Disordered Cell Integrity Signaling Caused by Disruption of the \textit{kexB} Gene in \textit{Aspergillus oryzae}\textsuperscript{†}

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We isolated the \textit{kexB} gene, which encodes a subtilisin-like processing enzyme, from a filamentous fungus, \textit{Aspergillus oryzae}. To examine the physiological role of \textit{kexB} in \textit{A. oryzae}, we constructed a \textit{kexB} disruptant (\textit{ΔkexB}), which formed shrunken colonies with poor generation of conidia on Czapek-Dox (CD) agar plates and hyperbranched mycelia in CD liquid medium. The phenotypes of the \textit{ΔkexB} strain were restored under high osmolarity in both solid and liquid culture conditions. We found that transcription of the \textit{mpkA} gene, which encodes a putative mitogen-activated protein kinase involved in cell integrity signaling, was significantly higher in \textit{ΔkexB} cells than in wild-type cells. The \textit{ΔkexB} cells also contained higher levels of transcripts for cell wall-related genes encoding \textit{β-1,3-glucanosyltransferase} and \textit{chitin synthases}, which is presumably attributable to cell integrity signaling through the increased gene expression of \textit{mpkA}. As expected, constitutively increased levels of phosphorylated \textit{MpkA} were observed in \textit{ΔkexB} cells on the CD plate culture. High osmotic stress greatly downregulated the increased levels of both transcripts of \textit{mpkA} and the phosphorylated form of \textit{MpkA} in \textit{ΔkexB} cells, concomitantly suppressing the morphological defects. These results suggest that the upregulation of transcription levels of \textit{mpkA} and cell wall biogenesis genes in the \textit{ΔkexB} strain is autoregulated by phosphorylated \textit{MpkA} as the active form through cell integrity signaling. We think that \textit{KexB} is required for precise proteolytic processing of sensor proteins in the cell integrity pathway or of cell wall-related enzymes under transcriptional control by the pathway and that the \textit{KexB} defect thus induces disordered cell integrity signaling.

Some secretory proteins of eukaryotic cells are converted from the precursor proteins to the mature proteins after modification through processes such as various glycosylation reactions and limited proteolysis within the Golgi apparatus. The modification process is well conserved from yeast to mammals, and the target proteins of the process are expanded to peptide hormones, neuropeptides, serum proteins, cell growth factors, and cell growth factor receptors. Therefore, elucidation of the protein modification process serves a clue to understand the physiological meaning of the posttranslational process. Kexin is a Ca\textsuperscript{2+}-dependent transmembrane serine protease that cleaves the secretory proproteins on the carboxyl side of Lys-Arg and Arg-Arg in a late Golgi compartment (15, 46). Kexin-like enzymes have been found from yeast to mammals (16, 19, 54, 65). Because filamentous fungi, including \textit{Aspergillus} species, secrete large amounts of proteins that are predicted to be modified through proteolysis in a Golgi compartment, kexins are thought to be key enzymes of the proteolytic process in the Golgi compartment of the fungi. \textit{Aspergillus} kexins are found in \textit{Aspergillus nidulans} (39) and \textit{A. niger} (29).

Disruption of the \textit{A. niger kexB} gene causes various morphological alterations such as shorter and multibranched hyphae (29, 60). Recent studies have shown that Kex2p in the dimorphic yeast \textit{Candida glabrata} is required for cell surface integrity (3). A bioinformatics approach, using the \textit{C. albicans} genome database, assigned 147 open reading frames (ORFs) as encoding potential substrates for Kex2p in \textit{C. albicans} (56). Among these ORF products, some predicted Kex2p targets were cell wall-related proteins and sensor proteins including \textit{GAS1} and \textit{WSC2} homologs. In light of the prediction from the bioinformatics approach using \textit{C. albicans} and the morphological defects of the \textit{A. niger kexB} disruptant, kexin activity in \textit{Aspergillus} species also seems to be concerned primarily with formation of the cell wall and morphogenesis. Therefore, maintenance of morphogenesis in fungi is likely to be one of the biologically important functions in which kexin plays some roles. However, the mechanism underlying the involvement of \textit{kexB} in morphogenesis in fungi is still unclear.

Although the \textit{Saccharomyces cerevisiae kex2-null} mutant (\textit{kex2Δ}) does not exhibit morphological defects compared with the phenotypes of other filamentous fungi, additional genetic defects in \textit{MPK1} (SLT2), together with the \textit{kex2A} mutation, cause lethality (62). Since the yeast \textit{MPK1} gene encodes a mitogen-activated protein (MAP) kinase in the cell integrity pathway that plays an essential role in maintaining the biogenesis and integrity of the cell wall (41), kexin seems to be involved in cell wall integrity in \textit{S. cerevisiae}. The cell integrity pathway is induced in response to several environmental stimuli, resulting in the increased expression of numerous genes, many of which encode integral cell wall proteins (glycosylphosphatidylinositol proteins, \textit{PIR} [proteins with internal repeats] family proteins, and others) or enzymes, including \textit{β-1,3-glucan synthases} (Fks1p and Fks2p) and \textit{chitin synthases}, required for

\textsuperscript{†} Supplemental material for this article may be found at http://ec.asm.org.
cell wall biogenesis in *S. cerevisiae* (13, 31, 67, 68). The *A. nidulans mpkA* gene that is a counterpart of the yeast *MPK1* (SLT2) was cloned and characterized (7). An *mpkA* deletion mutant (∆mpkA) was constructed, and its morphological defects suggested that the kinase is involved in germination of conidial spores and polarized growth. As described above, the apparent morphological changes observed in the *A. niger kexB* disruptant suggest the possibility that involvement of kexin in cell integrity is more significant in *Aspergillus* fungi than *S. cerevisiae*. Consequently, our studies focus on gaining an insight into the roles of kexin in morphogenesis and cell wall integrity in *Aspergillus* fungi.

