MPMI Vol. 20, No. 6, 2007, pp. 637–647. doi:10.1094/MPMI-20-6-0637.

Complementation of *Ustilago maydis* MAPK Mutants by a Wheat Leaf Rust, *Puccinia triticina* Homolog: Potential for Functional Analyses of Rust Genes

Guanggan Hu, Andrena Kamp, Rob Linning, Suresh Naik, and Guus Bakkeren

Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, BC VOH 1Z0, Canada

Submitted 10 October 2006. Accepted 20 December 2006.

From a large expressed sequence tag (EST) database representing several developmental stages of *Puccinia triticina*, we discovered a mitogen-activated protein kinase (MAPK) with homology to kinases with known pathogenic functions in other fungi. This PtMAPK1 is similar to the *Ustilago maydis* MAPK, Ubc3/Kpp2, but has a longer N-terminal extension of 43 amino acids (aa) with identities to *U. maydis* Kpp6, a homolog of Ubc3/Kpp2 with a 170-aa N-terminal extension. Ubc3/Kpp2 is involved in mating and subsequent pathogenic development, whereas Kpp6 functions during invasive growth in corn tissue. PtMAPK1, expressed from a *Ustilago* sp.-specific promoter, was able to complement a *ubc3/kpp2* deletion mutant and restore mating. It also substantially increased virulence on corn, measured as tumor formation, of a *kpp6* deletion mutant. Moreover, this construct restored to near-full pathogenicity a *ubc3/kpp2 kpp6* nonpathogenic double deletion mutant. Complementation of the *ubc3/kpp2* mutant with the complete *PtMAPK* gene and verification of expression by reverse-transcription polymerase chain reaction indicated that the rust promoter is recognized in *U. maydis*. Phylogenetically, these basidiomycete plant pathogens are related, which was reflected in comparison of *P. triticina* ESTs to *U. maydis* gene sequences. The *U. maydis* heterologous expression system allows functional analysis of rust genes, currently frustrated by the lack of efficient transformation and selection procedures.

Additional keywords: brown rust, *Uredinales*.

Rust fungi belong to the order *Uredinales*, estimated to include from 4,000 to 6,000 species belonging to 140 to 150 genera (Alexopoulos et al. 1996; Hahn 2000). The various rusts attack a wide variety of unrelated mono- and dicot plants and trees. Rust fungi are among the most devastating plant pathogens in the world and many are economically important, such as the cereal, bean, pine, coffee, carnation, and peanut rusts (Agrios 1997; Staples 2000). In particular, *Puccinia* spp., infecting mainly monocots (as do most *Ustilago* spp. or smuts), have been known since Biblical times because of the devastation they cause due to expanding cultivation of grains. Among the rusts studied extensively over the last 100 years is *Puccinia triticina* Eriks. (formerly *P. recondita* f. sp. *tritici*), causing leaf or brown rust on wheat and rye (wheat leaf rust [WLR]) (Bushnell and Roelfs 1984; Kolmer 1996; Roelfs and Bushnell 1985). Exquisite cell biological and light- and ultrastructural microscopic work on the many morphogenic stages, and numerous phylogenetic and (population) genetic studies, motivated by the dire need to continuously develop resistant host cultivars, have been published. However, the molecular study of cereal rust fungi has been severely hampered because of their obligate nature and the lack of an efficient transformation system, although the latter hurdle might be overcome (Schillberg et al. 2000; Webb et al. 2006).

Genomics, the generation and analysis of genome-wide resources such as expressed sequence tag (EST) databases, whole-genome sequencing, and physical and genetic maps, promises to overcome some of the research challenges obligate pathogens pose. Recently, several projects on rust fungi have been initiated, including in our laboratory, where a large-scale WLR EST database is being developed. The rusts have the most complicated life cycles in the fungal kingdom and can include many developmental stages and up to five different spore types produced on two unrelated plant hosts for the heteroecious, macrocyclic forms such as WLR (Horton et al. 2005). In an initial study, we have generated ESTs from five stages of fungal development, including resting and germinating urediniospores, an appressorium stage, and host invasive stages during compatible and incompatible interactions (G. Hu, R. Linning, and G. Bakkeren, *unpublished data*).

Mitogen-activated protein kinases (MAPKs) have been found in all organisms, including fungi, and are involved in transducing a variety of extracellular signals to regulate growth and differentiation processes. They are responsible for the phosphorylation of target transcription factors resulting in activation of specific genes (Raman and Cobb 2003; Schaeffer and Weber 1999; Xu 2000). Molecular genetic studies in many fungi have revealed homologs of MAPKs that fall into three main subgroups identified in *Saccharomyces cerevisiae* based on the following broad functions: osmoregulation and other stress responses (ScHog1, YSAPK-like); different developmental processes such as mating and filamentation, hyphal growth, conidiation, and conidial germination (ScFus3/Kss1 group, YERK1-like); and cell wall integrity, nutrient sensing, conidiation, and aerial hyphal growth (Sc Slt2 group, YERK2-like) (Fig. 1) (Kultz 1998; Saito and Tatebayashi 2004; Xu 2000). In pathogenic fungi, mutants in homologs of all three groups seem to affect pathogenesis, which is not surprising, considering the broad functions they impinge on.

Corresponding author: Guus Bakkeren; Telephone +1.250.494.6368; Fax: +1.250.494.0755; E-mail: BakkerenG@agr.gc.ca

Current address of G. Hu: The Michael Smith Laboratories, University of British Columbia, Vancouver, BC, V6T 1Z4 Canada.

Nucleotide sequence data is available in the GenBank database under the following numbers: Contig2832, DN956226 to DN956230 and EC387017 to EC387021; Contig6818, DN956227 and EC387023 to EC387038.

Ustilago maydis, the corn smut fungus, is the primary model basidiomycete plant pathogen. Single-haploid *U. maydis* basidiospores of opposite mating-type specificities at the *a* locus (*a1* or *a2*) are induced to mate after perception of each others' pheromones. Fusion produces the pathogenic dikaryon when they also harbor different allelic specificities at the *b* mating-type locus, resulting in a morphologic switch to a filamentous cell type necessary for invasive growth in corn tissues (Feldbrugge et al. 2004). In *U. maydis*, the pheromone response pathway appears to be a Ras/MAPK cascade. Individual components of this pathway have been identified, including Ubc4 (MAPKKK), Fuz7 (MAPKK), Ubc3/Kpp2 (MAPK), and a pheromone response factor, Prf1 (Andrews et al. 2000; Banuett and Herskowitz 1994; Hartmann et al. 1996; Mayorga and Gold 1999; Muller et al. 1999). *Ubc3/kpp2* deletion mutants are indistinguishable from wild-type cells in morphology and growth characteristics, indicating that this MAPK is a non-essential gene. However, crosses of *ubc3/kpp2* deletion mutants are impaired in the mating response, resulting in reduced virulence on corn (Mayorga and Gold 1999; Muller et al. 1999). Recently, Kpp6, a homolog of Ubc3/Kpp2 and a second MAPK from the YERK1 group, was identified in *U. maydis* (Brachmann et al. 2003). The *kpp6* deletion mutants, also indistinguishable from wild-type cells in morphology and growth characteristics, are not impaired in mating responses but seem to be affected mainly in penetration of host tissue. Indeed, crosses of compatible *kpp6* deletion mutants are only weakly pathogenic. On the other hand, crosses of compatible *ubc3/kpp2 kpp6* double mutants are essentially nonpathogenic (Brachmann et al. 2003).

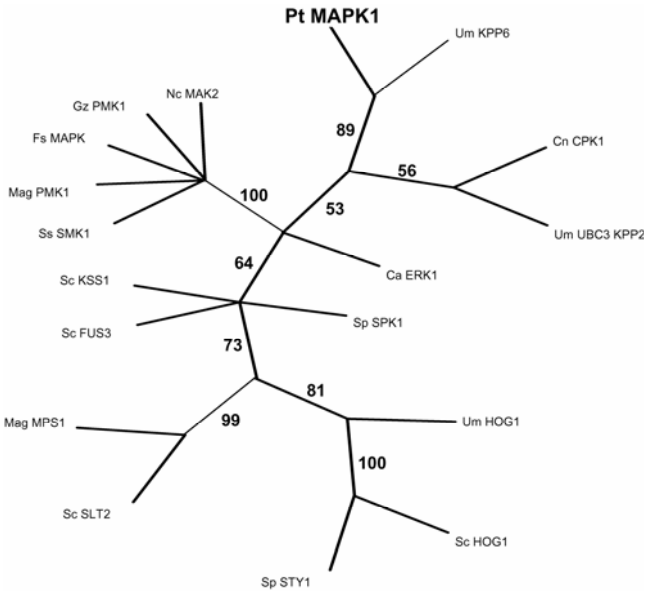

Fig. 1. Phylogenetic placement of the *Puccinia triticina* PtMAPK1 protein among a selection of fungal homologs (GenBank accessions in parenthesis): *Candida albicans,* Ca_ERK1 (P28869); *Cryptococcus* (*Filobasidiella*) *neoformans,* Cn_CPK1 (Q8NK05); *Fusarium solani,* Fs_MAPK (AAB72017); *Gibberella zeae,* Gz_PMK1 (AAL73403); *Magnaporthe grisea,* Mag_PMK1 (AAC49521); *M. grisea,* Mag_MPS1 (AAC63682); *Neurospora crassa,* Nc_MAK2 (AAK25816); *P. triticina,* Pt_MAPK1 (DQ026061); *Saccharomyces cerevisiae,* Sc_FUS3 (CAA49292); *S. cerevisiae,* Sc_HOG1 (CAA97680); *S. cerevisiae,* Sc_KSS1 (CAA97038); *S. cerevisiae,* Sc_SLT2 (CAA41954); *Schizosaccharomyces pombe,* Sp_SPK1 (CAA40610); *S. pombe,* Sp_STY1 (CAA61537); *Sclerotinia sclerotiorum,* Ss_SMK1 (AAQ54908); *Ustilago maydis,* Um_HOG1 (XP_759303); *U. maydis,* Um_KPP6 (CAD43731); *U. maydis,* Um_UBC3/KPP2 (AAF09452). Unrooted bootstrap consensus tree (full heuristic search, 50% majority rule) of 1,000 replicates; length, 1,722; 244 parsimony-informative characters out of 644 total.

