Characterization of the ERK homologue CpMK2 from the chestnut blight fungus *Cryphonectria parasitica*

Eun-Sil Choi,1 Hea-Jong Chung,1 Myoung-Ju Kim,1 Seung-Moon Park,1 Byeong-Jin Cha,2 Moon-Sik Yang1 and Dae-Hyuk Kim1

1Institute for Molecular Biology and Genetics, Basic Science Research Institute, Chonbuk National University, Jeonju, Chonbuk 561-756, Korea
2Department of Agricultural Biology, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea

The *Cryphonectria parasitica* gene *cpmk2*, which encodes a mitogen-activated protein kinase belonging to the yeast extracellular signalling-regulated kinase (YERK1) subfamily, was isolated and its biological function was examined. Disruption of *cpmk2* resulted in impaired pigmentation and abolished conidiation. Growth defects were observed in the *cpmk2* mutant grown on solid plates, but growth of the mutant appeared normal in liquid media, including EP complete and PD broth, suggesting that the *cpmk2* gene is involved in sensing and responding to growth conditions. The mutant's production of laccase, as measured by the size of the coloured area produced on tannic-acid-supplemented plates, was significantly reduced compared with the wild-type, but the intensity of the coloured area was unchanged, suggesting that the reduced laccase activity was owing to reduced growth on solid media rather than transcriptional downregulation. A dramatic reduction observed in the canker area produced by the *cpmk2* mutant compared with the wild-type, even more severe than that of a hypovirulent strain, can also be ascribed to defective growth on solid surfaces rather than to impairments in a virulence factor(s). Downregulation of the pheromone gene *Mf2/1* was also observed in the mutant, indicating a possible explanation for the regulation of the pheromone precursor gene in filamentous fungi and suggesting the presence of the yeast-like pheromone-responsive pathway in *C. parasitica*. Immunoblot analyses revealed that the phosphorylation level of CpMK2 increased in both virus-free and virus-containing strains in liquid cultures of up to 5 days old and decreased in older cultures. Moreover, the CpMK2 phosphorylation level increased in both strains after transfer from liquid to solid medium. However, levels of phosphorylated CpMK2 were similar in the two strains, suggesting that CpMK2, unlike CpMK1, is not under the direct control of a hypovirus.

INTRODUCTION

The chestnut blight fungus *Cryphonectria parasitica* has devastated the chestnut forestry of North America. However, infection of the fungus with a hypovirus is known to cause hypovirulence, resulting in biological control (Van Alfen et al., 1975; Anagnostakis, 1982; Nuss, 1992). *C. parasitica* strains containing the double-stranded (ds) RNA virus Cryphonectria hypovirus (CHV) display characteristic symptoms of hypovirulence and hypovirulence-associated changes such as reduced sporulation, laccase production, oxalate accumulation and pigmentation (Havir & Anagnostakis, 1983; Elliston, 1985; Rigling et al., 1989). Interestingly, the symptoms caused by hypoviral infection appear to be the result of aberrant expression of specific fungal genes in the hypovirulent strain, including genes for cutinase, laccase, cryparin and mating pheromones (Rigling & Van Alfen, 1991; Choi et al., 1992; Varley et al., 1992; Zhang et al., 1993, 1994; Allen et al., 2003; Allen & Nuss, 2004). Thus, *C. parasitica* and its hypovirus are a useful model system to study the mechanisms of fungal gene regulation by a mycovirus.

Since the phenotypic changes in fungal hosts induced by hypoviruses are pleiotropic but occur in a coordinated and specific manner, it was suggested that hypoviruses disturb one or more regulatory pathways (Nuss, 1996). Several
studies have demonstrated the implementation of a signal transduction pathway during viral symptom development. Genes modulated during this process encode components of various signal transduction pathways, including heterotrimeric G-proteins, a putative regulator of these G-proteins (Choi et al., 1995; Kasahara & Nuss, 1997; Kasahara et al., 2000), a novel kinase (Kim et al., 2002) and many others that are under investigation (Dawe & Nuss, 2001). Recently, we have demonstrated the hypoviral regulation of components of a mitogen-activated protein kinase (MAPK) signalling pathway in C. parasitica (Park et al., 2004).