*A. oryzae*, which is an economically important filamentous fungus, as well as *A. niger*, is used in the manufacture of fermented foods and in the production of enzymes for medical and food-grade use (10, 27, 71). Because its industrial importance has fortunately accelerated the establishment of the *A. oryzae* genomics, including an expressed sequence tag (EST) database and genome sequence information, *A. oryzae* genomics is now one of the most advanced platforms of fungal genomics and thus is also valuable for cell biology research on filamentous fungi (43; http://www.aist.go.jp/RIODB/ffdb/index.html). As a part of the *A. oryzae* genome projects, we manufactured *A. oryzae* cDNA microarrays consisting of approximately 2,000 cDNA clones by using information in the *A. oryzae* EST database (44). In the present study, we isolated an *A. oryzae kexB* knockout (∆kexB) strain that had remarkable morphological defects, and the mutant phenotypes, surprisingly, were suppressed under highly osmotic conditions. We investigated the function of *kexB* in morphogenesis by using the *kexB* knockout strain and our cDNA microarrays. Here we report (i) cloning of the *A. oryzae* kexin gene (*kexB*) and enzymatic characterization of KexB in the membrane fraction isolated from a KexB-overexpressing *A. oryzae* strain, (ii) construction of the ∆kexB strain and observation of its phenotype, (iii) comparison of gene expression profiles between the ∆kexB strain and the wild type under solid-culture conditions by using our cDNA microarrays and Northern blot analysis, and (iv) demonstration of constitutive upregulation of both transcription levels of the *mpkA* gene and phosphorylation levels of MpkA in the *A. oryzae* ∆kexB strain on the solid culture. Our results suggest that disruption of *kexB* in *A. oryzae* leads to morphological changes attributable to disordered cell integrity signaling. We discuss the contribution of *A. oryzae kexB* to the cell integrity pathway and morphogenesis.

### MATERIALS AND METHODS

**Strains, media, and growth conditions.** We used *A. oryzae* RIB40 (ATCC 42149) as the wild type and for constructing the *kexB* knockout mutant. This strain was also used for the *A. oryzae* EST and genome sequencing projects (43). *A. oryzae* niaD300 (niaD), a niaD mutant derived from RIB40, was used as a recipient strain for transformation and protein expression. These strains were grown in YPD complete medium (1% yeast extract, 2% polypeptone, 2% dextrose) supplemented with 0.1 M potassium nitrite, 500 mM chloride and either nitrite (NO₂⁻), hypoxanthine, glutamate, or ammonium chloride as a nitrogen source were added to CD medium.

Molecular cloning and sequencing of *kexB*. For subcloning, *Escherichia coli* XL1-blue (hsdR17 supE44 recA1 endA1 gryA6 thi-1 relA1 lacI*² Tn10, Ter*) cells and pBluescript II SK (+) plasmid (TOYOBO Inc., Tokyo, Japan) were used as host and vector, respectively, for DNA manipulation. The vector pGEM-T Easy (Promega Co., Tokyo, Japan) was used for TA cloning of PCR products. All basic molecular biology procedures were carried out as described by Sambrook et al. To clone the gene bySouthern analysis, we searched the *A. nidulans* EST database of the University of Oklahoma (http://www.genome.ou.edu/fungal.html) with the yeast (*S. cerevisiae*) KEX2 gene and found an approximately 600-bp homologous sequence. The nucleotide identity between the yeast KEX2 gene and the obtained *A. nidulans* DNA fragment was about 40%. PCR primers (5'-GGTTCTGGGAAGATCTACAATACG3' and 5'-GGTTACTTCCCGACAGCC3') were designed on the basis of the sequence of the fragment, and PCR was performed using genomic DNA from *A. nidulans* as the template. We obtained a 581-bp fragment, which we used as a probe for screening an *A. nidulans* cDNA library. Three positive clones were obtained from 2,000 plaques, and the positive clone with the longest insert was sequenced. The insert contains a 2,460-bp ORF encoding a single polypeptide comprising 819 amino acid residues. A FASTA search against the *A. oryzae* EST database (http://www.aist.go.jp/RIODB/ffdb/index.html) was performed with the full-length sequence of the *A. nidulans* kexB gene (DDBJ/EMBL/GenBank accession no. AB056726). Clone 6-58 of the *A. oryzae* EST database was homologous, and the DNA sequencing of this clone was completed using the ABI PRISM BigDye Terminator cycle-sequencing ready reaction kit version 2.0 (Applied Biosystems Japan Ltd., Tokyo, Japan) and an ABI PRISM 377 sequencer (Applied Biosystems Japan). The initiation codon of the *A. oryzae kexB* gene was predicted by comparison with that of the *A. niger kexB* gene (28) and on the basis of the discovery of a stop codon 33 bp upstream of the initiation codon in the *A. oryzae kexB* cDNA.

**Creation of the kexB-overexpressing strain.** The overexpression plasmid pnAKX1 was constructed as follows. The *kexB* gene was PCR amplified using Z-Taq DNA polymerase (TakaRa) and a pair of primers, 5'AAGCTTGATTTCAATCC3' and 5'AGAGTCGAGAACTTCTCCCGCGCATC3', which were designed to introduce a HindIII site (underlined). EST clone 6-58 of *A. oryzae* was used as the template. The amplified fragment was inserted into the pGEM-T Easy vector (Promega) and sequenced. The plasmid was digested with HindIII, and the digested fragment was ligated into the HindIII sites of the pNGA142 vector that has the gdsA142 promoter (20). The constructed expression plasmid was named pnAKX1. Transformation of *A. oryzae* niaD300 (niaD) was performed using the modified protoplast-polyethylene glycol method (20) and pnAKX1 digested with BamHI. The BamHI-digested pNGA142 also was introduced into the niaD300 strain as a control (pNGA strain). For protoplast formation, 5 mg of lysing enzyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 10 mg of cellulase Onozuka R-10 (Yakult Co., Tokyo, Japan) per ml, and 10 mg of Yatalase (TakaRa) per ml were used. Transformants were subcultured at least three times on CD agar plates to obtain homokaryotic strains.

**Enzyme assay.** The cells were grown in 50 ml of CD liquid medium with shaking for 2 days at 30°C. They were then transferred to CD liquid medium containing 2% maltose and cultured for 17 h at 30°C. They were collected using 1% glass beads and ground in a mortar on ice. The ground cells were resuspended in 0.2 M HEPES (pH 7.6) containing 1 mM EDTA and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was further centrifuged at 100,000 × g for 90 min at 4°C. The precipitate was resuspended in 50 mM HEPES (pH 7.6) containing 1 mM EDTA, 50 mM NaCl, 2% sodium deoxycholate, and 20% glycerol, and the mixture was centrifuged for 90 min at 4°C and 100,000 × g. The supernatants were pooled as the membrane protein fraction. Enzyme assays were performed as described previously (45), but 20 mM Tris-HCl (pH 7.0) was used as the assay buffer.

