The Journal of Microbiology (2011) Vol. 49, No. 3, pp. 418-430
DOI 10.1007/s12275-011-0193-3

The MpkB MAP Kinase Plays a Role in Post-karyogamy Processes as well as in Hyphal Anastomosis During Sexual Development in *Aspergillus nidulans*

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(Received May 25, 2010 / Accepted January 10, 2011)

Two genes encoding MAP kinase homologs, designated as *mpkB* and *mpkC*, were isolated from *Aspergillus nidulans* by PCR with degenerate primers. Deletion and over-expression mutants of *mpkC* showed no detectable phenotypes under any external stress tested. Deletion of *mpkB* caused pleiotropic phenotypes including a failure in forming cleistothecia under any induction conditions for sexual development, increased Hülle cell production, slow hyphal growth and aberrant conidiophore morphology. Over-expression of *mpkB* led to increased cleistothecium production. While the transcripts of *mpkB* and *mpkC* were constitutively synthesized through the entire life cycle, their size and amount differed with developmental stages. An outcross test using fluorescent protein reporters showed that the *mpkB* deletion mutant could not form heterokaryons with wild type. Protoplast fusion experiments showed that the fusant of the *mpkB* mutant with wild type could undergo normal sexual development. However, heterokaryotic mycelia that were produced from a fusant between two *mpkB* deletion mutants could not form cleistothecia, although they did appear to form diploid nuclei. These results suggest that the MpkB MAP kinase is required for some post-karyogamy process as well as at the hyphal anastomosis stage to accomplish sexual development successfully.

***Keywords*:** *Aspergillus nidulans*, MpkB MAP kinase, Hülle cell, post-karyogamy, anastomosis

MAP kinase (MAPK) which belongs to the eukaryotic protein kinase super-family, phosphorylates Ser and Thr residues in specific effector proteins (Rossomando *et al*., 1989; Boulton *et al*., 1991). Signal transduction networks allow cells to perceive changes in the intracellular environment and to receive an appropriate response. MAP kinase cascades are among the most intensively studied signal transduction systems and have been shown to participate in a diverse array of cellular programs, including differentiation, movement, division and death of cells, and so on. MAP kinase cascades, which are evolutionarily conserved in all eukaryotes, are typically organized in a three-kinase architecture consisting of a MAP kinase, a MAP kinase kinase (MAPK kinase), and a MAP kinase kinase kinase (MAPKK kinase). The MAP kinase family is grouped into three classes: stress-activated protein kinases (SAPKs), extracellular signal regulated kinases (ERKs), and the MAPK3 subgroup (Kultz, 1998). The activity of most of the MAP kinase family is transiently stimulated by a large variety of signals including mitogens, growth factors, cytokines, T cell antigens, pheromones, UV, ionized radiation, osmotic stress, heat shock, oxidative stress, and many other factors (Ferrell, 1996; Karin, 1996; Kyriakis and Avruch, 1996; Treisman, 1996; Woodgett *et al*., 1996; Cohen, 1997; Wurgler-Murphy and Saito, 1997).

MAP kinases in fungi have been intensively studied in the budding yeast *Saccharomyces cerevisiae*. At least five MAP kinase cascades in *S. cerevisiae* partially share MAPKK kinase or MAPK kinase in transmitting diverse extracellular signals to the nucleus (Gustin *et al*., 1998). For example, Ste11 MAPKK kinase is involved in mating, pseudohyphal growth and osmoregulation. Ste7 MAPK kinase activates Fus3 MAP kinase and Kss1 MAP kinase to accomplish the mating pheromone response and pseudohyphal growth, respectively, and Pbs2 MAPK kinase activates Hog1 MAP kinase in osmoadaptive responses (Posas *et al*., 1998). The sexual development of fungi is usually started with formation of a heterokaryotic cell by the fusion of two haploid hyphae (anastomosis). In heterothallic fungi, such as *S. cerevisiae*, the process of anastomosis is closely governed by the mating type. Moreover, mating pheromone signaling from mating type genes (*MAT*) activates a MAP kinase cascade and is an essential mechanism in the mating partner recognition process (Dohlman and Thorner, 2001). Activation of the pheromone signaling pathway is initiated by binding of an extracellular pheromone to the receptor Ste2 or Ste3 on the cell surface, which catalyzes the exchange of GDP for GTP on its cognate G protein α subunit (G_α) Gpa1, which in turn leads to its dissociation from the G beta-gamma ($G_{\beta\gamma}$) heterodimer. Free $G_{\beta\gamma}$ activates the downstream MAP kinase cascade, leading to new gene transcription, morphological changes, cell division arrest and ultimately cell fusion

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between two opposite cell types (Slessareva and Dohlman, 2006). Fus3 and Kss1, MAP kinases in the pheromone signaling pathway regulate the transcriptional activity of the transcription factor Ste12 by relieving the repression of two Ste12 inhibitors Dig1 and Dig2 (Cook *et al*., 1996; Tedford *et al*., 1997).

Most filamentous fungi have a more complicated life history than budding yeast in which they develop multicellular structures, such as asexual or sexual reproductive structures, during the life cycle. MAP kinases have been shown to be involved in several essential developmental processes, such as sporulation and pathogenicity in many filamentous fungi (Lengeler *et al*., 2000; Xu, 2000). A homothallic fungus, *Gibberella zeae*, was found to have two *MAT* idiomorphs in the genome and their expression was required for normal sexual development. Deletion of one of them resulted in loss of self-fertility of this fungus, causing it to be heterothallic (Lee *et al*., 2003). Similarly, *Aspergillus nidulans*, another homothallic fungus, contains a *MAT*-alpha locus and a high mobility group (HMG)-*MAT* locus that partly governs sexual differentiation (Paoletti *et al*., 2007). Moreover, a double deletion mutant of *gprA* and *gprB*, putative pheromone receptor genes in *A. nidulans*, eliminated fruiting body formation in the homothallic condition (Seo *et al*., 2004). Deletion of the *steC* gene that encodes a MAPKK kinase in *A. nidulans* showed pleiotropic phenotypes such as slow growth, altered conidiophore morphology, a defect in heterokaryon formation, and acleistothecial development. Western blot analysis for detection of a target that is *steC*-dependently phosphorylated, identified a MAP kinase with a molecular mass of about 30 kDa. Oxidative stress led to *steC*-dependent phosphorylation of another MAP kinase containing a tripeptide (Wei *et al*., 2003). Deletion of MAP kinase encoded by *mpkB*, which is a homolog of *S. cerevisiae FUS3*, led a defect in the formation of the sexual fruiting body (Paoletti *et al*., 2007), decreased sterigmatocystin gene expression and low mycotoxin levels. The mutation of *mpkB* also affected the expression of genes involved in penicillin and terrequinone A synthesis (Atoui *et al*., 2008). Interestingly, expression of sterigmatocystin gene and penicillin biosynthesis enzyme genes is regulated by VeA protein, encoded by the *veA* gene, and the VeA complex (Kato *et al*., 2003; Bayram *et al*., 2008). These results present the possibility that the pheromone/receptor system including the MAP kinase cascade could be active during sexual differentiation of these homothallic ascomycetes. To investigate factors governing developmental stages, we isolated the MAP kinase homologs, *mpkB* and *mpkC*, from *A. nidulans* by PCR amplification using degenerate primers. Here we report that the MpkB MAP kinase is an essential element for completing post-karyogamy processes as well as in hyphal anastomosis in sexual development of this fungus. In addition, the *mpkB* deletion mutant showed a low hyphal growth rate and aberrant conidiophores, indicating that this MAP kinase might also regulate vegetative growth and asexual reproduction, at least in part.

