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Functional analysis of Mpk1-mediated cell wall integrity signaling pathway in the thermotolerant methylotrophic yeast *Hansenula polymorpha*[§]

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Understanding the characteristics and regulation mechanisms of cell wall integrity (CWI) in yeast is important not only for basic research but also in biotechnological applications. We found significantly different CWIs in two representative strains of the thermotolerant methylotrophic yeast *Hansenula polymorpha*. Compared to the A16 strain (classified as *Ogataea polymorpha*), the DL1-L strain (classified as *Ogataea parapolymorpha*) has a thinner cell wall that was found to be more fragile following long-term cultivation and more sensitive to zymolyase. To gain a deeper insight into this difference, we compared the characteristics of the Mpk1p-mediated CWI signaling pathway in the two strains. While a DL1-L mutant deficient in Mpk1p (*mpk1*Δ) showed severe growth retardation at both normal and high growth temperatures and in the presence of cell-wall disrupting agents, the A16 *mpk1*Δ mutant displayed only a mild defect in cell growth. Sorbitol effect on rescuing growth retardation was different in the two *mpk1*Δ strains, which could partly be ascribed to subtle differences in the activation of HOG pathway. Among the cell wall disruptors evaluated, only caffeine clearly increased phosphorylation of Mpk1p in DL1-L, but not in A16. A transcriptome analysis of the DL1-L strain revealed that caffeine significantly increased the expression of a subset of cell-wall related genes in an Mpk1p-dependent manner, but not the expected Rlm1-target genes. Taken together, our data support an essential role for Mpk1p in maintaining CWI in *H. polymorpha*, although the requirement for Mpk1p and its regulation under diverse stress conditions varies depending on the strain background.

Keywords: *Hansenula polymorpha*, cell wall integrity, Mpk1, Hog1, caffeine

Introduction

The yeast cell wall is a glyco-structure containing polysaccharides and numerous glycoproteins (Lesage and Bussey, 2006), which play a role as a barrier for protein secretion, as well as for protection against environmental stresses (Mora-Montes, 2013). Proper maintenance of cell wall integrity (CWI) is important for cell viability and cell division. In yeast, the CWI signaling pathway monitors and modulates the organization and biosynthesis of the cell wall (Dichtl *et al.*, 2012). In baker's yeast *Saccharomyces cerevisiae*, alterations in cell wall architecture and environmental signals are detected by plasma membrane sensors (Levin, 2005). To initiate intracellular signal transduction, the sensors interact with the guanine nucleotide exchange factor Rom2 that acts on the small GTPase Rho1, which then binds to and activates protein kinase C (Pkc1p) to initiate a kinase cascade. Pkc1p mediated phosphorylation of the mitogen-activated protein kinase (MAPK) kinase kinase, Bck1p, activates two redundant MAPK kinases, Mkk1p and Mkk2p, which then phosphorylate MAPK Slt2p/Mpk1p (Martin *et al.*, 2000; Levin, 2005). Activated Mpk1p then phosphorylates the transcription factors, Rlm1 and SBF (Swi4/Swi6), leading to the alteration in the expression of genes involved in cell wall remodeling and G1-specific transcription, respectively (Jung *et al.*, 2002; Bermejo *et al.*, 2008; Levin, 2011). The role of Mpk1p MAPK in maintaining CWI is highly conserved in several yeast and filamentous fungal species, although there are some slight differences (Heilmann *et al.*, 2013; Delgado-Silva *et al.*, 2014; Donlin *et al.*, 2014; Valiante *et al.*, 2015).

Activation of CWI signaling is not restricted to a solitary stimulus, but can be induced by a number of events (Fuchs and Mylonakis, 2009). In *S. cerevisiae,* CWI signaling has been shown to be activated by a variety of chemicals that interfere with cell wall biosynthesis, such as glucan synthase inhibitors, calcofluor white, and congo red, by ER stress inducers, such as tunicamycin and dithiothreitol, and by heat shock (Chen *et al.*, 2005; Fuchs and Mylonakis, 2009). Osmoregulation is also associated with CWI signaling, in that rapid changes in the extracellular water activity can cause cell shrinkage or cell swelling, generating movement of the plasma membrane, as well as of the cell wall (Hohmann *et al.*, 2007). In *S. cerevisiae*, the osmoregulatory system is partly controlled by another MAPK cascade Cdc42-Ste20-Ste11-Pbs2-Hog1,

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Table 1. List of *H. polymorpha* strains used in this study

