Characterization of *CpSte11*, a MAPKKK gene of *Cryphonectria parasitica*, and initial evidence of its involvement in the pheromone response pathway

JIN-AH PARK, JUNG-MI KIM, SEUNG-MOON PARK AND DAE-HYUK KIM*

Institute for Molecular Biology and Genetics, Center for Fungal Pathogenesis, Chonbuk National University, Jeonju, Chonbuk 561-756, South Korea

SUMMARY

The gene *CpSte11* of *Cryphonectria parasitica*, which encodes a yeast Ste11 homologue, was cloned and characterized. Gene replacement analysis revealed a high frequency of *CpSte11* null mutants. When compared with the wild-type parent strain, *CpSte11* null mutants showed no difference in terms of growth rate or pigmentation. However, *CpSte11* null mutants showed a marked decrease in both the number and size of stromal pustules on chestnut twigs. The virulence test showed that, in comparison with those of the wild-type and virus-infected hypovirulent strains, *CpSte11* null mutants produced necrotic areas of intermediate size. Disruption of the *CpSte11* gene also resulted in defects in female fertility. Down-regulation of transcripts for the mating pheromone precursor gene, *Mf2/2*, and mating response transcription factors, such as *cpst12* and *pro1*, was observed in *CpSte11* null mutants. The down-regulation of *Mf2/2*, *cpst12* and *pro1* was also observed in the mutant phenotype of *Cpmk2*, a mating response Fus3-like mitogen-activated protein kinase (MAPK) gene, but not in the mutant of *Cpmk1*, a high-osmolarity glycerol Hog1-like MAPK gene. These results indicate that the cloned *CpSte11* gene is functionally involved in the mating response pathway and acts through downstream targets, including *Cpmk2*, *cpst12*, *pro1* and *Mf2/2*. However, the characteristics of the *CpSte11* null mutant were fully phenocopied only in the *cpst12* null mutant, but not in other studied null mutants of components of the putative mating response pathway.

INTRODUCTION

Cryphonectria parasitica (Murrill) Barr, the causal agent of chestnut blight, was responsible for the almost complete disappearance of chestnut forests in North America at the beginning of the 20th century (Van Alfen, 1982; Van Alfen *et al.*, 1975). Furthermore, disease severity has become more pronounced in other areas, including South Korea, where chestnut blight was once believed to

cause only minor problems (Ju *et al.*, 1999). However, strains containing double-stranded RNA (dsRNA) *Cryphonectria hypovirus 1* (CHV1) exhibit decreased virulence, a phenomenon referred to as 'hypovirulence' (Anagnostakis, 1982; Nuss, 1992; Van Alfen *et al.*, 1975). In addition, strains containing CHV1 show diverse hypovirulence-associated phenotypic changes, such as reduced sporulation, female fertility, pigmentation, laccase production and oxalate accumulation (Elliston, 1985; Havir and Anagnostakis, 1983; Rigling *et al.*, 1989). The molecular basis for these phenotypic changes involves alterations in host transcriptional profiles in response to hypovirus infection (Allen *et al.*, 2003; Allen and Nuss, 2004; Deng *et al.*, 2007a; Kang *et al.*, 2000; Kazmierczak *et al.*, 1996). Considering the broad spectrum of phenotypic changes resulting from coordinated gene regulation, the perturbation of the fungal host signal transduction pathway on virus infection has been suggested. Accordingly, several fungal signal transduction pathways appear to be perturbed by hypovirus infection (Chen *et al.*, 1996; Gao and Nuss, 1996; Kasahara and Nuss, 1997; Kim *et al.*, 2002; Park *et al.*, 2004; Turina *et al.*, 2006).

Mitogen-activated protein kinase (MAPK) signal transduction pathways are used by eukaryotic cells to transduce a wide variety of cellular signals through a stepwise phosphorylation relay. This cascade, which is conserved and occurs in a wide variety of organisms from yeast to humans (Herskowitz, 1995; Schaeffer and Weber, 1999), consists of three functionally interlinked protein kinases: MAPKKK (MAP kinase kinase kinase), MAPKK (MAP kinase kinase) and MAPK. Fungal MAPKs regulate numerous processes related to growth and differentiation (Xu, 2000). Moreover, MAPKs are involved in the pathogenicity of many plant pathogenic fungi (Zhao *et al.*, 2007). Although five different MAPK pathways have been identified and studied in *Saccharomyces cerevisiae*, three MAPK homologues, involved in the pheromone response pathway (Fus3/Kss1), the cell wall integrity pathway (Slit2) and the osmoregulation/stress response pathway (Hog1), are generally found in other higher fungi (Xu, 2000; Zhao *et al.*, 2007). Although the general functions of these three pathways are as listed above, they are anticipated to play more diverse roles in pathogenic fungi than their counterparts in *S. cerevisiae*. Indeed, depending on the fungus, different functions for the corresponding homologue in the MAPK pathway have been suggested. Thus, further studies on specific relationships between upstream inputs and downstream targets are needed to understand how

*Correspondence: Email: dhkim@chonbuk.ac.kr

extracellular stimuli are transduced to ultimately express a specific set of downstream target genes in fungi. Among three representative MAPK pathways, the MAPK signalling pathway, involved in the responsiveness to the mating pheromone, was of interest because the pheromone precursor genes were some of the initial genes shown to be down-regulated by CHV1 (Powell and Van Alfen, 1987a). In addition, the homologue of the yeast Fus3/Kss1 pathway appears to be important in the pathogenicity of plant pathogenic fungi, but plays less of or no role in the virulence of human pathogens (Sakaguchi *et al.*, 2010; Schamber *et al.*, 2010; Zhao *et al.*, 2007).

In *C. parasitica*, although several genes in MAPK pathways have been characterized (Choi *et al.*, 2005; Park *et al.*, 2004; Rostagno *et al.*, 2009; Turina *et al.*, 2006), no study on MAPKKK has been reported to date. Moreover, no study on the identification of signalling components in MAPK pathways to downstream transcription factors and target genes has been published. Compared with other MAPK pathways in *C. parasitica*, more is known about the putative mating response MAPK pathway. Accordingly, several putative downstream transcription factors, such as *cpst12* and *pro1*, and downstream target genes, such as *Mf2/1* and *Mf2/2*, encoding pheromone precursors have been analysed independently (Deng *et al.*, 2007b; Sun *et al.*, 2009; Zhang *et al.*, 1993; 1998). However, although CpMK2, the Fus3/Kss1 homologue from *C. parasitica*, has been characterized (Choi *et al.*, 2005), no other component in the mating response MAPK pathway has been studied. In addition, the signalling components and downstream effectors of the mating response pathway are not yet fully understood.