Materials and Methods

Strains, growth conditions, and nucleic acid manipulations *A. nidulans* VER7 (Han *et al*., 2001) was used as host strain for integration of manipulated genes. All *A. nidulans* strains used in this study (Table 1) were cultured on complex medium (CM) and minimal medium (MM). MM containing appropriate supplements was prepared as described elsewhere (Pontecorvo *et al*., 1953; Kafer, 1977). When necessary, 0.1% casamino acids (MMCA) or 0.1% yeast extract was added to MM. Asexual reproductive development was induced by spreading mycelial balls that had been grown for 14 h at 37°C in CM broth onto a plate. For inducing sexual development, plates on which the 14 h-grown mycelial balls had been spread were tightly sealed with parafilm and incubated for 24 h in the dark. The plates were further incubated for a given time after unsealing (Han *et al*., 2001). All nucleic acids were manipulated according to the procedures described elsewhere (Sambrook and Russell, 2001). Transformation and genetic manipulations of *A. nidulans* were also as described else-

Table 1. *A. nidulans* strains used in this study

Strain	Genotype	Source
FGSC A4	Wild type, *veA*$^+$	FGSC[a]
VER7	*pabaA1,yA2; ΔargB::trpC;trpC801,veA*$^+$	Han *et al*. (2001)
LBL03	*pyrG89;wA3;veA*$^+$	Han *et al*. (2001)
HSY2	*anA1,ΔargB::trpC,trpC801,veA*$^+$	FGSC A4 × VER7
VER7/pILJ16	*pabaA1,yA2;ΔargB::trpC,argB*$^+$*;trpC801,veA*$^+$	Harbored *argB*$^+$ plasmid
HSY2/pILJ16	*anA1,ΔargB::trpC,argB*$^+$*,trpC801,veA*$^+$	Harbored *argB*$^+$ plasmid
DMKB	*pabaA1,yA2;ΔargB::trpC;ΔmpkB::argB;trpC801,veA*$^+$	*mpkB* disrupted by *argB*
DMKB2	*anA1,ΔargB::trpC;ΔmpkB::argB;trpC801,veA*$^+$	*mpkB* disrupted by *argB*
DMKC	*pabaA1,yA2;ΔargB::trpC;ΔmpkC::argB;trpC801,veA*$^+$	*mpkC* disrupted by *argB*
OMPKB	*pabaA1,yA2;ΔargB::trpC;niiA(p)::mpkB;trpC801,veA*$^+$	*mpkB* O/E by *niiA* gene promoter
FLC8A32	*pyrG89;wA3;ΔmpkB::argB;argB?;trpC?;veA*$^+$	From fusant of LBL03 × DMKB
FLC1A2	*pabaA1,yA2;ΔmpkB::argB;argB?;trpC?;veA*$^+$	From fusant of LBL03 × DMKB
FLC6A1	*pyrG89,yA2;ΔmpkB::argB;argB?;trpC?;veA*$^+$	From fusant of LBL03 × DMKB
FLC2A11-GT[b]	*pabaA1,pyrG89,yA2;ΔmpkB::argB;argB?;trpC?;veA*$^+$*;pabaA*$^+$*,alcA(p)::histone h3::sgfp*	From fusant of LBL03 × DMKB
FLC2A21-GT[b]	*pabaA1,pyrG89,yA2;mpkB*$^+$*;veA*$^+$*;pabaA*$^+$*,alcA(p)::histone h3::sgfp*	From fusant of LBL03 × DMKB
FLC2A12-RT[c]	*pabaA1,pyrG89;wA3,mpkB*$^+$*;veA*$^+$*;pyrG*$^+$*,alcA(p)::histone h3::mrfp1*	From fusant of LBL03 × DMKB
FLC2A20-RT[c]	*pabaA1,pyrG89;wA3,ΔmpkB::argB;argB?;trpC?;veA*$^+$*;pyrG*$^+$*,alcA(p)::histone h3::mrfp1*	From fusant of LBL03 × DMKB

[a] FGSC (Fungal Genetics Stock Center, USA)
[b] These strains harbored the pSA11 plasmid. See 'Materials and Methods'.
[c] These strains harbored the pSR10 plasmid. See 'Materials and Methods'.

Table 2. PCR primers used in this study

Primer	The sequence of primer	Usage
MK1[a]	CTCTGCAGTNGCNATHAARAARATH	Cloning
MK2[a]	ATGGATCCAYMGNGAYYTNAARCC	Cloning
MK3[a]	ACGTCGACNGCNARNCCRAARTCRCA	Cloning
MK4[a]	ATTCTAGADATYTCNGGNGCNCRTA	Cloning
UXBPR	CATCTAGACCAAGCTCCTCATAAACTCTTCT	*mpkB* deletion
LCAPR	GTATCGATCGTCTCGCATAATACTTGAACAA	*mpkB* deletion
UARGP	GAGCTCCTCTTTTCCTTGTTC	*mpkB* deletion
LARGP	AAGAGGGGCAGCAAGCACAGAA	*mpkB* deletion
UXOPR	CCGCTCGAGGTGCTGACACCTTTCAGTTT	*mpkB* O/E
LAPPR	AAGGGCCCACTATAACGCTTTGTTCTTC	*mpkB* O/E
DEPR-up	TCCCCCGGGAGAACACTAACTCAAGCACGCTAT	*mpkC* deletion
DEPR-low	CCATCGATGAAAGGAGGTGGAGTAGAGAGCAA	*mpkC* deletion
FDCP-up	GCTCTAGAGCCAGAATCGAAACTCATTGAAAA	*mpkC* deletion
FDCP-low	CCGCTCGAGCGGAGACGGTACTGTGAATGACCAG	*mpkC* deletion
1st MKB5R	GGGTTCGCAGGCAAAACATCGAA	*mpkB* 5′-RACE
2nd MKB5R	CGCAGGCAAAACATCGAATGGTCA	*mpkB* 5′-RACE
1st MKB3	TTCCAGGTACGGGTGACGCA	*mpkB* 3′-RACE
2nd MKB3	GGTGATTCGCTTCGTCGGGT	*mpkB* 3′-RACE
PabaASacIIF	GATCCGCGGCTAGTCTTTATCATGCCCAT	Nucleus labeling
PabaASpeIR	GGCTCCGACTAGTTTGGGTACTGATTCAAGGT	Nucleus labeling
PyrGSacIIF	ATACCGCGGGTGGAGATAGGTCTGCGCCT	Nucleus labeling
PyrGSpeIR	GGCGGCACTAGTTAGAGACTTTTCAGTATTGGATG	Nucleus labeling
attB1HisF	GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGCTCGCACTAAGCAGACTG	Nucleus labeling
attB2HisR	GGGGACCACTTTGTACAAGAAAGCTGGGTAAGATCGCTCACCACGGAGG	Nucleus labeling

[a] MK1-MK4: Degenerate primers. N, H, R, Y, M and D indicate mixed base codes.
N, adenine, thymine, guanine, and cytosine; H, adenine, thymine, and cytosine; R, adenine and guanine; Y, thymine and cytosine; M, adenine and cytosine; D, adenine, thymine, and guanine.

where (Yelton *et al*., 1984; Timberlake, 1990). The nucleotide sequence was determined by an automated DNA sequencer, ABI PRISM 377 (KAIST Biomedical Research Center, Korea).

Isolation of genes encoding MAP kinase homologs

Regrettably, we could not refer to the *A. nidulans* genome database because this work was started before 2005. Therefore, we had to isolate MAP kinase homologs by using a traditional method, e.g. degenerate primer PCR from *A. nidulans* DNA. Degenerate PCR primers (see Table 2) were derived from the conserved amino acid sequence of fungal MAPKs. MK1 and MK2 were used as sense primers derived from subdomains II and VIb of MAPKs, respectively (Kultz, 1998). MK3 and MK4 were used as antisense primers derived from subdomains VII and VIII of MAPKs, respectively (Kultz, 1998). Four combinations of these primers were used for PCR amplification. Deduced amino acid sequences of PCR products were compared with that of other fungal MAP kinases in the GenBank database at NCBI. DNA fragments containing the whole MAP kinase gene were isolated by colony hybridization from the chromosome-specific genomic libraries constructed in cosmids pLORIST2 and pWE15 (Brody *et al*., 1991) using the PCR product as a probe.

Construction of deletion mutants

To delete *mpkB* from genomic DNA by one step gene replacement, the plasmid pDSCX was constructed by cloning the 2.6 kb *Sac*I/*Xha*I-digested fragment containing *mpkB* into pBluescriptII SK(+). A 1.5 kb fragment containing open reading frame (ORF) of *mpkB* was removed by inverse PCR. Two primers were designed to amplify in outward directions of *mpkB*, by which the *mpkB* ORF region was removed. Primer UXBPR was directed downstream of *mpkB* and contained the *Xba*I site and primer LCAPR was upstream of *mpkB* and contained the *Cla*I site. The PCR product using these two primers contained vector DNA and the flanking regions of the *mpkB* ORF. An approximately 3.0 kb *Cla*I/*Xba*I-digested fragment containing *argB* was ligated to the above PCR product, resulting in the plasmid pDSCXR which contained an *mpkB*-deleted cassette and *argB*. Another two PCR primers, UARGP and LARGP, amplified the deletion cassette from the flanking regions of the deleted *mpkB* toward *argB* using plasmid pDSCXR as a template, and the PCR product was used to obtain an *mpkB*-deletion mutant of *A. nidulans*. Deletion mutants of *mpkB* were screened by PCR and Southern hybridization. Deletions were confirmed by Northern blot analysis, which showed that *mpkB* was not expressed in these mutants, and by the recovery of retransformants in which the deletion mutants were retransformed with the wild type *mpkB* gene. *mpkC* was deleted using a procedure similar to that for the *mpkB* deletion. The plasmid pEHX, which was constructed by cloning an 8.0 kb *Eco*RI/*Hin*dIII-digested fragment containing the *mpkC* gene into pUC18, was used as a template for inverse PCR. Primer DEPR-up, directed downstream of *mpkC* and containing the *Sma*I site, and primer DEPR-low, directed upstream of *mpkC* and containing the *Cla*I site, were used for PCR amplification. The amplified PCR product with these two primers contained the pUC18 vector DNA flanked by the outside of the *mpkC* ORF region. An approximately 3.0 kb *Cla*I/*Sma*I-digested fragment containing *argB* was ligated to the above PCR product, which had been cut with *Cla*I/*Sma*I. The resulting plasmid, pAREHX, con-

tained an *mpkC*-deleted cassette. Another two PCR primers, FDCP-up and FDCP-low, amplified the deletion cassette from the outside of the deleted *mpkC* gene toward *argB*. The PCR product containing *argB* flanked on both sides by remaining *mpkC* gene was used obtain deletion mutants. Deletion mutants of *mpkC* were screened by PCR and confirmed by Southern hybridization as well as by Northern blot analysis.

