westfälische Wilhelms-Universität, Schlossgarten 3, D-48149 Münster, Germany; ²UMR 1932 CNRS-Aventis, Physiologie Cellulaire Végétale, Aventis Crop Science, 14 rue Pierre Baizet, F-69009 Lyon, France

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Claviceps purpurea is a common pathogen of a wide range of grasses and cereals that is able to establish a stable, balanced interaction with its host plant and is considered a biotroph. It does not form special penetration structures such as appressoria. To study the signaling processes involved in this special host–pathogen interaction, we have cloned a gene, *cpmk1*, encoding a mitogen-activated protein (MAP) kinase that shows significant homology to *Fus3* of *Saccharomyces cerevisiae* and to *pmk1* of *Magnaporthe grisea*. Using a gene-replacement approach, we isolated a Δ *cpmk1* mutant and characterized it in detail. Loss of CPMK1 has no obvious effect on vegetative properties (such as growth rate, morphology, and conidia formation); however, infection tests on rye show that the mutant is unable to colonize rye tissue, i.e., it appears to be completely nonpathogenic. Complementation of the mutant with a wild-type copy of *cpmk1* fully restores its pathogenicity, confirming that this MAP kinase is essential for infection of rye by *C. purpurea*. Transformation of the Δ *pmk1* mutant of *M. grisea* with a complete copy of *cpmk1* (including the *C. purpurea* promoter) fully restored its ability to form appressoria and its pathogenicity on barley. Although both fungi drastically differ in their pathogenic strategies, this result indicates that the signal pathway involving CPMK1 is highly conserved.

Claviceps purpurea is a ubiquitous pathogen of grasses and cereals that infects young florets and replaces the plant's ovarian tissue with sclerotia. The fungus is able to penetrate the plant's cuticle without the help of specific infection structures such as appressoria. Its intracellular growth follows the path of the pollen tube until it reaches the ovary base, where it taps into the vascular tissue. Although it can be easily grown in axenic culture, it is considered to be a biotroph, since it causes only limited host cell death during the colonization phase. It is able to maintain a continuous flow of phloem exudates for the production of conidia-containing honeydew (Tenberge 1999; Tudzynski et al. 1995). We have recently addressed several aspects of this specialized host–pathogen interaction by a combined cytological, biochemical, and molecular genetic approach involving, for instance, the role of cell wall-degrading enzymes (Giesbert et al. 1998; Müller et al. 1997; Tenberge et al. 1996; ten Have et al. 2001) and active-oxygen-scavenging

systems (Garre et al. 1998a, 1998b). Furthermore, we have initiated a broad, random approach to analyze infection-phase-specific gene expression, based on expressed sequence tag (EST) data and differential cDNA screening (Oeser et al., in press). We are particularly interested in the factors controlling the very early steps of infection, the directed growth in the plant tissue, and the establishment of a balanced interaction. As signal transduction is involved in all of these processes, we have initiated analyses of signal-transduction pathways in *C. purpurea*.

Signal pathway components are highly conserved within eukaryotic organisms, ranging from lower fungi to higher eukaryotes. Because of this high degree of homology, many fungal genes encoding components of such systems are known. In several host–pathogen systems, specific signaling components involved in the interaction have been identified, including subunits of heterotrimeric G proteins, components of the cyclic AMP (cAMP) pathway, or protein kinases (Lengeler et al. 2000). Especially members of the mitogen-activated protein (MAP) kinases have been shown to play an essential role in the early stages of infection in several plant-pathogenic fungi (Xu 2000). We used degenerate primers to clone a MAP kinase (MAPK) gene from *C. purpurea* (*cpmk1*); by targeted deletion and complementation, it was shown that this gene is essential for the penetration and colonization of host tissue by *C. purpurea*. In addition, the *cpmk1* gene can complement a mutant of the orthologous gene from *Magnaporthe grisea* (*pmk1*) and restore its ability to form appressoria and its pathogenicity. Thus, the signaling pathway containing *cpmk1* is highly conserved between these two fungi, even though they have completely different pathogenic strategies.

RESULTS

Isolation and characterization of the *cpmk1* gene from *C. purpurea*.

Degenerate primers based on the sequences of the *M. grisea* *pmk1* and *Aspergillus nidulans* *mpkB* genes were used to amplify a 490-bp genomic fragment from *C. purpurea*, the deduced peptide sequence of which is 95% identical to the PMK1 protein. The polymerase chain reaction (PCR) product was used as a probe to screen a *C. purpurea* genomic library. Of the 6,000 lambda clones screened, seven clones hybridized to the PCR probe and were characterized further. Restriction map analyses revealed a common 6.9-kb *Bam*HI fragment. This fragment was subcloned into pUC19, yielding plasmid pMKB2,

and sequenced. It contains an open reading frame of 1,406 bp, which is interrupted by three introns of 118, 107, and 116 bp (confirmed by cDNA sequencing, discussed below).

The 490-bp PCR fragment was also used as a probe to screen a *C. purpurea* cDNA library. Two cDNA clones hybridizing to the probe were further purified and sequenced. The transcription appeared to be initiated -397/-373 bp upstream of the predicted start codon, and only one major transcription termination site was found +352/+366 bp downstream of the stop codon (data not shown). The coding sequences of the cDNA clones are identical to the genomic exons of the gene, designated *cpmk1* (*Claviceps purpurea* MAP kinase 1). The sequence of *cpmk1* is available under GenBank accession number AJ318517.

The deduced peptide sequence, CPMK1, is 97% identical to CGK1 from *Colletotrichum gloeosporioides* and to FMK1 from *Fusarium oxysporum* f. sp. *lycopersici* and is 96% identical to PMK1 from *M. grisea*. Phylogenetic analyses showed that CPMK1 belongs to the same group as the protein Fus3 from *Saccharomyces cerevisiae* and the other fungal Fus3 homologues (Fig. 1). *S. cerevisiae* MAPK Slt2 and Hog1 and their fungal homologs, which are involved in the pathways responsible for the maintenance of cell wall integrity and the response to osmotic stress, segregate into two different phylogenetic groups.