**Creation of the kexB disruption mutant.** The plasmid for *kexB* gene disruption (pPAkexB-stop) was constructed as follows. The *kexB* fragment was obtained by PCR with primers 1 (5'-TAATGGCGGTTCGGAAGATCTACAATACG3') and 2 (5'-TAAGGTTGAAGACAAAAATGCTAAGGATCC3') by using EST clone 6-58 of *A. oryzae* as a template. These primers were designed to introduce PstI and HindIII sites (underlined) at restriction sites (above) and PstI at the end of gene (below). Because the restriction sites were lowerase, respectively, the fragment was digested with PstI and HindIII and ligated into the PstI-HindIII fragment of the pTRI vector (TakaRa), which contains the pyrithiamine resistance gene (*ptrA*). The constructed plasmid was named pPAkexB. In addition, a stop codon linker (5'-CTAGTGTTGATCTAAGTATCC-3') was inserted into the NehI site of pPAkexB. The linker had termination codons in every frame, and the pPAkexB-stop was named pPAkexB-stop (Fig. 1A). *A. oryzae* RIB40 (wild type) was performed as described above by using pPAkexB-stop digested with Xhol (Fig. 1B). *A. oryzae* transformants were screened for
Resistance to pyrithiamine (0.1 μg/ml) and were subcultured at least three times on CD agar plates containing pyrithiamine.

Candidates for the knockout strain were selected by colony PCR by using primer sets 1 (Δ- sense 1 [5'-GGTTGGAATCCGCGTAGCTTC-3'] and Δ-antisense 1 [5'-CCGAGCTCAGTGACTTCTCC-3']) and Δ-antisense 2 [5'-CCCATAATGCGGCTTTCGCAAAATGCG-3'] and Δ-antisense 1 and A. oryzae genomic DNA as a template. A. oryzae genomic DNA was isolated from mycelia grown in CD medium for 40 h at 30°C as described previously (66). If the XhoI-digested pΔeksBΔeks fragment was inserted into the Δeks region of the pMK4 plasmid and transformed into A. oryzae, the Δeks disruptant and wild-type strains were grown on CD agar plates at 30°C for 5 days, and 0.1 μg/ml pyrithiamine. Strains that were resistant to pyrithiamine (0.1 μg/ml) and were subcultured at least three times on CD agar plates containing pyrithiamine were obtained.

Northern blot hybridization. mRNAs were prepared as described above from the Δeks disruptant and wild-type strains cultured on CD agar plates at 30°C for 50, 70, and 105 h or a CD agar plate plus 0.8 M NaCl at 30°C for 105 h. mRNAs (50 ng each) were electrophoresed through a agarose-formaldehyde gel (44) and transferred to Hybond-N nylon membranes (Amersham Biosciences) by using 7.5 mM NaOH. Blotted membranes were hybridized with the probes for chsB, chsB, gelA, gelB, and gelC prepared by PCR using the primers 5'-AGGTCTATGGGAGGAGGACCA TACG-3' (forward) and 5'-CTTGTGATACATGCTAAGAGATGCG-3' (reverse), 5'-AGGCGCTCAACACCCTGTTAAGAAG-3' (forward) and 5'-AT GTTCTACGGCAGGTAGGAAAAGG-3' (reverse), 5'-ATATAAAAA TATCACGAGGATCTGCGC-3' (forward) and 5'-TCGGAAGAAATCTT GAAAACAAATAGGCC-3' (reverse), and 5'-CAGTATAGATCCACAGAT TACCCGAAAAGG-3' (forward) and 5'-GAAAGACATACATGGTTACG AAGGAAAGG-3' (reverse). The probe was removed from the membrane with 0.1% SDS at room temperature for 20 min and then twice in 1% SSC-0.1% SDS for 15 min at 60°C (55°C for the mpkA probe) and detected by autoradiography.

Molecular cloning and sequencing of mpkA and plasmid construction. A FASTA search against the A. oryzae EST database (http://www.nrrb.jp/pk/en /EST/divindex.html) was performed with the full-length sequence of the A. nidulans mpkA EST clone 6-58. After TA cloning of the PCR products, the DNA sequencing of this clone was completed using the ABI PRISM BigDye Terminator cycle-sequencing ready reaction kit version 2.0 and an ABI PRISM 377 sequencer. To examine the in vivo functionality of A. oryzae mpkA, S. cerevisiae strain TPN46 (MATa mpkAΔ: HIS3 ura3 leu2 trp1 his3 ade2 cam1) with a temperature-sensitive MPK1 allele (41) was used for complementation analysis. Expression plasmids used in this experiment were constructed with the expression vector pYES2 (Invitrogen), in which expression is under the control of the galactose-inducible GAL1 promoter (30). A fragment containing the complete ORF of the A. oryzae mpkA cDNA was digested with HindIII and XbaI and ligated into the HindIII-XbaI fragment of pYES2, resulting in the mpkA expression vector pYmpkA.

Creation of the mychis-tagged mpkA-expressing strain. The plasmids (pNmpkA3 and pNmpkA4) for expression of mpkA possessing a c-myc epitope and a polyhistidine tag (mychis-tagged) were created as follows. To construct the mychis-tagged vectors, a mychis fragment was digested with XhoI and Agel from pPI7LoC (Invitrogen) and ligated to the fragment of pSL1180 (Amersham Biosciences) digested with XhoI and Agel, resulting in pSLmychis. The mychis fragment derived from pSLmychis by Spel-Spel double digestion was ligated to the fragment from pNGA142 digested with Spel and Spel to generate pNmpkA. The mpkA fragment was obtained by PCR with primers 5'-AAGC TATCGGCTAGCTAAGACGAT-3' and 5'-GGGCGGCTTCATCTTCCTCG-3' (stop codon [TAA] replaced by the codon for Thr [AC; nucleotides that were changed to remove a nonsense codon are lowercase). These primers were designed to introduce HindIII and SaeI sites (underlined), respectively. Plasmid pYmpkA was used as the template. The amplified fragment was inserted into the pJEM-E Vector easy vector and sequenced. The resulting plasmid was transformed into the Δeks disruptant and the digested fragment into the HindIII and SaeI sites of the pNmpK gene vector, resulting in the vector pNmpkA3 expressing mychis-tagged mpkA. The fragment containing the
OF for mychis-tagged MpkA was digested with HindIII and Nhel from the pNmpkA4-mbm and ligated into the HindIII and XbaI sites of PYE52, resulting in the vector pNmpkA4-mbm expressing mychis-tagged MpkA.