Isolation of over-expression strains

The *niiA* promoter (Punt *et al*., 1995) was used to over-express *mpkB* at the transcription level. For *mpkB* over-expression, the entire *mpkB* ORF was isolated by PCR amplification with primers UXOPR and LAPPR, and then cloned into the *Apa*I/*Xho*I site of the plasmid pRB2-1, which contains the *argB* wild type gene that was used as a selective marker, and the promoter of *niiA*. The resulting plasmid pOMPKB was introduced by homologous recombination into *A. nidulans*. Over-expression strains were screened and confirmed by Southern hybridization. Over-production of the transcript was determined by Northern blot analysis. Transcription of *mpkB* was induced by 0.3% sodium nitrate and repressed by 0.2% ammonium tartrate as a nitrogen source (Johnstone *et al*., 1990).

External stresses applied to the deletion mutant of *mpkC*

Because the deduced amino acid sequence of MpkC showed high similarity to that of SAPKs such as HogA/SakA of *A. nidulans* and Hog1 of *S. cerevisiae*, we determined the impact of various environmental stresses on the phenotypes of deletion mutant and over-expression strains of *mpkC*. The stresses included sodium chloride, potassium chloride, sorbitol and glycerol (0.5, 1.0, 1.5, 2.0, and 3.0 M each) as osmotic agents and hydrogen peroxide (0.2 and 0.5%) as an oxidative agent. Two microliter aliquots (2×10^3 conidia of the *mpkC* mutant strain) were inoculated at the centers of CM or MM plates containing the above chemical agents and incubated at 37°C. CM or MM plates, in which the pH was adjusted to 4.5, 5.5, 6.5 and 7.5, were inoculated with 2×10^3 conidia at the centers, and then incubated at 37°C. In addition, light and temperature (25, 37, and 44°C) as physical agents were also applied to observe their effect on the morphology of the *mpkC* mutant strains.

Crossing test and protoplast fusion of the *mpkB* deletion mutants and wild type strain

To perform an outcross, conidia of two strains were inoculated into CM broth supplemented with selective nutrients in a test tube and incubated for 16 h at 37°C. The mixed hyphal disk was transferred to a MM plate without supplements. For convenient testing, parental strains used different auxotrophic (*pyrG89* and *pabaA1*) and conidial color (*wA3* and *yA2*) markers (Table 1). Protoplasts of wild type and *mpkB* deletion mutant were prepared from hyphal cells obtained by 14 h liquid culture in CM broth. For cell wall digestion, Novozym 234 (Novozymes) was used at 2 mg/ml in 0.8 M NaCl and 75 mM $CaCl_2$ osmotic stabilizing solution. After 2 h incubation at 30°C with 70 rpm shaking, hyphal debris was removed by filtration through glass-wool in a syringe. Protoplasts were collected by centrifugation at 1,100×g at room temperature and this protoplast precipitate was washed by adding a two-fold volume of STC buffer (1.4 M sorbitol, 10 mM Tris-HCl; pH 7.5, and 50 mM $CaCl_2$) and centrifuging at 1,100×g. Protoplast concentration was adjusted to 10^8/ml in STC buffer using a hemocytometer. To fuse the protoplasts of two parental strains, they were added to 30% (w/v) PEG 6,000 fusion solution in 50 mM $CaCl_2$, incubated for 30 min at 30°C, combined with 0.75% soft agar containing 0.75 M NaCl osmotic stabilizer and plated onto MM. Final concentration of each protoplast preparation was 10^6/ml.

Observation of Hülle cell in the *mpkB* deletion mutant

To observe Hülle cell production in a submerged culture, 10^6 conidia of wild type and *mpkB* deletion mutant were inoculated in CM broth and incubated at 37°C. The Hülle cells of several mycelial balls were observed by optical microscope. To quantify Hülle cell production, 10^6 conidia of wild type and *mpkB* deletion mutant were spread on a MPCA plate. The plate was sealed for 24 h for sexual induction or not sealed for asexual induction. The 24 h-sealed plates were unsealed and incubated for 6 days at 37°C for sexual development. Hülle cells in a 6 mm diameter agar block were counted as follows: the block was put into 200 μl Triton X-100 (0.08%) with glass beads, then vortexed for 90 sec, and then cells were counted using a hemocytometer on a microscope.

The 5′- and 3′-RACE PCR

Gene specific primers for amplification of 5′-end and 3′-end flanking regions were designed from the *mpkB* cDNA sequence. The 1st MKB5R and 2nd MKB5R of gene specific primers were paired with 5′-RACE adapter primer for the 5′-RACE and 1st MKB3 and 2nd MKB3 were paired with the oligo dT-adapter primer for the 3′-RACE. 10 μg of total RNA was extracted from mycelia 24 h after sexual induction and treated at 37°C for 1 h with 10 units of calf intestinal phosphatase (CIP) to remove free 5′-PO_4 from molecules and then reacted at 37°C for 1 h with 10 units of tobacco acid pyrophosphatase (TAP) to remove the cap structure from full length mRNA, leaving a 5′-monophosphate. One microgram of the RNA was ligated with 0.3 μg of the 5′-RACE adapter using T4 RNA ligase. The ligated mRNA was reverse transcribed (RT) at 42°C for 1 h using the 10 μM oligo dT-adapter and amplified with RACE-PCR primers using the primers described above. The RT reaction without TAP treatment and the PCR amplification without template DNA were used as negative RT and PCR controls.

Differential staining of nuclei with fluorescent proteins

For staining nuclei differentially, the plasmids used were pSA-sGFP (*argB* in pMT-sGFP was substituted by *pabaA*) and pSR-mRFP1 (*argB* in pMT-mRFP1 was substituted by *pyrG*) gateway destination vectors. The *pabaA* (locus ID ANID_06550.1 in *Aspergillus* genome database) was amplified by PCR using the *A. nidulans* FGSC A4 genomic DNA as a template and the PabaASacIIF and PabaASpeIR as primers and subcloned into pGEM-T Easy (Promega). The *pabaA* fragment was obtained by digestion with *Sac*II/*Spe*I, and ligated into the *Sac*II and *Spe*I sites between the *argB* of pMT-sGFP, resulting in pSA-sGFP (Toews *et al*., 2004). In the same manner as the pSA-sGFP, pSR-mRFP1 vector was also constructed from the fragment of *pyrG* gDNA (locus ID ANID_06157.1 in *Aspergillus* genome database) and pMT-mRFP1 vector. Primers employed for amplification of *pyrG* were PyrGSacIIF and PyrGSpeIR. The pMT-sGFP and pMT-mRFP1 vectors were obtained from Dr. Han (Woosuk University, Korea). The entry vector, pDONR-Histone H3 was constructed such that the Histone H3 cDNA (locus ID ANID_07033.1 in *Aspergillus* genome database) of *A. nidulans* FGSC A4 was amplified by PCR using the following primers attB1HisF and attB2HisR containing the attB sites and cloned into pDONR™221 (Invitrogen) with the BP clonase™ enzyme mix (Invitrogen), as described in the supplier's instructions. The destination vectors, pSA-sGFP and pSR-mRFP1 were recombined with pDONR-Histone H3 using the LR clonase™

enzyme mix (Invitrogen), as described by the supplier. The resulting expression plasmid clones were named pSA11 (*pabaA, alcA(p)::histone H3::sGFP, amp*^{r}) and pSR10 (*pyrG*, *alcA(p)::histone H3::mRFP1, amp*^{r}).

Fluorescence microscopy

sGFP and mRFP1 expressing strains were observed with a TCS SP2 (Leica) or LSM 510 META (Zeiss) confocal laser microscope. The excitation and emission peaks were: 405 nm and 470 nm (DAPI), 488 nm and 530 nm (sGFP), 612 nm and 680 nm (mRFP1), respectively.

Results

Molecular cloning of *mpkB* and *mpkC*

PCR using degenerate primers MK2 and MK4 produced two different DNA fragments whose deduced amino acid sequences showed high homology to that of fungal MAP kinase homologs. Southern hybridization of chromosome-specific cosmid libraries (Brody *et al*., 1991) with the two PCR products revealed that one each of the two DNA fragments was found in chromosome II and III respectively. Two cosmid clones, L17O08 and L15E03, isolated from the chromosome II-specific library, harbored a common fragment representing one PCR fragment and one cosmid clone, W18A08, containing the other fragment was isolated from the chromosome III-specific library (data not shown). We designated the gene from chromosome II as *mpkB* and the one from chromosome III as *mpkC* because the first MAP kinase gene isolated from *A. nidulans* had been named *mpkA* (Bussink and Osmani, 1999).