The MAPK signal transduction pathway is utilized by eukaryotic cells to transduce a wide variety of cellular signals through a stepwise phosphorylation relay. This cascade, which occurs in a wide variety of organisms from yeast to humans (Herskowitz, 1995; Schaeffer & Weber, 1999), consists of three functionally interlinked protein kinases: MEEK (MAP kinase kinase kinase), MEK (MAP kinase kinase) and MAPK. Fungal MAPks are involved in pathways that are required for numerous processes related to growth and differentiation (Xu, 2000). Moreover, MAPks are involved in the pathogenicity of many plant-pathogenic fungi (Xu, 2000). We previously demonstrated the presence of a HOG1-like MAPK, CpMK1, in C. parasitica, as well as hypovirus-mediated perturbation of its phosphorylation in response to hypertonic stress (Park et al., 2004).

In this study, we isolated from C. parasitica a member of a different class of MAPK, CpMK2, which is an ERK homologue. In addition, we addressed the putative biological functions of CpMK2 that are related to hypovirulence-associated traits and examined whether CpMK2 is affected by hypoviral infection.

METHODS

Fungal strains and growth conditions. The CHV1-713-containing hypovirulent C. parasitica strain UEPI and its isogenic virus-free strain EP155/2 (ATCC 38755) were maintained on potato dextrose agar plates containing 100 mg l-methionine l⁻¹ and 1 mg biotin l⁻¹ (PDAmb) under constant low light at 25 °C (Kim et al., 1995). Radial growth on plates was assessed by measuring the diameter of colonies. The culture conditions and preparation methods for the primary inoculum for liquid cultures were as previously described (Kim et al., 1995). To determine biomass, mycelia was filtered through Miracloth (Calbiochem), washed with sterile distilled water, blotted between dry paper towels until no excess water emerged, weighed to obtain the fresh weight and dried at 80 °C for a further 2 days as described previously (Kim et al., 1995) to obtain the dry weight.

Isolation and characterization of the MAPK cpmk2 gene. Degenerate primers specific for consensus nucleotide sequences corresponding to the most conserved amino acids within subdomains II/V/II of MAPks (Kultz, 1998) were designed as described previously (Park et al., 2004). The primers used were MK1-F1 (forward; 5'-GGTNGCNAATRAARAT-3') and MK1-R1 (reverse; 5'-GGYTNTANRTRTNCNRTG-3'). PCR was conducted as described by Park et al. (2004). The 310 bp PCR amplicon was cloned into the pGEM-T vector (Promega). Inserts containing positive clones were sequenced using the dideoxynucleotide method before being used as hybridization probes for genomic λ library screening according to standard procedures (Sambrook et al., 1989).

To obtain a DNA clone for cpmk2, RT-PCR was performed with the primers cMK2-F1 (forward; 5'-ATGTCAACGCCCTCCAGGCTCCC-3') and cMK2-R1 (reverse; 5'-GCATGATCCTCCTGATGAC-3'). The cDNA was sequenced using the dideoxynucleotide method and synthetic oligonucleotide primers.

Southern and Northern blot analysis. Genomic DNA from C. parasitica was extracted using the method of Churchill et al. (1990). DNA (10 μg) was digested with restriction enzymes, blotted onto nylon membrane and hybridized with a radioactively labelled cpmk2 fragment.

RNA was extracted from liquid cultures at 1, 3 and 5 days after inoculation and Northern blot analysis was conducted as described previously (Kim et al., 1995). RNA from cultures grown on solid medium was prepared using a previously described method (Park et al., 2004). The expression of cpmk2 was compared with that of the C. parasitica glyceraldehyde-3-phosphate dehydrogenase (Gpd) gene as an internal control (Choi & Nuss, 1990).

Heterologous expression of cpmk2 in Escherichia coli. The full-length cpmk2 protein CpMK1 was expressed in E. coli as a hexahistidine fusion protein and purified by nickel-affinity chromatography according to the manufacturer’s instructions (Novagen). A full-length CpMK2 cDNA was amplified by PCR using the primers 5’-CACATAGTGTCAGTCCCACAGCTCCA-3’ (forward) and 5’-CGGCGGCGGCACGATGCTCTGAGATGCTCC-3’ (reverse). The primers were modified to incorporate restriction sites (underlined) for Ndel and NotI, respectively. The full-length 1067 bp cpmk2 gene was fused between the Ndel and NotI sites in the expression vector pET-28a. The resulting recombinant plasmid was transformed into the E. coli strain BL21. Induction, purification and confirmation of recombinant CpMK2 were performed using an anti-hexahistidine antibody according to the manufacturer’s instructions (Novagen). E. coli-derived inclusion bodies, in which the recombinant CpMK2 was expressed, were solubilized and then refolded by stepwise dilution of the denaturants by dialysis (Creighton, 1990).