To add a niaD mutation to the A. oryzae ΔkexB strain, the nitrile auxotrophs derived from the ΔkexB strain were screened for resistance to chlorate by using a CD agar plate containing nitrite (NO₂⁻) instead of nitrate (NO₃⁻) as a nitrogen source in the presence of 500 mM chlorate. We further screened the niaD-mutating ΔkexB strains that were unable to utilize nitrate but could use nitrite, hypoxanthine, glutamate, or ammonium chloride as nitrogen sources. Strains (ΔkexB niaD) obtained from the selection were subcultured at least three times on the above-mentioned selection agar plates. Transformations of A. oryzae ΔkexB niaD and niaD300 (niaD) strains were performed as described above by using pNmpkA4-mbm digested with BamHI. A. oryzae transformants (ΔkexB-mpkA4-mbm and wt-mpkA4-mbm strains) were screened as prototrophs.

Transformants, ΔkexB-mpkA4-mbm and wt-mpkA4-mbm strains of A. oryzae, were obtained by colony PCR by using primers 5'-GCAAACGAAGTGCAAGACGAGTCG-3' and 5'-GTAGAATCACGAATGAGACCTTTGACGACC-3' to confirm that the BamHI-digested pNmpkA4-mbm fragment was inserted into the niaD locus of the genome. Genomic DNAs isolated from the transformants as described above were used as templates for colony PCR. When the BamHI-digested pNmpkA4-mbm fragment was inserted into the niaD locus, a 2,185-bp fragment was amplified by these primers.

Preparation of cell extracts and immunoblot analysis. Cell extracts were prepared by the same method as described for the mRNA isolation from solid cultures of the ΔkexB, ΔkexB-mpkA4-mbm, wild-type, and wt-mpkA4-mbm strains grown on CD agar plates at 30°C for 50, 70, and 105 h or a CD agar plate plus 0.8 M NaCl at 30°C for 105 h. The mycelia were ground to a fine powder in a mortar and immediately suspended in prewarmed SDS sample buffer (120 mM Tris HCl [pH 8.8], 5% SDS, 5% mercaptoethanol, 10% glycerol, 1 mM sodium vanadate) without dye. The samples were vortexed at 10 s quickly and boiled at 100°C for 10 min, and the cell debris was removed by centrifugation for 10 min at 15,000 × g. Each sample (50 µg of protein) was subject to SDS-polyacrylamide gel electrophoresis analysis. Protein concentrations were determined by the method of Schägger and Weißmann (64) with bovine serum albumin as a standard. After the transfer of proteins to a Pall Fluoro Trans W membrane (NIPPON Genetics Co. Ltd., Tokyo, Japan) using a semidyblotting apparatus (Bio-Rad), the membrane was used for immunoblotting with anti-phospho-p44/42 MAP kinase (Cell Signaling Technology, Inc., Beverly, Calif.) antibodies followed by immunonoconjugation with the alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibody (NACALAI TESQUE, Inc., Kyoto, Japan) and visualization of the immune complexes with the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. To detect the MpkA MAP kinase containing the mychis tag, immunoblots were probed with the anti-myc monoclonal antibody or anti-His monoclonal antibody (Co- vance Laboratories Inc., Vienna, Va.) and then an alkaline phosphatase-conjugated horse anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, Calif.). Immune complexes were detected as described above.

Nucleotide sequence accession number. The sequence data of the kexB genes of A. oryzae and A. nidulans have been submitted to the DDBJ/EMBL/GenBank database under accession no. AB056727 and AB056726, respectively. The sequence data of the mpkA gene of A. oryzae have been submitted to the DDBJ/EMBL/GenBank database under accession no. AB167718.

RESULTS

Cloning and characterization of the kexB gene from A. oryzae. To clone the kexin gene of A. oryzae, we searched for A. oryzae EST sequences homologous to the A. nidulans kexin-like gene by using the A. oryzae EST database (http://www.aist.go.jp/RIODB/fd/db/) and identified clone 6-58. We determined the complete nucleotide sequence of this EST clone, which contained a 2,511-bp ORF encoding a protein of 836 amino acid residues. The putative amino acid sequence showed about 70% identity to those of the putative kexin identified in A. nidulans (AB056726) and A. niger KexB (29), and thus the A. oryzae and A. nidulans genes both were designated kexB.

The catalytic triad, Ser-Asp-His, and several amino acids upstream and downstream from the catalytic domain were well conserved. The putative A. oryzae KexB also has a P-domain, which is necessary for enzymatic activities in other proteases (42, 55). Hydropathy analysis suggested the presence of two highly hydrophobic regions that are a signal peptide and transmembrane domain required for anchoring to Golgi membranes, similar to those in the amino acid sequence of yeast kexin. A propeptide with a Lys-Arg dibasic autocleavage site follows the signal peptide. In the putative cytoplasmic tail, 15 amino acids downstream from the transmembrane domain, we found the conserved peptide sequence YDFEMI, similar to that found in KexB of A. niger (29). The underlined amino acid residues are identical to the late-Golgi retention signal (consensus, YXXFXX1) in the cytoplasmic tail of the S. cerevisiae Kex2p (69).

To confirm whether the A. oryzae kexB gene product has proteolytic activities like those of known kexins, kexB was integrated at the niaD locus in the A. oryzae niaD300 strain and expressed using the A. oryzae glaA1-42 promoter. Southern blot analysis confirmed, in addition to the authentic kexB locus, the integration of an extra copy of kexB at the niaD locus in the transformant (data not shown). The transformed strain in which kexB was overexpressed did not show a detectable or noteworthy phenotype for hyphal development or morphology under induced conditions in either liquid or agar plate culture. We fractionated a cell lysate of the kexB-overexpressing strain and found kexin-like enzymatic activities in the membrane fraction. The solubilized membrane fraction from the kexB-overexpressing strain showed higher hydrolsis of fluorogenic peptides than did that from the control strain harboring the expression vector lacking the kexB insert (Table 1). In particular, A. oryzae KexB has a preference for peptides containing dibasic residues, as follows: Boc-Gln-Arg-Arg-MCA (4-methylcoumaryl-7-amide). Basic amino acids are in bold.

TABLE 1. Enzymatic activities for peptidyl-MCA substrates

| Peptide substrate | Cleavage activity (nkat/ml) |
|-------------------|--------------------------|
| Boc-Gln-Arg-Arg-MCA | 0.07 3.47 |
| Boc-Leu-Lys-Arg-MCA | 0.06 3.15 |
| Boc-Leu-Arg-Arg-MCA | 0.07 2.91 |
| Boc-Arg-Val-Arg-MCA | 0.05 2.39 |
| Boc-Val-Pro-Arg-MCA | 0.04 1.74 |
| Boc-Gly-Lys-Arg-MCA | 0.03 1.30 |
| Boc-Gly-Arg-Arg-MCA | 0.01 0.56 |
| Boc-Leu-Ser-Thr-Arg-MCA | 0.02 0.55 |
| Boc-Leu-Gly-Arg-MCA | 0.00 0.11 |
| Boc-Gln-Gly-Arg-MCA | 0.01 0.20 |
| Boc-Glu-Lys-Lys-MCA | 0.00 0.01 |

a The arrow indicates the cleavage site by A. oryzae KexB. Boc, N-butyloxycarbonyl-; MCA, 4-methylcoumaryl-7-amide. Basic amino acids are in bold.