Strain	Description	Reference
DL-1	wild-type (NRRL-Y-7560, ATCC26012)	Levine and Cooney (1973)
DL1-L	a *leu2* derivative of DL-1	Kang *et al.* (2002)
CBS4732	wild-type (CCY38-22-2, ATCC34438, NRRL-Y-5445)	De Morais and Maia (1959)
A16	a *leu2* derivative of CBS4732	Veale *et al.* (1992)
DL1-L+*LEU*	*leu2* [*LEU2*]	This study
A16+*LEU*	*leu2* [*LEU2*]	This study
DL1-L *mpk1Δ*	*leu2 mpk1*::*LEU2*	This study
A16 *mpk1Δ*	*leu2 mpk1*::*LEU2*	This study
DL1 *mpk1Δ*[*MPK1*]	*leu2 mpk1*::*LEU2* [pHINZL-HpMPK1u]	This study
A16 *mpk1Δ*[*MPK1*]	*leu2 mpk1*::*LEU2* [A16pHINZL-HpMPK1u]	This study
DL1-L[*MPK1F*]	*leu2* [pHINZL-HpMPK1F]	This study
A16[*MPK1F*]	*leu2* [A16pHINZ-HpMPK1F]	This study

termed the high osmolarity glycerol (HOG) pathway (Saito and Posas, 2012).

Hansenula polymorpha is a thermotolerant methylotrophic yeast that can utilize methanol as the sole energy and carbon source and can grow at temperatures up to 48°C (Gellissen *et al.*, 2005; van der Klei *et al.*, 2006). It is a useful model organism that has been used to study the mechanisms of peroxisomal biogenesis and degradation, the regulation of methanol metabolism, nitrate assimilation, and the stress response (Ishchuk *et al.*, 2009). *H. polymorpha* has been also used as a host for the production of various heterologous proteins (Kim *et al.*, 2015). Based on its ability to grow at temperature of up to 48°C and to ferment xylose, *H. polymorpha* has recently gained attention as a good candidate strain that can be used for simultaneous saccharification and fermentation (Ishchuk *et al.*, 2009; Dmitruk and Sibirnyi, 2013). To date, three representative *H. polymorpha* strains of independent origin, DL-1 (synonymous with ATCC26012, NRRL-Y-7560) (Levine and Cooney, 1973), CBS4732 (synonymous with ATCC34438, NRRL-Y-5445, CCY38–22–2) (De Morais and Maia, 1959) and NCYC495 (synonymous with CBS1976, ATCC14754, NRLL-Y-1798, VKM-Y-1397) (Wickerham, 1951), are widely used for basic and applied research.

The CBS4732 and NCYC495 strains have been recently reclassified as *Ogataea polymorpha*, while the DL-1 strain has been assigned to *Ogataea parapolymorpha* (Kurtzman and Robnett, 2010; Suh and Zhou, 2010; Kurtzman, 2011). The full genomes of *O. polymorpha* CBS4732 (Ramezani-Rad *et al.*, 2003), NCYC495 (Riley *et al.*, 2016), *O. parapolymorpha* DL-1 (Ravin *et al.*, 2013) have been sequenced, revealing that the *O. polymorpha* and *O. parapolymorpha* genomes are approximately 10% divergent over their whole genome sequence. The sequence identities of the open reading frames for selected genes range between 94.5 and 97.2%, with an average value of 96.6% (Kunze *et al.*, 2009). The sequence differences are observed to be much more significant at the 5′- and 3′-untranslated regions, and so might be involved in controlling gene expression. Although these *H. polymorpha* strains have been previously unresolved based on the basic features of fermentation and growth reactions, which are typically used for the identification of yeast species, a few notable differences in their growth phenotypes, such as different levels of resistance to orthovanadate (Kim *et al.*, 2002) and distinct consumption rates for glycerol and methanol (Kim *et al.*, 2004), have been observed. Here, we have carried out a comparative analysis of CWI signaling pathway in two representative *H. polymorpha* strains, *O. parapolymorpha* DL1-L (a *leu2* derivative of DL-1) and *O. polymorpha* A16 (a *leu2* derivative of CBS4732), focusing on an analysis of mutant strains lacking the Mpk1p MAPK, and demonstrate that there is a con-