In the current study, we cloned a yeast Ste11 homologue, a mating response MAPKKK, from *C. parasitica* and examined its biological functions. In addition, its relationships with possible downstream transcriptional factors and targets were examined to determine the pathway specificity and its role as a component in a specific regulatory hierarchy.

RESULTS

Characteristics of the *CpSte11* gene

Three different MAPKKK genes were identified in the genome of *C. parasitica* by inspection of the draft genome sequence (<http://genome.jgi-psf.org/Crypa2/Crypa2.home.html>). Polymerase chain reaction (PCR) amplification of the MAPKKK gene that showed high similarity to other MAPKKKs in the mating response pathway resulted in the expected 4.4-kbp fragment. Southern blot analysis of total genomic DNA under high stringency with the cloned PCR amplicon as a probe suggested that a single copy of the cloned gene was present in the *C. parasitica* genome (data not shown). Based on the genomic sequence analysis, a near full-length cDNA clone was obtained using reverse

Fig. 1 Characteristics of the *CpSte11* gene from *Cryphonectria parasitica*. Schematic organization of CpSTE11 and its homologues from other fungi: *Saccharomyces cerevisiae* Ste11, *Schizosaccharomyces pombe* Byr2, *Cryptococcus neoformans* CnSte11, *Magnaporthe grisea* Mst11, *Fusarium graminearum* FgSte11 and *Pneumocystis carinii* Mekk. The sterile α motif (SAM) domain, Ras-association (RA) domain and C-terminal kinase domain are represented by white, black and grey boxes, respectively.

transcription-polymerase chain reaction (RT-PCR), with the primer pair CpSte11-mF1 and CpSte11-mR1, at nucleotide positions -6 to 12 and 2795 to 2813 (relative to the start codon), respectively, and the resulting 2752-bp amplicon was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). A sequence comparison with the corresponding genomic sequence revealed that the cloned gene contained two exons, with an intervening sequence of 67 bp.

Sequence analysis of the cloned cDNA showed an open reading frame (ORF) of 916 amino acids, with an estimated molecular mass of 101.6 kDa and a *pI* value of 8.72 (GenBank accession number for *CpSte11* is AEC04750), named '*CpSte11*' (a yeast Ste11 homologue in *C. parasitica*). Analysis of the deduced *CpSte11* gene product (CpSTE11) revealed the presence of three conserved domains: an N-terminal 'sterile α motif' (SAM) domain, related to a phosphorylation site between amino acids 66 and 128, a Ras-association (RA) domain of the ubiquitin (UBQ) superfamily between amino acids 266 and 353, and the C-terminal catalytic domain of serine/threonine protein kinases of the protein kinase C (PKC)-like superfamily between amino acids 652 and 912 (Fig. 1).

Regulation of *CpSte11* gene expression

Northern blot analysis using RNA samples from standard liquid cultures revealed that *CpSte11* was expressed at very low levels in virus-free EP155/2 and its isogenic virus-containing UEP1 (data not shown). Thus, quantitative real-time RT-PCR was used for the expression profile. As shown in Fig. 2, the expression of *CpSte11* increased as growth proceeded in EP155/2, reached a peak at 3 days of growth, and then decreased gradually thereafter. However, significant down-regulation in the expression level of *CpSte11* was observed in the hypovirulent UEP1 strain, indicating that hypovirus infection altered the accumulation of the *CpSte11* transcript in *C. parasitica*.

Fig. 2 Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *CpSte11* transcript levels relative to levels of glyceraldehyde-3-phosphate dehydrogenase (*gpd*). The values on the y-axis were normalized to the *CpSte11* transcript levels in 1-day cultured EP155/2 strain, with standard deviations, based on three independent measurements of two independent RNA preparations of the same sample, indicated by the error bars. The wild-type EP155/2 strain and its isogenic hypovirus-infected UEP1 strain are represented by filled and open bars, respectively.

Construction of a *CpSte11* null mutant

To examine the effects of deleting the *CpSte11* gene, a *CpSte11* null mutant was constructed by site-directed recombination during integrative transformation. A linear DNA containing a disrupted *CpSte11* gene with 1095-bp 5' and 1322-bp 3' flanking regions was used to transform the virus-free *C. parasitica* EP155/2 strain. In total, 54 stable single-spored transformants were screened by PCR using the outer gene-specific and inner *hph* primers, which corresponded to -1611 to -1591 and 1000 to 1017 (relative to the start codon of *CpSte11* and *hph*, respectively). Six transformants showed a PCR amplicon of the expected size, 2.5 kb, corresponding to disrupted alleles of the *CpSte11* gene. To confirm the pure replacement of the wild-type allele with the disrupted allele, these six transformants were further examined by Southern blot analysis (Fig. 3). As shown in Fig. 3, the hybridization pattern of *SalI*-digested genomic DNA of the *CpSte11* null mutant with a probe prepared using the 1.1-kb *EcoRI*-digested *CpSte11* fragment differed from the wild-type, suggesting that the transforming vector had integrated at the *CpSte11* locus by site-directed homologous recombination. Moreover, *SalI*-digested genomic DNA of the transformants showed a hybridizing band at 5.3 kb, corresponding to the expected size of the replaced allele, and the probe also hybridized to a 0.4-kb *SalI/BamHI*-*hph* fragment. In addition, the hybridization pattern of *EcoRI*- and *SacI*-digested genomic DNA of the transformants with the 1.1-kb probe showed hybridizing bands at 2.3 kb and 7.2 kb, instead of 8.7 kb and 5.2 kb, respectively, in the wild-type (Fig. 3), in good agreement with the results of *SalI* digestion, indicating that the *CpSte11*

Fig. 3 Restriction map and Southern blot analyses of *CpSte11* null mutants and wild-type EP155/2. (A) Restriction map of the *CpSte11* genomic region and the expected replaced allele of the *CpSte11* gene as a result of double homologous recombination. Boxes indicate the genomic region for the construction of the gene replacement vector pDSTE11, which replaced the internal 1932 bp with the hygromycin B resistance gene (*hph*), resulting in 1095- and 1322-bp fragments as the 5' and 3' flanking regions, respectively. Arrows indicate the direction of transcription. E, *EcoRI*; S, *SalI*; Sa, *SacI*. (B) Southern blot analysis of the wild-type EP155/2 strain (lane 7) and the *CpSte11* null mutants TdSTE11-3, -17, -56, -74, -78 and -85 (lanes 1–6). Enzymes used to digest the DNA samples are indicated above the lane numbers and the probe is indicated on the restriction map in (A). Numbers on the right and left indicate the size in kilobases. The *CpSte11* null mutants underwent the desired replacement at *CpSte11*, as indicated by the expected changes in the sizes of the bands that hybridized with the probe.

gene was replaced with part of the transforming vector, rather than being disrupted.