Southern blot analyses using *cpmk1* cDNA as a probe under low stringency conditions revealed only one 8-kb *EcoRI* fragment and two *HindIII* fragments of 5 kb and 0.9 kb in the wild-type strain 20-1 (data not shown). This is consistent with the presence of a single copy of the *cpmk1* gene in the genome of *C. purpurea*.

Fig. 1. Comparison of CPMK1 from *Claviceps purpurea* to other fungal mitogen-activated protein (MAP) kinases. The deduced peptide sequence of *cpmk1* from *C. purpurea* (GenBank accession no. AJ318517) was compared with the MAP kinases PMK1 (U70134), MPS1 (AF020316), and OSM1 (AF184980) from *Magnaporthe grisea*; GMK1 from *Gaeumannomyces graminis* (AF258529); CGK1 from *Colletotrichum gloeosporioides* (AB047033); CMK1 from *Colletotrichum lagenarium* (AF174649); FMK1 from *Fusarium oxysporum* f. sp. *lycopersici* (AF286533); FsmAPK from *Fusarium solani* (U52963); BMP1 from *Botrytis cinerea* (AF205375); MAP1 from *Blumeria graminis* (AF301165); CHK1 from *Cochliobolus heterostrophus* (AF178977); MPKB from *Emericella nidulans* (AF198118); MAPK from *Pneumocystis carinii* (AF043941); Ubc3/KPP2 from *Ustilago maydis* (AAF09452/AAF15528); MAPK from *Lentinula edodes* (AF173376); and Fus3 (M31132), Slt2 (X59262), and Hog1 (L06279) from *Saccharomyces cerevisiae*. The phylogram was constructed with the MegAlign program (DNA Star, Madison, WI, U.S.A.).

***cpmk1* Is expressed in planta.**

Reverse-transcription (RT)-PCR analyses were performed in order to determine whether *cpmk1* is expressed during rye infection. A 465-bp PCR fragment was amplified with the primers K9 and K10 (Fig. 2A) and a cDNA clone as the template (Fig. 2B, lane 2). Because of the presence of two introns in this region, a longer fragment (690 bp) was amplified with the same primer pairs and genomic DNA as the template (Fig. 2B, lane 1). RT-PCR analyses were performed with the primers K9 and K10 and with mRNA extracted from rye infected with the *C. purpurea* wild-type strain for 5, 10, 15, and 20 days. The PCR products, separated with an agarose gel, were difficult to detect by using ethidium bromide staining. Hybridization with a *cpmk1* cDNA probe revealed the presence of the predicted 465-bp fragment at 10, 15, and 20 days postinoculation (dpi) (Fig. 2B, lanes 4 to 6), corresponding respectively to the stages of honeydew production, differentiation, and maturation of the sclerotia (the upper band occurring in lanes 4 and 6 is derived from contaminating genomic DNA in these specific RNA samples; note the same bands in the control [Fig. 2C]). The expression level of *cpmk1* seems to reach a maximum at 10 dpi. No signal could be detected with the mRNA extracted at 5 dpi (Fig. 2, lane 3). The lack of expression of *cpmk1* at this stage could be attributed to both the low ratio of fungal/plant mRNA and the general low expression level of *cpmk1*. By using a comparable RT-PCR approach, transcripts of the class II hydrophobin gene *chp1* (S. Moore and P. Tudzynski, unpublished data) were detected in all stages of rye infection and appeared, according to ethidium bromide staining of the amplification products, to be much more abundant than the *cpmk1* transcripts (Fig. 2C). *cpmk1* Appears thus to be expressed in planta, though at a very low level.

CPMK1 is essential for pathogenicity.

Mutant strains lacking an active *cpmk1* gene were generated using the gene-replacement vector pMIR (Fig. 3A). Transformants were selected on medium containing phleomycin, and their genomic DNA was subjected to PCR with the primers K5 and pAN2 (Fig. 3A). Two out of the 246 transformants analyzed exhibited the 1,320-bp fragment, consistent with the replacement of *cpmk1* with the phleomycin resistance construct. The Δ *cpmk1* transformants called M1.5 and M1.245 were genetically purified using single-spore isolation. One isolate of each knock-out transformant, lacking the wild-type copy of the *cpmk1* gene as revealed by PCR with the primers K5 and K7 (Fig. 3A), was subjected to Southern blot analysis with the left flank of the replacement vector as a probe (Fig. 3B). A 3.6-kb *PstI* fragment and a 1.3-kb *KpnI* fragment corresponding to *cpmk1* were revealed in both the wild-type strain and the transformant M1.28 carrying an ectopic insertion of the replacement construct (Fig. 3B, lanes 1, 4, 5, and 8). Neither signal was detected in the knock-out transformants (Fig. 3B, lanes 2, 3, 6, and 7). Instead, consistent with a replacement event, a 1.2-kb *PstI* fragment was detected in the M1.5 and M1.245 strains. However, the presence of an additional 5-kb *PstI* fragment (Fig. 3B, lane 3) revealed that the M1.245 transformant does contain an additional ectopic copy of the replacement construct. Both Δ *cpmk1* strains exhibited *KpnI* fragments longer than the expected 5.4-kb fragment, indicating that the *KpnI* site on the right flank of the *cpmk1* locus was lost during the recombination process.