We report here the identification and characterization of the first rust MAPK gene, designated *PtMAPK1*, from *P. triticina*. The availability of deletion mutants of homologs in *U. maydis* prompted us to investigate the possibility of using this related fungus as a "surrogate" system for the functional testing of rust genes. We show here that the *PtMAPK1* gene is a functional homolog of both *ubc3/kpp2* and *kpp6* and that *U. maydis* can be used as a heterologous expression system for *P. triticina* open reading frames (ORFs) and even complete genes.

RESULTS

Cloning and description of *P. triticina MAPK1*.

A large-scale wheat leaf rust EST project yielded a database in which many partial cDNA clones were provisionally annotated (G. Hu, R. Linning, and G. Bakkeren, *unpublished data*). Two consensus sequences derived from two contigs, contig2832 (9 members, discussed below) and contig6818 (18 members, see Material and Methods), appeared in a BLASTx search to have homology to several fungal MAPKs. Both consensus sequences (representing 3′ partial gene sequences) were most similar to an MAPK from *Gibberella zeae* (GzCon[4565] in the COGEME database), although contig2832 produced an *e* value of e-109 and contig6818 an *e* value of 2e-19, reflecting their divergence. Contig2832 also was similar to the corn smut fungus, *U. maydis*, MAPKs Kpp6 (GenBank accession number CAD43731; um02331 in the Munich Information Center for Protein Sequences (MIPS) Ustilago Maydis database and Ubc3/Kpp2 (GenBank accession numbers AAF09452 and AAF15528, respectively). The translated consensus sequence from contig6818 also was weakly related to Mak-2 from *Neurospora crassa* and others and showed 48 identities with the translated sequence from contig2832 over the C-terminal 55–amino acid (aa) protein available (87%). When comparing the nucleotide sequences for this C-terminal coding region, 104 of 138 bases were identical (75%); in the 3′ untranslated region, only 174 of 451 nucleotides (39%) were shared. We have not further analyzed this potential MAPK, but it is possible that it represents a homolog of PtMAPK1 in the YERK1 group.

Because no genomic library was available for this *P. triticina* strain, the 5′ end of *PtMAPK1*, including the promoter, was obtained by polymerase chain reaction (PCR) from genomic DNA (Zhang and Gurr 2002) (discussed below). Primers designed to the gene-specific promoter region allowed the amplification, cloning, and analysis of the complete genomic copy of the MAPK gene, which was designated *PtMAPK1*. The ORF codes for 405 aa and the complete protein was most similar to the *U. maydis* MAPK, Kpp6. The molecular phylogeny indicated that PtMAPK1 is included in the clade that is represented by the *S. cerevisiae* Fus3/Kss1 proteins and, therefore, likely involved in mating (pheromone response), morphogenesis, or pathogenic development (Fig. 1).

The level of conservation between the PtMAPK1 protein, the two *U. maydis* homologs, Ubc3/Kpp2 and Kpp6, and the *Magnaporthe grisea* homolog, PMK1, is illustrated in Figure 2. With 405 aa, PtMAPK1 is longer than the 354 aa reported for Ubc3/Kpp2 (Mayorga and Gold 1999; Muller et al. 1999), and the 356-aa-long homolog PMK1 from *M. grisea* (Xu et al. 1996). It is shorter than the *U. maydis* Kpp6 homolog of 533 aa which, compared with Ubc3/Kpp2, has a 154-residue-long amino-terminal extension. This extension is thought to be a different protein domain attached through an alanine linker and of various lengths among isolates (Brachmann et al. 2003). However, the approximately 45-aa-long amino terminal extension of PtMAPK1 does not harbor an alanine linker and is not homologous to the Kpp6 extension, although 10 identical and 5 similar amino acids can be identified. When disre-

garding the 154-long amino-terminal extension, Kpp6 displays 65% identity and 75% similarity to PtMAPK1 when directly compared, whereas Ubc3/Kpp2 shares 62% identical and 73% similar amino acids with PtMAPK1. Indeed, when comparing a large group of MAPK homologs from a variety of fungi, molecular phylogenetic calculations indicated that the rust MAPK1 is more related to *U. maydis* Kpp6 than to Ubc3/Kpp2 (Fig. 1).

PtMAPK1 has a calculated molecular weight of 46 kDa and an isoelectric point of 7.24 (EMBOSS suite of programs). No introns could be detected when comparing the genomic sequences to the consensus cDNA sequences. This is similar to what has been reported for other fungal MAPKs, such as for *U. maydis* (Brachmann et al. 2003; Mayorga and Gold 1999; Muller et al. 1999). The sequence analysis of the presumed promoter region revealed a TATA box at position –166 and a CCAAAT (CAAT) motif at position –291. Similar deduced consensus elements can be found at various positions in the promoters of several *U. maydis* genes (Basse and Farfsing 2006), including in the promoters of *ubc3/kpp2* and *kpp6*.

```
MgPMK1          ------------------------------------------------------------
UmUbc3/Kpp2     ------------------------------------------------------------
PtMAPK1         ------------------------------------------------------------
UmKpp6          MSIANASSSTASHDLDAADADPCPSACTSVSALCNMSTLPSPRPFGYRPTYVPAMVASGV 60

MgPMK1          ------------------------------------------------------------
UmUbc3/Kpp2     ------------------------------------------------------------
PtMAPK1         ------------------------------------------------------------
UmKpp6          SAVTVTTNSHRPPSKSHSISSVDSLASVSSNAPSTPTHDADDVRFEMAVIPKLVRASADA 120

MgPMK1          -------------------------------------------------MSRANPPSNSSGS 13
UmUbc3/Kpp2     -------------------------------------------------MSHAHGQQPN 10
PtMAPK1         -------MASAAVALPSSATGGSMNPTAPASTSVNATTINITNAPANKPKQAGSRAVGEP 53
UmKpp6          YLDRVGKPLPPSPAPQIVATSATTKTNAQAARSVAAAAAAAAAAAAVPPSHSALRNENHK 180

MgPMK1          RKISFNVSEQYDIQDVVGEGAYGVVCSAIHKPSGQKVAIKKITPFDHSMFCLRTLREMKL 73
UmUbc3/Kpp2     HSTTFKVGETYKVVDVVGEGAYGVVCSAIHVPSSSRVAIKKITPFDHSMFCLRTLREIKL 70
PtMAPK1         RRVRFNVGSKYHVMDVIGEGAYGVVCSAIHRPTGQKVAIKKIVPFDHSMFCLRTLRELKL 113
UmKpp6          NAISFRVGSKYKVCEIIGEGAYGVVCSAIHRATGQKVAIKKIQPFEHQMFALRTLRELKL 240
                .   *.*.. *.: :::************** .:..:****** **:*.**.******:**

MgPMK1          LRYFNHENIIS----ILDIQKPRSYETFNEVYLIQELMETDMHRVIRTQDLSDDHCQYFI 129
UmUbc3/Kpp2     LRHFNHENIIS----ILDIVKPDDYDSFSEVYLIQELMETDMHRVIRTQELSDDHCQYFI 126
PtMAPK1         LKYFQEHNVSENIVSIVDIIRPPTIEAFKEVYLIQELMETDMHRVIRTQVLSDDHCQYFI 173
UmKpp6          LRFFQECDVSENIISILDIIKPSTYEAFTEVYLVQELMETDLHRVIRTQELSDDHCQYFT 300
                *:.*:. :: .    *:** :*   ::*.****:*******:******* *********

MgPMK1          YQTLRALKAMHSANVLHRDLKPSNLLLNANCDLKVCDFGLARSAASQENN-SGFMTEYVA 188
UmUbc3/Kpp2     YQTLRGLKALHSAQVLHRDLKPSNLLLNANCDLKICDFGLARSANQPEAEGTGFMTEYVA 186
PtMAPK1         YPTLRAMKALHSADVIHRDLKPSNLLLNANCDLKVCEFGLARSIR-TAEQETGFMTEYVA 232
UmKpp6          YQTLRALKPMHCADVIHRDLKPSNVLLNANCDLKVCDFGLARSVLTADQD-TGFMTEYVA 359
                * ***.:*.:*.*:*:********:*********:*:******      : :********

MgPMK1          TRWYRAPEIMLTFKEYTKAIDVWSVGCILAEMLSGKPLFPGKDYHHQLTLILDVLGTPTM 248
UmUbc3/Kpp2     TRWYRAPEIMLTFKEYTKAIDVWSVGCILAEMLAGKPLFPGRDYHHQLSLTLEILGTPSL 246
PtMAPK1         TRWHRAPEIMLTFKQYTKAIDVWSVGCILGEMLSGRPLFPGRDYHHQLTLILDVLGTPTL 292
UmKpp6          TRWYRAPEIMLTFKQYTKAIDAWAVGCILAEMLTGRPLFPGRDYHQQLSLILDVLGTPTL 419
                ***:**********:******.*:*****.***:*:*****:***:**:* *::****::

MgPMK1          EDYYGIKSRRAREYIRSLPFKKKVPFRTLFPKTSDLALDLLEKLLAFNPVKRITVEEALK 308
UmUbc3/Kpp2     DDFYAITSTRSRDYIRALPFRKRRNLSLMFPNANPLAVDLMEKCLTFSPRKRITVEEALA 306
PtMAPK1         DEFYAINSRRSRDYIRALPLRKKRPFATIYPNASPLAIDFLTKTLTFDPKKRLTVEEALQ 352
UmKpp6          EEFQNINSRRSRDYIRSMPFRKRREFRTLFPKASPEAIDFLQKTLTFDPRNRLTVEECLQ 479
                :::  *.* *:*:***::*::*:  :  ::*::.  *:*:: * *:*.* :*:****.*

MgPMK1          HPYLEPYHDPDDEPTAPPIPEEFFDFDKHKDNLSKEQLKQFIYQEIMR------ 356
UmUbc3/Kpp2     HPYLEPYHDPEDEPTAEPLDPSFFDFDYCKEQLSRSELKRLIYNEIMR------ 354
PtMAPK1         HPYLEAYHDPEDEPTAPPLDEDFFAFDRQKDEISKEELKRLLFEEINSFHFTT- 405
UmKpp6          HPYLSAYHDPDDEPGAPRLDPDFFYFDMQKESITKEDLRKELWYQVQEFQPLLR 533
                ****..****:*** *  :  .** **  *:.:::.:*:: :: ::
```

Fig. 2. Protein sequence alignment of several mitogen-activated protein kinase (MAPK) homologs. Compared were the *Magnaporthe grisea* PMK1, the *Ustilago maydis* Ubc3/Kpp2, the *Puccinia triticina* PtMAPK1, and the *U. maydis* Kpp6. The amino acid positions are indicated as well as the extent of the homologies: asterisks denote positions which have a single, fully conserved residue and the double and single dots indicating conservation within a defined group of amino acids with a high and low score, respectively.