As shown in Fig. 3, the six selected transformants exhibited identical hybridizing patterns, indicating that all transformants resulted from the same gene replacement of the *CpSte11* gene with the *hph* gene in the transforming vector.

Phenotypic characteristics of *CpSte11* null mutants

In the *CpSte11* null mutants, no difference in radial growth was observed under standard growth conditions (under constant

Fig. 4 Colony morphology of the *CpSte11* null mutant strains. Colonies after 15 days of culture are shown. The strains indicated are the wild-type EP155/2, six *CpSte11* null mutants (TdSTE11-3, -17, -56, -74, -78 and -85) and the complemented strain of TdSTE11-17 (TcSTE11-17) (A). (B) Corresponding hypovirus-transferred strains of (A) (UEP1, TdSTE11-3-V, -17-V, -56-V, -74-V, -78-V, -85-V and TcSTE11-17-V).

low-level light at 25 °C; Fig. 4A). No change in pigmentation was observed as a result of a loss of function of the *CpSte11* gene. However, asexual sporulation of the *CpSte11* null mutants differed from that of the wild-type strain EP155/2. The numbers of conidia per plate produced by the *CpSte11* null mutants were significantly higher than those of EP155/2 (Table 1).

The laccase activity of the strains was examined on plates containing tannic acid. Compared with the wild-type, no significant change in the dark-brown-coloured zones was observed in the *CpSte11* null mutants, suggesting that laccase production was unaffected by the *CpSte11* mutation (data not shown). No significant changes in temperature sensitivity, osmosensitivity and responses to oxidative stress were observed in the *CpSte11* null mutants compared with the wild-type (data not shown). These results suggest that responses to stress, including tannic acid, temperature, hypertonic conditions and oxidative stress, were not markedly changed by the loss of function of the *CpSte11* gene.

To ensure that the phenotypic change attributed to the *CpSte11* mutation was caused by the gene replacement event, the enhanced conidiation of two randomly selected *CpSte11* null mutants was complemented *in trans* with a wild-type allele of

CpSte11. The geneticin-resistant transformants that had received a wild-type *CpSte11* produced comparable numbers of conidia to those of the wild-type EP155/2 (Fig. 4). PCR analyses revealed that the complemented transformants contained an additional wild-type *CpSte11* allele. Thus, in addition to multiple mutants showing the associated phenotypes, functional complementation using a wild-type *CpSte11* gene unequivocally confirmed that the phenotypic change in the mutant was caused by the disruption of *CpSte11*.

Effect of hypoviral infection on colony morphology in *CpSte11* null mutants

To examine the biological functions of the *CpSte11* gene related to hypovirus infection, we compared the phenotypic changes between virus-free and virus-containing isogenic *CpSte11* null mutants. The transfer of hypovirus CHV1-713 to the *CpSte11* null mutants resulted in a lower growth rate, with less aerial mycelia showing dark brown rather than bright yellow pigmentation, as well as reduced conidiation (Fig. 4B). Moreover, the appearance of fast-growing and normal-pigmented sectors, indicating virus curing, as observed in the *pro1* null mutant (Sun *et al.*, 2009), did not occur in colonies of the virus-containing *CpSte11* null mutants. Stability in vertical inheritance of the hypovirus was examined by isolation of dsRNA from 20 randomly selected single-spored progenies of the newly infected *CpSte11* null mutants. All but one showed the presence of dsRNA on ethidium bromide-stained gel (data not shown). These results suggest that the horizontal and vertical transmission of the hypovirus does not require the presence of the *CpSte11* gene, and that *CpSte11* is not implicated in the maintenance of hypovirus infection.

Impaired colonization of the *CpSte11* null mutant on chestnut bark

The *CpSte11* null mutants differed from the wild-type EP155/2 in their ability to colonize chestnut twigs embedded in agar. When inoculated on the agar substrate adjacent to a sterile chestnut twig, all *CpSte11* null mutants showed severely retarded colonization, characterized by a decreased number of stromal pustules and less extended stroma. Stromal pustules of the *CpSte11* null mutants erupted primarily around broken bark or prominent lenticels (Fig. 5). Thus, the *CpSte11* null mutants seemed to have difficulty in effectively colonizing and erupting through the bark of sterile chestnut twigs.

The *CpSte11* null mutants were inoculated on excised chestnut tree bark to test whether disruption of the *CpSte11* gene affected virulence. The sizes of the necrotic areas on excised bark induced by the *CpSte11* null mutants were smaller than those of the wild-type strain EP155/2, indicating that the *CpSte11* null mutants were less virulent than EP155/2 (Table 1). Thus, the *CpSte11* null

Strain	Characteristic	Canker area (mm ²)†	No. of conidiospores/mL‡
EP155/2	Wild-type	127.88 ± 20.26a§	2.37 × 10 ⁸ ± 2.13 × 10 ⁸ a§
TdSTE11-3	This study	64.76 ± 6.24b	9.01 × 10 ⁸ ± 3.32 × 10 ⁸ b
TdSTE11-17	This study	62.57 ± 11.30b	6.43 × 10 ⁸ ± 1.47 × 10 ⁸ b
TdSTE11-56	This study	66.58 ± 14.87b	7.28 × 10 ⁸ ± 1.50 × 10 ⁸ b
TdSTE11-74	This study	49.71 ± 16.07b	1.06 × 10 ⁹ ± 3.43 × 10 ⁸ b
TdSTE11-78	This study	54.66 ± 21.75b	7.98 × 10 ⁸ ± 2.70 × 10 ⁸ b
TdSTE11-85	This study	53.23 ± 8.27b	9.58 × 10 ⁸ ± 3.49 × 10 ⁸ b
TcSTE11-17	This study	111.25 ± 22.55a	1.53 × 10 ⁸ ± 3.13 × 10 ⁸ a
UEP1	Hypovirulent	21.24 ± 2.55c	2.23 × 10 ⁶ ± 0.53 × 10 ⁶ c

*Culture conditions have been described previously (Kim *et al.*, 1995).

†The 5-mm agar plugs with mycelium were placed on excised bark, and canker areas were measured 7 days after inoculation, as described previously (Lee *et al.*, 1992). Data are the averages of replicates from four trials of each virulence test per strain.

‡The conidia from each plate were harvested with 10 mL of sterile water, and the number of conidia per plate was determined using a haemocytometer. Four replicates of each strain were used and the experiment was repeated three times.

§Values are means ± standard deviation. Values followed by the same letter are not significantly different ($P < 0.05$), as determined by Duncan's multiple-range test.

Fig. 5 Stroma pustules on chestnut twigs. The wild-type strain EP155/2 and the complemented strain of TdSTE11-17 (TcSTE11-17) were able to effectively colonize and erupt through the sterile chestnut twig, whereas the *CpSte11* null mutant strains showed a decreased number of stromal pustules and less extended stroma.

mutants had difficulty in effectively colonizing and growing under the bark of sterile chestnut twigs.