Pathogenicity of the Δ *cpmk1* mutant strain M1.5 (carrying no additional ectopic integration) was assayed by using rye infection tests. Rye flowers were inoculated with conidial suspension of the M1.5, M1.28 (an ectopic transformant carrying an intact *cpmk1* gene), and wild-type strains and monitored for 3 weeks. Honeydew was visible on the rye florets infected with

the M1.28 and 20-1 strains after 6 to 9 dpi. In assays with both strains, sclerotia fully differentiated within 21 dpi (Fig. 4A to C). In contrast, no disease symptoms could be observed on the flowers infected with the $\Delta cpmk1$ strain (Fig. 4A). Some of the infected flowers even produced seeds, indicating that the development of the plant is not affected by the presence of the fungus. A fluorescent microscopical analysis of ovaries infected with the $cpmk1$ mutant did not reveal any fungal hyphae within the style tissue at 5 dpi, whereas in the wild-type infection, the hyphae penetrated the stigmatic hairs soon after germination and grew downward to the style (Fig. 5A). Later, massive hyphal bundles could be observed growing within the style toward the ovary (data not shown). In the mutant, only epiphytic hyphae could be detected growing on the outer surface of the stigma (Fig. 5B to D). Although the mutant conidia could germinate on the stigma surface, they obviously were unable to penetrate.

In order to determine whether the deletion of $cpmk1$ only affects the ability of the mutant to penetrate the host tissue, spores of the mutant M1.5 were introduced inside the floret tissue with a needle. No symptoms of the ergot disease appeared and the $\Delta cpmk1$ transformant remained completely nonpathogenic, whereas the same infection conditions led to a normal time course of disease development with the ectopic transformant M1.28 and the wild-type strain (data not shown). Obviously, the mutant is also significantly impaired in colonizing the host tissue after wounding.

To prove that the lack of pathogenicity of the M1.5 strain is because of the deletion of $cpmk1$, complementation experiments were performed. A 3.6-kb genomic fragment, including the $cpmk1$ coding region and 1.0 kb and 1.1 kb of the $cpmk1$ promoter and terminator regions, respectively, was cloned into the *Xba*I and *Sac*I sites of the pCB1635 vector, which carries a glufosinate resistance cassette (*bar*). The plasmid obtained, pM1CXS, was used to transform the *C. purpurea* $\Delta cpmk1$ mutant strain M1.5. Transformants were selected on glufosinate-containing plates. Twenty transformants were analyzed in detail; they were shown to be both phleomycin and glufosinate resistant, indicating that they carry both the replacement and the complementation constructs. PCR analyses with the primers K9 and K12 revealed that 18 transformants contain the complete $cpmk1$ coding region (data not shown). Two of them (M1C.6 and M1C.8) were further analyzed by Southern hybridization with the pM1P *Eco*RI insert as a probe. A 3.4-kb *Eco*RV fragment and a 1.0-kb *Pst*I fragment, characteristic for the replacement of $cpmk1$ with the phleomycin resistance cassette, were detected in the M1.5 recipient strain and in the M1C.6 and M1C.8 transformants (data not shown). Both the M1C.6 and M1C.8 strains showed one 2.5-kb *Eco*RV fragment corresponding to the $cpmk1$ gene under the control of its own promoter and terminator regions. They carry several copies of the complementation vector, as revealed by the presence of several *Pst*I fragments hybridizing to the probe. The 4.4-kb *Eco*RV fragment and the 3.6-kb *Pst*I fragment, corresponding to the wild-type $cpmk1$ gene, were detected in the 20-1 strain but not in the M1C.6, M1C.8, and M1.5 strains, indicating that no cross-contamination of the transformants with the wild-type strain occurred (data not shown).

Conidial suspensions of the M1C.6 and M1C.8 strains were used to infect rye flowers. The honeydew production started between 6 and 8 dpi. The spores rescued from the honeydew produced by both transformants were shown to be both phleomycin and glufosinate resistant, i.e., the positive infection was not due to a wild-type contamination. As in infection with the wild-type strain, sclerotia differentiated within 21 dpi (Fig. 4D). The time course of infection was comparable to the one obtained with the wild-type strain 20-1 (Fig. 4D); M1C.6 and

M1C.8 thus appear to be as pathogenic as the wild-type strain. This demonstrates that $cpmk1$ complements the pathogenicity defect of the $\Delta cpmk1$ mutant M1.5 and confirms that $cpmk1$ is essential for the pathogenicity of *C. purpurea* on rye.

***cpmk1* Restores the appressoria formation and pathogenicity functions of an *M. grisea* $\Delta pmk1$ mutant.**

In order to determine whether the MAP kinase CPMK1 from *C. purpurea*, a non-appressoria-forming fungus, can restore the functions of its orthologue PMK1 in *M. grisea*, the pM1CXS plasmid, carrying $cpmk1$ under the control of its own regulatory regions, was introduced into the *M. grisea* $\Delta pmk1$ mutant strain nn78. The transformants were selected on glufosinate-containing plates. As a control, $\Delta pmk1$ transformants carrying

Fig. 2. *cpmk1* Expression during infection of rye by *Claviceps purpurea*. **A**, Scheme of the structure of the *cpmk1* gene and cDNA showing the positions of the coding sequence (CDS, white box), promoter (P), and terminator (T) regions. The introns are represented by hatched boxes. The positions of the primers K9 and K10 used for reverse-transcription-polymerase chain reaction (RT-PCR) are indicated with black triangles. **B**, RT-PCR analysis of *cpmk1*: PCR was performed with genomic DNA (lane 1) and a *cpmk1* cDNA plasmid (lane 2) as a control. First-strand cDNAs were generated from total RNA extracted from rye ovaries infected for 5, 10, 15, and 20 days (lanes 3 to 6, respectively) and from noninfected rye ovaries (lane 7). This cDNA was then used as a template for PCR with the gene-specific primers K9 and K10. To prove the identity of the PCR products, the agarose gel was blotted on nylon membrane and hybridized with a 32 P-labeled *cpmk1* probe. The GeneRuler 1-kb DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) (left side) was used to estimate the size of the PCR products. **C**, Control RT-PCR: the same templates as in **B** were used for RT-PCR with a primer pair specific for a gene expressed constitutively in planta, *cph1*, coding for a class II hydrophobin (S. Moore and P. Tudzynski, unpublished data). A low-molecular-weight, unspecific amplification product appears in lane 7.

the pCB1635 vector containing only the glufosinate resistance cassette were generated in parallel.