Kinase activity of CpMK2. MAPK activity of the E. coli-expressed CpMK2 was assayed by measuring the incorporation of [γ-32P]ATP into myelin basic protein (MBP) as described previously (Park et al., 2004). To test for autophosphorylation, purified CpMK2 was used in a kinase assay without MBP. A kinase assay of cell-free extracts was conducted as described previously (Kim et al., 2002).

Immunoblot analysis. Anti-CpMK2 antibody was obtained as described previously (Kim et al., 2002). Purified recombinant CpMK2 (100 μg) was injected into an 8-week-old BALB/c mouse, which was boosted with the same amount of the CpMK2 emulsified in incomplete Freund’s adjuvant 10 days after the initial injection. Polysera were obtained 5 days after the booster injection and Western blot analysis was conducted according to standard procedures (Sambrook et al., 1989).

To determine whether mycoviruses specifically affect the signal cascade that involves CpMK2, levels of phosphorylated CpMK2 were examined by immunoblotting with an antibody specific for doubly phosphorylated p44/42 MAPK (phospho-p44/42 MAP kinase antibody) according to the manufacturer’s instructions (Cell Signalling Technology). To examine the induction of phosphorylation owing to changes in the culture medium, mycelia grown in liquid culture were weighed, transferred onto PDAmb and harvested at the appropriate
time to measure the phosphorylation level of CpMK2. The non-phosphorylation-specific p44/42 MAP kinase antibody (Cell Signalling Technology) was also applied to verify the equal loading of protein.

**Construction of a replacement vector and fungal transformation.** The replacement vector pDMk2, which was designed to favour double-crossover integration events, was constructed as follows. A 2-0 kb KpnI fragment containing the full-length cpmk2 ORF was ligated into KpnI-inactivated pBluescript II SK (+) and the resulting plasmid was used as a template for inverted PCR using the primers 5'-GTCGACGGCAGACAAACATAGGGACCC-3' and 5'-GTCGAC-CCCTGGACCATCTCCTCAAC-3', which incorporate the restriction site for SalI (underlined). The PCR amplicon was digested with SalI and ligated. The resulting plasmid was further digested with SalI and fused with a 2-4 kb SalI fragment of pDH25 (Cullen et al., 1987) carrying the hygromycin phosphotransferase gene (hph) cassette. In the replacement vector pDMk2, the hph cassette was inserted between sites 113 and 553 of the cpmk2 gene relative to the start codon and was flanked by approximately 630 and 930 bp, respectively, of 5' and 3' sequences. KpnI-digested linear pDMk2 was then used to transform the virus-free EP155/2 strain.

Functional complementation of the cpmk2 mutant using a wild-type allele was performed. The complementing vector pCmk2 was constructed by the insertion of a 2-6 kb blunt-ended SalI fragment of pSV50 containing the benomyl resistance cassette (Orbach et al., 1986) into blunt-ended SalI-digested pCpmk2, which carried a 7-5 kb SalI fragment containing the full-length cpmk2 gene. The resulting vector was then used to transform the cpmk2 mutant.

Protoplast preparation and transformation were performed as described previously (Churchill et al., 1990; Kim et al., 1995). Transformants were selected from agar plates that were supplemented with 150 μg hygromycin B ml⁻¹ (Calbiochem) or 1-5 μg benomyl ml⁻¹ (DuPont) as appropriate, passaged three or four times on selective media and single-spore-isolated, as described previously (Kim et al., 1995, 2002). PCR and Southern blot analysis were conducted with genomic DNA from the transformants to confirm the replacement and complementation of the cpmk2 gene in trans.

**Characteristics of the cpmk2 mutant.** The phenotypic and molecular characteristics of the cpmk2 mutant were compared with those of the wild-type EP155/2 and the hypovirulent UEP1 strains. Phenotypic changes in pigmentation, conidiation and mating capability were measured as described previously (Kim et al., 1995). Laccase activity was gauged by growing the strains on Bavendamm’s medium (0-5 % malt extract, 2-0 % malt extract, 2-0 % agar) and assessing the resulting colouration of the medium (Rigling et al., 1989). Laccase activity of culture filtrate was determined as described previously (Kim et al., 1995, 1996). Expression of the virus-regulated cryparin (Crp), laccase (lac1) and mating pheromone (Mf2/1) genes was examined by Northern blot analysis (Kim et al., 1995, 2002).