Nucleotide sequences of genomic DNA and cDNA showed that *mpkB* (deposited to NCBI GenBank accession no. AF198118) contained a 354 amino acid-ORF interrupted by four introns (Fig. 1A). The nucleotide sequence of *mpkB* was matched to that of a gene (locus ID ANID_03719.1) found in the *A. nidulans* genome database. The amino acid sequence of MpkB showed 92-93% identity to that of MAK-2 of *Neurospora crassa* (Pandey *et al*., 2004), FMK1 of *Fusarium oxysporum* (Di Pietro *et al*., 2001; accession no. AF286533), MAPK of *Leptosphaeria maculans* (accession no. AY118109), PTK1 of *Pyrenophora teres* (Ruiz-Roldan *et al*., 2001; accession no. AF272831) and CPMK1 of the *Claviceps purpurea* (Mey *et al*., 2002; accession no. AJ318517). These MAP kinases play roles in signaling for conidiation, pathogenicity, appressorium formation and/or colonization of host tissues. MpkB also had some similarity to Spk1 (68% identity) of *Schizosaccharomyces pombe* and Fus3 (60% identity) and Kss1 (60% identity) of *S. cerevisiae* (Toda *et al*., 1991; Neiman *et al*., 1993). These MAP kinases are involved in the regulation of sporulation, pheromone responses, and conjugation (Elion *et al*., 1990; Gartner *et al*., 1992; Gotoh *et al*., 1993). MpkB had the TEY motif that is a dual-phosphorylation site for MAPK kinase that is conserved in the ERK subfamily (Kultz, 1998).

mpkC (deposited to NCBI GenBank accession no. AF195773) had a 415 amino acid-ORF interrupted by eight introns (Fig. 1B). The nucleotide sequence of *mpkC* also matched a gene (locus ID ANID_04668.1) in the *A. nidulans* genome database. The amino acid sequence of MpkC showed 77-85% identity to that of MAPK of *Cryphonectria parasitica* (accession no. AY166687), Osm1 of *Magnaporthe grisea* (accession no. AF184980), Os-2 of *Neurospora crassa* (accession no. AF297032), and HwHog1 of *Hortaea werneckii* (accession no. AF516914). MpkC had the TGY motif that is also a dual-phosphorylation site for MAPK kinase and is conserved in the SAPK subfamily (Kultz, 1998).

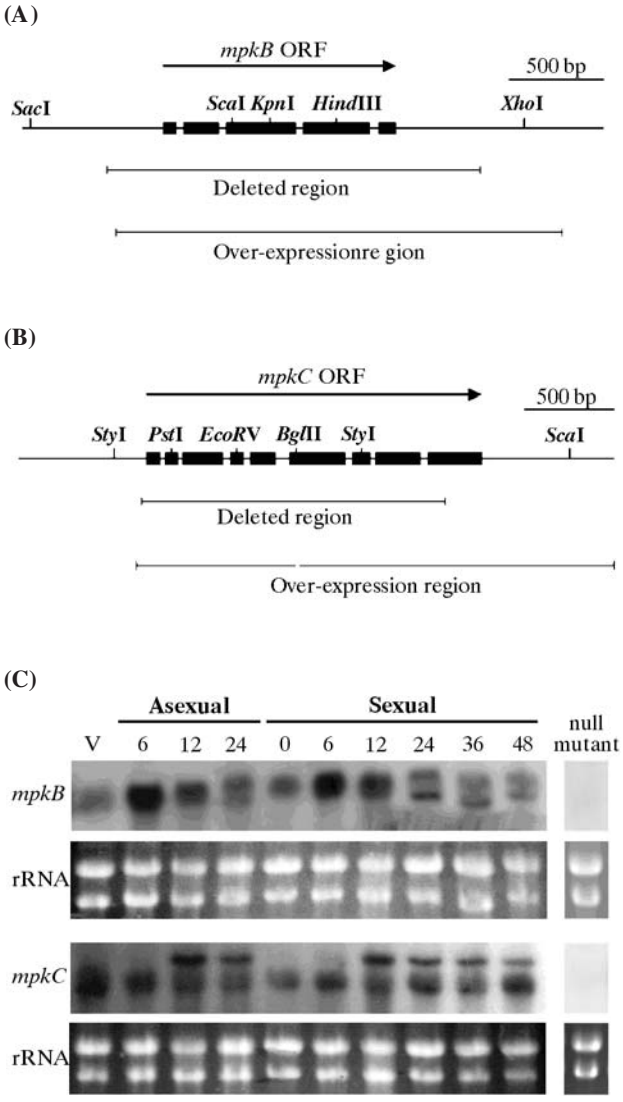

Fig. 1. The physical map and expression of *mpkB* and *mpkC*. (A) and (B) Deleted regions of both genes were replaced with *argB* to construct a knockout cassette, and over-expression regions of both genes were fused to the promoter of the nitrite reductase gene (*niiA*) to create over-expression strains (See 'Materials and Methods'). Black boxes and interrupted regions represent exons and introns, respectively, of *mpkB* and *mpkC* open reading frames (ORFs). (C) The wild type *A. nidulans* FGSC A4 was cultured in CM broth for 14 h and then mycelial balls were harvested to isolate total RNA. A part of the harvested mycelial balls was transferred to MM plates with or without casamino acid (0.1%) to induce sexual or asexual development, respectively. MM plates containing casamino acid were tightly sealed, incubated for 24 h and then unsealed. V means total RNA from 14 h grown mycelial balls and the numbers in the upper row indicate the harvest time (h) after transferring mycelial balls onto (asexual) and unsealing MM plates (sexual).

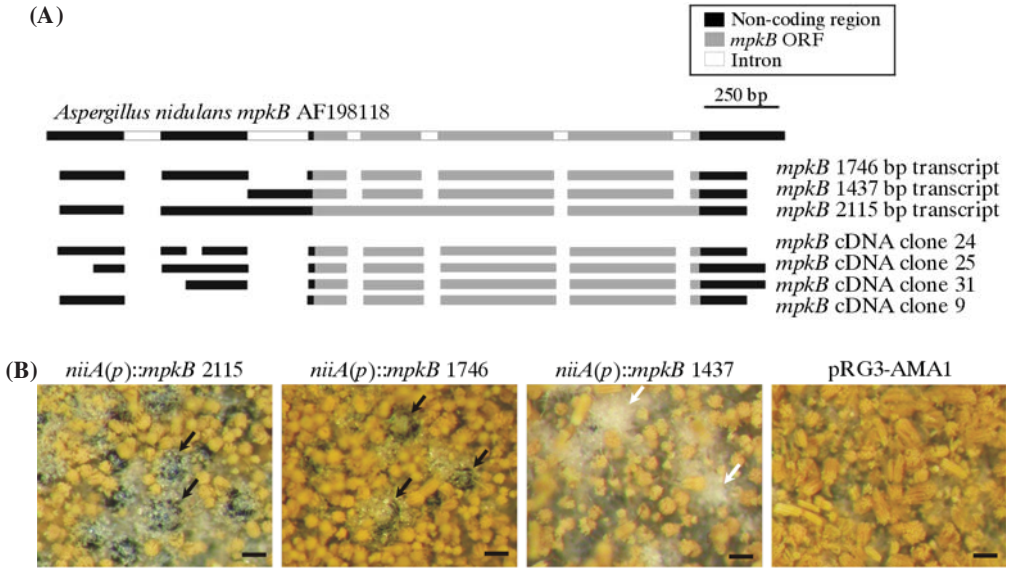

Fig. 2. Various transcripts of *mpkB* isolated by RACE-PCR and from a cDNA library. (A) Total mRNA for the RACE-PCR was isolated from the culture incubated for 24 h after induction of sexual development and the cDNA library was constructed from the mRNA of 12 h culture after sexual induction. The gray boxes indicate exons of the *mpkB* ORF, black boxes represent non-coding regions and white boxes are introns of *mpkB*. Three transcripts named as *mpkB* 1746, *mpkB* 1437, and *mpkB* 2115 were produced by RACE-PCR and four transcripts isolated from a cDNA library are *mpkB* cDNA clones 24, 25, 31, and 9. Note that cDNA clones 25 and 31 have an alternative poly-adenlylation site. (B) The *mpkB* transcript clones were introduced into an *mpkB* deletion strain (FLC6A1). Transformants with the small size of transcript (*niiA(p)::mpkB*1437) did not produce cleistothecia but formed nest-like hyphal aggregates (white arrows). The pRG3-AMA1 plasmid is a positive control of pyrimidine prototrophy for transformation. Black arrows indicate mature cleistothecia. Scale bar is 50 μm.