Southern blot analyses, with a *cpmk1* cDNA clone as a probe, showed that the $\Delta pmk1/cpmk1$ transformants T1 to T4 carry the 2.5-kb *EcoRV* fragment characteristic for the *cpmk1* gene. Only one *XbaI* fragment was revealed in the strains T1 and T2, indicating that only one copy of the complementation vector integrated into the genome. No signal could be detected in the $\Delta pmk1$ recipient strain, the transformants containing the pCB1635 vector, and the wild-type strain (data not shown).

Fig. 3. Inactivation of *cpmk1* using a gene-replacement approach. **A**, The replacement vector pMIR was constructed by cloning the 3' and 5' parts of *cpmk1* on each side of a phleomycin resistance cassette (*ble*) in the pAN8-1UM plasmid (details in text). The resistance cassette, excised with a *SacII-KpnI* restriction, was used to transform the *Claviceps purpurea* wild-type strain 20-1 (top). $\Delta cpmk1$ Mutants were generated following the disruption of the wild-type gene (WT) by homologous recombination through a double-crossover event. The *cpmk1* coding sequence is represented with a white box. The sense of transcription of *cpmk1* is indicated with a black arrow. The grey arrow shows the orientation of the phleomycin resistance cassette within the replacement construct. The positions of the primers K5, K7, and pAN2, used for the detection of *cpmk1* wild-type copy and for the identification of the $\Delta cpmk1$ mutants, respectively, are indicated with black triangles. Abbreviations for restriction enzymes: P = *PstI* and K = *KpnI*. **B**, Southern blot analysis of genomic DNA extracted from the wild-type strain 20-1 (lanes 1 and 5), the $\Delta cpmk1$ mutants M1.5 (lanes 2 and 6) and M1.245 (lanes 3 and 7), and the transformant M1.28 carrying an ectopic integration of the replacement construct (lanes 4 and 8). The DNA was digested with *PstI* (lanes 1 to 4) and *KpnI* (lanes 5 to 8). The blot was probed with the 1-kb *SacII-NotI* fragment from *cpmk1* corresponding to the 3' flank of the replacement vector (dotted box shown in A).

Appressoria differentiation tests were performed in vitro with two different hydrophobic surfaces, plastic slides and onion epidermis. The germinating spores of the transformants T1 to T4 (carrying the M1CXS vector) were able to differentiate melanized appressoria comparable to the ones obtained with the wild-type strain on both surfaces (Fig. 6A, C, and D). In contrast, the spores of the transformants carrying the pCB1635 vector and the $\Delta pmk1$ recipient strain produced long germ tubes but did not develop any infection structure (Fig. 6B). Thus, the expression of the heterologous gene *cpmk1* from *C. purpurea* restores the appressoria formation function in the *M. grisea* $\Delta pmk1$ mutant strain.

Four transformants carrying *cpmk1* were tested for their ability to infect barley. Conidial suspensions of the $\Delta pmk1/cpmk1$ strains T1 to T4 were spread on the surface of barley leaves. Infection tests using the $\Delta pmk1$ recipient strain, a transformant carrying the pCB1635 vector alone, and the wild-type strain Guy 11 were performed in parallel as a control. Comparable lesions were observed at 5 dpi on the leaves infected with the $\Delta pmk1/cpmk1$ transformants and with the wild-type strain (Fig. 7). No lesions developed on the leaves infected with the *M. grisea* $\Delta pmk1$ mutant strain and with the transformants carrying the vector pCB1635 (Fig. 7).

These data prove that the *cpmk1* gene from *C. purpurea*, under the control of its own 5' and 3' regulatory regions, complements the $\Delta pmk1$ deficiency in *M. grisea*.

DISCUSSION

MAP kinases, a special family of serine/threonine protein kinases, are known to mediate the adjustment of intracellular activities of eukaryotic cells to environmental changes; they are activated by a MAP kinase cascade that has been shown to be highly conserved in a wide variety of eukaryotic organisms (Schaeffer and Webber 1999). In *S. cerevisiae*, five MAP kinase-mediated signal-transduction pathways have been identified, three of which have been shown to be also active in filamentous fungi: Fus3/Kss1 is involved (in *S. cerevisiae*) in mating responses and filamentous growth, Slr2 is responsible for cell integrity, and Hog1 is involved in stress response (especially high osmotic pressure). In the phytopathogenic filamentous fungus *M. grisea*, homologs of these three MAP kinases have been described: *pmk1* ($\triangleq$ Fus3), *osm1* ($\triangleq$ Hog1), and *mps1* ($\triangleq$ Slr2). Only *pmk1* and *mps1* have been shown to be involved in the pathogenicity of this fungus: *pmk1* mutants are impaired in appressoria formation, penetration, and invasive growth; *mps1* mutants are defective in penetration but are able to invade the host's tissue after wounding. Currently, *pmk1/Fus3* homologues have been described in several plant-pathogenic fungi (Xu 2000), and in most cases, they play an essential role in pathogenesis, though the phenotypes of deletion mutants differ with respect to impact on vegetative properties such as conidiation, etc.

Here, we present for the first time a functional analysis of a *pmk1/Fus3* homologue, *cpmk1*, which has been isolated from a biotrophic, highly organ-specific, non-appressoria-forming fungus, the ascomycete *C. purpurea*. We have obtained a knockout mutant showing a drastic phenotype: the inactivation of this signaling pathway has no impact on vegetative properties (growth, conidiation, and germination of conidia), but it renders the mutant completely nonpathogenic. The lack of detectable hyphae in the style tissue and the complete absence of any host reaction (the florets can produce normal karyopses; no browning occurs, as has been observed in the case of another set of apathogenic mutants defective in polygalacturonase activity [Oeser et al., in press]) indicates that no penetration of the host's cuticle occurs, although this is difficult to monitor