Table 2. List of plasmids used in this study

Plasmid	Description	Reference
pTHpLEU2-NS(c)	pGEM-T-based vector containing the *HpLEU2* gene	Kim *et al.* (2013)
pHIGAZ-6HA	pUC-based vector containing a zeocin marker, the *HpGAP* promoter, and six copies of hemagglutinin (HA) tag	Kim *et al.* (2013)
pTHpMPK1DL	pGEM-T-based vector containing the DL1*mpk1*::*HpLEU2* disruption cassette	This study
pHINZ-HpMPK1H	pUC-based vector containing HA-tagged *MPK1* of DL1-L strain under its native promoter with a zeocin marker	This study
pHINZL-HpMPK1H	pUC-based vector containing HA-tagged *MPK1* of DL1-L strain under its native promoter with zeocin and *HpLEU2* markers	This study
pHINZL-HpMPK1u	pUC-based vector containing *MPK1* from the DL1-L strain under its native promoter with zeocin and *HpLEU2* markers	This study
A16pHINZL-HpMPK1u	pUC-based vector containing *MPK1* from the A16 strain under its native promoter with zeocin and *HpLEU2* markers	This study
pHINZL-HpMPK1F	pUC-based vector for expression of FLAG-tagged *MPK1* from the DL1-L strain with zeocin and *HpLEU2* markers	Kim *et al.* (2013)
A16pHINZL-HpMPK1F	pUC-based vector for expression of FLAG-tagged *MPK1* from the A16 strain with zeocin and *HpLEU2* markers	This study

siderable difference in the level of resistance to cell wall stressors and activation of Mpk1p under various stress conditions, although Mpk1p is essential for maintaining CWI in *H. polymorpha*.

Materials and Methods

Yeast strains, plasmids, and culture conditions

The *H. polymorpha* strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Yeast cells were grown at 37°C in yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% Bacto Peptone, and 2% glucose). The transformation of *H. polymorpha* was performed according to the modified lithium acetate-dimethyl sulfoxide (DMSO) method (Hill *et al.*, 1991). A selective synthetic complete (SC) media (0.67% yeast nitrogen base [YNB] without amino acids, 2% glucose, 0.77 g/L drop-out supplement without leucine [TaKaRa Bio]), or YPD media containing 80 or 100 μg/ml zeocin (Invitrogen), or 100 or 200 μg/ml hygromycin B (Sigma) were used for the selection of various yeast transformants.

Gene disruption and complementation of *HpMPK1*

To construct the *HpMPK1* disruption in *H. polymorpha* DL1-L and A16 strains, the N- and C-terminal fragments of *HpMPK1* were PCR-amplified with two pairs of primers, HpMPK1D_1F (5′-ATGGACCAGTATCACGTC-3′)/HpMPK1D_2B (5′-AGACTCGAGTGCTAGCTCAGAAAGC CCTGGTTCT-3′) and HpMPK1D_3F (5′-TGAGCTAGC ACTCGAGTCTGTGGGGTGTATCTTG-3′)/HpMPK1D_ 4B (5′-TCGTAGAGCATTTCCTGC-3′), respectively, using *H. polymorpha* DL1-L genomic DNA as the template, respectively. Next, using fusion PCR with the primers HpMPK1D_ 1F and HpMPK1D_ 4B, the N-terminal fragment was combined with the C-terminal fragment of *HpMPK1*. The fused N-/C-terminal fragment of *HpMPK1* was sub-cloned into pGEM-T easy vector (Promega) to generate pTHpMPK1D. The *HpLEU2* gene fragment was excised from pTHpLEU2-NS(c) (Kim *et al.*, 2013) using *Nhe*I and *Spe*I, and inserted into pTHpMPK1D digested with *Nhe*I, generating pTHpMPK1DL. The *HpMpk1*Δ::*HpLEU2* cassette, obtained from the plasmid pTHpMPK1DL by *Not*I digestion, was introduced into the *H. polymorpha* DL1-L and A16 strains, respectively, to replace the genomic *HpMPK1* gene by double homologous recombination (Supplementary data Fig. S1A).