Construction and characterization of the kexB disruptant. To further study the in vivo functionality of A. oryzae kexB, we constructed the kexB disruptant (ΔkexB) strain by homologous recombination with pPΔkexB-stop (Fig. 1A), which contained ptrA and a truncated kexB fragment carrying a stop codon...
linker downstream of the XhoI site (Fig. 1B). The stop codon linker had multiple stop codons to cover all reading frames. The ΔkexB strain was isolated from about 400 colonies of the transformants by the colony PCR method as described by van Zeijl et al. (66) with primers 1 and 2, as described in Materials and Methods. The ΔkexB candidate was further confirmed by PCR and Southern analysis (Fig. 1C). The probes for both kexB and ptrA indicated the expected two hybridization signals to digested genomic DNA isolated from the ΔkexB candidate and the single hybridization signal to that from the wild-type strain, suggesting that the homologous recombination successfully took place at the authentic kexB locus. Reverse transcription-PCR failed to reveal transcripts derived from kexB in the ΔkexB strain (data not shown).

The ΔkexB strain formed shrunken colonies and scarcely differentiated conidia on CD agar plates (Fig. 2A). The detailed morphology of the ΔkexB strain was further compared with that of the wild type by using scanning electron microscopy (Fig. 2C to G). ΔkexB cells grown for 4 days on CD agar plates formed neither conidiophores nor conidia. The hyphae of the ΔkexB strain became finer, denser and hyperbranched in comparison with those of the wild type. Hyphal tips of the ΔkexB strain were thicker and multibranched (Fig. 2F). The disruptant grown in CD liquid culture medium also showed highly branched mycelia (data not shown). We carried out a rescue experiment in which the mutant phenotypes of the A. oryzae ΔkexB (ΔkexB niaD) strain were almost fully restored by transformation with the pNAKX1 plasmid containing the wild-type kexB gene (see Fig. S1 in the supplemental material). However, the A. oryzae ΔkexB (ΔkexB niaD) strain transformed with the pNGA142 vector without the kexB insert maintained the mutant phenotypes (see Fig. S1 in the supplemental material). These results suggested that the phenotypes of the ΔkexB strain are attributable to the defect of the kexB gene. Surprisingly, although the ΔkexB strain exhibited the various described morphological defects on CD agar plates, the defects were suppressed on high-osmolarity CD agar plates containing 0.8 M sodium chloride (Fig. 3), 0.8 M potassium chloride, or 1.2 M sorbitol (data not shown). The morphological defects of the ΔkexB strain also were restored in a high-osmolarity CD liquid culture medium (data not shown).

cDNA microarray analysis of the ΔkexB disruptant. Because the ΔkexB strain showed the various described phenotypes, we wondered how transcription profiles reflected the phenotypes and therefore compared the gene expression profiles of the ΔkexB and wild-type strains by using A. oryzae cDNA microarrays. We analyzed transcripts from the two strains cultured on CD agar plates for 105 h at 30°C as well as on CD agar plates containing 0.8 M NaCl (Table 2). On CD agar plates, a large number of genes were more upregulated in the ΔkexB cells than in wild-type cells whereas gene expression levels of the ΔkexB strain were similar to those of the wild type in the presence of high osmotic pressure (~0.8 M NaCl). The ΔkexB type (lanes 3, 4, 7, and 8). The enzymes used were PstI and SphI (lanes 1 and 3), ApaLI and NspV (lanes 2 and 4), SpeI (lanes 5 and 7), and PstI (lanes 6 and 8). Hybridization was performed with the kexB probe (see Materials and Methods) (left) and the ptrA probe (right).
strain exhibited 4.9- and 55-fold-lower levels of brlA (70) and rodA (57) transcripts, respectively, than did the wild type. Because brlA encodes a transactivator to promote the formation of conidia and rodA encodes a hydrophobic protein necessary to form conidiophores, the downregulation of brlA expression in the ΔkexB strain might be one of the reasons for its poor generation of conidia. We were unable to assign identities to other genes that showed markedly reduced transcript levels in the ΔkexB strain, because the annotations of some genes in the cDNA microarray remain unknown.

Because of the morphological defects of the ΔkexB strain, we paid further attention to the following eight genes which are involved in cell wall biogenesis: chsC (50), chsA (unpublished data; DDBJ/ENBL/GenBank accession no. BAB85683), chsB (51), chsY, chsZ (9), gelA, gelB (48), and fksA (34) (Fig. 4). chsC, chsA and -B, chsY, and chsZ encode type I, III, V, and VI chitin synthases, respectively (9). According to tBlastx analysis, gelA and gelB are predicted to be the A. oryzae counterparts of the A. fumigatus gel1 and gel2 genes, which encode putative glucanosyltransferases that are thought to be involved in cell wall biosynthesis (49). fksA is the putative A. oryzae β-1,3-glucan synthase gene (34). On CD agar plates, transcription of chsC, chsB, and gelB of the ΔkexB strain was markedly upregulated and their transcription levels were 3.2, 4.6, and 4.3 times higher, respectively, than those of the wild type. In contrast, high osmotic pressure (0.8 M NaCl) in the culture plate suppressed the upregulation of these three genes in the ΔkexB strain.