Expression of *PtMAPK1*.

In our EST database, *PtMAPK1* transcripts were identified in resting urediniospores and in urediniospores germinated in vitro over water; *PtMAPK1* was not encountered among ESTs from an appressorium stage on wheat leaves, from infected compatible leaves at 6 days after inoculation just before sporulation, or from an incompatible interaction at 24 h after inoculation (G. Hu, R. Linning, and G. Bakkeren, *unpublished data*). Analysis of relative expression levels in several of these stages was performed using quantitative reverse-transcription polymerase chain reaction (RT-PCR) (discussed below). Primers to *PtMAPK1* (Table 1) were demonstrated to be unique to this gene, in that they did not amplify sequences from the homologs in contig6818 or from wheat total genomic DNA (data not shown). We searched for potential reference genes that might be expressed at similar rates during the different stages by comparing their representation in our EST database. Out of several tested, a histone H4 gene proved the most reliably expressed among the stages. The amount of *PtMAPK1* mRNA detectable in urediniospores was 9- to 50-fold less after 6 h of germination in vitro over water (Fig. 3). At 48 h after inoculation, when the first plant cells are invaded and the infection starts to become established during a compatible interaction, *PtMAPK1* seems to be an additional sixfold downregulated on average. During subsequent fungal growth up to 6 days after infection (before sporulation), expression seems more or less maintained.

Complementation of mating defects in *U. maydis ubc3/kpp2* deletion mutants.

The close homology of PtMAPK1 to Ubc3/Kpp2 prompted us to verify its function by attempting to complement a *U. maydis ubc3/kpp2* deletion mutant impaired in mating interactions. The *ubc3/kpp2* mutation essentially abolishes the ability of *U. maydis* strains to respond to mating pheromone from a partner having an opposite *a*-locus specificity by putting out conjugation hyphae and, to a lesser degree, they are compromised in transmitting mating pheromone signals. Mating between two *ubc3/kpp2* mutants is severely reduced, but cytoplasmic fusion is not abolished in cells that are in close enough proximity (Mayorga and Gold 1999). Post-fusion events also are affected, such as the dimorphic switch, producing a reduced number of straight-growing, dikaryotic filaments which are measured in a visual plate mating assay (Muller et al. 1999). The results of plate mating tests between wild-type, mutant strains and various transformants are shown in Figure 4A and tabulated in Table 2. Single-haploid strains, either mutant or transformed, never showed a fuzzy phenotype on charcoal plates. Pairings between an *ubc3/kpp2* deletion mutant and a wild-type strain of opposite *a*-locus specificity gave a reduced reaction, as was noted before (Mayorga and Gold 1999; Muller et al. 1999) (Fig. 4A). This intermediate reaction also was seen when an *ubc3/kpp2* deletion mutant expressed the *PtMAPK1* ORF from the *U. maydis* Hsp70 promoter in only one of the mating pairs. For example, 2/58 (pUmPtMAPK1) mated to 2/59 (pUBleX1, carrying only an empty vector control) produced the same level of fuzzy phenotype reaction (Fuz) as 2/58 (pUBleX1) mated to 2/59 (pUmPtMAPK1). Heterologous complementation of MAPK function in only one mating type allowed the strain to respond to mating pheromone from the other partner, resulting in an intermediate mating response seen as a partially fuzzy colony phenotype.

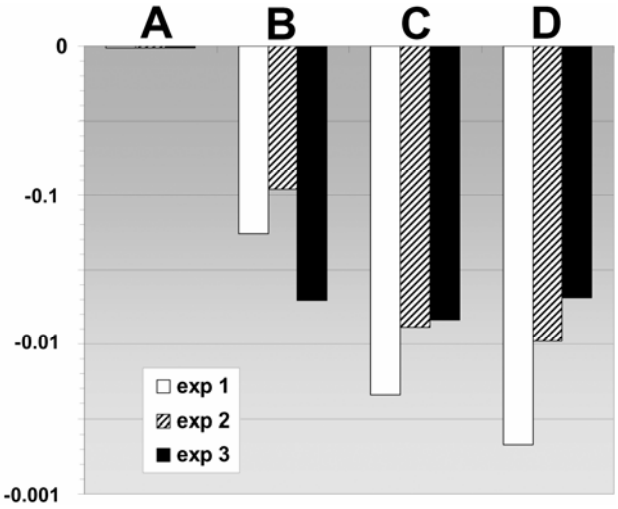

Fig. 3. Relative measurement of *PtMAPK1* RNA levels during various life cycle stages. Quantitative reverse-transcription polymerase chain reaction data were normalized to *Puccinia triticina* histone H4 levels (see Materials and Methods) and presented in reference to stage A on a log10 scale. Stages: resting urediniospores (A), urediniospores germinated in vitro over water for 6 h (B), and compatible in planta stage 48 h after infection (C) and 6 days after infection (D).

Table 1. Oligonucleotide primers used in this study

Genes targeted[a]	Primer no.	Sequence	Amplicon size (bp)
Pt MAPK1 gene cloning	162	5′-TCGATGGCTTCAAATCCCGATGG	
	163	5′-GCGAGCCAATCCAAATTCGCAG	
	227	5′-TGGCATGCGAGCAGGGGCCGAGTAGGTC[b]	1976
	228	5′-GCGCATGCACAGTTCATTTATTGATTAGTTCGGAGAGG[b]	
	229	5′-TGAGATCT**ATG**GCTTCTGCTGCTGTAGCTCTCCCG[c]	1218
	230	5′-GCAGATCT**TCA**AGTGGTAAAGTGAAAGCTG[d]	
RT assay			
Pt MAPK1	424	5′-TCGGCAGATGTGATCCATCG	728
	434	5′-AGTGGAGCTCGACGACTAAGAGG	
Ustilago maydis succinate dehydrogenase	401	5′-GTCGCTATTCAACGTCAGCAACGGTCTTCG	745
	402	5′-GAGCGAAAAGGTGTTCTCGAGCTTCTGTC	
Quantitative RT-PCR			
Pt MAPK1	473	5′-CAACGCTTCACCCTTGGCTATCGA	385
	474	5′-CGAAGGGATTGTGAATGGCGCA	
Pt histone H4	481	5′-GTCAAGCGTATCTCCGGCCTGA	281
	482	5′-TAGACGACATCGAGCGAGGTGA	

[a] MAPK = mitogen-activated protein kinase, RT = reverse transcription, and PCR = polymerase chain reaction.
[b] *Sph*1 site underlined.
[c] Start codon in bold; *Bgl*II site underlined.
[d] Stop codon in bold; *Bgl*II site underlined.

Mating interactions were restored to near-wild-type levels when we paired transformants each harboring a *PtMAPK1* ORF driven from the *U. maydis* Hsp70 promoter (pUmPtMAPK1) (Fig. 4A). However, complementation was better when the construct was present on an episomal plasmid than when stably integrated in the genome (pUmPtMAPKInt) (Table 2). This might be caused by a "gene dosage effect" because the autonomously replicating sequence element has been estimated to allow replication of episomal plasmids to approximately 25 copies per cell (Tsukuda et al. 1988).

Interestingly, mutants also are partially complemented by episomal plasmids harboring the native *PtMAPK* gene (Fig. 4B); for the integrated constructs, the level of mycelial colony phenotype was close to background levels produced by the empty-vector controls (Table 2). To verify recognition of potential rust promoter elements and subsequent transcription by the *U. maydis* machinery, an RT-PCR analysis was performed (Fig. 4C and D). PtMAPK1 primers specific for *P. triticina* (lanes 11 to 13) revealed transcripts from both single-transformed *U. maydis* cells (lane 6) and mated-transformed cells (lane 8); only the latter displayed clearly visible mycelial growth (Fig. 4B). The RT-PCR results indicate that (certain) *P. triticina* promoter elements are recognized by the *Ustilago* transcription machinery.

Restoration of *U. maydis* pathogenicity.