To generate the DL1-L *mpk1*Δ strain complemented with the wild-type *MPK1* gene (DL1 *mpk1Δ*/*MPK1*), the 1.33 kb *HpMPK1* gene fragment containing its 0.54 kb promoter but lacking the stop codon, was amplified by PCR from DL1-L genomic DNA using the primers *Psi*I*Not*I-HpMPK1F (5′-ATCCCTTATAAGCGGCCGCAGAGCTTGCCGACAAG AA-3′) and HpMPK1-*Eco*RI_R (5′-CCGAATTCCTGGTA CTGGAAGTTGTAG-3′). The PCR product was digested with *Psi*I/*Eco*RI and ligated into *Hpa*I/ *Eco*RI-digested pHIGAZ-6HA (Kim *et al.*, 2013), generating a HA-tagged *HpMPK1* vector containing its native promoter, pHINZ-HpMPK1H. The *HpLEU2* gene was excised from pTHpLEU2-NS(c) by *Not*I, and inserted into the *Not*I site of pHINZ-HpMPK1H generating pHINZL-HpMPK1H. To construct an expression vector for untagged HpMpk1 protein, the PCR fragment containing the C-terminus with the stop codon of the *HpMPK1* gene was obtained from *H. polymorpha* DL1-L genomic DNA using the primer pair HpMPK1D_3F and HpMPK1R-stop-*Afl*II (5′-TATCTTAAGTCACTGGTACTGGAAGTTG-3′). The PCR product was cloned as a *Sph*I/*Afl*II fragment into pHINZL-HpMPK1H, generating pHINZL-HpMPK1u, which was cut at the *Hpa*I site within the *HpMPK1* promoter, and integrated by homologous recombination into the genomic *HpMPK1* locus of the DL1 *mpk1*Δ strain, resulting in the DL1 *mpk1Δ*[*MPK1*] strain (Supplementary data Fig. S1B). To generate the *mpk1*Δ[*MPK1*] complemented strains of A16, each of the *HpMPK1* gene fragments containing its 0.54 kb promoter and a 1.33 kb ORF with the stop codon, was amplified by PCR from A16 genomic DNA, using the primers *Psi*I*Not*I-HpMPK1F and HpMPK1R-stop-*Afl*II. Each PCR product was cloned as a *Not*I/*Afl*II fragment into pHINZL-HpMPK1u, generating A16pHINZL-HpMPK1u. The *Hpa*I-digested A16pHINZL-HpMPK1u was integrated into the native *MPK1* locus of the A16 *mpk1*Δ strain, respectively, generating the A16 *mpk1*Δ[*MPK1*] complemented strain.

Construction of expression vectors and strains for FLAG-tagged HpMpk1p

Construction of the FLAG-tagged HpMPK1 vector expressing HpMpk1p from the DL1-L strain has been previously described (Kim *et al.*, 2013). To generate the FLAG-tagged HpMPK1 vector expressing the HpMpk1p from the A16 strain, the DNA fragment containing the 0.56 kb promoter, and the full length ORF without the stop codon of *HpMPK1* was PCR-amplified from the A16 genomic DNA, using the primers *Psi*I*Not*I-HpMPK1F and HpMPK1R-*Asc*I (5′-TAT GGCGCGCCCTGGTACTGGAAGTTGTAG-3′). The PCR product was digested with *Not*I/*Asc*I and ligated into *Not*I/*Asc*I-digested pHINZL-HpMPK1F (Kim *et al.*, 2013), generating A16pHINZL-HpMPK1F. Each plasmid was cut at the *Sph*I or the *Hpa*I site within the *HpMPK1* ORF for linearization and integrated by homologous recombination into the genomic *HpMPK1* locus of the DL1-L or A16 strain, respectively.

Western blot analysis of MAPK phosphorylation

To detect the expression of phospho-Mpk1p, the *H. polymorpha* strains were grown to an OD_{600} of 1.0 in YPD medium at 37°C, and then one-half of the cells was harvested, while the second half was treated with 20 mM caffeine, 0.2 mg/ml calcofluor white (CFW), 10 mg/ml congo red (CR), 1 M NaCl, or 1 M sorbitol for 2 h. The cells were lysed by vortexing with glass beads (425–600 μm in diameter, Sigma) in a phosphatase inhibitor lysis buffer. Supernatants were separated by centrifugation at 2,000 × *g* for 5 min at 4°C and analyzed by 10% SDS-PAGE. The anti-phospho-p44/p42 MAPK and anti-phospho-p38 MAPK antibodies (Cell Signaling Technology) were used to detect phosphorylated Mpk1 and phospho-Hog1 proteins, respectively, and an anti-FLAG antibody (Sigma) and an anti-ScHog1 antibody (Santa Cruz Biotechnology) were used to monitor the level of Mpk1 and