Northern blot analysis of the ΔkexB strain. Cell wall biogenesis in S. cerevisiae is thought to be under the control of

| Relative expression ratios (ΔkexB/wild type) (fold) | No. of genes in cells grown on**a** |
|---------------------------------------------------|-----------------------------------|
| CD                                               | CD + 0.8 M NaCl                    |
| >4.0                                              | 68                                |
| 2.0–4.0                                           | 291                               |
| 0.5–2.0                                           | 1,087                             |
| 0.25–0.5                                          | 48                                |
| <0.25                                             | 27                                |

**a** After three independent analyses, 1,521 spots on the cDNA microarrays for CD plates and 1,762 spots on the cDNA microarrays for CD plates with 0.8 M NaCl were statistically verified by using the Genomic profiler program.
signal transduction pathways including the cell integrity pathway. The *S. cerevisiae* MPK1 (SLT2) gene encodes a MAP kinase in the cell integrity pathway (26). In *S. cerevisiae*, when cell wall biosynthesis is inhibited or the cell wall is damaged, transcription levels of *MPK1* and genes required for cell wall biogenesis are simultaneously upregulated by the transcription factor Rlm1p, which is a phosphorylation target of Mpk1p (13, 31, 67, 68). Bussink et al. (7) isolated the *A. nidulans mpkA* gene which is the counterpart of yeast MPK1. Recently we confirmed that expression of *A. nidulans mpkA* cDNA in a temperature-sensitive *S. cerevisiae mpkl* disruptant suppressed the mpk1 disruption mutation, suggesting that *A. nidulans mpkA* functionally complements yeast MPK1 (T. Fujioka, K. Furukawa, O. Mizutani, K. Abe, and T. Nakajima, unpublished data). We also found an *A. oryzae mpkA* gene homolog in the *A. oryzae* EST database and *A. oryzae* genome information through homology searches with the nucleotide sequence of *A. nidulans mpkA*. Because our cDNA microarray analyses showed upregulation of the transcription levels of genes involved in cell wall biogenesis in *A. oryzae* ΔkexB, we further examined whether transcription of *mpkA* is also upregulated in this strain. We performed transcriptional analyses of *mpkA* and cell wall-related genes such as *chsC*, *chsB*, *gelA*, and *gelB* by Northern blotting at various times after inoculation (Fig. 5A and B). The wild-type strain began to form conidiophores and conidia 70 h after inoculation and had formed fully mature conidiophores with plenty of conidia at 105 h on CD plates. Transcription of the histone H2B gene seemed to be constitutive and was used as a control at all time points. Transcription levels of *chsC*, *chsB*, and *gelB* were higher in the ΔkexB strain than in the wild type at the three time points assayed. As expected, the transcription of *mpkA* in the ΔkexB strain also was increased at all time points. However, the transcription levels of *gelA* were almost same in both strains at all time points. Because cell integrity signaling is inactivated by high osmotic stress in *S. cerevisiae* (11, 25, 26), we examined whether high osmotic stress downregulates the high level of transcription of *mpkA* in the ΔkexB strain to the transcription level of *mpkA* in the wild-type strain. We performed transcriptional analyses of *mpkA* by Northern blotting under conditions of high osmosis (Fig. 5C). As expected, high osmotic stress apparently downregulated the transcription level of *mpkA* in the ΔkexB strain to a level similar to that in the wild-type strain. Therefore, the morphological defects concomitant with the marked upregulation of *mpkA* and the osmoreponsive suppression of the morphological defects with the simultaneous downregulation of transcription of *mpkA* and genes for cell wall biogenesis may indicate that kexB is involved in a signal transduction pathway, particularly cell integrity signaling.

**Cloning of the mpkA gene from A. oryzae and time course of MpkA phosphorylation in the ΔkexB strain.** Since transcription levels of the *mpkA* gene in the ΔkexB strain are constitutively upregulated under normal culture conditions and downregulated in the presence of osmotic stress, we further examined phosphorylation levels of MpkA protein in the ΔkexB strain under normal and high-osmosis conditions. From the *A. oryzae* cDNA library, we isolated a positive clone by using PCR. We determined the complete nucleotide sequence of this clone, which contained a 1,272-bp ORF encoding an MpkA protein of 423 amino acid residues. The catalytic domain of MpkA possesses all the subdomains found in protein kinases (23). In addition, MpkA has a TEY tripeptide dual phosphorylation motif characteristic of MAP kinases, which is known to be required for activation of MAP kinases. The putative amino acid sequence showed about 90% identity to that of the putative MpkA identified in *A. nidulans* (7), and thus the *A. oryzae* gene was designated *mpkA*.

Because MpkA of *A. oryzae* is also related to the yeast Mpk1p, we investigated the in vivo functionality of MpkA and its derivative mychis-tagged MpkA by using an *S. cerevisiae mpkl* mutant. We confirmed that expression of both *A. oryzae mpkA* and mpKAh genes in the *S. cerevisiae mpkl* disruptant...

![Fig. 4. Expression levels of genes involved in cell wall biogenesis.](image)

**FIG. 4.** Expression levels of genes involved in cell wall biogenesis. The bar graphs indicate the expression levels of the genes encoding cell wall biogenesis-related proteins from wild-type and *A. nidulans mpkA* disruptant. The gray and white bars indicate the relative intensities of transcription of the genes in the wild-type and ΔkexB strains, respectively, on the basis of the cDNA microarray analyses. The relative intensities of the examined genes were calculated using the intensity of histone H2B (histone) as an internal standard (1.0). The genes were as follows: 1, *chsC* (chitin synthase C) (50); 2, *chsA* (chitin synthase A) (our unpublished data; DDBJ/ENBL/GenBank accession no. BAB85683); 3, *chsB* (chitin synthase B) (51); 4, *chsY* (chitin synthase Y) (9); 5, *chsZ* (chitin synthase Z) (9); 6, *gelA* (glycosylphosphatidylinositol-anchored glucanosyltransferase) (48); 7, *gelB* (glycosylphosphatidylinositol-anchored glucanosyltransferases) (48); and 8, *chsC* (β1-3 glucan synthesis) (34).

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suppressed the temperature sensitivity attributed to the \textit{mpk1} mutation, suggesting that both \textit{mpkA} and \textit{mpkA}\textit{mh} functionally complement the yeast \textit{MPK1} (data not shown). To demonstrate whether MpkA is phosphorylated in the \textit{ΔkexB} strain, we examined the phosphorylation levels of MpkA and MpkAmh expressed in \textit{ΔkexB} cells grown on the CD agar plates. We detected a significant increase in the phosphorylation levels of both MpkA and MpkAmh in the \textit{A. oryzae} \textit{ΔkexB} cells grown for 105 h on the CD agar plates by using anti-phospho-p44/42 MAP kinase antibodies (Fig. 6A, lanes 1 and 2), although MpkA in the wild-type strain was scarcely phosphorylated, regardless of osmotic stress (lanes 3, 4, 7, and 8). The phosphorylation levels of MpkA and MpkAmh in the \textit{ΔkexB} strain were apparently downregulated by high osmotic stress (lanes 5 and 6), with concomitant downregulation of the transcription levels of \textit{mpkA} (Fig. 5C). Phosphorylation of MpkA was observed when the wild-type cells were subjected to hypotonic stress (Fig. 6A, lanes 9 and 10), indicating that MpkA is capable of transducing the same types of stress signals through the putative cell integrity pathway as well as that of \textit{S. cerevisiae}. The \textit{ΔkexB}-\textit{mpkA}\textit{mh} and \textit{wt}-\textit{mpkA}\textit{mh} strains used as controls of expression quantity of MpkA showed the same phenotypes as the \textit{ΔkexB} and the wild-type strains on the CD agar plate culture, respectively. Furthermore, the phosphorylation levels of MpkAmh and the authentic MpkA in the \textit{ΔkexB-mpkA}\textit{mh} cells significantly increased at the three culture time points examined whereas MpkA and its derivative in the \textit{wt-mpkA}\textit{mh} strain were poorly phosphorylated (Fig. 6B). The phosphorylation levels of MpkAmh and the authentic MpkA in the \textit{ΔkexB-mpkA}\textit{mh} strains at 70 and 105 h of cultivation were higher than those at 50 h. Although the phosphorylation levels of MpkAs at each growth stage