because of the complex structure of the rye's highly branched stigma. The penetration process in the *C. purpurea*-rye interaction is not yet fully understood. Detailed transmission and scanning electron microscopic studies have never given any evidence for the presence of appressorialike structures (Tenberge 1999; K. B. Tenberge, *unpublished data*); penetration normally occurs shortly after germination of conidia, in the majority of cases, through the stigmatic hairs, but in rare cases, almost any other part of the rye floret can be used as a point of entry. Despite the lack of appressoria (the main target of the PMK1-dependent pathway) in this system, the phenotype is almost identical to that of $\Delta pmk1$ of *M. grisea*, indicating that the basic penetration mechanism could be related, in spite of the lack of apparent morphological differentiation. Alternatively, MAP kinase cascades represent evolutionary old signaling pathways involved in sensing the host's surface, initiating penetration programs that have been divergently adapted to the different hosts/organs involved. In addition, the *cpmk1* mutant cannot invade the host tissue after wounding; obviously, in the *C. purpurea* system, as in the foliar pathogens *M. grisea* (Xu and Hamer 1996), *Colletotrichum lagenarium* (Takano et al. 2000), *Pyrenophora teres* (Ruiz-Roldán et al. 2001), the necrotrophic fungus *Botrytis cinerea* (Zheng et al. 2000), and the root pathogen *F. oxysporum* (Di Pietro et al. 2001), the factors necessary for invasive growth are controlled by this Fus3 homologous signaling pathway.

Since only one pure $\Delta cpmk1$ mutant was obtained (the second one, M1.245, carries an additional ectopic copy), the correlation between the observed phenotype and the deletion of *cpmk1* was confirmed by complementation of the mutant by the wild-type gene.

The functional equivalence of *cpmk1* and *pmk1* was demonstrated by the interspecies complementation experiment: *cpmk1* fully restored the wild-type phenotype in a $\Delta pmk1$ mutant, even under the control of the *C. purpurea* promoter. This was not to be expected, since in a comparable experiment with various hydrophobin genes for complementation of the $\Delta mpg1$ mutant of *M. grisea*, an *M. grisea* promoter fusion was a prerequisite for successful complementation in most cases (Kershaw et al. 1998). Little is known about the transcriptional regulation of the *pmk1*-like genes. In contrast to *ptk1* from *P. teres* (Ruiz-Roldán et al. 2001), which is constitutively expressed, Xu (2000) reported that the *M. grisea pmk1* protein is particularly abundant during appressoria differentiation. Differences between the temporal expression patterns of *pmk1*-like genes could contribute to the partial complementation of the pathogenic behavior of the $\Delta fmk1$ mutant from *F. oxysporum* by *M. grisea pmk1* and to the dose-dependent complementation of its invasive growth (Di Pietro et al. 2001). However, *pmk1* fully restores the ability of the *Colletotrichum lagenarium* $\Delta cmk1$ mutant to develop infection structures (Takano et al. 2000), indicating that the regulatory pathways controlling the expression of both genes in these two pathogens are similar; this is not surprising, since they have comparable appressoria-dependent infection processes. On the other hand, because of the completely different infection strategies, the obviously strict conservation of the functional homology of the *M. grisea* and the *C. purpurea* systems was not to be expected. The conservation is high enough to allow a correct expression of the gene in *M. grisea*, even under the control of the *C. purpurea* promoter, and the full complementation of the *pmk1* deficiency with a single-copy integration of *cpmk1*.

In contrast to yeast (and higher eukaryotes), the analysis of MAP kinase cascades in filamentous fungi is still in its infancy. Among the few upstream elements of the tripartite Fus3/Kss1-like pathways known in filamentous fungi are the MAPK kinase (MAPKK) Ubc5 (Fuz7) from *Ustilago maydis* (Andrews et al.

2000), the CgMEK1 from *Colletotrichum gloeosporioides* (Kim et al. 2000), and the MAPKK kinase (MAPKKK) Ubc4 from *U. maydis* (Andrews et al. 2000). As in the filamentous growth of *S. cerevisiae*, which is controlled by both the cAMP/PKA- and Kss1-dependant pathways, cAMP signaling acts in parallel to the PMK1 pathway to trigger fungal differentiation and pathogenesis in several filamentous fungi. In *M.*

Fig. 4. Pathogenicity assay on rye plants. Rye florets were infected with conidia from the wild-type strain (WT), the $\Delta cpmk1$ mutant M1.5 ($\Delta cpmk1$), the transformant M1.28 carrying an ectopic integration of the replacement construct (E), or the M1.5-complemented transformants M1C.6 (C1) and M1C.8 (C2) carrying ectopic copies of the wild-type gene *cpmk1* ($\Delta cpmk1/cpmk1$). **A**, Rye ears infected with the wild type, the knock-out mutant, and the ectopic transformants after 3 weeks. **B and C**, Magnifications of the ears infected with the wild type and the ectopic transformant, respectively. **D**, Ears infected with the wild type and complemented transformants after 4 weeks. Arrows indicate the positions of sclerotia.

grisea, cAMP was shown to trigger the differentiation of appressoria, and the $\Delta pmk1$ mutants still exhibited swelling but did not form melanized appressoria in response to the presence of exogenous cAMP (Xu and Hamer 1996). *U. maydis uac1* mutants, which are affected in adenylate cyclase activity, show a constitutive filamentous growth and are impaired in pheromone response and virulence. The MAPK gene *ubc3* was identified as a suppressor mutation of the *uac1* phenotype and was cloned by complementation (Mayorga and Gold 1999). The $\Delta ubc3$ mutant is defective in filamentous growth; the solopathogenic haploid strain $\Delta kpp2$ (*kpp2* = *ubc3*) showed a reduced virulence on maize (Müller et al. 1999). In contrast, the formation of appressoria in *P. teres*, which could not be induced in the $\Delta ptk1$ mutant by the addition of cAMP, seems to strictly depend on the PTK1 MAPK pathway (Ruiz-Roldán et al. 2001).