**RESULTS**

**Characteristics of the cpmk2 gene.**

Using degenerate primers derived from the conserved subdomains II and VIb of MAPKs (Kultz, 1998), a 310 bp DNA fragment was amplified and sequenced. Sequence analysis of 210 clones led to the identification of a cDNA fragment encoding a Fus3/Kss1 MAPK homologue from C. parasitica that is distinct from a previously identified gene, cpmk1, which is from a subfamily of the yeast stress-activated protein kinases (YSAPK) (Park et al., 2004). The sequence of this fragment is highly similar to the pmk1 gene of Magnaporthe grisea, with 83 and 97 % identity at the nucleotide and amino acid levels, respectively, in contrast to the 62 and 43 % identity to cpmk1. Southern blot analysis indicated that the gene is present as a single copy in the C. parasitica genome (data not shown). A genomic library prepared from C. parasitica strain EP155/2 was screened with the PCR product as a probe, and three positive clones out of 40 000 were identified. The 7-5 kb SalI-digested λ clone selected for further analysis contained a full-length C. parasitica MAPK gene that was designated cpmk2 (GenBank accession no. AK262368). Sequence analysis of a cpmk2 cDNA obtained using RT-PCR indicated that the cpmk2 gene consists of four exons with three intervening sequences, of 75, 49 and 60 bp. Putative CAAT and TATA boxes were observed at positions −284 and −83, relative to the start codon, in the promoter region of cpmk2. The deduced CpMK2 sequence consists of 355 codons with an estimated molecular mass of 41-3 kDa and a pI of 7-52.

The predicted sequence of CpMK2 contains all 11 conserved protein kinase subdomains and the characteristic MAPK dual phosphorylation sites (TGY, residues 183-185) upstream of the YRAPE domain (Nishida & Gotoh, 1993). A homology search indicated that CpMK2 is highly related to the fungal MAPKs CMK1 from Colletotrichum lagenarium (96 % identity), PMK1 from Magnaporthe grisea (94 %) and FUS3 from Saccharomyces cerevisiae (58 %) and belongs to the yeast extracellular signal-regulated kinase 1 (YERK1) subfamily. CpMK2 has only 45 % identity to C. parasitica CMK1, the functional homologue of S. cerevisiae HOG1, which demonstrates the phylogenetic difference between the two C. parasitica MAPKs.

Northern blot analysis revealed that, similar to the pmk1 gene, cpmk2 is expressed at very low levels in the C. parasitica strain EP155/2 and its isogenic hypovirulent strain UEP1. The low expression level complicates efforts to detect any transcriptional regulation by the hypovirus (data not shown).

**Kinase activity of E. coli-expressed CpMK2**

Full-length Cpmk2 was expressed in E. coli. Following gentle purification and renaturation, a single 50-0 kDa Cpmk2 band was observed in SDS-PAGE, slightly larger than the calculated mass owing to the addition of the hexahistidine tag for purification purposes. Autophosphorylation of the renatured Cpmk2 and its phosphorylation of a common substrate of MAPK, MBP (Fig. 1), indicated that the protein has kinase activity and belongs to a subgroup of the MAPK proteins.

**Construction of a cpmpk2 mutant**

To perform functional analysis of CpMK2, a cpmk2 mutant was constructed by site-directed recombination during integrative transformations. A linear PCR fragment

http://mic.sgmjournals.org
containing the disrupted cpmk2 gene was used to transform the virus-free C. parasitica strain EP155/2. Three hundred single-spored transformants were screened first by PCR using the inner and outer primers, which were used to amplify both wild-type and replacement alleles of cpmk2. All but one of the transformants showed the expected 1-3 kb fragment corresponding to the wild-type allele of cpmk2, and one putative transformant (TdMK2-1) exhibited only the 3-2 kb fragment of a disrupted allele (data not shown). Construction of the cpmk2 mutant was further confirmed by Southern blot analysis. As shown in Fig. 2, hybridization of cpmk2 5’- and 3’-flanking region probes to a 7-9 kb fragment in TdMK2-1 differed from the hybridization to 3-9 and 2-0 kb bands, respectively, that was observed in the wild-type.