mpkB and *mpkC* were constitutively transcribed with a variable transcript size

To examine the expression profile of *mpkB* during the life cycle, total RNA from vegetative hyphae, or from mycelia after induction of asexual or sexual development, was isolated and hybridized with an *mpkB*-specific probe. The *mpkB* transcripts, whose size ranged broadly from about 1.6 to 2.5 kb, appeared at all life stages and accumulated to a high level at 6 h of following initiation of both the asexual and sexual developmental stages. Furthermore, the size of the transcript varied as asexual and sexual developments proceeded. At 24 h after induction of both asexual and sexual development, larger and smaller transcripts of *mpkB* appeared on the Northern blot (Fig. 1C). The 1.7 kb *mpkC* transcript was constitutively detected through the whole life cycle. However, during both asexual and sexual development, a larger, 2.7 kb transcript accumulated by 12 h after induction of the development (Fig. 1C). We isolated cDNA varieties of *mpkB* through RACE-PCR, and from the cDNA library that was constructed from the mRNA of a 12 h culture after sexual induction, to characterize these transcripts. RACE-PCR gave us 2,115 bp, 1,746 bp, and 1,437 bp *mpkB* transcripts from the total mRNA of the culture incubated for 24 h after induction of sexual development. These transcripts seemed to be alternatively spliced (Fig. 2A). The 2,115 bp transcript had four non-spliced introns, the 1,746 bp transcript of might be fully processed, and the 1,437 bp transcript of was truncated near the putative translation initiation site (Fig. 2A). Each transcript was cloned into the pRG3-AMA1 expression vector under the control of the *niiA* promoter and transformed into the *mpkB*-deletion mutant strain FLC6A1. The transformants harboring the 2,115 bp or 1,746 bp transcript showed flawless sexual development. However, the transformant bearing the 1,437 bp transcript could not finish sexual development. It produced only young primordia and nest-like structures when incubated more than two weeks after induction of sexual development (Fig. 2B). Four transcripts that were isolated from the cDNA library also contained alternatively spliced mRNAs, in addition to two different poly-adenylated sites (Fig. 2A).

mpkC mutants did not show any particular characteristics in various culture conditions

Because amino acid sequences of MpkC were homologous to those of HogA/SakA and other SAPKs, we first examined the effect of osmotic shocks conferred by various concentrations of salts and organic solutes on the cellular morphology of the deletion mutant and over-expression strains. Salts and solutes including sodium chloride, potassium chloride, glycerol, sorbitol, and others did not noticeably affect the strains. An oxidative stress conferred by hydrogen peroxide and hydrogen ion concentration as well as physical stresses by light and heat also did not lead to any particular phenotypic changes of the vegetative growth or development in both mutant strains (data not shown).

Deletion of *mpkB* caused slow hyphal growth and aberrant conidiophore morphology

Six of the ten transformants having the *mpkB*-deletion cassette were confirmed by Southern blot analysis to be missing the entire ORF of *mpkB* from their genome (data not shown).

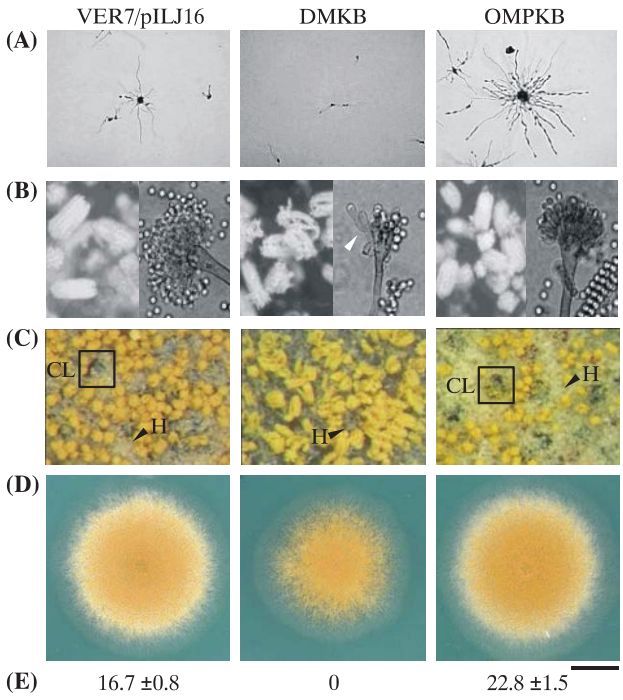

Fig. 3. Effect of deletion or over-expression of *mpkB* on vegetative growth and reproductive developments. (A) A young colony (17 h incubation) originated from a single conidium of the *mpkB*-deletion mutant (DMKB) showed relatively slower hyphal extension when compared with the wild type (VER7/pILJ16) and the over-expression mutant (OMPKB). (B) The deletion mutant produced many misshapen conidiophore heads resembling the *medusa* mutant (white arrow heads). (C) Confluent culture (1×10^5 conidia per plate) of the deletion mutant showed no cleistothecia but produced Hülle cells (H). Insets in VER7/pILJ16 and OMPKB indicate cleistothecia (CL). (D) Conidia of each strain were center-point inoculated on MM plates and incubated for 4 days. (E) The number of cleistothecia per mm^2 was counted from the culture after sexual induction. Data obtained from 30 different microscopic fields of three plates of each strain were averaged. Values are Mean±SD. Scale bar represents 120 μm (A), 50 μm and 10 μm (left and right, respectively, in B), 100 μm (C) and 1 cm (D).

A deletion mutant strain, DMKB, was examined for morphological and developmental characteristics. Colonies originating from single conidia of the deletion mutant and the wild type were compared with each other to observe the impact of *mpkB* on hyphal growth, conidiation and colony morphology. The diameter of the deletion mutant colony was smaller than that of wild type and an over-expression mutant (Figs. 3A and D), indicating that colony extension rate of the deletion mutant is slower than that of the wild type. The deletion mutant colony showed a different morphology from that of the wild type and had an irregular surface morphology that resembled a sectioned colony (Fig. 3D). This irregular surface morphology might be due to formation of the aberrant conidiophores observed in the deletion mutant. Although conidiation in the deletion mutant appeared to be normal, many conidiophores showing aberrant morphology, such as having a small number of metulae and phialide and forming secondary conidiophores on a primary conidiophore vesicle (Fig. 3B). The bundles of conidial chains on the conidiophore vesicle were disorderly and the length of conidial chains was shorter than that of the wild type (Fig. 3B).

mpkB-deletion mutant formed Hülle cells but not cleistothecia

To gain an insight into the function of *mpkB* in sexual development, the deletion mutant was cultured under the conditions for induction of sexual development. No a single cleistothecium was observed for the deletion mutant even when it grew for more than 14 d (Figs. 3C and E). Hyphal aggregates, which ultimately develop into cleistothecial initials in normal sexual development, were observed at a very early developmental stage but never underwent further development into later stages in the deletion mutant (Fig. 3C). Any known conditions favoring sexual development, such as supplementing lactose or glycerol as a sole carbon source, high concentration of glucose (3%), addition of calcium chloride or oxalic acid,

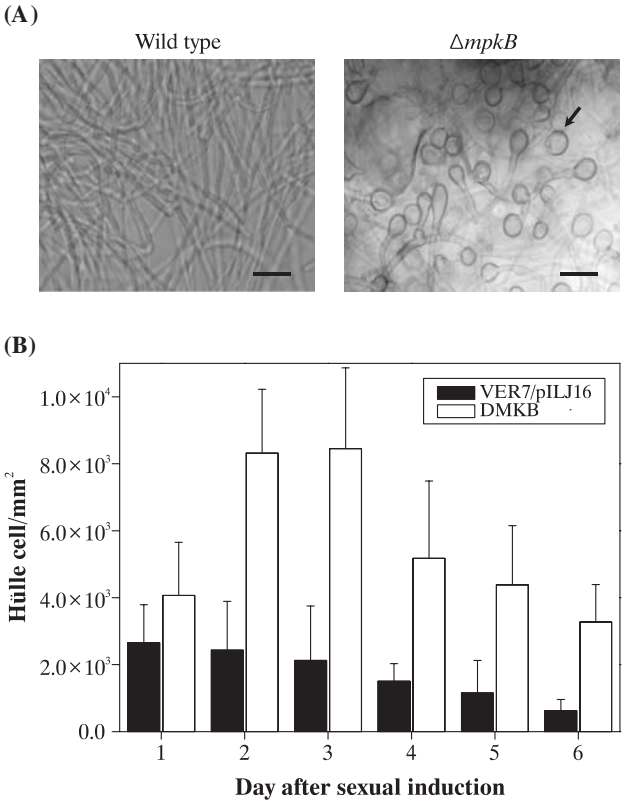

Fig. 4. Production of Hülle cell in wild type VER7/pILJ16 and *mpkB* mutant DMKB. (A) Wild type and *mpkB* deletion mutant strains incubated in CM broth for 48 h were observed with an optical microscope. Hülle cells in the mycelial ball are indicated by black arrow. (B) Deletion of *mpkB* caused a different pattern in the production and degradation of Hülle cells during the sexual developmental stage. Black and white bars indicate wild type (VER7/pILJ16) and *mpkB* mutant (DMKB), respectively. Illustrated are the means and standard errors (error bars) which were calculated from the values of 16 separate agar blocks for each strain (See 'Materials and Methods'). Statistical significance was determined by the one-way ANOVA analysis of variance ($P<0.05$). Scale bar in the panel (A) is 20 μm.