With the exception of the transcription factor Prf1 from *U. maydis* (Hartmann et al. 1996; Kahmann et al. 1999), not much information is available about the proteins acting downstream of the PMK1-like MAPKs. In *Colletotrichum lagenarium*, expression studies have revealed putative target genes: the temporal transcription patterns of three melanin biosynthetic genes were affected in the nongerminating conidia of the $\Delta cmk1$ mutant (Takano et al. 2000) and the mRNA levels of an endopeptidase lyase were greatly reduced in the $\Delta fmk1$ mutant from *F. oxysporum* (Di Pietro et al. 2001). Interestingly, the expression of an endopolygalacturonase gene in *S. cerevisiae* depends on the Kss1 pathway (Madhani et al. 1999), suggesting that the transcriptional control of genes coding for secreted enzymes may involve comparable regulatory pathways in yeast and filamentous fungi.

The identification of a signaling pathway controlling pathogenicity in *C. purpurea* on rye opens several new pathways for

the study of this interaction: by comparison of gene expression in the wild type and the $\Delta cpmk1$ mutant, the identification of target genes is possible, thus allowing a more detailed understanding of this specialized system. In a first approach, the genes that have been identified in our lab will be tested in order to determine whether they are under the control of this MAP kinase pathway (e.g., polygalacturonase/cellulase/xylanase genes and several active-oxygen-scavenging enzyme genes). Furthermore, a random approach using a general differential screening system is also possible. The use of a reporter gene linked to a promoter of a target gene could allow the identification of signals triggering this pathway, as well as signaling components upstream of CPMK1, such as the receptor or receptors. We recently isolated a *C. purpurea* *Slr2* homologue, called *cpmk2*, that shows high homology to the *M. grisea* gene *mps1*; the joint analysis of these MAP kinase cascades will yield valuable insights into the highly specialized infection process of the biotrophic fungus *C. purpurea*.

MATERIALS AND METHODS

Strains and culture conditions.

The wild-type *C. purpurea* (Fr.:Fr.) Tul. strain used in these experiments was 20.1, a putative haploid derivative of standard field isolate T5, isolated from rye (*Secale cereale* L.; Hohenheim, Germany) and obtained by haploidization-inducing benomyl treatment (Hüsken et al. 1999). For conidia harvesting and DNA isolation, mycelia were cultivated on Mantle agar (16 g of agar per liter) with 100 g of sucrose per liter (Mantle and Nisbet 1976) at 28°C for 12 to 14 days. The fertile rice-pathogenic *M. grisea* strain Guy11 (Nottingham and Silué 1991) was used as a wild-type strain in this study, and the $\Delta pmk1$ mutant strain nn78 (Xu and Hamer 1996) was used for

Fig. 5. Effect of the inactivation of *cpmk1* on the ability of *Claviceps purpurea* to penetrate and colonize rye ovaries. Rye ovaries were infected in vitro with conidial suspensions from the **A**, wild-type and **B, C, and D**, $\Delta cpmk1$ mutant M1.5 strains. The ovaries were collected at 5 days postinoculation, stained with aniline blue, and observed using epifluorescence microscopy. Arrows indicate the positions of hyphae.

the complementation experiments. The fungus was grown routinely on complete medium (Talbot et al. 1993) and stored as described by Kershaw and colleagues (1998). *Escherichia coli* strain TOP10F⁺ (Invitrogen, Carlsbad, CA, U.S.A.) was used for all the subcloning experiments. *E. coli* strains LE392 and XL1Blue (Stratagene, La Jolla, CA, U.S.A.) were employed for the propagation of *C. purpurea* genomic and cDNA lambda clones, respectively.

Nucleic acid extraction and analysis.

Standard recombinant DNA methods were performed according to Sambrook and colleagues (1989) and Ausubel and colleagues (1987). Genomic DNA from *C. purpurea* was prepared from lyophilized mycelium according to Cenis (1992). *M. grisea* genomic DNA extraction was performed according to Daboussi and colleagues (1989). For Southern blot analysis, 5 to 10 µg of restriction-digested chromosomal DNA or PCR products were electrophoresed in 0.8 to 1.6% agarose gels with salt-free buffer (Sambrook et al. 1989), blotted onto positively charged nylon filters (Hybond-N⁺; Amersham, Braunschweig, Germany), and hybridized to radioactivity-labeled DNA probes in Denhardt's hybridization solution (Sambrook et al. 1989). Filters were washed for 10 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) and for 10 min in 1× SSC, 0.1% SDS. The hybridization and washing temperatures used were 57 and 65°C for low and high stringency conditions, respectively. DNA sequencing was carried out as described by Moore and colleagues (2002). Protein and DNA sequence alignment, editing, and organization were done with DNA Star (Madison, WI, U.S.A.). Sequence analysis was done using BLAST at National Center for Biotechnology Information, Bethesda, MD, U.S.A. (Altschul et al. 1990).

PCR was done as described by Sambrook and colleagues (1989), using the Red Taq Polymerase (Sigma, Milwaukee, WI, U.S.A.). All primers were synthesized by MWG-Biotech (München, Germany). The amplification products were cloned with the PCR 2.1 TOPO-Cloning Kit from Invitrogen.

Cloning of *cpmk1*.

A *cpmk1* 490-bp internal fragment was amplified by PCR with the degenerate primers MAPK5a (5' GARATGAAGCTVCTB-CGRTACTTAAAYC 3') and MAPK4 (5' CATDATYTCDGG-DGDCDCGRATCC 3') (R = A or G; V = A, C, or G; B = C, G, or T; Y = C or T; and D = A, G, or T) and genomic DNA from *C. purpurea* strain 20-1. The amplification product, cloned into the PCR 2.1 TOPO vector (Invitrogen), was excised by *EcoRI* digestion and used as a probe to screen a genomic library of strain T5 (Smit and Tudzynski 1992) by plaque filter hybridization (Sambrook et al. 1989). Out of the 6,000 lambda clones screened, seven hybridized to the PCR probe and were further purified (Sambrook et al. 1989). They all carry overlapping genomic region, as revealed by restriction and Southern blot analyses. A 6.9-kb *BamHI* fragment from the phage no. 2, carrying the complete *cpmk1* gene, was cloned into pUC19, giving the pMKB2 plasmid, and completely sequenced. The 490-bp PCR fragment was also used as a probe to screen a *C. purpurea* cDNA library (λZAP express, Stratagene) (S. Moore, unpublished data) by plaque filter hybridization (Sambrook et al. 1989); two cDNA clones were obtained, excised, and sequenced.