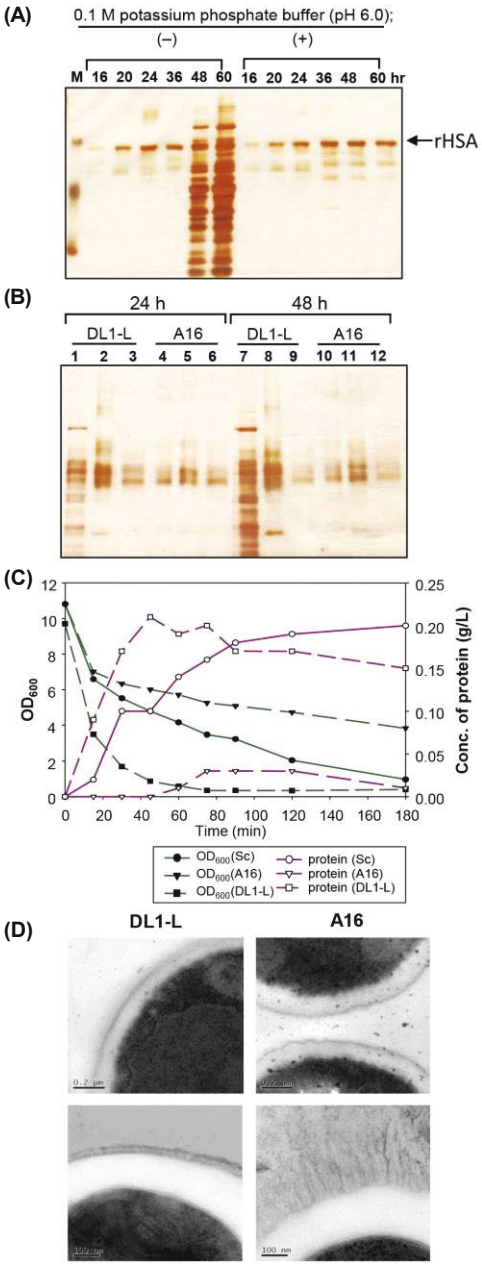

Fig. 1. Comparative cell wall integrity and structural analysis of DL1-L and A16 strains. (A) pH-dependent autolytic phenotype of the DL1-L strain. The *H. polymorpha* DL1-L strain producing recombinant human serum albumin (rHSA) was cultivated in YCG medium (1% yeast extract, 2% casamino acids, 2% glucose) buffered without or with 0.1 M potassium phosphate (pH 6.0) for the indicated times, and the culture supernatants were separated by SDS-PAGE and silver stained. The band representing rHSA is indicated. (B) Silver staining of the culture supernatants from DL1-L and A16 strains cultured in YPD (lanes 1, 4, 7, and 10), YPD + 1 M sorbitol (lanes 2, 5, 8, and 11), and YPD + 0.5 M KCl (lanes 3, 6, 9, and 12). (C) Comparison of the sensitivity to zymolyase. The yeast cell density OD_{600} and protein concentrations were measured at the indicated incubation times over the period 0–180 min. *Saccharomyces cerevisiae* Y2805 (Sc) was also analyzed for comparison. (D) Transmission electron micrographs of the cell surface of DL1-L and A16 cells. The *H. polymorpha* cells were cultured on YPD agar for 48 h and suspended in water. After fixation, the cell samples were applied to glow-discharged carbon-coated copper grids, and stained with 2% (w/v) uranyl acetate. The images were recorded with a Tecnai G2 Spirit Twin microscope (FEI) at an acceleration voltage of 120 kV.

Hog1 proteins, respectively. The anti-beta actin antibody (Abcam) was used to assess beta actin levels as a loading control.

Quantitative real-time PCR analysis (qRT-PCR) analysis

Total RNA from yeast cells was isolated using the hot phenol extraction method (Chen *et al.*, 2003). The RNA was treated with DNase I according to the manufacturer's instructions (Thermos Fisher Scientific), and cDNA was synthesized from 1 μg of total RNA using the M-MLV reverse transcriptase (Invitrogen) and oligo (dT) primers, according to the manufacturer's instructions. qRT-PCR was conducted using a CFX96 Real-Time PCR detection system (Bio-Rad) using 1 ng of the synthesized cDNA and gene-specific primers (Supplementary data Table S1) with the Maxima SYBR Green qPCR Master Mix (Fermentas). The specificity of the amplification was confirmed by melting curve analysis of a single peak. Each sample was analyzed in triplicate, and data were normalized to the endogenous control, *H. polymorpha ACT1* using the primers HpACT1F-RT/HpACT1B-RT.

RNA-Seq analysis

The *H. polymorpha* DL1-L strain was grown to an OD_{600} of 1.0 in YPD medium at 37°C, and then treated with 20 mM caffeine for 2 h. Total RNA was isolated using the hot phenol extraction method (Chen *et al.*, 2003). All subsequent procedures used for RNA sequencing were conducted by ChunLab. Genes with fold changes greater than 1.5-fold were functionally classified using the Munich Information Center for Protein Sequences (MIPS) FunCat (Ruepp *et al.*, 2004). Processed data were deposited in the Gene Expression Omnibus (GEO) database with accession number GSE106861.