Table 3. Cleistothecium formation by the outcross and protoplast fusion

Cross combination	Cleistothecium production	
	In out cross	In protoplast fusant
Wild type × Wild type (VER7/pILJ16 × LBL03)	Yes	Yes
Wild type × Δ*mpkB* (LBL03 × DMKB)	NC[a]	Yes
Δ*mpkB* × Δ*mpkB* (FLC1A2 × FLC8A32)	NC[a]	No

[a] No cross was successful in this out cross due to *mpkB*-deletion

or blocking light and aeration (which favors induction of sexual structures) could not produce cleistothecia in the deletion mutant. This result indicated that the MpkB MAP kinase has an essential role during successful sexual reproduction. When the *mpkB* deletion mutant strain was incubated in submerged culture, more Hülle cells formed as compared with wild type (Fig. 4A). We counted the number of Hülle cells on an MM agar block. Hülle cells were produced more in the deletion mutant of *mpkB* than in wild type and increased until three days after sexual induction, whereas the number of Hülle cells produced in wild type started to decrease from one day after production (Fig. 4B).

Over-expression of *mpkB* enhanced cleistothecium formation

Colony morphology, conidiation pattern and hyphal growth of the over-expression mutant did not seem to be different from those of the wild type. Because deletion of *mpkB* caused an acleistothecial condition, it led us to determine whether MpkB had a positive role in sexual development. The number of cleistothecia was examined after induction of sexual development in wild type, deletion mutant, and over-expression mutant. The over-expression mutant formed 36% more cleistothecia than the wild type (Fig. 3E), suggesting that the MpkB MAP kinase might have a function in enhancing cleistothecium formation. However, the submerged culture of the over-expressed strain did not form a cleistothecial structure inside mycelial balls (data not shown), in contrast to that cleistothecial structures were observed inside mycelial balls of the over-expression mutant of *veA* (Kim *et al*., 2002).

Lack of *mpkB* prevented cell fusion but showed no effect on nuclear fusion

When we had conducted a conventional outcross between wild type and deletion mutant to isolate an *mpkB*-deleted strain that included other genotypes, we failed to get cleistothecia. To circumvent this failure we tried protoplast fusion between the wild type LBL03 and the deletion mutant DMKB. Because the LBL03 strain had white conidia (*wA3*) and a pyrimidine auxotrophy (*pyrG89*) and the DMKB strain had yellow conidia (*yA2*) and a *p*-aminobenzoic acid auxotrophy (*pabaA1*), the fusant was a balanced heterokaryon carrying nuclei from both parents, able to grow on unsupplemented MM. A heterokaryotic fusant obtained from the protoplast fusion between LBL03 and DMKB developed mature cleistothecia and ascospores (Table 3 and Fig. 5A), suggesting that the acleistothecial phenotype of the *mpkB* deletion is recessive and the MpkB MAP kinase is essential for complete hyphal fusion (anastomosis). Segregants from ascospores of this fusant were used to isolate additional *mpkB*-deletion strains that harbored convenient genetic markers for the following experiments. Protoplast fusion was performed between two *mpkB* deletion mutants using strains FLC1A2 (*pabaA1*, *yA2*, Δ*mpkB*) and FLC8A32 (*pyrG89*, *wA3*, Δ*mpkB*). The fusant obtained from two *mpkB* deletion mutants could not produce any cleistothecia (Table 3 and Fig. 5B). Whereas most fusants formed colonies in which both yellow and white conidia were produced simultaneously, a few colonies containing a sector of green conidia were also obtained from this forced outcrossing (data not shown). These green conidia were likely produced as a result of diploidization or parasexual genetic recombination between two *mpkB* deletion mutants, indicating that a nuclear fusion probably occurred between two mutant nuclei in the fusant.

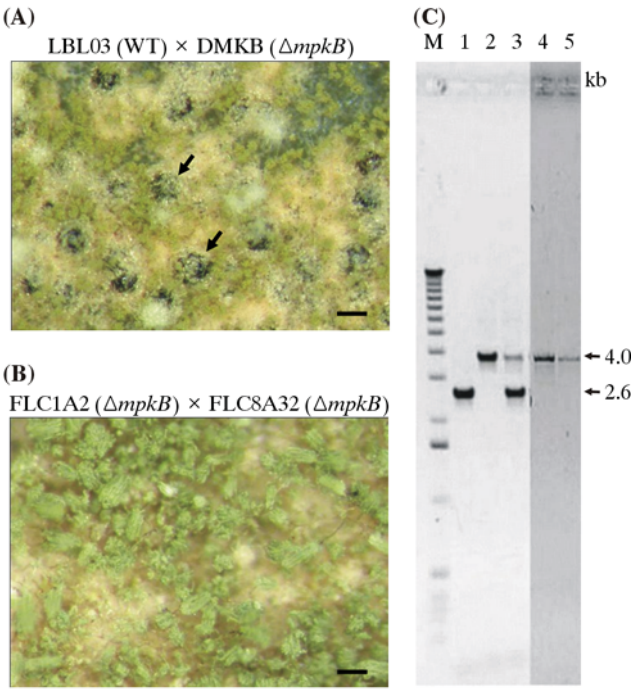

Fig. 5. Heterokaryon formation and sexual development by protoplast fusion. (A) The fusant of Δ*mpkB* and wild type formed green conidia and black cleistothecia (arrows) on minimal medium. (B) The fusant of two *mpkB* deletion strains (FLC1A2 and FLC8A32) produced green conidia and nest-like structures but no cleistothecia. (C) Heterokaryotic mycelia confirmed by PCR amplification of the different size of wild type (2.6 kb) and disrupted *mpkB* genes (4.0 kb). Lanes: M, 1 kb ladder; 1, LBL03; 2, DMKB; 3, the fusant of LBL03 and DMKB; 4, FLC1A2; 5, the fusant of FLC1A2 and FLC8A32. Scale bar in panels (A) and (B) is 50 μm.

To determine whether the absence of MpkB caused the defect in anastomosis, we took advantage of the visual properties of chromogenic nuclei that were stained by histone 3 fused with green or red fluorescent protein. Histone-fluorescence protein reporter analysis showed that hyphal fusion in the outcrossing experiment between two wild type strains (FLC2A21-GT×FLC2A12-RT) was successful (Fig. 6). However,

the outcrosses in which at least one partner contained an *mpkB* deletion (FLC2A21-GT×FLC2A20-RT and FLC2A11-GT×FLC2A20-RT) produced only homokaryotic hyphae (Fig. 6). Forced outcrossing experiments by protoplast fusion were also performed in the strains containing fluorescent nuclei. Whereas all fusants derived from the combination between *mpkB* deletion mutant and wild type showed heterokaryotic hyphae containing red, green and some yellow fluorescent nuclei (data not shown) and produced cleistothecia as expected, the fusant between two *mpkB* deletion mutants while also showing red, green and yellow fluorescent nuclei, could not produce cleistothecia (Table 3 and data of fluorescent nuclei not shown).

Discussion

The size of *mpkB* and *mpkC* transcripts was variable in the developmental stages

Northern blot analysis showed that the *mpkB* and *mpkC* transcripts, whose sizes ranged broadly, appeared at all life stages.