U. maydis ubc3/kpp2 deletion mutants are moderately attenuated in pathogenicity (Mayorga and Gold 1999; Muller et al. 1999). However, because *kpp6* deletion mutants are more severely attenuated in pathogenicity and *ubc3/kpp2 kpp6* double mutants are essentially nonpathogenic (Brachmann et al. 2003) (Figs. 5 and 6A, C, and E), we tested whether the PtMAPK constructs could restore virulence and pathogenicity in these mutants. Indeed, virulence was increased in the *kpp6* deletion mutants when the rust MAPK ORF was expressed from the

Table 2. Mating tests between *Ustilago maydis* strains and transformants[a]

Strain 1	Mated with strain 2	Fuz score[b]
521 wild type	523 wild type	+++
2/58 [*a1b1* Δ*ubc3/kpp2-1*]	2/59 [*a2b2* Δ*ubc3/kpp2-1*]	±
2/58 (+ pUBleX1)	2/59 (+ pUBleX1)	±
2/58 (+ pUmPtMAPK1)	2/59 (+ pUBleX1)	+
2/58 (+ pUmPtMAPK1)	2/59 (+ pUmPtMAPK1)	++
2/58 [+ pUBleX1Int]	2/59 [+ pUBleX1Int]	–
2/58 [+ pUmPtMAPKInt]	2/59 [+ pUBleX1Int]	+
2/58 [+ pUmPtMAPKInt]	2/59 [+ pUmPtMAPKInt]	++[c]
2/58 (+ pCM60)	2/59 (+ pCM60)	±
2/58 (+ pPtMAPK1)	2/59 (+ pCM60)	+
2/58 (+ pPtMAPK1)	2/59 (+ pPtMAPK1)	++
2/58 [+ pUBle3]	2/59 [+ pUBle3]	±
2/58 [+ pPtMAPK1Int]	2/59 [+ pUBle3]	+
2/58 [+ pPtMAPK1Int]	2/59 [+ pPtMAPK1Int]	+

[a] Constructs between parentheses indicate episomal plasmids; square brackets indicate stably integrated constructs.
[b] Fuz score indicates the colony phenotype appearing after co-spotting 5 µl of late-log culture of each cell type.
[c] Fuz reaction had a more spiky appearance.

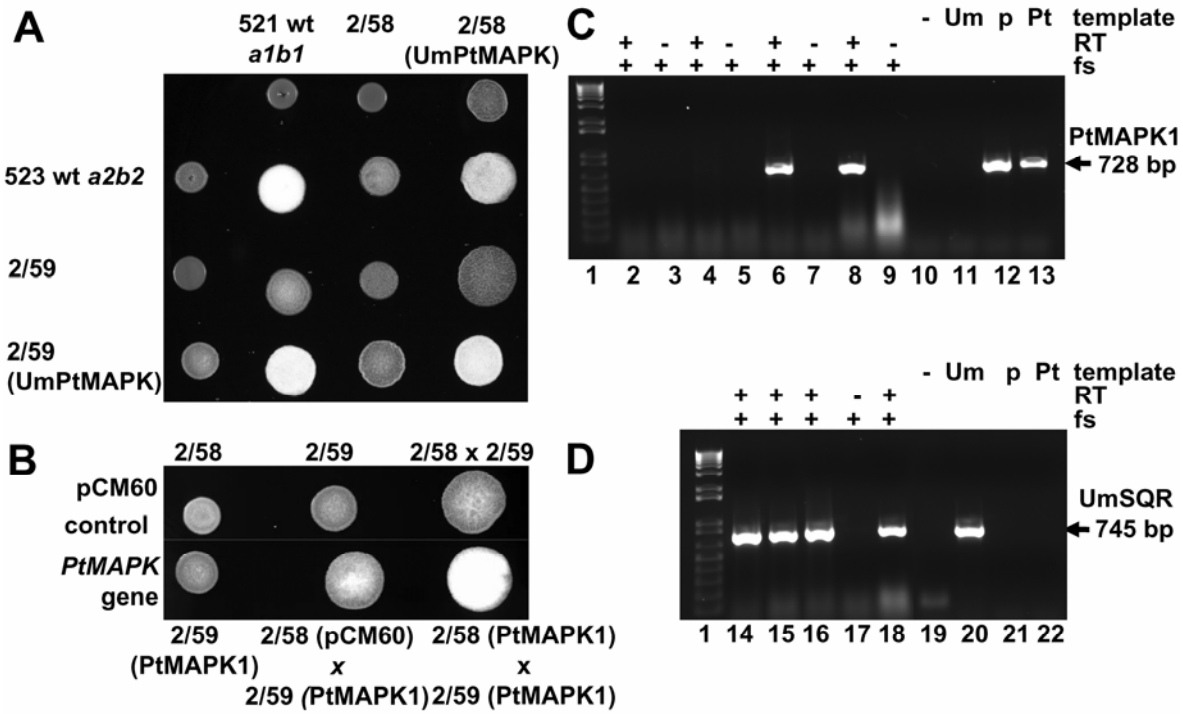

Fig. 4. Complementation of the *Ustilago maydis ubc3/kpp2* deletion mutant with the *Puccinia triticina* mitogen-activated protein kinase (MAPK)1 homolog and restoration of mating interactions. Mating assay on charcoal plates: white "fuzzy" mycelium ("Fuz" reaction) are straight-growing aerial dikaryotic hyphae produced upon fusion of cells of opposite mating type and is indicative of a successful mating interaction. *U. maydis* MAPK deletion mutants 2/58 (*a1b1* Δ*ubc3/kpp2*) and 2/59 (*a2b2* Δ*ubc3/kpp2*) are compromised in mating. **A,** Transformants harboring episomal plasmids with the PtMAPK open reading frame under the control of the *U. maydis* Hsp70 promoter. Various controls are spotted as single cell lines across the top and left-hand rows. **B,** Top row left and middle show individually spotted mutants harboring the episomal vector control which were mated in the colony on the right. Lower row shows transformants harboring episomal plasmids with the complete rust gene, *PtMAPK1*, individually (left) and mated (middle and right). **C** and **D,** Verifying expression of *PtMAPK1* from its endogenous rust promoter in *U. maydis* (from panel B). Reverse-transcription polymerase chain reaction (RT-PCR) experiments using **C,** *PtMAPK1*-specific primers or **D,** primers for a *U. maydis* control gene, succinate dehydrogenase (UmSQR). The expected PCR products in base pairs are indicated by the arrows. DNA templates as indicated: fs, first-strand cDNA; –RT, omission of reverse transcriptase as control. Lane 1: marker; lanes 2, 3, and 14: 2/59 (pCM60); lanes 4, 5, and 15: 2/58 (pCM60) mated with 2/59 (pCM60); lanes 6, 7, 16, and 17: 2/59 (pPtMAPK2); lanes 8, 9, and 18: 2/58 (pPtMAPK2) mated with 2/59 (pPtMAPK2); lanes 10 and 19: no DNA control (–); lanes 11 and 20: total *U. maydis* genomic DNA (Um); lanes 12 and 21: plasmid pPtMAPK2 (p); lanes 13 and 22: total *P. triticina* genomic DNA (Pt).

U. maydis Hsp70 promoter, and this effect seemed more pronounced in integrated constructs (compare Figs. 5A and B and 6A to D). The effect of an integrated complete *PtMAPK1* gene in the single *kpp6* deletion mutant was not significant (data not shown).

The effect of PtMAPK1 in the double mutants (*ubc3/kpp2 kpp6*) was much more dramatic. Even when present in only one transformed mating partner, the integrated construct harboring *Ustilago* Hsp70 promoter-driven pUmPtMAPK1 was sufficient to restore the pathogenic potential to near-wild-type levels (Figs. 5C and 6E and F). Integrated copies of the complete *PtMAPK1* gene did not seem to restore pathogenicity (Fig. 5D), suggesting that PtMAPK1 is not present in high enough amounts, possibly due to weak rust promoter activity. We have not tested activity of the *PtMAPK1* gene on an episomal vector in the double mutants. Interestingly, in the double mutants, transformants harboring episomal plasmids expressing PtMAPK from the *Ustilago* Hsp70 promoter grew very slowly and eventually could not be maintained, whereas the same mutants with episomal plasmids without inserts still could be cultured by repeated transfers on selective solid medium.

DISCUSSION

For the first time, we report on the isolation of an MAPK gene from a rust fungus, an understudied and recalcitrant group of basidiomycete obligate pathogens. We named this wheat leaf rust gene *PtMAPK1* and revealed that it codes for a protein with close homology to the *U. maydis* kinases, Ubc3/Kpp2 and Kpp6 from the YERK1 subfamily. In *U. maydis*, these MAPKs play a role in mating interactions, and plant tissue invasion and pathogenic development after mating, respectively. However, several experiments conducted by Brachmann and associates (2003) led these authors to suggest possible functional redundancy between Kpp6 and Ubc3/Kpp2; *ubc3/kpp2* deletion mutants are not completely mating deficient, possibly complemented for by the action of an as yet unidentified MAPK, possibly Kpp6 (Muller et al. 1999), and *kpp6* deletion mutants are weakly virulent on corn leaves, whereas *ubc3/kpp2 kpp6* double mutants are nonpathogenic (Brachmann et al. 2003). PtMAPK1 was able to complement *U. maydis ubc3/kpp2*, *kpp6*, and *ubc3/kpp2 kpp6* double mutants to restore mating interactions, virulence, and pathogenicity to near wild-type levels, proving we have isolated an active, functional MAPK equivalent from the YERK1 subgroup.