Phenotype of the cpmk2 mutant

In the cpmk2 mutant, aerial hyphae development was drastically reduced and conidiation was completely abolished. In addition, the mutant displayed a defect in radial growth, as measured by the diameter of colonies on a variety of solid media. As shown in Fig. 3, the wild-type EP155/2 strain formed three morphologically distinct hyphal types on agar plates. Sparse hyphae with large diameters, referred to as feeding hyphae, penetrated the agar, an interwoven hyphal mat with generally vertically orientated hyphae formed at the agar surface, and narrower, more cytoplasmically dense aerial hyphae that locally differentiated into pycnidia formed above the mat. In contrast, during the initial growth of the cpmk2 mutant, the invasive feeding hyphae growing within the agar media were thinner and, behind these hyphae, a flat mycelial mat formed with dark-brown rather than bright-yellow pigmentation. However, almost no aerial hyphae were observed during the early growth of this strain. Later, the growth became even more retarded, and growth always ceased before the colony reached the edge of the plate. At this time, the mutant began to form a dense mycelial mat with no sign of spor-bearing structures; the colonies had discrete margins and reduced diameters and were more densely packed compared with the wild-type. The mutant was further distinguished from the wild-type strain by radial creases in the mycelial mat and indentation of the medium surface at the colony margin.

Although the cpmk2 mutant produced many fewer aerial hyphae and exhibited a slower growth rate than the wild-type on solid media, it grew as robustly as the wild-type strain in liquid media. Primary inocula were obtained from cultures grown on cellophane overlaying PDamb, 0-5 g freshly harvested mycelial mat was homogenized in 50 ml sterile medium and the resulting slurry was used to inoculate 1 l liquid medium (Kim et al., 1995). Liquid cultures of the wild-type and the cpmk2 mutant showed similar growth curves, as measured by fresh and dry weight as described previously (Carpenter et al., 1992), and reached the stationary growth phase at 4 days after inoculation (Fig. 3b).
Since different media were initially used for the solid (PDAmb) and liquid (EP complete) cultures, the media components of the liquid (PDmb) and solid (EP complete plus 1.5% agar) cultures were then switched. In these media, the cpmk2 mutant again had a reduced radial growth rate on solid medium but a mycelial mass increase similar to that of the wild-type in liquid culture (data not shown). These results indicate that the growth inhibition is dependent on the solid property of the culture medium rather than the components of the medium.

To ensure that any phenotypic changes attributed to the cpmk2 mutation were owing to the gene replacement event, the altered growth rate of TdMK2-1 was complemented in trans on solid medium with a wild-type allele of cpmk2. The benomyl resistance cassette (benR) was introduced into the pBluescript II KS (+) vector containing the 7.5 kb SalI fragment with the entire cpmk2 gene. The resulting vector, pCmk2, was used to transform the cpmk2 mutant TdMK2-1. Benomyl-resistant transformants of the cpmk2 mutant that had received a wild-type cpmk2 gene showed a normal growth rate and abundant pycnidia on solid media (Fig. 4). PCR analyses revealed that all of the complemented transformants contained an additional wild-type allele of cpmk2 (data not shown). Thus, functional complementation using a wild-type cpmk2 gene confirmed unequivocally that the phenotypic changes in the mutant were due to the disruption of cpmk2.

Immunoblot assay of CpMK2 phosphorylation

An immunoblot assay was used to determine whether hypoviral perturbation of phosphorylation occurs for CpMK2 as described previously for CpMK1 (Park et al., 2004). Since CpMK2 contains the residues TEYVATR surrounding the dual phosphorylation site (underlined) and an identical sequence is present in YERK, the phospho-p44/42 MAPK (Erk1 and Erk2) antibody was employed. As shown in Fig. 5(a), two major bands immunospecific to the phospho-p44/42 MAPK antibody appeared in cell-free extracts from liquid cultures. The 42 kDa band appeared to be specific to the phosphorylated CpMK2, as no corresponding band was observed in the cpmk2 mutant (Fig. 5a) and antibody against CpMK2 reacted to a protein of the same size, 42 kDa (data not shown). The upper band, although immunospecific to the phospho-p44/42 MAPK antibody, was not specific to the phosphorylated CpMK2, as a corresponding band was present in the cpmk2 mutant (Fig. 5a). It is most likely that the 44 kDa band is another MAPK distinct from CpMK1 (YSAPK) and CpMK2, based on the immunospecific reaction to the phospho- as well as non-phospho-p44/42 MAPK antibodies. The phosphorylation of CpMK2 in both virus-free and virus-containing strains peaked 5 days after inoculation of the liquid culture and then decreased. However, no significant difference in the amount of phosphorylated CpMK2 between the virus-free EP155/2 and the hypovirulent UEP1 strains was observed. Since the cpmk2 mutant showed a difference from the wild-type EP155/2 on solid media but not in liquid media, strains from 1.5-day-old liquid cultures were harvested, excess medium was removed by blotting between sterile paper towels, the strains were transferred to PDAmb and cell-free extracts were prepared before and 1, 2 and 4 h after the transfer. As shown in Fig. 5(b), phosphorylation of CpMK2 was induced dramatically within the first hour of