Mating ability of the *CpSte11* null mutant

The *CpSte11* null mutants were examined for their capability to serve as either male or female parent in sexual crosses with an opposite mating type strain EP6 (Mat-1). Spermatization of strain EP6 with conidia from wild-type EP155/2 or the two *CpSte11* null mutants resulted in a similar number of perithecia and viable ascospores. However, when the *CpSte11* null mutants were grown

as a female and crossed with conidia from strain EP6, no mating product was observed. Although efficient colonization may be important for the mating process, it appears unlikely to be a necessary prerequisite for mating to occur, because the cryparin mutant, showing defects in eruption of stromal pustules, mated well as a female partner. Thus, the *CpSte11* null mutants have defects in mating ability as a female partner.

Changes in the expression of other genes in the putative mating pathway of the *CpSte11* null mutants

Because three different genes, *Mf2/2*, *cpst12* and *pro1*, have been analysed and identified as important components in the putative mating pathway, the expression patterns of these genes were analysed in the *CpSte11* null mutant by real-time RT-PCR. As shown in Fig. 6, all three genes were down-regulated in the *CpSte11* null mutant, indicating that the *CpSte11* gene positively controls these three genes. In addition, the down-regulation of two transcription factors, *cpst12* and *pro1*, and a downstream target gene, *Mf2/2*, was observed in the *Cpmk2* null mutant, a mutant in the mating response MAPK gene. However, no significant change in the expression of the two transcription factors or the *Mf2/2* gene was observed in the null mutant of the *Cpmk1* gene, which is another MAPK gene in the high osmotic response pathway (Park *et al.*, 2004).

Western blot analysis using phospho-specific and phospho-nonspecific MAPK antibodies was conducted to determine changes in the phosphorylation of CpmK2. As CpmK2 contains the characteristic residues TEYVATR surrounding the dual phosphorylation site (*italic*), and the identical sequence is present in ERK1 (p44) and ERK2 (p42), the phospho-p44/42 MAPK (Erk1 and Erk2) antibody was employed, as described previously (Choi *et al.*, 2005). As shown in Fig. 7, phospho-nonspecific antibodies

Table 1 Characteristics of null mutants compared with the wild-type, complemented and hypovirulent strains.*

Fig. 6 Changes in expression of *Mf2/2*, *cpst12* and *pro1* in the *CpSte11* null mutants compared with the wild-type (EP155/2), hypovirulent (UEP1), *Cpmk1* and *Cpmk2* mutant strains. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of *Mf2/2* (A), *cpst12* (B) and *pro1* (C) transcript levels relative to levels of glyceraldehyde-3-phosphate dehydrogenase (*gpd*). The values on the y-axis were normalized to the transcript levels of the corresponding gene in 5-day cultured EP155/2 strain, with standard deviations, based on three independent measurements of two independent RNA preparations, indicated by the error bars.

revealed two major bands immunospecific to the p44/42 MAPK antibody, among which the 42-kDa band was demonstrated to be specific to CpmK2 (Park *et al.*, 2004). However, the level of the protein product of the *Cpmk2* gene in the *CpSte11* null mutant was lower than in the wild-type. Furthermore, phosphorylation of CpmK2 was reduced significantly based on the near-absence of phospho-specific CpmK2 bands in the *CpSte11* null mutant. Although differences in the levels of reduction of mRNA accumulation existed among two transcription factors and a downstream target gene, the specific down-regulation of all three genes in both *CpSte11* and *Cpmk2* null mutants and Western blot analysis suggested that *CpSte11* specifically affects a signalling cascade involving *Cpmk2*, and that the *CpSte11* gene controls downstream target genes through the action of the *Cpmk2* gene.

DISCUSSION

In eukaryotic cells, including fungi, the MAPK cascade is well conserved and involved in the transduction of a variety of

extracellular signals regulating different developmental processes. The sequential activation of the MAPK cascade eventually results in the activation of transcription factors and the expression of sets of downstream target genes in response to external stimuli. Although five different MAPK pathways have been identified in *S. cerevisiae*, three representative MAPK pathways, pheromone response, cell wall integrity and osmoregulation/stress response, exist in fungal pathogens (Zhao *et al.*, 2007). The analysis of a *C. parasitica* genome database has revealed the presence of at least six MAPKKK-, three MAPKK- and four MAPK-related genes. BLAST searches have indicated that, among the six MAPKKKs, two show high similarity (*E*-value < 1e-177) to the known MAPKKKs involved in the pheromone response and cell wall integrity. The others show similarity to other MAPKKK-related proteins with, as yet, uncharacterized functions. The three MAPKKs appear to be homologues for the components in the three known MAPK pathways (Rostagno *et al.*, 2009). Among the four MAPK-related genes, one has high similarity to *Ime2*, a meiosis induction MAPK-related kinase of *S. cerevisiae* (Garrido *et al.*, 2004). The other

Fig. 7 Immunoblot assay of phosphorylation of CpMK2 in the *CpSte11* null mutant strains. Autoradiogram using the phospho-nonspecific (A) and phospho-specific (B) p44/42 mitogen-activated protein kinase (MAPK) antibodies. Lanes 1, 2 and 3 contain protein samples from the wild-type strain EP155/2, TdSTE11-3 and TdSTE11-17, respectively. The arrowhead indicates CpMK2.

three MAPKs show high similarity to the corresponding homologues of the pheromone response, cell wall integrity and osmoregulation/stress response MAPK pathways. Like other filamentous fungi, *C. parasitica* appears to have only one pheromone response MAPK, which is homologous to yeast Fus3. In *C. parasitica*, homologues to Fus3 and Hog1 have been characterized previously (Choi *et al.*, 2005; Park *et al.*, 2004) and hypoviral regulation of MAPKK belonging to the cell wall integrity pathway has been demonstrated (Turina *et al.*, 2006). However, no study at the level of MAPKKK has yet been reported. Among the different putative MAPKKKs, the deduced amino acid sequence of the *CpSte11* gene shows the presence of highly conserved N-terminal regulatory and C-terminal serine/threonine protein kinase domains, hallmark features of this class of enzyme. In addition, the sequence shows high homology to the known pheromone response MAPKKKs, such as yeast Ste11 and Byr2, further suggesting that the cloned *CpSte11* gene is the pheromone response MAPKKK. The preservation of these conserved domains in CPMKKK indicates that, although the response to external stimuli may differ from that of other fungi, homologues of the signalling pathway, via a MAPKKK cascade, exist and protein–protein interactions probably occur in a similar way.