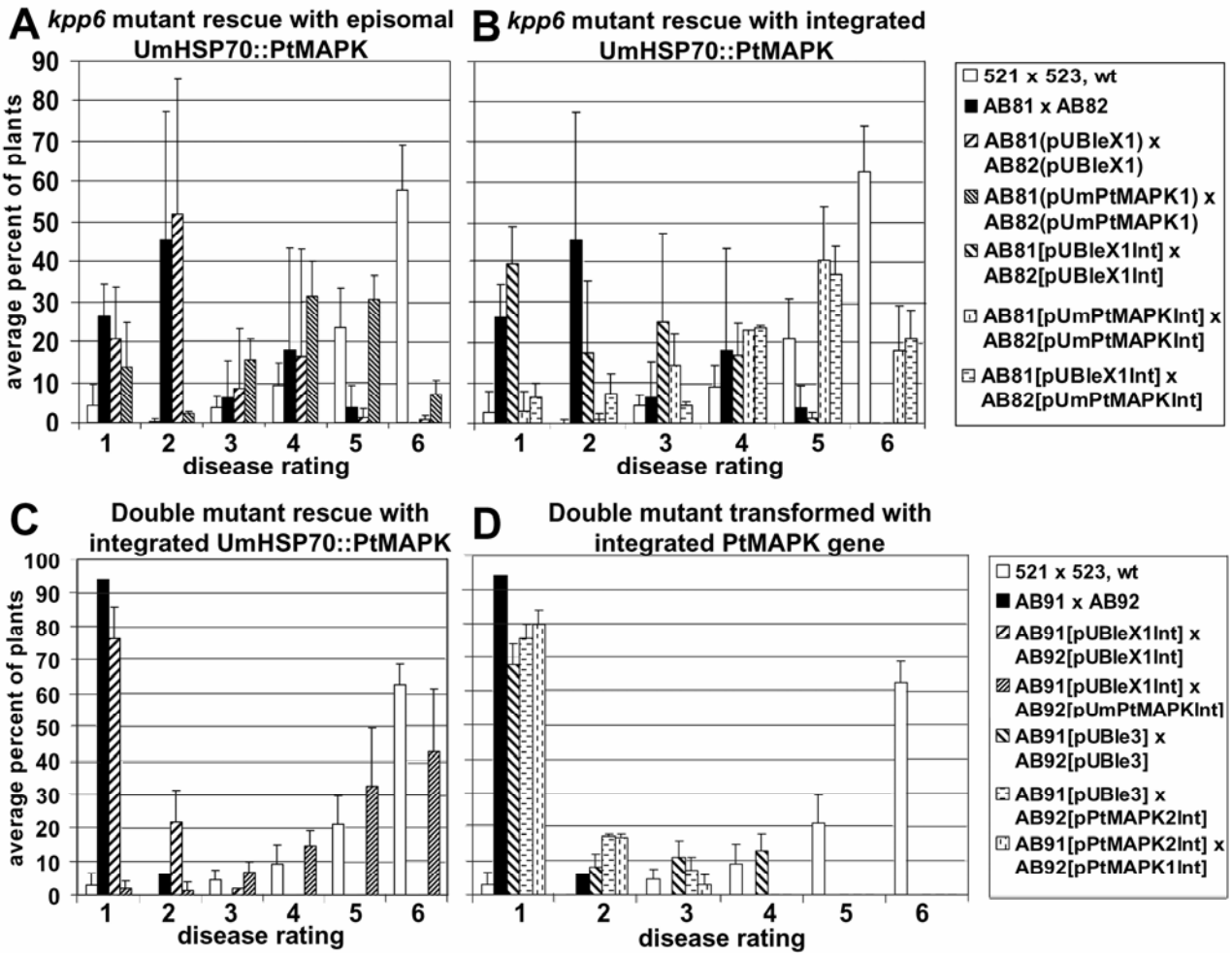

Fig. 5. Level of restoration of virulence on corn plants of single and double *Ustilago maydis* mitogen-activated protein kinase (MAPK) mutants by various *Puccinia triticina* MAPK constructs. Inoculations were performed with mated partners each having identical mutations and plasmids, except in D, where AB92 (*a2b2 kpp2-1 kpp6* [pPtMAPK2Int]) was mated with AB91 (*a1b1 kpp2-1 kpp6* [pUBle3]) harboring only the vector. **A,** *U. maydis kpp6* single mutants complemented with episomal plasmids as indicated in the legends. **B,** *U. maydis kpp6* single mutants complemented with integrated constructs. **C,** *U. maydis kpp2-1 kpp6* double mutants complemented with integrated constructs. **D,** *U. maydis kpp2-1 kpp6* double mutants complemented with integrated constructs carrying the complete *PtMAPK1* gene. The Y axis indicates the average percentage of plants showing the disease rating as indicated on the X axis: 1 = no visible symptoms, 2 = bleached streaks on leaves around the inoculation marks, 3 = anthocyanin production on leaves bearing inoculation marks (considered a plant stress response upon attempted invasion of plant tissue) (Banuett and Herskowitz 1996), 4 = leaf tumors, 5 = stem tumors, and 6 = plant death due to disease.

In complementation experiments, the increase in virulence of the *kpp6* deletion mutants was more pronounced when the PtMAPK ORF expressed from the *U. maydis* Hsp70 promoter was present on integrated plasmids (presumably a single-copy insertion) than on episomal plasmids (multiple copies) (compare Figs. 5A and B and 6A to D). Although we did not measure RNA or protein levels in these transformants, this discrepancy could indicate that *Ustilago* cells are sensitive to overexpression of the *kpp6* gene. However, decreased virulence also could be caused by loss of the episomal plasmid early in infection due to lack of selection. Proper regulation seemed less critical for the *ubc3/kpp2* gene, as suggested by the experiments with the *ubc3/kpp2* deletion mutants, where episomal plasmids still increased complementation compared with integrative constructs, indicating some level of tolerance to possible higher levels of PtMAPK1. The *U. maydis* double mutant most likely lacks any YERK1-like MAPKs and presumably does not respond well to any effectors of such signaling pathways. This lack, however, obviously is not lethal, possibly because a MAPK from a related subgroup can partially substitute and rescue some of the processes, or such signaling pathways are not essential during certain growth stages. Nevertheless, it is likely that these Δ*ubc3/kpp2* Δ*kpp6* double mutants require controlled levels of these MAPKs. This was corroborated by the fact that we were unable to maintain Δ*ubc3/kpp2* Δ*kpp6* double mutants when selecting for episomal vectors expressing PtMAPK from the *Ustilago* Hsp70 promoter. It is likely that these episomal vectors, present in approximately 25 copies per cell (Tsukuda et al. 1988), increase the level of PtMAPK to a toxic threshold, overcompensating or interfering with other signaling pathways.

In *U. maydis*, *kpp6* has a basic low level of expression in haploid basidiospores which increases upon pheromone stimulation (Brachmann et al. 2003). In mated cells or in cells with an active bE/bW heterodimer (heterozygosity at the *b* mating-type gene complex), this level of expression is maintained, as well as throughout the infection. However, *kpp6* seems to be turned off in maturing teliospores in tumors based on fluorescence of a *kpp6* promoter-GFP fusion. *Kpp2* is constitutively expressed (Brachmann et al. 2003). *U. maydis* does not produce structures of equivalent biological function to the rust urediniospores. Wheat infection occurs by dikaryotic urediniospores which produce new urediniospores; only upon unfavorable conditions are minute amounts of teliospores produced on stem segments. Teliospores in *P. triticina* produce haploid basidiospores as in *U. maydis*; however, these infect the alternate host to produce pycnia. Haploid pycniospores produced in pycnia of opposite or different mating types will mate by cross-fertilization to produce the dikaryotic aeciospores (Alexopoulos et al. 1996; Horton et al. 2005). The expression of *PtMAPK1* during these latter stages was not investigated, partly because of the difficult task of obtaining sufficient material for testing. We were able to isolate RNA from appressoria; however, the amounts were too low to obtain reliable quantitative RT-PCR data.

What would the role of PtMAPK1 be in leaf rust? Relative to the histone H4 gene, which presumably can be considered a housekeeping gene, *PtMAPK1* is expressed to a higher level in resting urediniospores and its message seems to rapidly decrease upon spore germination. Assuming no metabolic activity takes place in resting spores, this suggests a build-up of the pool of *PtMAPK1* mRNA immediately prior to urediniospore maturation or desiccation in anticipation of the need of PtMAPK protein upon spore germination. This normally is followed within 6 h by appressorium formation, invasion of the host through the stomatal opening at approximately 12 hai, and subsequent plant cell breach from within the substomatal cavity between 24 to 48 hai. A burst of PtMAPK1 upon germination could be consistent with a role during initial stages of germination and early plant infection. A strong induction of the gene would be necessary upon urediniospore maturation or desiccation, in which process PtMAPK1 also could have additional roles; increased expression of *PtMAPK1* was not observed yet at 6 days after infection. Interestingly, teliospores resulting from compatible *U. maydis ubc3* deletion mutants showed a 100× decrease in germination rate (Mayorga and Gold 1999), suggesting that this MAPK might have a function in this spore stage. MgFus3, another MAPK from the ascomycete wheat pathogen *Mycosphaerella graminicola*, seemed to play a role