Results

Differing characteristics in cell wall integrity and structure of *H. polymorpha* strains

While cultivating *H. polymorpha* recombinant strains harboring the human serum albumin expression vector pYHSA173, we observed that the *H. polymorpha* DL1-L strain, a derivative of the DL-1 strain (*O. parapolymorpha*), was more fragile than the A16 strain, a derivative of the CBS strain (*O. polymorpha*) (Heo *et al.*, 2003). The DL1-L strain showed a pH-dependent autolytic phenotype, which could be rescued by supplementing 1 M sorbitol or 0.5 M KCl into the culture medium (Fig. 1A and B). Moreover, the DL1-L strain displayed a higher sensitivity to zymolyase than the A16 strain (Fig. 1C). These observations led us to speculate that the two *H. polymorpha* strains might have distinct cell wall structures. In general, most filamentous fungi and yeast have a common cell wall structure, which is comprised of an inner layer of chitin and β-glucan matrix and an outer layer decorated with a dense coat of mannan fibrils (Klis *et al.*, 2006; Chaffin, 2008; Jin, 2012; Mora-Montes, 2013). Interestingly, electron microscopy images of the cell wall architecture of the two *H. polymorpha* strains revealed that the A16 strain has a slightly thicker inner layer (~100–150 nm) and a much longer fibrillar polysaccharide layer, aligned perpendicularly to inner

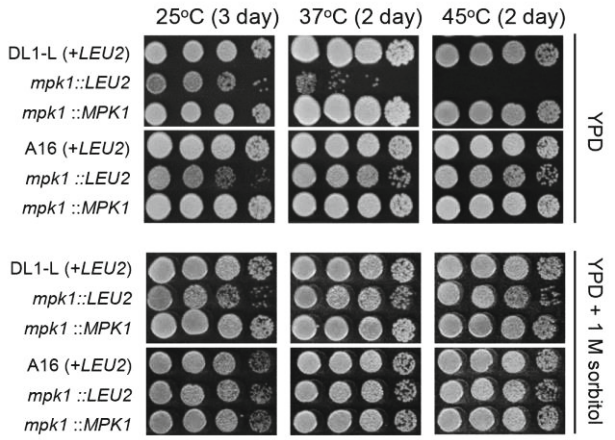

Fig. 2. Effect of temperature on the growth of *H. polymorpha mpk1*Δ mutants. A series of 10-fold dilutions of *H. polymorpha* strains were spotted on YPD plates without (top panel) or with 1 M sorbitol (bottom panel). Yeast cells were incubated at three different temperatures (25°C, 37°C, and 45°C) for the days indicated.

layer (~200 nm), than the DL1-L strain (inner layer, ~100 nm; outer layer, ~30 nm) (Fig. 1D).

Roles of Mpk1p in cell wall integrity under heat stress in *H. polymorpha*

Damage to cell wall or environmental changes that affect cell wall integrity (CWI) activates the MAP kinase pathway to repair or compensate for the cell wall defects (Popolo *et al.*, 2001). In an effort to better understand the molecular mechanisms involved in maintenance of the cell wall integrity of *H. polymorpha*, we investigated the role of *H. polymorpha* Mpk1p (HpMpk1p), a key MAP kinase in maintaining CWI, in responding to various challenges that result in cell wall damage. The amino acid sequences (442 aa) of the Mpk1 proteins in two *H. polymorpha* strains were almost identical, except for one amino acid, and shared 86% identity with *S. cerevisiae* Mpk1p (ScMpk1p) (Supplementary data Fig. S2). The Mpk1 protein, which has been extensively characterized in *S. cerevisiae* (Soler *et al.*, 1995; Kim *et al.*, 2007), contains an ATP binding site (GXGXXG) located at the N-terminus, the TEY motif containing its regulatory phosphorylation sites, and the DEP motif in the kinase domain at the C-terminus, which is then followed by a glutamine-rich region that is common among transcriptional activators (Titz *et al.*, 2006). These functional protein motifs in ScMpk1p were well conserved in the HpMpk1 proteins (Supplementary data Fig. S2), but it is notable that HpMpk1 proteins contain a significantly shorter stretch of consecutive glutamine residues, and lack 50 amino acids in the C-terminal region. It has previously been reported that the consecutive glutamine stretch was dispensable for ScMpk1p function, but lack of the C-terminus portion resulted in ScMpk1p nearly devoid of transcriptional induction (Kim *et al.*, 2007).