In addition to the current study on the *CpSte11* gene, female fertility has been hampered in mutants of the *cpst12*, *pro1* and *Mf2/2* genes (Deng *et al.*, 2007b; Sun *et al.*, 2009; Zhang *et al.*, 1993). Moreover, molecular phenotypes showing the down-regulation of all three genes in the *CpSte11* null mutant, the down-regulation of *cpst12*, *pro1* and *Mf2/2* genes in the *Cpmk2* null mutant and a reduced level of phosphorylation of CpMK2 in

the *CpSte11* null mutant suggest that all four genes are under the control of the *CpSte11* gene, acting through *Cpmk2*, and are likely to be important components in the same putative mating pathway. On the basis of comparative analyses of homologues in other fungi, the epistatic relationship of these five genes is probably in the order *CpSte11*, *Cpmk2*, *cpst12/pro1* and *Mf2/2*, resembling a regulatory cascade of a MAPK pathway, its downstream transcription factors and target genes. However, apart from mating incompetency as a female partner, phenotypic changes in colony morphology appear to be different depending on the gene. In terms of asexual conidiation, *CpSte11* and *cpst12* mutants reveal enhanced asexual sporulation, whereas *Mf2/2* and *pro1* mutants show reduced conidiation (Deng *et al.*, 2007b; Sun *et al.*, 2009; Zhang *et al.*, 1993). The *Cpmk2* null mutant shows abolished conidiation and phenotypic changes in the growth pattern related to culture conditions (liquid vs. solid media) (Choi *et al.*, 2005). Enhanced conidiation is in sharp contrast with the function of MAPKKK ChSte11 of *Cochliobolus heterostrophus* (Izumitsu *et al.*, 2009). Impaired colonization of the *CpSte11* null mutants on chestnut bark has also been observed in the *cpst12* null mutant. Because phenotypic changes, with fewer stromal pustules, have been observed in the cryparin mutant (Kazmierczak *et al.*, 2005), we examined the expression pattern of the cryparin gene in the *CpSte11* null mutants. No difference in the expression pattern of the cryparin gene was observed compared with wild-type EP155/2 (data not shown), suggesting that the impaired colonization in this study is specifically caused by the loss of function of *CpSte11*. Neither viral instability nor growth defects on the plate, which occurred in the *pro1* and *Cpmk2* mutants, respectively, was observed in the *CpSte11* null mutants.

The phenotypic changes, such as enhanced conidiation, impaired colonization on chestnut bark and mating incompetency as a female partner, in the *CpSte11* null mutant were fully reproduced only in the *cpst12* mutant (Deng *et al.*, 2007b), and not in the *pro1* mutant (Sun *et al.*, 2009), even though both transcription factors are apparently under the control of the mating response pathways involving the *CpSte11* and *Cpmk2* genes (Choi *et al.*, 2005). As summarized in Fig. 8, one possible explanation for this finding is that the protein product of the *pro1* gene acts as a heterooligomeric complex with that of the *cpst12* gene, whereas the *cpst12* gene can act without *pro1*, reminiscent of yeast transcriptional regulation by transcription factors of *Ste12* and *Tec1* (Chen and Thorner, 2007). Accordingly, the expression of the downstream target genes of *pro1* is dependent only on the heterodimeric complex, whereas the presence of *cpst12* is essential for both heterodimeric and homodimeric complexes. Thus, a loss-of-function mutant in *CpSte11* results in the simultaneous reduction of both transcription factors, which is reproduced in the *cpst12* mutant. Likewise, the lack of a specific signalling component within the same putative mating pathway does not show the same phenotype, but rather different phenotypes, some of which

Fig. 8 Schematic diagram of the pheromone response mitogen-activated protein kinase (MAPK) signalling pathway in *Cryphonectria parasitica*. Rectangles, genes; ovals, phenotypes; arrows, activation; T-bars, inhibition.

may even appear to be opposite changes, i.e. enhanced conidiation in the *CpSte11* null mutant versus abolished conidiation in the *Cpmk2* null mutant. In addition, the mutation of a specific signalling component *Cpmk2* has a more profound effect than *CpSte11* on the expression of a putative target gene *cpst12*. However, the phenotypes of the *Cpmk2* null mutant are more dissimilar from those of the *cpst12* mutant than the *CpSte11* null mutant. These results indicate that a given signalling component in the putative mating pathway of *C. parasitica* can act independently of other elements and can be used in more than one pathway within the same cell to regulate different developmental processes. Thus, specificity and cross-pathway interactions may involve the modulation of the activity of a component by its association with different accessory proteins. It should be noted that the characteristic growth and developmental retardation of the *Cpmk2* null mutant are observed only on agar plates and not in liquid culture. The wild-type *C. parasitica* strain forms three morphologically distinct hyphal types on agar plates: feeding hyphae that appear as sparse hyphae with large diameters and penetrate the agar; an interwoven hyphal mat that forms at the agar surface with generally vertically oriented hyphae; and narrower, more cytoplasmically dense, aerial hyphae that locally differentiate into pycnidia formed above the mat. The growth and developmental retardation of the *Cpmk2* null mutant are primarily a result of a defect in the development of feeding hyphae (Choi *et al.*, 2005), reminiscent of defects in the invasive growth of the budding yeast.

In *C. parasitica*, the mating process (perithecia formation) occurs only on chestnut bark and not on any axenic culture. Thus, colonization on chestnut bark is important for a successful mating process (female fertility). Pathogenicity also requires successful

colonization of host tissue. However, recent studies on two downstream transcription factors *cpst12* and *pro1* have revealed that both are involved in female fertility, but show differences in pathogenicity between the two null mutants (Deng *et al.*, 2007b; Sun *et al.*, 2009), indicating that mating and pathogenicity can be somewhat independent of each other. In addition, female sterility appears to be under different downstream control mechanisms, because transcription factor *pro1* regulates *pro41* as in *Sordaria macrospora*, whereas *cpst12* does not affect *pro41* gene expression (Sun *et al.*, 2009). The current study suggests the presence of downstream target genes of a *pro1* heteromeric complex, which appears to be independent of the *cpst12* heteromeric complex including pathogenicity. Thus, the characterization of the mechanism of MAPK-directed developmental commitment and cell fate determination in *C. parasitica* will be of interest to understand colonization-, mating- and pathogenicity-specific pathways.

EXPERIMENTAL PROCEDURES

Fungal strains and growth

The CHV1-713-containing hypovirulent *C. parasitica* strain UEP1 and its isogenic virus-free strain EP155/2 (ATCC 38755) were maintained on potato dextrose agar containing L-methionine (100 mg/L) and biotin (1 mg/L) (PDAMB) plates under constant low light at 25 °C (Kim *et al.*, 1995). Culture conditions and methods for preparing the primary inoculum for liquid cultures have been described previously (Kim *et al.*, 1995). Radial growth on plates was assessed by measuring the diameter of the colonies. The mycelium was collected and lyophilized as described previously until use (Powell and Van Alfen, 1987b).