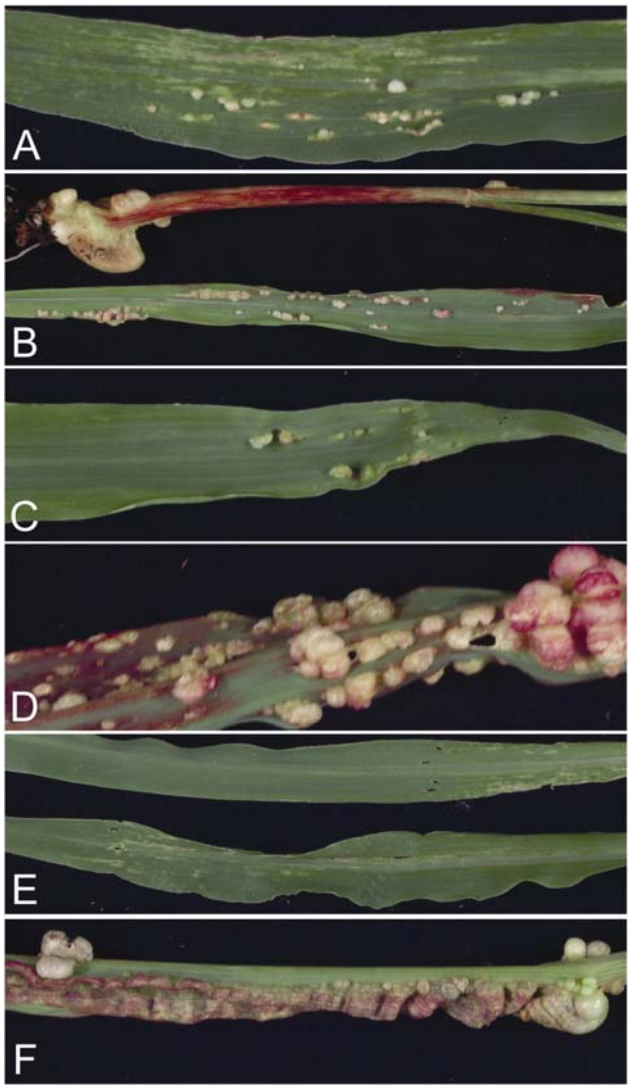

Fig. 6. Symptom formation on corn leaves 2 weeks after inoculation with various deletion mutants and rescued transformants. **A,** episomal vector control in *Ustilago maydis* single mutants, AB81 (*a1b1* Δ*kpp6*, pUBleX1) × AB82 (*a2b2* Δ*kpp6*, pUBleX1); **B,** rescue of *U. maydis* single mutants by episomal plasmid, AB81 (*a1b1* Δ*kpp6* pUmPtMAPK) × AB82 (*a2b2* Δ*kpp6* pUmPtMAPK); **C,** integrative vector control in *U. maydis* single mutants, AB81 (*a1b1* Δ*kpp6* [pUBleX1Int]) × AB82 (*a2b2* Δ*kpp6* [pUBleX1Int]); **D,** rescue of *U. maydis* single mutants by integrative construct, AB81 (*a1b1* Δ*kpp6* [pUmPtMAPK]) × AB82 (*a2b2* Δ*kpp6* [pUmPtMAPK]); **E,** integrative vector control in *U. maydis* double mutants, AB91 (*a1b1* Δ*kpp2-1* Δ*kpp6* [pUBleX1Int]) × AB92 (*a2b2* Δ*kpp2-1* Δ*kpp6* [pUBleX1Int]); and **F,** rescue of *U. maydis* double mutants by integrative construct, AB91 (*a1b1* Δ*kpp2-1* Δ*kpp6* [pUBleX1Int]) × AB92 (*a2b2* Δ*kpp2-1* Δ*kpp6* [pUmPtMAPKInt]). Plasmids between parentheses are episomal; constructs stably integrated appear between square brackets.

in leaf invasion through stomata and possibly production of pycnidia (Cousin et al. 2006). Pmk1 regulates appressorium formation in *Magnaporthe grisea* (Xu 2000; Xu and Hamer 1996). *Kpp6* deletion mutants seem to have a much reduced appressorial penetration function but still allow the penetration of short mycelium in leaf tissues in 3% of cases after inoculation. Moreover, when infiltrated into leaf tissue, thereby bypassing the need for penetrating the epidermis, a *kpp6*^{T335A,Y357F} mutant (which cannot be activated through phosphorylation) still cannot cause disease. These data suggest that Kpp6 is needed for post-penetration invasive growth (Brachmann et al. 2003).

It has been suggested that the presence of two close homologs of the YERK1 subfamily MAPKs represents the situation in basidiomycetes, whereas only one YERK1 MAP kinase is found in the genomes of filamentous ascomycetes (Brachmann et al. 2003). It is tempting to speculate that one of these MAPKs is involved in stabilization and maintenance of the dikaryotic state which is a hallmark of basidiomycete fungi that grow in a filamentous fashion. In *U. maydis,* the maintenance of the dikaryotic state has been shown to be under *a*-locus control to some degree (Banuett and Herskowitz 1989; Hartmann et al. 1996) and Kpp2 was shown to have some role in post-fusion events (Brachmann et al. 2003). That would explain why *U. maydis kpp6* expression is very low in haploid basidiospores, induced in preparation for and upon mating, and turned off during sporulation; teliospore formation involves nuclear fusion and generates true diploids.

The complete *PtMAPK1* gene on episomal plasmids was able to complement the *ubc3/kpp2* mutation (Fig. 4B and C), whereas integrated constructs did not show this effect (Table 2). This indicates that at least some of the *P. triticina* expression signals are recognized in *Ustilago* spp., possibly leading to some weak promoter activity but compensated for by the higher copy number of episomal plasmids. It also is possible that some promoter elements are not recognized or need species-, temporal-, or stage-specific transcription factors for full expression. Overall, this result indicates that it might be possible to test other rust clones harboring complete genes in a functional genomics approach for their ability to complement known *Ustilago* mutants. Conceivably, rust clones could reveal novel functions such as causing changes in virulence or host range when transferred into wild-type *Ustilago* spp.

Surrogate organisms for functional analyses of rust genes have been tested before. A plasma membrane H(+)-ATPase, a thiamine (vitamin B1) biosynthesis gene, and a hexose transporter gene, HXT1, from the bean rust *Uromyces fabae*, have been expressed to successfully complement homologous mutations in *S. cerevisiae* and *Schizosaccharomyces pombe* (Sohn et al. 2000; Struck et al. 1998; Voegele et al. 2001). We have chosen *Ustilago maydis* because it also is a plant-pathogenic basidiomycete fungus and is a much closer relative. Many of the leaf rust ESTs we generated are most similar to genes in *U. maydis* (G. Hu and G. Bakkeren, *unpublished*). We also study *U. hordei*, a barley smut which is a model for the small grain-infecting smut fungi; this group also includes *U. avenae* and *U. tritici*, infecting oat and wheat, respectively (Bakkeren et al. 2000; Menzies et al. 2003). *U. hordei* is closely related to *U. maydis*, and all tools and methods developed for *U. maydis* work in *U. hordei* (Bakkeren and Kronstad 1993, 1996; Gold et al. 1994). The tractability of these related smuts pathogenic on the same host complex as the cereal rusts, which include, in addition to wheat leaf rust, wheat stem rust (*P. graminis* f. sp. *tritici*), wheat stripe rust (*P. striiformis*), and oat crown rust (*P. coronata*), promises to present a viable avenue along which to pursue molecular analyses of genes from the obligate rust pathogens.

MATERIALS AND METHODS

Strains, plasmids, culturing, transformation, and mating procedures.

A list of plasmids and strains used and constructed during this study is given in Table 3. Bacterial strains *Escherichia coli* DH5α or DH10B/r (Invitrogen Canada Inc., Burlington, ON, Canada) were used for all cloning steps. Plasmid pCM60 is derived from pHL10 (Holden et al. 1989) by cloning the 3.1-kb *Hin*dIII fragment containing a hygromycinB-resistance cassette driven by the strong *U. maydis* heat shock protein Hsp70 promoter, in vector pUC18 (Fermentas Canada, Burlington, ON, Canada). Plasmid pUBleX1 is an episomal vector that can drive expression of ORFs cloned in the unique *Bgl*II site using the Hsp70 promoter and terminator elements; it is the progenitor of pBleX1 (Gold et al. 1994), lacking the multiple cloning site and having retained the original 850-bp *Xba*I fragment upstream of the promoter elements. pUBleX1Int is an integrative

Table 3. Plasmids and strains used and constructed

Plasmids, strains	Description, genotype[a]	Reference
pUBle3	Integrative backbone plasmid, *zeo*^{r}	Bakkeren et al. 1993
pUBleX1	Episomal expression plasmid, *zeo*^{r}	This study
pUBleX1Int	Integrative expression plasmid, *zeo*^{r}	This study
pCM60	Episomal backbone plasmid, *hyg*^{r}	This study
pUmPtMAPK1	PtMAPK ORF in *Bgl*II site of pUBleX1; episomal, *zeo*^{r}	This study
pUmPtMAPK1Int	PtMAPK ORF in *Bgl*II site of pUBleX1Int; integrative, *zeo*^{r}	This study
pPtMAPK1	Complete PtMAPK gene in *Sph*I site of pCM60; episomal, *hyg*^{r}	This study
pPtMAPK2	Idem as pPtMAPK1, but insert in reverse orientation	This study
pPtMAPK1Int	Complete PtMAPK gene in *Sph*I site of pUBle3; integrative, *zeo*^{r}	This study
pPtMAPK2Int	Idem as pPtMAPK1Int, but insert in reverse orientation	This study
Ustilago maydis strains		
521	*a1b1* wild type	Kronstad and Leong 1989
523	*a2b2* wild type	Kronstad and Leong 1989
2/58 (=FB1Δkpp2-1)	*a1b1* Δ*ubc3/kpp2-1::nat*^{r}	Mayorga and Gold 1999; Muller et al. 1999
2/59 (=FB2Δkpp2-1)	*a2b2* Δ*ubc3/kpp2-1::nat*^{r}	Mayorga and Gold 1999; Muller et al. 1999
AB81	*a1b1* Δ*kpp6::hyg*^{r}	Brachmann et al. 2003
AB82	*a2b2* Δ*kpp6::hyg*^{r}	Brachmann et al. 2003
AB91	*a1b1* Δ*kpp2-1::nat*^{r} Δ*kpp6::hyg*^{r}	Brachmann et al. 2003
AB92	*a2b2* Δ*kpp2-1::nat*^{r} Δ*kpp6::hyg*^{r}	Brachmann et al. 2003

[a] MAPK = mitogen-activated protein kinase, ORF = open reading frame; Δ = deletion of respective gene by marker exchange with resistance cassette as indicated. *hyg*^{r} = hygromycinB resistance, *nat*^{r} = nourseothricin resistance, and *zeo*^{r} = zeomycin or bleomycin (Zeocin) resistance.

vector derived from pUBleX1 by deleting its 400-bp *U. maydis* autonomously replication sequence-containing *Ssp*I fragment. pUBle3 is an integrative vector based on pGEM3, expressing zeocin-resistance under the control of *Ustilago*-specific Hsp70 promoter and terminator elements (Bakkeren and Kronstad 1993).