---

**Fig. 3.** (a) Radial growth rate on PDAmb. Numbers to the left indicate the age (in days) of cultures on PDAmb. The strains examined, virus-free wild-type (EP155/2) and the cpmk2 mutant (TdMK2-1), are indicated above the panels. (b) Biomass (dry weight) was monitored as a function of time after inoculation of EP155/2 (●, solid line) or TdMK2-1 (■, dotted line) in liquid medium. Each point represents data from a single flask. Representative results from one of three similar experiments are shown.

http://mic.sgmjournals.org 1353
the transfer and then remained at a steady level, which was in good agreement with the growth defect of the cpmk2 mutant on solid media but not in liquid medium. In contrast, no induction was observed in the upper immunospecific band, suggesting that CpMK2 responds specifically to the environmental change from liquid to solid culture and that the growth defect of the cpmk2 mutant on hard surfaces results from the absence of a responsive CpMK2. No difference in the induction pattern of phosphorylated CpMK2 was observed in the EP155/2 and UEP1 strains. Equal amounts of CpMK2 and cell-free extract were confirmed with the Western blot analysis using the non-phosphorylation specific p44/42 MAPK antibody and a Bradford assay followed by Coomassie blue staining of parallel gels, respectively.

Laccase activity and virulence assays

The laccase activity of the strains was examined on plates containing tannic acid. In comparison to the wild-type, the cpmk2 mutant was reduced in size but produced a brown-coloured area of comparable intensity, indicating that it produces less laccase enzyme. The severe growth inhibition of the cpmk2 mutant on tannic-acid-containing plates suggests that the reduced laccase production is the result of growth inhibition owing to a loss-of-function mutation in the mutant (Fig. 6a). In contrast, laccase activities using culture filtrates of EP complete media showed no difference between the cpmk2 mutant and wild-type EP155/2 (Fig. 6b), which is consistent with the observation of no growth difference in liquid media.

Pathogenicity tests performed using bark excised from a chestnut tree (Lee et al., 1992) indicated that the cpmk2 mutant is less virulent than EP155/2 (Table 1). The size of the necrotic area induced on excised bark by the cpmk2 mutant was even smaller than that of a hypovirulent strain, indicating that the cpmk2 loss-of-function mutant resulted in a significant decrease in virulence, even more severe than that of CHV1-EP713. The complemented strain, however, was as virulent as the wild-type EP155/2.

Expression of virus-regulated fungal genes in the cpmk2 mutant

Northern blot analysis indicated that the expression of the mating pheromone precursor gene was severely reduced in the cpmk2 mutant (Fig. 7). Moreover, due to the phenotypic changes of severe growth inhibition on solid media and abolished conidiation, we were not able to observe any mating products when the cpmk2 mutant (Mat-2) mated with an appropriate EP6 strain (Mat-1). Expression of the
other virus-regulated fungal genes examined, cryparin (Fig. 7) and laccase 1 (data not shown), showed no changes.

DISCUSSION

Mutations related to reduced conidiation, pigmentation and vegetative growth have been reported in several studies of *C. parasitica* signal-pathway-related genes (Zhang et al., 1993; Choi et al., 1995; Gao & Nuss, 1996; Kim et al., 2002). However, the *cpmk2* phenotypes of changes in growth pattern related to culture conditions (liquid vs solid media) and a complete lack of conidiation are unusual. Similar phenotypes have been observed previously in strains with mutations in genes related to G-proteins (Choi et al., 1995; Gao & Nuss, 1996; Segers & Nuss, 2003). Therefore, it will be of interest to examine the direct relationship between the CpmK2 pathway and the G-protein-coupled signal-transduction pathway in *C. parasitica*.