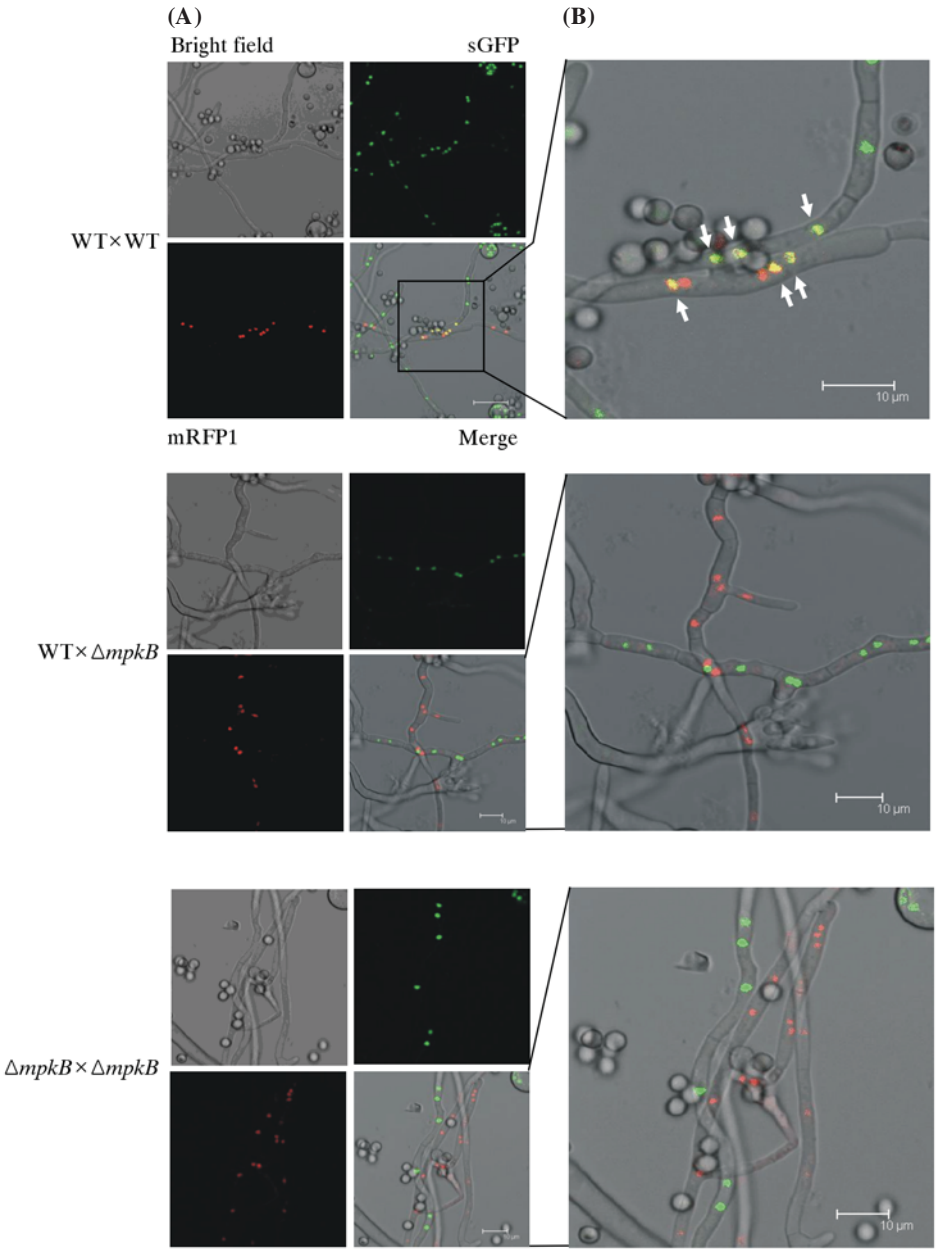

Fig. 6. Observation of heterokaryons by outcross between histone H3::sGFP and histone H3::mRFP1 strains. (A) Outcross test performed with WT×WT (FLC2A21-GT×FLC2A12-RT), WT×Δ*mpkB* (FLC2A21-GT×FLC2A20-RT) and Δ*mpkB*×Δ*mpkB* (FLC2A11-GT×FLC2A20-RT) combinations. Bright field and fluorescence images show the same region, at same time. (B) The merging of two fluorescent images in (A) shows in higher magnification. White arrows indicate hyphal fusion and heterokaryotic cell in WT×WT outcross. For outcrossing, conidia of two parental strains were inoculated onto a 22×22 mm cover-slip, covered with 2% glycerol MM and incubated for 16 h at 37°C.

These results suggest that MpkB might be active throughout the whole life cycle, including vegetative growth. This hypothesis is supported by the fact that the deletion mutant of *mpkB* showed slow colony expansion, misshapen conidiophores and acleistothecial phenotype. However, another report on an *mpkB* deletion mutant has shown that the *mpkB* transcript detected by semi-quantitative RT-PCR appeared only under conditions for sexual induction (Paoletti *et al*., 2007). This discrepancy might be due to the different techniques used for detecting the transcript or the different backgrounds of the strains. Indeed, we have isolated *mpkB* cDNA from the cDNA library that were constructed with poly-A RNAs isolated from the hyphal mass of a 14 h culture, indicating that this gene is also transcribed during vegetative growth (Fig. 2A). The parental strain VER7 used in this study has a wild type allele of *veA* that encodes a transcriptional activator enhancing sexual development in this fungus (Kim *et al*., 2002). In contrast, the strain TN02A7 used by Paoletti *et al*. (2007) has a mutation in the *veA* gene. When we determined the transcription of *mpkB* in *veA* over-expression and deletion mutant strains, the *mpkB* transcript pattern of these strains was not different from that of the wild type strain (data not shown).

Interestingly, the transcripts of *mpkB* and *mpkC* were variable in size and larger one appeared after 24 h and 12 h, respectively, in both asexual and sexual reproductive developments (Fig. 1C). In order to investigate the significance of multiple transcript forms during development we isolated cDNAs of *mpkB* from a 24 h culture after sexual induction, by RACE-PCR, and determined whether they could complement the acleistothecial phenotype of the *mpkB* deletion strain. RACE-PCR showed that there were at least three different *mpkB* transcripts produced by alternative splicing at the flanked region of the 5′-end and introns in the ORF of the gene (Fig. 2A). When cDNAs of these different transcripts were introduced into the *mpkB* deletion mutant strain, cDNA from the shortest transcript (1,437 bp) could only partially complement the defect in sexual development, whereas the transformants with larger ones (2,115 bp and 1,746 bp) could produce normal fruiting bodies (Fig. 2B). Alternative pre-mRNA splicing is recognized as a significant contributor to proteome diversity in higher metazoans (Ast, 2004). The examination of expressed sequence tag (EST) libraries and genome sequences from some fungi has revealed numerous examples of exon skipping and alternative splice site selection (Ebbole *et al*., 2004; Loftus *et al*., 2005). Although we don't know the exact reason why the spliced variants characteristically appear at a new developmental stage, we can speculate that the transcription of *mpkB* might be spatiotemporally regulated by internal stimuli during development that turn on some signals required for developmental progression. Whereas the smallest transcript appeared throughout the whole life cycle, the large ones accumulated only during development leading to reproduction. So far we do not have direct evidence that the smaller transcript observed by northern blot is the 1,437 bp transcript or that the larger ones are the 2,115 bp or 1,746 bp transcripts. An experiment that attempted to isolate cDNA of *mpkB* from other libraries showed some alternatively spliced transcripts and two alternative poly-adenylated sites (Fig. 2A).

Deletion mutant of *mpkB* shows pleiotropic phenotypes in vegetative growth and both sexual and asexual reproductive developments

Deletion of *mpkB* caused pleiotropic effects on vegetative growth and development in *A. nidulans*. The *mpkB* deletion mutant was compared with a wild type and over-expression mutant with respect to hyphal growth, conidiophore morphology and sexual reproduction. The hyphal growth of the *mpkB* deletion mutant is slower than that of the wild type and over-expression mutant, indicating that *mpkB* may be involved in signaling for hyphal elongation. It has been reported that a MAP kinase, MpkA, is involved in hyphal growth (Bussink and Osmani, 1999; Furukawa *et al*., 2005). The severity of structural changes of hyphae in the *mpkB* deletion mutant is less than that of the *mpkA* deletion mutant. Although direct evidence for phosphorylation of proteins controlling hyphal growth by MpkA has not been reported yet, MpkA might be a main signal transducer in hyphal growth (Furukawa *et al*., 2005). The MpkB MAP kinase might partly affect the activity of the genes involved in hyphal growth in addition to MpkA. Another phenotype of the *mpkB* deletion mutant was that conidiophores developed an improper morphology. Metulae and phialides were differentiated in reduced number on the vesicle of some conidiophores in the deletion mutant (Fig. 3B), resulting in formation of misshapen conidiophore heads as shown in the *medA15* mutant (Clutterbuck, 1969). This phenotype is similar to that of the mutant of *steC* that encodes a MAPKK kinase (Wei *et al*., 2003). This mutant strain also fails to initiate cleistothecium formation. Anti-phospho antibody p44/p42, which is able to detect the dual phosphorylated tripeptide motif, TEY, identified the presumptive target MAP kinase of SteC MAPKK kinase (Wei *et al*., 2003). A candidate for the target of the SteC MAPKK kinase would be a 30 kDa protein that was quite different from the predicted molecular weight of MpkA or MpkB. Although this finding suggests that there exists a MAP kinase other than MpkA and MpkB as a target of SteC, the phenotypic resemblance of the *mpkB* deletion mutant to the *steC* mutant suggests that MpkB could be downstream from SteC. A possible explanation for this hypothesis is the transcript diversity of *mpkB* or posttranslational modification of MpkB. Although we do not have any experimental evidence of posttranslational modification, the various sizes of the *mpkB* transcript appeared during development (Fig. 1C). A *Neurospora crassa* mutant in which *mak-2*, an *mpkB* ortholog, was deleted showed similar phenotypes to the *mpkB* deletion mutant. This *mak-2* deletion mutant of *N. crassa* exhibits slow growth, defective conidiation, shortened aerial hyphae, lack of vegetative hyphal fusion, and female sterility (Pandey *et al*., 2004). Resemblance of mutant morphology and high similarity (92% identity) in amino acid sequences between Mak-2 and MpkB suggest that these fungi, which possess two different sexual life styles, heterothallic and homothallic, may share common cellular roles for MAP kinase in growth and development.