Cloning and characterization of a MAPKKK gene, *CpSte11*

The genome database of *C. parasitica* (*Cryphonectria parasitica* EP155 v2.0) was screened for the yeast Ste11 homologue and PCR amplification was performed with primers CpSte11-gF1 (forward) (5'-CTAGCGGAGACGGGCTGC-3') and CpSte11-gR1 (reverse) (5'-GGGTCTCTTGGTATGGTC-3'). The resulting 4.4-kb PCR amplicon was cloned into the pGEM-T Easy Vector (Promega) and sequenced using the dideoxynucleotide method with universal and synthetic oligonucleotide primers.

To obtain a cDNA clone of *CpSte11*, RT-PCR was performed with primers CpSte11-cF1 (forward) (5'-GTCGACAGCGGGTGTGT-3') and CpSte11-cR1 (reverse) (5'-GGTCTCGACCTGCTGACTG-3'). The resulting cDNA amplicon was cloned and sequenced.

Southern blot 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 *EcoRI*, *SalI* and *SacI*, blotted onto a nylon membrane and hybridized with radioactive probes (Sambrook *et al.*, 1989).

Target gene	Forward sequence (5'–3')	Reverse sequence (5'–3')	Product size (bp)
<i>gpd</i>	CCGTCACGACCCCTTCAT	GTTGCCGTGTTGAGAGTCATACTT	71
<i>Mf2/2</i>	TTCAAACCAACCATCAAAATGC	GCCGTTGACTCCCATGGA	66
<i>cpst12</i>	GCATTCTCAGATCAGACAACCT	AGGTTCACTCCATCGACTCCAT	77
<i>pro1</i>	CAATGCCAATCCGAAACAAA	GCACTTCTTCTCCGGAGTCT	85
<i>CpSte11</i>	CCTGAAGGAGATGGGTATTGACA	GAGTCCTCATTCCGCTTCTTG	111

The temporal expression pattern of *CpSte11* was examined by quantitative real-time RT-PCR using total RNA extracted from cultures at 1, 2, 3, 5 and 7 days after inoculation, as described previously (Kim *et al.*, 2002). *CpSte11* transcript levels were compared using glyceraldehyde-3-phosphate dehydrogenase (*gpd*) of *C. parasitica* as an internal control (Choi and Nuss, 1990).

Immunoblot analysis

To determine whether *CpSte11* is specifically required for the phosphorylation of CpMK2, the levels of phosphorylated CpMK2 were examined by immunoblotting with an antibody specific for doubly phosphorylated p44/42 MAPK (phospho-p44/42 MAPK antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA), as described previously (Choi *et al.*, 2005). Total soluble protein was extracted from 5-day cultured mycelia and a nonphosphorylated-specific p44/42 MAPK antibody (Santa Cruz Biotechnology) was also used to verify the loading of equal amounts of protein.

Quantitative analysis of transcript accumulation using real-time RT-PCR

To examine the expression levels of target and internal control genes, quantitative RT-PCR was performed using a GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) and a SYBR green mixture RT kit (Applied Biosystems). A single cycle to reverse transcribe 1 µg of RNase-free RQ1 DNase-treated RNA was followed by real-time PCR using 2 µL of reverse-transcribed cDNA and 150 nm each of forward and reverse primers. Analyses were conducted at least twice, in triplicate for each transcript, from at least two independent RNA preparations of the same sample with primers specific for *gpd* and the target genes. Primer pairs for each gene are indicated in Table 2. Transcript abundance, relative to the amount of *gpd*, in the sample was calculated using the comparative threshold cycle method, as described previously (Parsley *et al.*, 2002). RNA was extracted from liquid cultures as described previously (Kim *et al.*, 1995).

Construction of a replacement vector and fungal transformation

The replacement vector pDSTE11, designed to favour double-crossover integration events, was constructed as follows: a 4.4-kb fragment containing the full-length *CpSte11* ORF was ligated into the pGEM-T Easy Vector, and the resulting plasmid was used as template for inverted PCR employing the primers 5'-GGTCTAGACTCAATCTTCGTATCAATGCTTA-3' and 5'-GGTCTAGACTCACCTCGTACTTCCAGACCA-3', which incorporate restriction sites for *Xba*I (italic). The PCR amplicon was digested with

Table 2 Primers for quantitative real-time polymerase chain reaction (PCR) analysis.

*Xba*I and religated. The resulting plasmid was further digested with *Xba*I and fused with a 2.3-kb *Xba*I fragment of pBSII*hph* harbouring the 2.4-kb *Sal*I fragment of pDH25 containing a hygromycin phosphotransferase gene cassette (*hph*). In the replacement vector, pDSTE11, the *hph* cassette was inserted between sites –492 and 1440 bp of the *CpSte11* gene, relative to the start codon, and was flanked by approximately 1095 and 1322 bp of 5' and 3' sequences, respectively. *Not*I-digested linear pDSTE11 was then used to transform the virus-free EP155/2 strain.

Functional complementation of the *CpSte11* null mutant was performed using a wild-type allele. PCR amplification of the 5.6-kb *CpSte11* gene was performed with the primers 5'-GGACTAGTACTGTTCACTAGCGGAG ACG-3' and 5'-CGGAATTCCAATGCTCACCCACGAAGACG-3' to amplify a fragment between sites –1591 and 3966 bp of the *CpSte11* gene, relative to the start codon, incorporating *Spe*I and *Eco*RI sites (italic), respectively. The resulting 5.6-kb PCR amplicon was cloned into the *Spe*I/*Eco*RI-digested pBluescriptII KS (+) (Stratagene, La Jolla, CA, USA). The complementing vector, pCSTE11, was constructed by insertion of the 5.6-kb *CpSte11* gene into the *Spe*I/*Eco*RI-digested recombinant pBluescriptII KS (+) carrying the 1.7-kb *Xho*I fragment of pSD1 containing the geneticin resistance cassette (Nguyen *et al.*, 2008). The resulting vector was then used to transform the *CpSte11* null mutant.

Protoplast preparation and transformation were performed as described previously (Churchill *et al.*, 1990). Transformants were selected from agar plates supplemented with 150 µg/mL hygromycin B (Calbiochem, San Diego, CA, USA) or 120 µg/mL geneticin (Invitrogen, Carlsbad, CA, USA), as appropriate, passaged three or four times on selective media and single spore isolated, as described previously (Churchill *et al.*, 1990; Kim *et al.*, 2004). PCR and Southern blot analyses were conducted with genomic DNA from the transformants to confirm the replacement and *in trans* complementation of the *CpSte11* gene.