\begin{itemize}
\item \textbf{FIG. 5.} Gene expression analysis of \textit{chsC}, \textit{chsB}, \textit{gelA}, \textit{gelB}, and \textit{mpkA} by Northern blotting. (A) Time course of the phenotypic change of the \textit{ΔkexB} and wild-type strains on CD agar plates. The left, middle, and right panels show colonies cultivated for 50, 70, and 105 h, respectively. (B) Gene expression analysis of \textit{chsC}, \textit{chsB}, \textit{gelA}, \textit{gelB}, and \textit{mpkA} over time (50, 70, and 105 h) by Northern blotting. A histone gene was used as a control. \textit{wt}, wild type. (C) Gene expression analysis of \textit{mpkA} on CD agar plates plus 0.8 M NaCl for 105 h by Northern blotting. A histone gene was used as a control.
\end{itemize}
were different in the \( \Delta \text{kexB-never} \text{mpkA} \text{mh} \) cells, the MpkA in the \( \Delta \text{kexB-mpkA} \text{mh} \) were remarkably phosphorylated at all time points after inoculation, suggesting that the KexB defect in \( \text{A. oryzae} \) causes constitutive activation of the cell integrity pathway.

**DISCUSSION**

In the present study, we cloned the \( \text{A. oryzae} \text{ kexB} \) gene, which encodes 836 amino acid residues and confirmed that the encoded protein has processing activity (Table 1). To study the \( \text{kexB} \) function, we constructed a \( \text{kexB} \) gene disruptant strain (\( \Delta \text{kexB} \); Fig. 1). The \( \Delta \text{kexB} \) strain showed shrunken colonies with poor generation of conidia on CD agar plates (Fig. 2), and hyperbranched mycelia occurred in liquid culture. Interestingly, the morphological defects derived from the \( \Delta \text{kexB} \) gene- type were restored under conditions of high osmotic stress (Fig. 3).

In \( \text{A. oryzae} \text{ kexB} \), the transcription levels of genes for cell wall biogenesis and an mpkA homolog that presumably encodes a MAP kinase involved in cell integrity signaling were higher than those in the wild type (Table 2; Fig. 4 and 5), and high osmotic pressure downregulated the transcription levels of \( \text{A. oryzae mpkA} \) and the cell wall-related genes in the \( \Delta \text{kexB} \) strain to levels similar to those in the wild type (Fig. 4 and 5). Then we cloned the \( \text{A. oryzae mpkA} \) gene and confirmed that its expression suppressed the temperature sensitivity of the \( \text{S. cerevisiae mpkl} \) disruptant (data not shown), suggesting the in vivo functionality of \( \text{A. oryzae MpKA} \). As expected, constitutively elevated phosphorylation levels of MpkA in \( \Delta \text{kexB} \) cells on the CD agar plate culture were demonstrated by using anti-phospho-p44/42 MAP kinase antibodies, and high osmotic stress downregulated the increased phosphorylation levels of MpkA in the \( \Delta \text{kexB} \) strain to the same as those observed in the wild type (Fig. 6). These results suggest that the upregulation of transcription levels of mpkA and cell wall-related genes in the \( \Delta \text{kexB} \) strain is mediated by phosphorylated MpkA as an active form through cell integrity signaling.

The phenotypes of \( \text{A. oryzae} \text{ kexB} \) are different from those of \( \text{kexB} \) disruptants of \( \text{A. niger} \) (29, 60) and \( \text{A. nidulans} \) (K. Furukawa, O. Mizutani, T. Fujioka, Y. Yamagata, K. Abe, K. Gomi, and T. Nakajima, unpublished data). The \( \text{A. niger kexB} \) disruptant formed conidiophores and conidia on agar plates (29). The \( \text{A. nidulans} \text{ kexB} \) strain showed shrunken colonies on agar plates but had differentiated conidiophores and conidia. According to the phenotypes of the \( \Delta \text{kexB} \) strains of these three \( \text{Aspergillus} \) species, the function of the \( \text{kexB} \) gene in \( \text{A. oryzae} \) is probably more essential for cell growth and especially for cell wall biogenesis. The differences of the \( \Delta \text{kexB} \) phenotypes among these three \( \text{Aspergillus} \) species may be attributable to the processing targets of KexB in each species but not to the substrate specificity of each KexB protein, because the substrate specificity of the \( \text{A. oryzae} \text{ kexB} \) product was similar to those of \( \text{A. niger} \) (29) and \( \text{A. nidulans} \) (39). Moreover, it is noteworthy that the various morphological phenotypes derived from the \( \Delta \text{kexB} \) genotype in \( \text{A. oryzae} \) were restored under high osmotic stress in both solid and liquid culture. However, the number of conidia in the \( \Delta \text{kexB} \) strain was 70% of that in the wild type even under high osmotic pressure, suggesting that osmotic suppression of \( \Delta \text{kexB} \) phenotypes is incomplete. Although high osmotic pressure did not suppress the phenotypes of \( \text{kexB} \) gene disruptants of \( \text{S. cerevisiae} \) or \( \text{C. albicans} \) (36, 56), the osmotic restoration of \( \Delta \text{kexB} \) phenotypes occurs in \( \text{A. oryzae} \) and \( \text{A. nidulans} \) (Furukawa et al., unpublished). It remains unknown whether the \( \Delta \text{kexB} \) phenotypes of \( \text{A. niger} \) are suppressed under high osmotic conditions.