U. maydis strains were grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit) or in PDA broth at 30°C. *U. maydis* cells were converted to spheroplasts and were transformed by chemical treatment using polyethylene glycol 4000 as previously described (Wang et al. 1988). DNA (5 μg) was mixed with 15 μg of heparin in a 5-μl volume and was used to transform 10^6 cells in a 50-μl volume; integrative plasmid constructs were linearized with *Eco*R1 (pUble3 and derivatives) or *Xho*I (pUBleX1Int and derivatives) prior to transformation. Transformants were selected on complete medium plates (Holliday 1974) with hygromycinB at 300 μg/ml (Calbiochem; Biosciences Inc., La Jolla, CA, U.S.A.) or Zeocin at 50 μg/ml (Cayla Laboratories, France) (zeomycin, equivalent to phleomycin or bleomycin). Mating interactions were tested by mixing 5 μl of culture of each of two candidate strains on complete medium plates amended with 1% charcoal (Holliday 1974), sealing with parafilm to prevent gas exchange, and incubating at room temperature for 48 h; the presence or absence of infection hyphae (Fuz) was verified using a compound microscope and indicated mating or no mating, respectively.

P. triticina race 1 (designation BBB: avirulent on a set of 3 × 4 = 12 North-American standard cultivars) (Long and Kolmer 1989) was used. Stages sampled in triplicate were as follows: resting urediniospores, urediniospores germinated for 12 h over a water surface in petri dishes, a haustorial developmental stage before sporulation, at 6 days postinoculation (leaf surface was "cleaned" of ungerminated spores by dipping leaves in a 20% cellulose acetate in acetone solution and pealing the resulting film off), and a compatible interaction 48 h postinoculation, when the fungus has penetrated through stomata (cleaned leaves, as above). Inoculation was done on 10-day-old seedlings of wheat cultivar Thatcher as described (Hu and Rijkenberg 1998).

Gene isolation and DNA manipulations.

DNA manipulations were performed essentially as described (Ausubel et al. 2005). The generation of the *P. triticina* cDNA libraries and EST sequences will be described elsewhere (G. Hu, R. Linning, and G. Bakkeren, *unpublished data*). Plasmids and constructs for cloning, transformation or sequencing were isolated and purified with a QIAprep Spin Miniprep Kit according to the manufacturer's protocol (QIAGEN, Mississauga, ON, Canada). Oligonucleotide primers were synthesized on a Beckmann Oligo 1000M DNA synthesizer and purified according to the manufacturer's recommendation, or obtained from Invitrogen. The 5′ genomic region of *PtMAPK1*, including the promoter, was obtained by the method of Zhang and Gurr (2002) using PtMAPK1-specific primers 162 and 163 (Table 1). To make the constructs, specific parts of the gene were amplified by PCR from 25 ng of total genomic *P. triticina* DNA using various primers. To generate the complete *PtMAPK1* gene, primers 227 and 228 were used and the PtMAPK1 ORF was amplified from primers 229 and 230. PCR reactions were performed using *Taq* DNA polymerase in standard buffer containing 1.5 mM $MgCl_2$, 0.3 μM each primer, and 200 μM each dNTP. Amplification conditions were as follows: 5 min at 96°C; 35 cycles of 30 s at 96°C, 30 s at 55°C, and a 3-min extension at 72°C; followed by an 8-min extension at 72°C. PCR products were purified over columns (QIAquick PCR Purification Kit; QIAGEN), digested with restriction enzymes or made flush with T4 DNA polymerase as required, purified again, and subsequently used for cloning. *PtMAPK1* was first cloned as a blunt-end PCR product in the dephosphorylated Sma1-site of pBlueScriptIIKS (Stratagene, La Jolla, CA, U.S.A.) using a TOPO cloning kit (Invitrogen). The 1,989-bp *Sph*I fragment subsequently was cut out and cloned in two orientations in the *Sph*I site of vector pCM60 to create pPtMAPK1 and -2, and in vector pUBle3 to create pPtMAPK1Int and pPtMAPK2Int (Table 3). The PtMAPK1 ORF was cloned as a 1,230-bp *Bgl*II fragment in the correct orientation in the *Bgl*II site of expression vectors pUBleX1 to create episomal plasmid pUmPtMAPK1, and in pUBleX1Int to create the integrative construct pUmPtMAPK1Int (Table 3).

Genomic DNA was isolated from *P. triticina* urediniospores germinated in petri dishes over water with 10^{-7} M nonanol (nonyl alcohol; Sigma-Aldrich, Oakville, ON, Canada) and small pieces of filter paper soaked in 10^{-4} M nonanol adhered in the lid (nonanol is a volatile compound that stimulates urediniospore germination otherwise inhibited under "overcrowding" conditions) (French 1992). A mat of germ tubes was collected and ground up in liquid nitrogen in a mortar. This produced a powder from which total genomic DNA was extracted using a Plant DNA Extraction Kit (QIAGEN) following the manufacturer's protocol.

Quantitative and regular RT-PCR.

Total RNA was isolated from freeze-dried colony material (RT-PCR experiments) or from freshly frozen cell material (quantitative RT-PCR experiments) by grinding with sterile sand and extraction in a solution of phenol-guanidine isothiocyanate (TRI Reagent; BIOCAN Scientific, Mississauga, ON, Canada) according to instructions provided by the manufacturer. DNaseI treatment and first-strand synthesis using SuperscriptII reverse transcriptase and random hexamer primers were performed using kits from Invitrogen as described by the manufacturer. RT-PCR conditions were 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM each dNTPs, 1 μM each primer, and 0.5 units of recombinant Taq polymerase (Invitrogen) in 25 μl of total volume using the following profile on a MyCycler (BioRad): 3 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 65°C, and 2 min at 72°C; followed by an extension of 10 min at 72°C. *P. triticina*-specific *MAPK1* primers were 424 and 434; as control, the *U. maydis* succinate dehydrogenase gene (um00844, GenBank accession XM_398459) was employed using primers 401 and 402. For quantitative RT-PCR, 2 μg of total RNA was treated with 2 units of DNaseI (Amplification Grade, Invitrogen), reverse-transcribed with 200 units SuperscriptIII (a fraction was withheld as "minus RT control"), and remaining RNA digested with RNaseH. The first-strand cDNA samples were diluted to 12.5 ng/μl (based on input RNA). Quantitative RT-PCR was performed on a Mx3000P qPCR System (Stratagene) with primer pairs as indicated in Table 1; the length and uniqueness of the amplicons was verified by agarose gel electrophoresis and melting curve comparison within the analysis software. Transcript levels were derived from the accumulation of SYBR green fluorescence using the following components: 20 μl of volume containing 1× AmpliTaq Gold II buffer plus 0.5 unit of AmpliTaq Gold (PE BioSystems, Foster City, CA, U.S.A.), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 150 nmol each primer, 7.5% glycerol, 3% dimethyl sulfoxide, 1/40,000 dilution of SYBR green and 1/30,000 dilution of ROX as reference dye (both from Stratagene), and 25 ng of cDNA. PCR conditions were 10 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; followed by a melting curve program. The threshold cycle values for *PtMAPK1* and *P. triticina* histone H4 each were converted to amounts of cDNA present in the sample relative to

a standard curve of threshold cycle values for each gene generated by serial dilutions of known quantities of total *P. triticina* genomic DNA using the same PCR cocktail. Amounts of MAPK product subsequently were normalized to amounts of histone H4 product.

Sequence analysis and bioinformatics.

DNA sequencing was done using the BigDye Terminator Cycle Sequence chemistry (Applied Biosystems, Foster City, CA, U.S.A.) and the standard –21M13 forward or reverse primers and products were analyzed on an ABI310 Genetic Analyzer (Applied Biosystems). *PtMAPK1* and EST sequences have been deposited in GenBank (accession numbers in parentheses). *PtMAPK1* (DQ026061). Contig2832 is composed of nine partial cDNA clones:

PT0054c.B09.BR (DN956226),
PT0062c.A03.BR (DN956228),
PT0065d.G07.BR (DN956229),
PT0113d.A04.BR (DN956230),
PT0241.N06--PT0062c.A03.TB (EC387017),
PT0241.N18--PT0062c.A03.TB (EC387018),
PT0271.A19--PT0062c.A03.TB (EC387019),
PT0271.N06--PT0062c.A03.TB (EC387020), and
PT0281.N06--PT0062c.A03.C21 (EC387021).

Contig6818 is composed of 18 partial cDNA clones:

PT0061a.H01.B7 (DN956227),
Pta.13a.R.pta (EC387022),
PT0241.P14--PT0061a.H01.TB (EC387023),
PT0241.P02--PT0061a.H01.TB (EC387024),
PT0201.I17--PT0061a.H01.CPTR (EC387025),
PT0105.E01.CR (EC387026),
PT0105.E01.C7 (EC387027),
PT0092d.D02.B21 (EC387028),
PT0067b.H07.BR (EC387029),
PT0067a.D07.BR (EC387030),
PT0066b.B01.BR (EC387031),
PT0066a.H09.BR (EC387032),
PT0065d.B10.BR (EC387033),
PT0065b.D11.BR (EC387034),
PT0065b.B12.BR (EC387035),
PT0062b.C06.BR (EC387036),
PT0061a.H01.TB (EC387037), and
PT0053a.G12.BR (EC387038).

Protein sequence data from a variety of MAPKs was retrieved from GenBank and piled up using the clustalW program, version 1.83 (Chenna et al. 2003). Phylogenetic analysis using parsimony (PAUP version 4.0 for MacIntosh computer) (Swofford 2002) in full heuristic search mode was used for the generation of the phylogenetic tree (1,000 replicates in bootstrap analysis).

Pathogenicity tests.