Characteristics of the *CpSte11* null mutant

The phenotypic and molecular characteristics of the *CpSte11* null mutant were compared with those of the wild-type EP155/2 and hypovirulent UEP1 strains. Phenotypic changes in pigmentation, conidiation and mating capability were measured as described previously (Kim *et al.*, 2002; Powell and Van Alfen, 1987b). A virulence test using excised chestnut tree bark was conducted according to Lee *et al.* (1992). Laccase activity was measured by growing the strains on Bavendamm's medium (0.7% tannic acid, 1.5% malt extract, 2.0% agar) and assessing the resulting coloration of the medium (Rigling *et al.*, 1989). The laccase activity of the culture filtrate was determined as described previously (Kim *et al.*, 2002).

To examine alterations in the expression levels of the putative target genes involved in the mating response pathway, including *Mf2/2*, *cpst12*, *pro1* and *Cpmk2*, quantitative real-time RT-PCR was performed.

Transmission of the dsRNA virus

Virus transmission was performed as described previously (Cortesi *et al.*, 2001). Briefly, mycelial plugs of the virus-containing strain UEP1 were co-cultured with adjacent mycelial plugs of virus-free recipient transformants on PDAMB medium. After 5 days of co-culture, putatively fused mycelia at the border between each pair of strains were transferred to hygromycin-containing PDAMB and examined for the occurrence of a sector showing different phenotypes. Mycelia in the sector were successively transferred to fresh hygromycin-containing medium, and strains were single spored to select virus-infected recipient transformants. The presence of hypovirus was confirmed by purification of dsRNA from single-spored isolates.

Isolation of dsRNA from *C. parasitica*

dsRNA was isolated according to a modified version of the procedure of Kim *et al.* (2008). The UEP1 and dsRNA-transmitted transformants were grown on a cellophane membrane overlaying PDAMB for 7 days at 25 °C. Mycelia (0.2 g wet weight) were homogenized in extraction buffer [$2 \times$ STE: 0.2 M NaCl, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA), plus 2% sodium dodecylsulphate (SDS), 1% sodium bisulphate] using a bead beater (Biospec Products, Inc., Bartlesville, OK, USA). Following two successive phenol extractions, dsRNA was isolated by affinity chromatography using CC41 resin. Isolated dsRNA was analysed by electrophoresis in a 0.8% agarose gel.

ACKNOWLEDGEMENTS

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant, funded by the South Korean government (R11-2008-062-02002-0). We thank Alice C. L. Churchill (Cornell University, Ithaca, NY, USA) for providing the sequence information of a cloned *CpSte11* gene fragment, which was used to initiate this study, prior to the availability of the genome sequence. We thank the Center for Fungal Pathogenesis and the Institute of Molecular Biology and Genetics at Chonbuk National University, Chonbuk, South Korea for kindly providing facilities for this research.

REFERENCES

- Allen, T.D. and Nuss, D.L. (2004) Specific and common alterations in host gene transcript accumulation following infection of the chestnut blight fungus by mild and severe hypoviruses. *J. Virol.* **78**, 4145–4155.
- Allen, T.D., Dawe, A.L. and Nuss, D.L. (2003) Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus *Cryphonectria parasitica* to infection by virulence-attenuating hypoviruses. *Eukaryot. Cell*, **2**, 1253–1265.
- Anagnostakis, S.L. (1982) Biological control of chestnut blight. *Science*, **215**, 466–471.
- Chen, B., Gao, S., Choi, G.H. and Nuss, D.L. (1996) Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. *Proc. Natl. Acad. Sci. USA*, **93**, 7996–8000.
- Chen, R.E. and Thorne, J. (2007) Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta*, **1773**, 1311–1340.
- Choi, E.S., Chung, H.J., Kim, M.J., Park, S.M., Cha, B.J., Yang, M.S. and Kim, D.H. (2005) Characterization of the ERK homologue CpMK2 from the chestnut blight fungus *Cryphonectria parasitica*. *Microbiology*, **151**, 1349–1358.
- Choi, G.H. and Nuss, D.L. (1990) Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase gene from *Cryphonectria parasitica*. *Nucleic Acids Res.* **18**, 5566.
- Churchill, A.C.L., Ciuffetti, L.M., Hansen, D.R., Vanetten, H.D. and Van Alfen, N.K. (1990) Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. *Curr. Genet.* **17**, 25–31.
- Cortesi, P., McCulloch, C.E., Song, H., Lin, H. and Milgroom, M.G. (2001) Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics*, **159**, 107–118.
- Deng, F., Allen, T.D., Hillman, B.I. and Nuss, D.L. (2007a) Comparative analysis of alterations in host phenotype and transcript accumulation following hypovirus and mycoreovirus infections of the chestnut blight fungus *Cryphonectria parasitica*. *Eukaryot. Cell*, **6**, 1286–1298.
- Deng, F., Allen, T.D. and Nuss, D.L. (2007b) Ste12 transcription factor homologue CpST12 is down-regulated by hypovirus infection and required for virulence and female fertility of the chestnut blight fungus *Cryphonectria parasitica*. *Eukaryot. Cell*, **6**, 235–244.
- Elliston, J.E. (1985) Characteristics of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. *Phytopathology*, **75**, 151–158.
- Gao, S. and Nuss, D.L. (1996) Distinct roles for two G protein alpha subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. *Proc. Natl. Acad. Sci. USA*, **93**, 14 122–14 127.
- Garrido, E., Voss, U., Muller, P., Castillo-Lluya, S., Kahmann, R. and Perez-Martin, J. (2004) The induction of sexual development and virulence in the smut fungus *Ustilago maydis* depends on Crk1, a novel MAPK protein. *Genes Dev.* **18**, 3117–3130.
- Havir, E.A. and Anagnostakis, S.L. (1983) Oxalate production by virulent but not by hypovirulent strains of *Endothia parasitica*. *Physiol. Plant Pathol.* **23**, 369–376.
- Herskowitz, I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–197.
- Izumitsu, K., Yoshimi, A., Kubo, D., Morita, A. and Tanaka, C. (2009) The MAPKK kinase ChSte11 regulates sexual/asexual development, melanization, pathogenicity, and adaptation to oxidative stress in *Cochliobolus heterostrophus*. *Curr. Genet.* **55**, 439–448.
- Ju, Y.J., Park, K.H., Kim, D.H. and Cha, B.J. (1999) Survey on chestnut blight caused by *Cryphonectria parasitica*. In: Annual Meeting of the Korean Society of Plant Pathology, Andong, South Korea, p. 13.
- Kang, H.S., Choi, J.W., Park, S.M., Cha, B.J., Yang, M.S. and Kim, D.H. (2000) Ordered differential display from *Cryphonectria parasitica*. *Plant Pathol. J.* **16**, 142–146.
- Kasahara, S. and Nuss, D.L. (1997) Targeted disruption of a fungal G-protein beta subunit gene results in increased vegetative growth but reduced virulence. *Mol. Plant-Microbe Interact.* **10**, 984–993.
- Kazmierczak, P., Pfeiffer, P., Zhang, L. and Van Alfen, N.K. (1996) Transcriptional repression of specific host genes by the mycovirus *Cryphonectria hypovirus 1*. *J. Virol.* **70**, 1137–1142.
- Kazmierczak, P., Kim, D.H., Turina, M. and Van Alfen, N.K. (2005) A hydrophobin of the chestnut blight fungus, *Cryphonectria parasitica*, is required for stromal pustule eruption. *Eukaryot. Cell*, **4**, 931–936.
- Kim, D.H., Rigling, D., Zhang, L. and Van Alfen, N.K. (1995) A new extracellular laccase of *Cryphonectria parasitica* is revealed by deletion of Lac1. *Mol. Plant-Microbe Interact.* **8**, 259–266.
- Kim, M.J., Choi, J.W., Park, S.M., Cha, B.J., Yang, M.S. and Kim, D.H. (2002) Characterization of a fungal protein kinase from *Cryphonectria parasitica* and its transcriptional upregulation by hypovirus. *Mol. Microbiol.* **45**, 933–941.
- Kim, M.J., Kwon, B.R., Park, S.M., Chung, H.J., Yang, M.S., Churchill, A.C.L., Van Alfen, N.K. and Kim, D.H. (2008) Promoter analysis of the cell surface-abundant and hypoviral-regulated cryparin gene from *Cryphonectria parasitica*. *Mol. Cells*, **26**, 496–502.
- Kim, M.J., Park, S.M., Kim, Y.H., Cha, B.J., Yang, M.S. and Kim, D.H. (2004) Deletion of a hypoviral-regulated *cpgk1* gene in a chestnut blight fungus, *Cryphonectria parasitica*, results in microcolonies. *Fungal Genet. Biol.* **41**, 482–492.
- Lee, J.K., Tattar, T.A., Berman, P.M. and Mount, M.S. (1992) A rapid method for testing the virulence of *Cryphonectria parasitica* using excised bark and wood of American chestnut. *Phytopathology*, **82**, 1454–1456.
- Nguyen, Q.B., Kadotani, N., Kasahara, S., Tosa, Y., Mayama, S. and Nakayashiki, H. (2008) Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. *Mol. Microbiol.* **68**, 1348–1365.
- Nuss, D.L. (1992) Biological control of chestnut blight: an example of virus-mediated attenuation of fungal pathogenesis. *Microbiol. Rev.* **56**, 561–576.