Expression studies.

For expression analysis during the parasitic cycle of ergot, total RNA from rye ovaries infected for 5, 10, 15, and 20 days with *C. purpurea* wild-type strain and from noninfected ovaries was prepared by using the RNagents total RNA Isolation Sys-

tem from Promega (Madison, WI, U.S.A.). RT-PCR was carried out as described previously (Müller et al. 1997). One microgram of mRNA was converted to cDNA by using the reverse-transcriptase "Superscript" (Gibco BRL, Grand Island, NY, U.S.A.) and the primer K10 (5' GGCCTTCAACGCACGCA-GCGTCTGG 3'), binding 635 bp downstream of the *cpmk1* predicted start codon; the product was taken up in 21 µl of double-distilled H₂O. The PCR was done with 5 µl of cDNA template using 1 µM gene-specific primers K9 (5' GCTACC-CCGCCGAACCCTCCCC 3'), binding 55 bp upstream of the translation initiation site, and K10 (Fig. 2, top). RT-PCR expression analysis of a class II hydrophobin gene, *cph1* (S. Moore and P. Tudzynski, unpublished data), was used as a control with the gene-specific primers RTHF (5' TCCTTCAT-CATCTCCATCATGG 3') and RTHR (5' GAAATGCACAGA-ACACCCTGTC 3').

Construction of the replacement and complementation vectors.

For constructing the *cpmk1* replacement vector, the genomic region located between -536 and +473 bp, respective to the predicted start codon, was amplified using PCR and subcloned with the *SacII*-*NotI* sites of the vector PCR 2.1 TOPO downstream of the phleomycin resistance cassette (*ble*) into the corresponding *SacII*-*NotI* restriction sites of the pAN8-1UM vector, giving the pM1P plasmid. The pM1R replacement plasmid was obtained following the cloning of a 860-bp fragment corresponding to the region -196 to +666 bp, respective to the stop codon, excised from the pMKB2 vector with a *HindIII* digest upstream of the fungal selection marker (*ble*) into the pM1P vector. The linear replacement construct was excised with a *SacII*-*KpnI* restriction and used to transform the *C. purpurea* wild-type strain 20-1 (Fig. 3A). The complementation vector was obtained by cloning a 3.6-kb *XbaI*-*SacI* fragment from the pMKB2 plasmid into the corresponding restriction sites of the pCB1635 vector (Sweigard et al. 1997), carrying a resistance cassette to the glufosinate. The plasmid obtained, called pM1CXS, carries the coding sequence of *cpmk1*, 1.0 and 1.1 kb of its 5' and 3' flanking regions, respectively. The circular vector was used to transform the *C. purpurea* Δ *cpmk1* mutant strain M1.5.

Fig. 6. Complementation of the appressoria differentiation defect of the Δ *cpmk1* mutant strain of *Magnaporthe grisea* by *Claviceps purpurea* *cpmk1*. Thirty- to fifty-microliter droplets of conidial suspensions from the *M. grisea* strains **A**, Guy 11 (wild type) and **B**, nn78 (Δ *cpmk1*), and **C** and **D**, the nn78 transformants carrying the *cpmk1* gene from *C. purpurea* (Δ *cpmk1*/*cpmk1*) were placed on the hydrophobic face of onion epidermis previously flattened on glass slides and incubated with humidity overnight at room temperature. Appressoria formation was followed by using light microscopy. Arrows indicate appressoria.

Transformation and analysis of fungal transformants.

Transformation of *C. purpurea* was performed according to Smit and Tudzynski (1992), with the following modifications: the recipient strains, 20-1 and M1.5, were grown for 3 days on cellophane sheets on Mantle agar (instead of liquid culture). For the generation of protoplasts, 10 mg of Lysing Enzyme (Sigma) per ml and 2.5 mg of Driselase (Sigma) per ml were used instead of Novozym (Interspex, Foster City, CA, U.S.A.).

For the selection of phleomycin-resistant transformants, the protoplasts were mixed with BII agar medium (Esser and Tudzynski 1978) and modified for transformation (BII/8: pH 8, lacking Fe and containing 20% saccharose and 12 g of agar per liter). After 4 h of regeneration at 28°C, the protoplasts were overlaid with 10 ml of BII/8 containing 100 µg of phleomycin per ml (Cayla, Toulouse, France; final concentration of 33 µg/ml) and 16 g of agar per liter. After 3 to 5 days of growth at 28°C, phleomycin-resistant colonies were transferred to BII/8 plates containing 100 µg of phleomycin per ml and subjected to at least one round of single-spore isolation to obtain homokaryotic transformants. In order to obtain glufosinate-resistant transformants, Czapek Dox agar (Merck, Darmstadt, Germany) modified for transformation (pH 5.2, lacking glucose and containing 20% saccharose) was mixed with the transformed protoplasts. A 10-ml overlay of Czapek Dox agar containing 600 µg of glufosinate per ml (glufosinate-ammonium PESTANAL; Riedel-de Haën, Rdh Laborchemikalien GmbH & Co. KG, Seelze, Germany; final concentration of 200 µg/ml); was added after 24 h of regeneration at 28°C. After 10 days incubation at 28°C, the glufosinate-resistant colonies were transferred to modified Czapek Dox plates (pH 5.2, 10% saccharose, no glucose) containing 300 µg of glufosinate per ml.

Fig. 7. Complementation of the pathogenicity defect of the $\Delta pmk1$ mutant strain from *Magnaporthe grisea* on barley by *Claviceps purpurea* *cpmk1*. Conidial suspensions from the *M. grisea* strains Guy 11 (wild type, WT), nn78 ($\Delta pmk1$), the nn78 transformants carrying the *cpmk1* gene from *C. purpurea* ($\Delta pmk1/cpmk1$, T1 to T4), and the nn78 control transformants carrying the pCB1635 vector (C) were spread on the surface of barley leaves in vitro and incubated at 26°C. The picture was taken at 5 days postinoculation. A control with water (H₂O) was performed in parallel.