Ten-day-old corn seedlings (cv. Improved Golden Bantam; Seed Centre Inc., Edmonton, Alberta, Canada) grown in Premier ProMix BX–general use growth medium each were treated with 100 µl of a cell suspension containing 10^5 cells of each mating type, injected into the culm just above the soil line. Plants were maintained in a greenhouse under a day-night cycle of 15 and 9 h, respectively, at 21 and 17°C, day and night, respectively. Disease symptoms were scored 15 days after infection. Experiments were repeated three times and were performed with at least two separate transformants per series.

ACKNOWLEDGMENTS

The authors thank S. Gold and R. Kahmann for supplying the *U. maydis* deletion strains; and S. Gold, J.-R. Xu, and P. Wiersma for critical comments on the manuscript.

LITERATURE CITED

Agrios, G. N. 1997. Plant Pathology, 4th ed. Academic Press, San Diego, CA, U.S.A.

Alexopoulos, C. J., Mims, C. W., and Blackwell, M. 1996. Introductory Mycology,4th ed. John Wiley & Sons, Inc., New York.

Andrews, D. L., Egan, J. D., Mayorga, M. E., and Gold, S. E. 2000. The *Ustilago maydis ubc4* and *ubc5* genes encode members of a MAP kinase cascade required for filamentous growth. Mol. Plant-Microbe Interact. 13:781-786.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 2005. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Bakkeren, G., and Kronstad, J. W. 1993. Conservation of the *b* mating-type gene complex among bipolar and tetrapolar smut fungi. Plant Cell 5:123-136.

Bakkeren, G., and Kronstad, J. W. 1996. The pheromone cell signaling components of the *Ustilago a* mating-type loci determine intercompatibility between species. Genetics 143:1601-1613.

Bakkeren, G., Kronstad, J. W., and Levesque, C. A. 2000. Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. Mycologia 92:510-521.

Banuett, F., and Herskowitz, I. 1989. Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. Proc. Natl. Acad. Sci. U.S.A. 86:5878-5882.

Banuett, F., and Herskowitz, I. 1994. Identification of fuz7, a *Ustilago maydis* MEK/MAPKK homolog required for a-locus-dependent and -independent steps in the fungal life cycle. Genes Dev. 8:1367-1378.

Banuett, F., and Herskowitz, I. 1996. Discrete developmental stages during teliospore formation in the corn smut fungus, *Ustilago maydis*. Development 122:2965-2976.

Basse, C. W., and Farfsing, J. W. 2006. Promoters and their regulation in *Ustilago maydis* and other phytopathogenic fungi. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 254:208-216.

Brachmann, A., Schirawski, J., Mueller, P., and Kahmann, R. 2003. An unusual MAP kinase is required for efficient penetration of the plant surface by *Ustilago maydis*. EMBO (Eur. Mol. Biol. Organ.) J. 22:2199-2210.

Bushnell, W. R., and Roelfs, A. P., eds. 1984. The Cereal Rusts. Vol. I. Origins, Specificity, Structure and Physiology. Academic Press, Inc., Orlando, FL, U.S.A.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31:3497-3500.

Cousin, A., Mehrabi, R., Guilleroux, M., Dufresne, M., van der Lee, T., Waalwijk, C., Langin, T., and Kema, G. H. J. 2006. The MAP kinase-encoding gene *MgFus3* of the non-appressorium phytopathogen *Mycosphaerella graminicola* is required for penetration and in vitro pycnidia formation. Mol. Plant Pathol. 7:269-278.

Feldbrugge, M., Kamper, J., Steinberg, G., and Kahmann, R. 2004. Regulation of mating and pathogenic development in *Ustilago maydis*. Curr. Opin. Microbiol. 7:666-672.

French, R. C. 1992. Volatile chemical germination stimulators of rust and other fungal spores. Mycologia 84:277-288.

Gold, S. E., Bakkeren, G., Davies, J. E., and Kronstad, J. W. 1994. Three selectable markers for transformation of *Ustilago maydis*. Gene 142:225-230.

Hahn, M. 2000. The rust fungi. Cytology, physiology and molecular biology of infection. Pages 267-306 in: Fungal Pathology. J. W. Kronstad, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Hartmann, H. A., Kahmann, R., and Bolker, M. 1996. The pheromone response factor coordinates filamentous growth and pathogenicity in *Ustilago maydis*. EMBO (Eur. Mol. Biol. Organ.) J. 15:1632-1641.

Holden, D. W., Kronstad, J. W., and Leong, S. A. 1989. Mutation in a heat-regulated hsp70 gene of *Ustilago maydis*. EMBO (Eur. Mol. Biol. Organ.) J. 8:1927-1934.

Holliday, R. 1974. *Ustilago maydis*. Pages 575-595 in: Handbook of Genetics. R. C. King, ed. Plenum, New York.

Horton, J. S., Bakkeren, G., Klosterman, S. J., Garcia-Pedrajas, M., and Gold, S. E. 2005. Genetics of morphogenesis in Basidiomycetes. Pages 353-422 in: Applied Mycology and Biotechnology. D. K. Arora and R. Berka, eds. Elsevier, Dordrecht, The Netherlands.

Hu, G., and Rijkenberg, F. H. J. 1998. Scanning electron microscopy of early infection structure formation by *Puccinia recondita* f. sp. *tritici* on and in susceptible and resistant wheat lines. Mycol. Res. 102:391-399.

Kolmer, J. A. 1996. Genetics of resistance to wheat leaf rust. Annu. Rev. Phytopathol. 34:435-455.

Kronstad, J. W., and Leong, S. A. 1989. Isolation of two alleles of the *b* locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. U.S.A. 86:978-982.

Kultz, D. 1998. Phylogenetic and functional classification of mitogen- and stress-activated protein kinases. J. Mol. Evol. 46:571-588.

Long, D. L., and Kolmer, J. A. 1989. A North American system of nomenclature for *Puccinia recondita* f. sp. *tritici*. Phytopathology 79:525-529.
Mayorga, M. E., and Gold, S. E. 1999. A MAP kinase encoded by the *ubc3* gene of *Ustilago maydis* is required for filamentous growth and full virulence. Mol. Microbiol. 34:485-497.
Menzies, J., Bakkeren, G., Matheson, F., Procunier, J.-D., and Woods, S. 2003. Use of inter-simple sequence repeats and amplified fragment length polymorphisms to analyze genetic relationships among small grain-infecting species of *Ustilago*. Phytopathology 93:167-175.
Muller, P., Aichinger, C., Feldbrugge, M., and Kahmann, R. 1999. The MAP kinase kpp2 regulates mating and pathogenic development in *Ustilago maydis*. Mol. Microbiol. 34:1007-1017.
Raman, M., and Cobb, M. H. 2003. MAP kinase modules: many roads home. Curr. Biol. 13:R886-888.
Roelfs, A. P., and Bushnell, W. R., eds. 1985. The Cereal Rusts. Vol. II: Diseases, Distribution, Epidemiology and Control. Academic Press, Orlando, FL, U.S.A.
Saito, H., and Tatebayashi, K. 2004. Regulation of the osmoregulatory HOG MAPK cascade in yeast. J. Biochem. 136:267-272.
Schaeffer, H. J., and Weber, M. J. 1999. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cell. Biol. 19:2435-2444.
Schillberg, S., Tiburzy, R., and Fischer, R. 2000. Transient transformation of the rust fungus *Puccinia graminis* f. sp. *tritici*. Mol. Gen. Genet. 262:911-915.
Sohn, J., Voegele, R. T., Mendgen, K., and Hahn, M. 2000. High level activation of vitamin B1 biosynthesis genes in haustoria of the rust fungus *Uromyces fabae*. Mol. Plant-Microbe Interact. 13:629-636.
Staples, R. C. 2000. Research on the rust fungi during the twentieth century. Annu. Rev. Phytopathol. 38:49-69.
Struck, C., Siebels, C., Rommel, O., Wernitz, M., and Hahn, M. 1998. The plasma membrane H(+)-ATPase from the biotrophic rust fungus *Uromyces fabae*: molecular characterization of the gene (*PMA1*) and functional expression of the enzyme in yeast. Mol. Plant-Microbe Interact. 11:458-465.
Swofford, D. L. 2002. PAUP v4.0, Phylogenetic Analysis Using Parsimony. Sinauer Associates, Inc., Sunderland, MA, U.S.A.
Tsukuda, T., Carleton, S., Fotheringham, S., and Holloman, W. K. 1988. Isolation and characterization of an autonomously replicating sequence from *Ustilago maydis*. Mol. Cell. Biol. 8:3703-3709.
Voegele, R. T., Struck, C., Hahn, M., and Mendgen, K. 2001. The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*. Proc. Natl. Acad. Sci. U.S.A. 98:8133-8138.
Wang, J., Holden, D. W., and Leong, S. A. 1988. Gene transfer system for the phytopathogenic fungus *Ustilago maydis*. Proc. Natl. Acad. Sci. U.S.A. 85:865-869.
Webb, C. A., Szabo, L. J., Bakkeren, G., Garry, C., Staples, R. C., Eversmeyer, M., and Fellers, J. P. 2006. Transient expression and insertional mutagenesis of *Puccinia triticina* using biolistics. Funct. Integr. Genomics 6:250-260.
Xu, J. R. 2000. MAP kinases in fungal pathogens. Fungal Genet. Biol. 31:137-152.
Xu, J. R., and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. Genes Dev. 10:2696-2706.
Zhang, Z., and Gurr, S. J. 2002. A "step down" PCR-based technique for walking into and the subsequent direct-sequence analysis of flanking genomic DNA. Methods Mol. Biol. 192:343-350.

AUTHOR-RECOMMENDED INTERNET RESOURCES

COGEME (Consortium for the Functional Genomics of Microbial Eukaryotes) database: cogeme.ex.ac.uk/index.html
GenBank database: www.ncbi.nlm.nih.gov/entrez/query.fcgi
MIPS (Munich InformationCenter for Protein Sequences) *Ustilago maydis* database: mips.gsf.de/genre/proj/ustilago/