To explore the effects of *HpMPK1* deletion on cell wall integrity in the DL1-L and A16 strains, we constructed a *mpk1* null mutant (*mpk1*Δ) in each strain by replacing the *MPK1* gene with *LEU2*. The *LEU2* gene was also introduced into

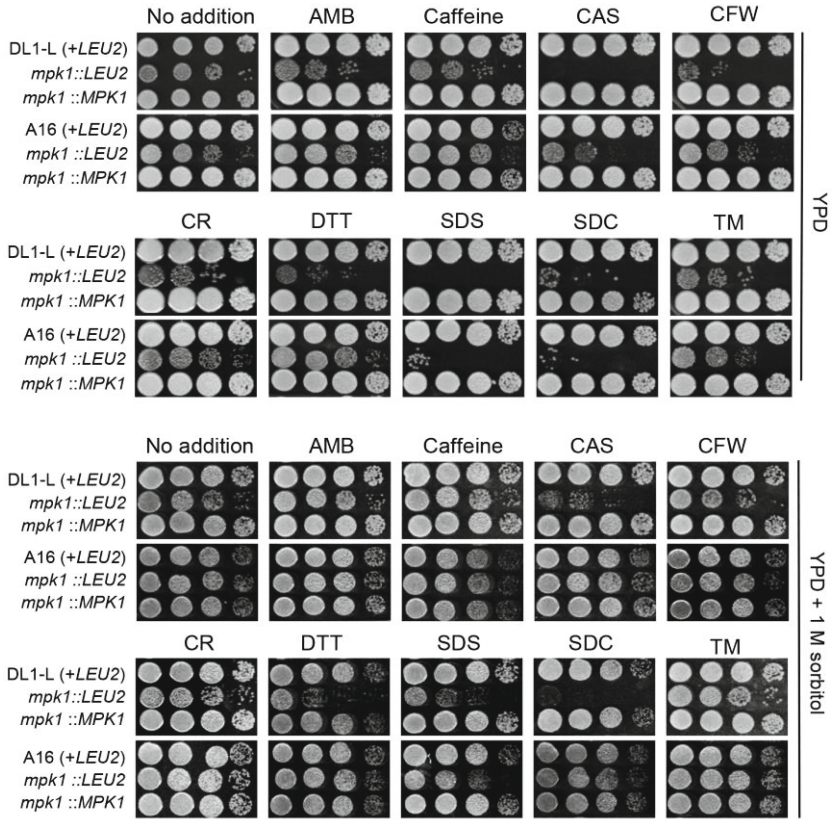

Fig. 3. Effect of cell wall disrupting agents on the growth of *H. polymorpha mpk1*Δ mutants. Yeast cells were spotted on YPD plates alone, or YPD plates supplemented with 0.6 μg/ml amphotericin B (AMB), 5 mM caffeine, 0.2 μg/ml caspofungin (CAS), 0.1 mg/ml calcofluor white (CFW), 1.5 mg/ml congo red (CR), 10 mM dithiothreitol (DTT), 0.002% sodium dodecyl sulfate (SDS), 0.01% sodium deoxycholate (SDC), or 30 ng/ml tunicamycin (TM), without or with 1 M sorbitol, respectively. Yeast cells were incubated for three days at 25°C.

each of the wild-type strains to complement for stress sensitivity caused by the leucine auxotroph (Cohen and Engelberg, 2007; Swinnen *et al.*, 2015). In *S. cerevisiae*, deletion of *MPK1* resulted in a temperature-dependent cell lysis defect, which could be suppressed in the presence of osmotic stabilizers (Lee *et al.*, 1993). To test whether HpMpk1p is involved in CWI signaling in response to heat stress in *H. polymorpha*, we grew the *mpk1*Δ mutants at low (25°C), normal (37°C), and high (45°C) temperatures. While the DLl-L *mpk1*Δ mutant displayed severe growth defects at 37°C and 45°C, the A16 *mpk1*Δ mutant showed only slightly decreased growth compared to the wild type strain at these temperatures (Fig. 2, Top panel), suggesting that in *H. polymorpha* Mpk1p plays a role in CWI signaling triggered by heat stress, but in a strain-specific way. Re-introduction of the respective *HpMPK1* into each of *mpk1*Δ mutant strains, in order to restore Mpk1 protein expression, completely rescued the temperature-related growth defects (Fig. 2). In both *mpk1*Δ mutants, the growth defect at high temperatures could be suppressed by the addition of the osmotic protector, 1 M sorbitol, suggesting that the temperature sensitive growth defects were mostly due to their inability to maintain cell wall integrity (Fig. 2, Bottom panel).