MAPKs constitute a family of Ser/Thr kinases that are highly conserved in organisms ranging from yeast to humans. Three MAPKs exist in the model phytopathogenic fungus *M. grisea*. Two of these, PMK1 and MPS1, were identified as orthologues of FUS3/KSS1 and SLT2 of *S. cerevisiae*, respectively, and have been shown to be important for the pathogenicity of this fungus. A sequence comparison suggests that CpmK2 is a homologue of PMK1. To date, all plant-pathogenic fungal PMK1/FUS3 homologues that have been functionally analysed have been implicated in pathogenicity but not in defects in vegetative growth in axenic culture, implying that the PMK1/FUS3 homologue is part of a general pathogenicity-related MAPK signal chain in pathogenic fungi. The *cpmk2* mutant

![Fig. 6. Laccase activity assay. (a) Colonies were grown on tannic-acid-containing medium as described by Rigling et al. (1989). In this assay, the level of brown colouration produced by each strain correlates with the laccase activity. Numbers 1, 2, 3 and 4 respectively indicate strains EP155/2, UEP1, the *cpmk2* mutant (TdMK2-1) and *cpmk2*-complemented TcMK2-1. (b) Extracellular laccase activities monitored as a function of time after inoculation of liquid media. Each point represents data from a single flask. Representative results are shown for EP155/2 (○), TdMK2-1 (△), TcMK2 (■) and UEP1 (●).](http://mic.sgmjournals.org

**Table 1.** Characteristics of transformed, wild-type, hypovirulent and complemented strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristic</th>
<th>Canker area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP155/2</td>
<td>Wild-type</td>
<td>172.6±17.2</td>
</tr>
<tr>
<td>TdMK2-1</td>
<td>Transformed</td>
<td>53.1±8.7</td>
</tr>
<tr>
<td>UEP1</td>
<td>Hypovirulent</td>
<td>90.3±11.2</td>
</tr>
<tr>
<td>TcMK2-1</td>
<td>Complemented</td>
<td>158.5±19.4</td>
</tr>
</tbody>
</table>

G-protein-coupled MAPK cascade has been observed in response to mating pheromones in the fission yeast *Schizosaccharomyces pombe*. Therefore, it will be of interest to determine the canker area, 5-mm agar plugs containing mycelium were placed on excised bark and the resulting canker areas were measured 7 days after inoculation as described previously (Lee et al., 1992). The data are the means±SD of replicates from at least two trials of each virulence test per strain.

![Fig. 7. Molecular characteristics of the *cpmk2* mutant. Northern blot analysis of the *Mf2/1*, *Crp* and *Gpd* genes is shown. Total RNA from liquid-grown cultures was extracted on the days after inoculation listed above each lane. The strains are indicated above the line. Equal loading of RNA samples was confirmed in a parallel blot that was hybridized with the *Gpd* probe as an internal control and by ethidium-bromide staining of the gel to visualize rRNA.](http://mic.sgmjournals.org

http://mic.sgmjournals.org
produces drastically reduced canker areas, a phenotype that is even more severe than that of the hypovirulent strain. If pathogenic determinants are defined as factors that affect only pathogenicity, CpMK2, which when disrupted results in defective vegetative growth on solid surfaces, may not be a specific pathogenic determinant in the strict sense. The *C. parasitica* CpMK2 pathway might differ from those of other pathogenic fungi and may be essential for appropriate fungal growth on solid surfaces.

In addition to phenotypic changes, pheromone gene expression was severely repressed in the *cpmk2* mutant, and this effect was not due to growth inhibition, as a similar reduction in pheromone gene expression was also observed when the mutant was grown in liquid culture. Although pheromone-related gene expression and the pheromone-responsive pathway are well-characterized in yeast, few details exist on the regulation of pheromone gene expression in filamentous fungi. The structures of all of the pheromones in the ascomycetes are of the two types described in *S. cerevisiae*. Genes of two classes of pheromones were identified recently from three heterothallic filamentous fungi, *C. parasitica*, *M. grisea* and *Neurospora crassa*, and from the homothallic fungus *Sordaria macrospora* (Zhang et al., 1998; Shen et al., 1999; Poggeler, 2000; Bobrowicz et al., 2002; Turina et al., 2003). The first complete set of pheromone precursor genes to be identified in a filamentous ascomycete was from *C. parasitica* (Zhang et al., 1998), and three pheromone precursor genes, *Mf1/1, Mf2/1* and *Mf2/2*, have been reported to exist in both *C. parasitica* mating types (Zhang et al., 1998; Turina et al., 2003). The expression of the pheromone precursor genes is mating-type-specific; for example, *Mf1/1* is expressed only by *Mat-1* strains, and the genes display transcriptional suppression, characteristic of a virus-containing hypovirulent strain. The downregulation of pheromone gene expression owing to alterations in the expression of CpMK2, which belongs to a family of pheromone-responsive MAPKs, could imply that pheromone gene expression is dependent on the MAPK pathway, in *S. cerevisiae*, and that a MAPK cascade similar to the *S. cerevisiae* FUS3/KSS1 MAPK pathway exists in *C. parasitica*. Based on the significant similarities in the structures and expression patterns of the pheromone precursor genes of *C. parasitica* and the ascomycetous yeasts, it is expected that the features of the mating system of yeasts are conserved in *C. parasitica* and that pheromone expression in *C. parasitica* is regulated by the mating-type locus, which encodes transcriptional regulators (Zhang et al., 1998; Shen et al., 1999). Therefore, the finding that disruption of CpMK2 results in inhibited pheromone gene expression could provide clues to intriguing connections between possible mating signals and a MAPK pathway in a phytopathogenic fungus.