- Park, S.M., Choi, E.S., Kim, M.J., Cha, B.J., Yang, M.S. and Kim, D.H. (2004) Characterization of HOG1 homologue, Cpmk1, from *Cryphonectria parasitica* and evidence for hypovirus-mediated perturbation of its phosphorylation in response to hypertonic stress. *Mol. Microbiol.* **51**, 1267–1277.
- Parsley, T.B., Chen, B., Geletka, L.M. and Nuss, D.L. (2002) Differential modulation of cellular signaling pathways by mild and severe hypovirus strains. *Eukaryot. Cell*, **1**, 401–413.
- Powell, W.A. and Van Alfen, N.K. (1987a) Differential accumulation of poly(A)+ RNA between virulent and double-stranded RNA-induced hypovirulent strains of *Cryphonectria (Endothia) parasitica*. *Mol. Cell. Biol.* **7**, 3688–3693.
- Powell, W.A. and Van Alfen, N.K. (1987b) Two nonhomologous viruses of *Cryphonectria (Endothia) parasitica* reduce accumulation of specific virulence-associated polypeptides. *J. Bacteriol.* **169**, 5324–5326.
- Rigling, D., Heiniger, U. and Hohl, H.R. (1989) Reduction of laccase activity in dsRNA-containing hypovirulent strains of *Cryphonectria (Endothia) parasitica*. *Phytopathology*, **79**, 219–223.
- Rostagno, L., Crivelli, G. and Turina, M. (2009) Study of mRNA expression by real time PCR of Cpkk1, Cpkk2 and Cpkk3, three MEKs of *Cryphonectria parasitica*, in virus-free and virus-infected isogenic isolates. *J. Phytopathol.* **158**, 409–416.
- Sakaguchi, A., Tsuji, G. and Kubo, Y. (2010) A yeast STE11 homologue CoMEKK1 is essential for pathogenesis-related morphogenesis in *Colletotrichum orbiculare*. *Mol. Plant–Microbe Interact.* **23**, 1563–1572.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schaeffer, H.J. and Weber, M.J. (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.* **19**, 2435–2444.
- Schamber, A., Leroch, M., Diwo, J., Mendgen, K. and Hahn, M. (2010) The role of mitogen-activated protein (MAP) kinase signaling components and the Ste12 transcription factor in germination and pathogenicity of *Botrytis cinerea*. *Mol. Plant Pathol.* **11**, 105–119.
- Sun, Q., Choi, G.H. and Nuss, D.L. (2009) Hypovirus-responsive transcription factor gene *pro1* of the chestnut blight fungus *Cryphonectria parasitica* is required for female fertility, asexual spore development, and stable maintenance of hypovirus infection. *Eukaryot. Cell*, **8**, 262–270.
- Turina, M., Zhang, L. and Van Alfen, N.K. (2006) Effect of *Cryphonectria* hypovirus 1 (CHV1) infection on Cpkk1, a mitogen-activated protein kinase kinase of the filamentous fungus *Cryphonectria parasitica*. *Fungal Genet. Biol.* **43**, 764–774.
- Van Alfen, N.K. (1982) Biology and potential for disease control of hypovirulence of *Endothia parasitica*. *Annu. Rev. Phytopathol.* **20**, 349–362.
- Van Alfen, N.K., Jaynes, R.A., Anagnostakis, S.L. and Day, P.R. (1975) Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. *Science*, **189**, 890–891.
- Xu, J.R. (2000) Map kinases in fungal pathogens. *Fungal Genet. Biol.* **31**, 137–152.
- Zhang, L., Churchill, A.C., Kazmierczak, P., Kim, D.H. and Van Alfen, N.K. (1993) Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. *Mol. Cell. Biol.* **13**, 7782–7792.
- Zhang, L., Baasiri, R.A. and Van Alfen, N.K. (1998) Viral repression of fungal pheromone precursor gene expression. *Mol. Cell. Biol.* **18**, 953–959.
- Zhao, X., Mehrabi, R. and Xu, J.R. (2007) Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot. Cell*, **6**, 1701–1714.