In *A. nidulans*, initiation of sexual development takes place shortly after conidiation begins (Champe *et al*., 1994). Fusion of vegetative hyphae forms the cleistothecial initials that come from hyphal aggregates surrounded with Hülle cells and eventually develop into cleistothecia in which numerous asci form. The Hülle cell, which has a thick-walled globular shape,

has a nursing role for cleistothecium maturation and envelops the cleistothecial initials (Hermann *et al*., 1983). Although cleistothecium development is accompanied by Hülle cell formation in *A. nidulans*, the formation of Hülle cells is not likely a prerequisite for cleistothecium formation. Hyphal aggregates that were observed in the *mpkB*-deletion mutant never develop primordia or cleistothecia at all, even when grown for more than 14 d. However, Hülle cells were abundantly formed on the mycelial mat of the *mpkB*-deletion mutant. A report had shown that an *mpkB* deletion mutant did not produce Hülle cells under sexual induction conditions (Paoletti *et al*., 2007). This phenotypic difference might be due to strain differences as described earlier. The observation that deletion of *veA* resulted in no Hülle cell production under any sexual induction condition (Kim *et al*., 2002) supports this hypothesis. However, the *steC* deletion strain that had a *veA1* background could produce Hülle cells during sexual development (Wei *et al*., 2003), implying that Hülle cell formation is not likely depended on the MAP kinase cascade. Interestingly, the *mpkB* deletion strain in this study produced many more Hülle cells in submerged culture as compared to wild type (Fig. 4A). We counted the number of Hülle cells during sexual development on the plate. Whereas Hülle cell formation in wild type reached its highest level by 24 h after sexual induction and started to decrease thereafter, the *mpkB* deletion strain produced two times more Hülle cells than wild type by 72 h after sexual induction and only then started to decrease (Fig. 4B). This suggests that MpkB may have a role in regulating the nourishing activity of Hülle cells during cleistothecium maturation through breaking down their thick cell walls. Other explanations are possible to describe this phenomenon. The hypoxic sexual induction condition used in the experiment would cause an enhancing activity for Hülle cell production under the MpkB-free condition.

Roles of the MpkB MAP kinase in sexual reproduction

Wild type *A. nidulans* is able to undergo self and non-self hyphal fusion to form heterokaryons and develop sexual structures (Champe *et al*., 1994). Mutants generated from the same strain are usually compatible with each other because they have identical *het* loci which determine vegetative compatibility (Dales *et al*., 1993). Interestingly, the *mpkB* deletion mutation caused not only self incompatibility for formation of heterokaryotic hyphae, but also non-self incompatibility. Using fluorescent histone protein, we confirmed that *mpkB* mutants were defective in self and non-self hyphal fusion (anastomosis) and heterokaryon formation (Fig. 6). However, sexual development occurs in forced heterokaryons obtained by protoplast fusion between wild type and *mpkB* mutant but not in the forced heterokaryon between two *mpkB* mutants (Table 3 and Fig. 5). This result shows that the MpkB MAP kinase has to exist in both mating partners simultaneously to have successful hyphal fusion. We can speculate that the cell wall of each mutant partner had altered so that cell-to-cell fusion could not be established as an early process of heterokaryon formation. A report that an *mpkB* deletion mutant showed a hypersensitivity to micafungin which is an inhibitor of cell wall synthesis (personal communication with Dr. K. Abe of Tohoku University, Japan) supports this hypothesis. Moreover, the fact that forced fusion using protoplasts of two *mpkB* mutant strains could not overcome the sexual defect indicates that the MpkB MAP kinase is also required for a process following anastomosis to complete the formation of cleistothecia in the heterokaryon. In *S. cerevisiae*, the Fus3 MAP kinase controls the transduction of the pheromone signal in haploid cells for an initial mating process, and also loss of the MAK-2 MAP kinase in *Neurospora crassa* shows defects of hyphal fusion and ascus development (Gustin *et al*., 1998; Pandey *et al*., 2004). Other MAP kinases of plant or human pathogenic fungi, Kpp2 in *Ustilago maydis*, Mgv1 in *Fusarium graminearum*, Mps1 in *Magnaporthe grisea*, and Cpk1 in *Cryptococcus neoformans* involved pheromone gene expression, cell fusion for mating process and/or pathogenicity (Xu *et al*., 1998; Müller *et al*., 1999; Hou *et al*., 2002; Davidson *et al*., 2003). The phenotype of the *mpkB* deletion mutant resembled that of the *steC*-deletion mutant of *A. nidulans*. Deletion of SteC, homolog of *S. cerevisiae* Ste11 MAPKK kinase, showed an inhibition of heterokaryon formation and a block of cleistothecium development (Wei *et al*., 2003). Furthermore, the *mpkB* mutant shares other phenotypes of the *steC* mutant such as slower hyphal growth rate and altered conidiophore morphology. Hence, these observations strongly suggest that MpkB could be a member of the phosphorylation signaling pathway from the SteC MAPKK kinase and is involved in hyphal fusion for heterokaryon formation and ascospore production in filamentous fungi.

We have also questioned if the MpkB MAP kinase is required for nuclear recognition or nuclear fusion. By using our histone-fluorescence protein reporter analysis, we had expected to confirm the possibility of nuclear fusion (karyogamy) in the forced heterokaryon hyphae between wild type and *mpkB* mutant or both *mpkB* mutants. Unfortunately, we could not determine whether the yellow fluorescent nucleus is actually a diploid or the result of *trans*-complementation effects between two fluorescent protein tagged nuclei in heterokaryons (data not shown). However, we observed that heterokaryotic fusants between two *mpkB* mutants (FLC1A2 and FLC8A32) produced yellow, white and green conidial colors. The green color of asexual spores should be the result of diploid formation or of parasexual recombination between two different nuclei. This result indicates that karyogamy must happen in the fusant without MpkB MAP kinase. Therefore, the MpkB MAP kinase is dispensable during nuclear recognition and nuclear fusion. Nevertheless, the forced heterokaryon fusant between two *mpkB* mutants absolutely could not produce cleistothecia and ascospores. Hence, we propose that MpkB MAP kinase also plays an essential role following karyogamy, such as during zygote structure formation, meiotic division or ascospore development. In addition, double deletion of two putative pheromone receptor genes in *A. nidulans*, *gprA* and *gprB*, resulted in self-sterility, but they could produce cleistothecia when crossed with each other or with wild type, indicating that both putative pheromone receptor genes are required for homothallic (self) mating (Seo *et al*., 2004). Also, in a recent study on the mating type (*MAT*) genes of *A. nidulans*, *MAT1* (*MATα* domain locus) or *MAT2* (HMG box domain locus), a single deletion mutant showed defects in sexual development (Paoletti *et al*., 2007). Paoletti *et al*. (2007) also proposed that sexual differentiation of *A. nidulans* is correlated with increased expression of pheromone response path-

way genes, such as mating type genes (*MAT1* and *MAT2*), pheromone precursor gene (*ppgA*), pheromone receptor genes (*preA* [*gprB*] and *preB* [*gprA*]), G-protein subunit genes (*fadA*, *sfaD*, and *gpgA*), MAP kinase cascade component genes (*steC* and *mpkB*) and transcription factor gene (*steA*). However, the pheromone response signaling pathway of homothallic fungi requires additional investigation.

Deletion of *mpkC* did not show any discernible phenotypes

When *mpkC*, a SAPK gene highly homologous to *hogA*, was disrupted, no phenotypic characters were observed under the various osmotic and oxidative stresses. This contrasts with the *Aspergillus fumigatus* deletion strain of *mpkC*, an ortholog of *A. nidulans mpkC*. *A. fumigatus mpkC* is required for utilization of polyalcohol sugars such sorbitol and mannitol as a sole carbon source and is involved in germination (Reyes *et al*., 2006). Although MpkC has been suggested as having a redundant role in osmoadaptation in combination with HogA, little is yet known about its cellular function (Furukawa *et al*., 2005). Further sophisticated experiments should be carried out to characterize the function of this kinase.

Acknowledgements

We thank Dr. A. J. Clutterbuck (Glasgow University, Scotland) for his kind gift of plasmid pILJ16 and Dr. Kap-Hoon Han (Woosuk University, Korea) for the fruitful support for confocal laser microscopy and plasmids. This work was supported by a grant from Korea Research Foundation (KRF 005-2003-I-C00029) to K.-Y. J.. S.-C. J. was supported by a post-doctorial fellowship of the Korea Research Foundation Grant endowed to the RCBM of Chonbuk National University (KRF-2006-005-J03104).

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