The phleomycin-resistant transformants carrying a homologous integration of the replacement construct were identified by PCR with the primers K5 (5' GTGTCCACTGTCCTCGTCGCC 3') and pAN2 (5' CCGTAACACCCAATACGCCG 3') (Fig. 3A) and 10 to 100 ng of genomic DNA. The presence of the wild-type gene copy in the $\Delta pmk1$ mutants was checked with the primers K5 and K7 (5' GCTGCCGAACGAGCCAA-GCCG 3'), which give rise to a 1,480-bp fragment with the wild-type strain. The presence of the wild-type *cpmk1* coding sequence in the M1.5 transformants ($\Delta pmk1/cpmk1$) was verified by PCR with the primers K11 (5' GGAGGTATGTACTC-CGTAGGTAGG 3') and K12 (5' GGCCCTGTCAGGACGG-ACGAGC 3'), binding to the regions 579 bp upstream of the *cpmk1* translation initiation site and 141 bp downstream of the stop codon. The resistance of the M1.5 transformants to phleomycin was tested on BII/8 agar. The identity of M1.5 ($\Delta pmk1$) as the recipient strain was confirmed by Southern blot analysis of the M1C.6 and M1C.8 transformants.

Preparation of the protoplasts and transformation of *M. grisea* were performed according to Daboussi and colleagues (1989). Transformants were selected for glufosinate resistance in modified "CM" medium (Sweigard et al. 1992) containing 20% saccharose and 40 µl of glufosinate per ml. Transformants were purified by monoconidial isolation and analyzed by Southern blot. Part of the *M. grisea* *pmk1* gene, corresponding to the region +358 to +1179 bp respective to the start codon, was amplified with the primers MK1 (5' CTCCGCGAGATGAAGCT-GCTCCG 3') and MK2 (5' CGACGGTGATGGGCTTGA-CGGG 3') and with genomic DNA from strain Guy 11 as a template. The 820-bp amplification product was cloned into the PCR2.1 TOPO vector (Invitrogen) and used as a probe to reveal the *pmk1* wild-type gene copy.

C. purpurea pathogenicity assays.

Rye plants were cultivated in growth chambers as described by Smit and Tudzynski (1992). Florets of blooming ears (60 to 80 per ear; altogether 40 ears in five independent experiments) were inoculated with conidial suspensions as described by Tenberge and colleagues (1996). In order to avoid cross-contamination, directly after inoculation, the ears were covered with paper bags equipped with cellophane windows. To introduce the fungus inside the plant (wounding test), rye florets were infiltrated with the conidial suspension by using a syringe with a needle (25 gauge $\times$ 5/8 in., U-40 insulin, Terumo syringe; Terumo Europe N. V., Leuven, Belgium). Pictures were taken 3 to 4 weeks postinoculation.

Aniline blue staining and fluorescence microscopy observations.

An in vitro infection system was used (modified from Heslop-Harrison and Heslop-Harrison 1981; J. Scheffer, unpublished data). Rye ovaries were isolated from blooming rye ears and put onto Hoagland solution (Hewitt 1966; Hoagland and Arnon 1938) modified for barley shoot culture (for 1 liter: MES buffer, 5 ml [2-morpholino ethanesulfonic acid, 19.5 g/liter; NaOH, 2.0 g/liter; pH 6.5]; Ca(NO₃)₂ 4 H₂O, 94 mg; KNO₃, 66 mg; MgSO₄ 7 H₂O, 52 mg; KH₂PO₄, 38 mg; H₃BO₃, 2.86 mg; ZnSO₄ 7 H₂O, 0.22 mg; CuSO₄ 5 H₂O, 0.1 mg; Na₂MoO₄ 2 H₂O, 0.05 mg; and FeEDTA, 12 mg) and solidified with 16 g of agar per liter. Stigmas were inoculated with suspensions containing 2×10^6 conidia per ml by using cotton tips and were incubated at room temperature for 5 days. The KOH-aniline blue staining of the fungus was realized as described by Hood and Shew (1996), except that the ovaries were incubated in 1 M KOH overnight at room temperature. The infected ovaries were observed using epifluorescence microscopy (Microscope Leica DMRBE [Leica Instruments, Heerbrugg,

Switzerland] with PixelFly Digital camera [PCO Computer Optics GmbH, Kelheim, Germany], filter block A [band pass filter {BP} 340-380, reflection short pass filter {RSP} 400, long pass filter {LF} 425], UV light 340 to 380 nm).

Appressoria differentiation assays and barley infection tests.

M. grisea conidia were collected in water, filtered through glass wool, centrifuged for 10 min at $5,000 \times g$ and 4°C , and resuspended into water. The final concentration of the spores was adjusted to 2×10^4 per ml.

Appressoria differentiation tests were performed using two types of hydrophobic surfaces, vinyl slides Rinzl (PolyLabo, Strasbourg, France) and onion epidermis. Prior to use, the peeled inner epidermis of onion was washed for 5 min in chloroform and then in water and flattened onto glass slides so that the hydrophobic sides faced upward (M. J. Kershaw and N. J. Talbot, *personal communication*). The slides, placed into petri dishes, were inoculated with 30- to 50- μl droplets of conidial suspensions and incubated overnight at room temperature under high humidity. Appressoria differentiation was followed by using light microscopy. Barley infection tests were performed as described by Clergeot and colleagues (2001). Seedlings from the barley cultivar Express (Aventis CropScience, Lyon, France) were cultivated for 2 weeks at 12°C (with 12 h of light per day). Leaf segments 3 cm long were placed onto agar plates (1%) containing 2 mg of kinetin per liter (Sigma) and wetted with water by using cotton tips. The conidial suspensions were spread on the leaf surface in the same way. The lesions appeared and started to sporulate after 4 to 7 days of incubation at 26°C .

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