In fungal signalling pathways, interactions frequently exist between the cAMP signalling and MAPK pathways involved in mating, morphogenesis, virulence and stress responses (Kronstad et al., 1998). Although the cAMP signalling and MAPK pathways act in parallel during filamentous growth in *S. cerevisiae* and appressorium formation in *M. grisea*, many examples of antiparallel cooperation have also been observed, such as negative regulation by the cyclic AMP pathway but positive regulation by the MAPK pathway in the induction of *ste11* in *S. pombe* and *prf1* in *Ustilago maydis* (Kronstad et al., 1998). Moreover, there has been considerable progress in the understanding of the regulation of intracellular cAMP levels in filamentous fungi, including *M. grisea* and *C. parasitica*. Briefly, a mutation in the *M. grisea* pmk1 gene, a homologue of *cpmk2*, results in defects in appressorium formation and invasive growth in rice plants, causing avirulence. Since the *pmk1* mutants remain responsive to exogenous cAMP but do not form appressoria, it was suggested that cooperative signalling between the cAMP- and MAPK-dependent pathways is required for surface recognition and infection structure formation (Xu & Hamer, 1996). In addition, of the three Gα subunit genes in *M. grisea*, mutants with disruptions in *magB* but not *magA* or *magC* show reduced appressorium formation, conidiation and virulence (Liu & Dean, 1997). These effects can be suppressed by exogenous cAMP, implicating MAGB in the cAMP signalling pathway (Xu, 2000). Therefore, one can speculate that the regulatory cascade from the G-protein to the two independent but tightly coordinated and cooperative cAMP- and MAPK-dependent pathways is required for appressorium formation and that this cooperative signalling might be a common feature in fungal pathogens (Xu & Hamer, 1996; Kronstad et al., 1998). Likewise, targeted disruption of the genes for two *C. parasitica* Gα subunits, *cpg-1* and *cpg-2*, showed that the *Gα* subunit encoded by *cpg-1*, but not *cpg-2*, has roles in fungal reproduction, virulence and vegetative growth. Moreover, *CPG-1* cosuppression resulted in constitutively elevated cAMP levels, consistent with the prediction that CPG-1 negatively regulates adenylyl cyclase (Chen et al., 1996). Since the phenotype of the *cpmk2* mutant does resemble the *cpg-1* mutant in two interesting ways, complete loss of conidiation and differential growth on solid and liquid media, it is conceivable that CpMK2 may be subject to regulation through the G-protein-coupled signalling pathway. Accordingly, it can again be speculated that the cAMP signalling and MAPK pathways interact cooperatively during the processes of conidiation and surface sensing in *C. parasitica*. It will be of interest to disrupt the adenylyl cyclase gene to examine the resulting phenotypic changes.

Fungal growth is the consequence of many metabolic processes, including various sensing and response processes. Therefore, the absence of signalling can result in severe growth defects through disruption of a required signal relay. Based on the time of induction of elevated phosphorylation of CpMK2, this phosphorylation event may be implicated in the fungal response to environmental stimuli, such as the sensing of hard surfaces. However, the lack of differences in the basal and induction levels of phosphorylated CpMK2 between the virus-free EP155/2 and virus-containing UEP1 suggests that the normal regulation of the
phosphorylation level of CpMK2 in *C. parasitica* is independent of hypoviral infection.

**ACKNOWLEDGEMENTS**

This work was supported by a 1999 Korea Research Foundation Grant (99-005-1D0070). We thank the Research Center for Industrial Development of BioFood Materials at Chonbuk National University for kindly providing the facilities for this research.

**REFERENCES**