In our comprehensive and comparative analysis of gene expression between the \( \text{A. oryzae} \text{ kexB} \) and wild-type strains by using cDNA microarrays, the transcription levels of a large number of genes were higher in the \( \Delta \text{kexB} \) strain than in the wild type on CD agar plates (Table 2). These results imply that disruption of the \( \text{kexB} \) gene affects the transcription levels of a broad range of genes and that KexB of \( \text{A. oryzae} \) probably processes key proteins required for maintenance of normal morphogenesis and cell growth. In addition, we found that the expression levels of \( \text{chsC, chsB,} \) and \( \text{gelB} \) were markedly higher in the \( \text{A. oryzae} \text{ kexB} \) strain than in the wild type (Fig. 4).
Cell wall biogenesis in *S. cerevisiae* is thought to be under the control of various signal transduction pathways including the cell integrity pathway (26). Cell integrity signaling is activated, with different timing and kinetics, by hypoosmotic shock (11, 32), by heat shock (32), during bud emergence (72), on exposure to mating pheromone (6, 72), and on various treatments leading to perturbation of the cell wall (4, 35, 67). The pathway organizes changes in cellular morphology by controlling the expression of genes encoding enzymes involved in cell wall metabolism. The central pathway concerned with cell integrity is the Mpk1p MAP kinase cascade, and Mpk1p phosphorylates and consequently activates the transcription factor Rlm1p, whose transcriptional targets are genes encoding cell wall-biogenesis proteins, cell wall proteins, and Mpk1p itself (13, 31, 68). Northern blot analyses of cell wall-related genes and *mpkA* revealed that transcription levels of the examined genes were simultaneously upregulated in *A. oryzae* genome sequences (O. Mizutani, K. Abe, and T. Nakajima, unpublished data). The cell integrity pathway in the ΔkexB strain is constitutively activated (A), although the pathway is not activated (resting) unless the wild-type strain senses some stress such as hypoosmolarity (B). *A. oryzae* KexB is predicted to be required for precise proteolytic processing of sensor proteins in the cell integrity pathway and/or of cell wall-related enzymes whose genes are under transcriptional control by the pathway.

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We propose the following two scenarios to explain why and how cell integrity signaling is activated in the *A. oryzae* ΔkexB strain. In the first scenario, if KexB processes enzymes required for cell wall biogenesis, then the ΔkexB strain fails to process these enzymes, resulting in perturbation of cell wall assembly. Various sensor proteins might detect perturbation of the cell wall as a stressor in *A. oryzae* ΔkexB and consequently activate the cell integrity pathway via G-protein reactions (26). Putative ORFs homologous to the yeast sensors occur among *A. oryzae* genome sequences (O. Mizutani, K. Abe, and T. Nakajima, unpublished data). The cell integrity pathway in the ΔkexB strain is constitutively activated (A), although the pathway is not activated (resting) unless the wild-type strain senses some stress such as hypoosmolarity (B). *A. oryzae* KexB is predicted to be required for precise proteolytic processing of sensor proteins in the cell integrity pathway and/or of cell wall-related enzymes whose genes are under transcriptional control by the pathway.

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related genes. The morphological defects of the \( \Delta kexB \) strain might be caused by upregulation of cell wall-related genes through activation of cell integrity signaling. A bioinformatics approach using the \( C. albicans \) genome database assigned 147 ORFs as putative Kex2p substrates, and these ORFs included a gene encoding a Wsc2p homologue that seems to be a sensor protein in the cell integrity pathway (56). Applying this analogy to \( A. oryzae \), the sensor/signal-like proteins of the pathway in \( A. oryzae \) might be processed by KexB.

Although KexB may process cell wall-related enzymes and/or cell surface sensors (Fig. 7), we currently prefer the first scenario in light of the following predictions. Although the yeast KEX2 disruptant (\( kex2\Delta \)) has no noteworthy morphological phenotype, the Pkc1-Mpk1 pathway is known to be activated in the \( kex2\Delta \) strain, resulting in perturbation of cell wall structure (62). Consequently, additional defects of \( A. oryzae \) synthetic lethality suggests that the perturbation of cell wall-related genes. The morphological defects of the \( kex2\Delta \) strain might be upregulated by the influence of another signal transduction pathway.

The \( kex2\Delta \) \( mpk1\Delta \) phenotype of \( S. cerevisiae \) is rescued by growth on high-osmolality medium (62). The phenotype of the \( A. oryzae \) \( kexB \) strain also was restored under high osmotic pressure (Fig. 3). We propose the following two explanations for why the phenotypes of the \( \Delta kexB \) strain were suppressed under high osmotic pressure. First, instead of KexB, perhaps other processing proteases inducible under high osmotic pressure suppress the \( \Delta kexB \) mutation, YPS1 and YPS2, which encode aspartyl proteases, are multicopy suppressors of the \( \Delta kex2 \) mutation in \( S. cerevisiae \) (14, 36). The expression level of YPS1 in \( S. cerevisiae \) cells treated for 10 min with 0.4 M NaCl is 15.7 times higher than that in cells without the NaCl treatment (58). We found two genes homologous to YPS1 and YPS2 in \( A. oryzae \) (38), and NaCl may induce these homologs and suppress the \( \Delta kexB \) mutation. Second, the restoration of the \( \Delta kexB \) phenotypes might depend on mechanisms mediated by osmotic stress, such as a cascade similar to the yeast Hog1 MAP kinase pathway (22, 26, 33, 59). The cell integrity pathway in \( S. cerevisiae \) is thought to be negatively regulated by osmotic pressure (11, 25, 26), and activation of the Hog1p pathway mediates adaptive rearrangements in cell wall composition and architecture in \( S. cerevisiae \) and \( C. albicans \) (1, 18). A similar scenario might explain the osmotic restoration of the \( \Delta kexB \) phenotypes in \( A. oryzae \). To verify restoration of the altered phenotypes by increased osmotic pressure in \( \Delta kexB \), in vivo functional studies, such as exploration of suppressors of the \( \Delta kexB \) mutation or construction of a mutant in which the HOG pathway is constitutively activated in the \( \Delta kexB \) genetic background, are necessary and in progress.

In conclusion, we predict that \( A. oryzae \) KexB is required for precise proteolytic processing of sensor proteins in the cell integrity pathway or of cell wall-related enzymes whose genes are under transcriptional control by the pathway. Thus, the KexB defect leads to disordered cell integrity signaling.

ACKNOWLEDGMENTS

We thank Katsuya Gomi and Hiroyuki Horiiuchi for helpful suggestions. We also thank Osumi Hatamoto, Hiroshi Maeda, Junichi Marmure, Taruji Satou, Tatsumitsu Maruyama, Yoshikihiko Matsuoka, Kanako Suzuki, Motoaki Sano, and Masayuki Machida for helpful discussions and/or technical assistance.

This work was supported in part by a Grant-in-Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan (BDP-03-VI-1-7).

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