Roles of Mpk1p in resistance to cell wall disrupting agents in *H. polymorpha*

We also analyzed the growth phenotypes of the DL1-L and A16 *mpk1*Δ mutant strains in the presence of various cell wall disrupting agents at 25°C. Both *mpk1*Δ mutant strains were highly sensitive to cell wall destabilizers such as the fungal (1→3)-β-D-glucan synthesis inhibitor caspofungin (CAS), and the chitin binding agent calcofluor white (CFW), as well as to ionic detergents such as sodium dodecyl sulfate (SDS) and sodium deoxycholate (SDC). However it was notable that the DL1-L *mpk1*Δ strain generally displayed a higher sensitivity to these agents than the A16 *mpk1*Δ strain. While the DL1-L *mpk1*Δ mutant also showed moderately retarded growth in the presence of the antifungal amphotericin B (AMB), as well as the cell wall stressors caffeine and congo red (CR), the growth of the A16 *mpk1*Δ mutant was almost completely unaffected by these chemicals, except for a slight inhibition of growth in the presence of CAS (Fig. 3, Top panel). It is interesting that tunicamycin (TM) and dithiothreitol (DTT), two well-known ER stress inducers (Cheon *et al.*, 2011), affected the growth of the *mpk1*Δ mutant strains in different ways. The DL1-L *mpk1*Δ mutant grew very poorly in the presence of DTT, but the growth of the A16 *mpk1*Δ mutant was not inhibited by DTT. Both the DL1-L and A16 *mpk1*Δ mutants displayed mild sensitivity to TM. Next, we examined whether the sensitivity of the *mpk1*Δ mutant strains to the cell wall and ER stressors could be rescued by supplementation with an osmotic stabilizer, such as sorbitol. While 1 M sorbitol suppressed the growth defect of the A16 *mpk1*Δ mutant in media containing each agent, it only marginally rescued the sensitive phenotype of the DL1-L *mpk1*Δ strain for CAS, SDS, SDC, and DTT (Fig. 3, Bottom panel). Overall, these data indicate that the Mpk1 protein is required for *H. polymorpha* to survive when exposed to cell wall disrupting agents. Nevertheless, it is interesting that the requirement of Mpk1p to maintain CWI varies depending on the strain background.

Differential expression of HOG pathway genes in *H. polymorpha* strains

To investigate the reasons for the different effects of 1 M sorbitol on suppressing the growth defects in the DL1-L and A16 *mpk1*Δ strains, we examined the phosphorylation and activation of Hog1p in these *H. polymorpha* strains following treatment with 1 M NaCl or 1 M sorbitol (Fig. 4A). The Hog1-dependent signal pathway is responsible for responding to osmotic shock in fungal species (Hohmann *et al.*, 2007). While NaCl treatment resulted in the phosphorylation of Hog1p in both the DL1-L and A16 strains, sorbitol treatment induced only a slight degree of Hog1p phosphorylation only in the A16 strain. To confirm the differential activation of the HOG pathway by NaCl and sorbitol, we analyzed the transcript levels of several downstream candidate targets, which are known to be regulated by the HOG signaling pathway in *S. cerevisiae* (Hawle *et al.*, 2007; Hawley and Warburton, 2007; Katz *et al.*, 2007). It is clear that NaCl and sorbitol

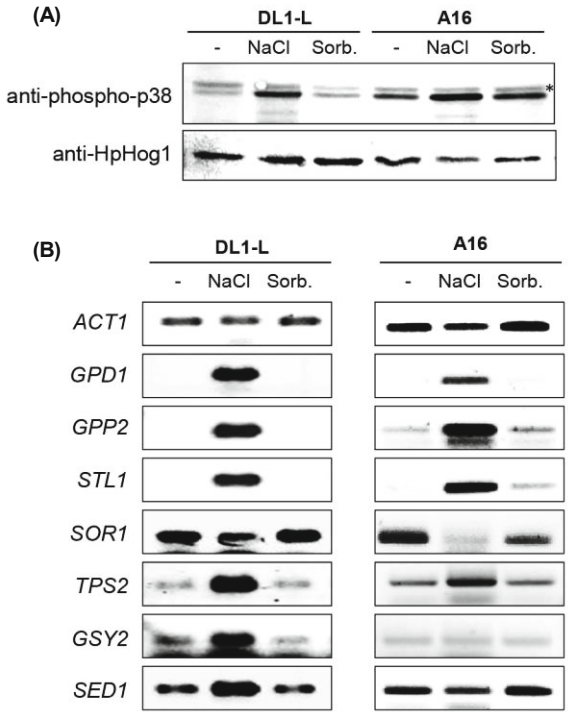

Fig. 4. Analysis of HOG pathway activation in *H. polymorpha* under osmotic stress conditions. (A) Phosphorylation analysis of Hog1 proteins in the *H. polymorpha* DL1-L and A16 strains. Yeast cells were grown to early exponential phase in YPD, and treated with 1 M NaCl or 1 M sorbitol for 2 h. Immunoblotting was conducted using an anti-phospho-p38 antibody to detect phospho-Hog1p, and with an anti-Hog1 antibody as a loading control. The asterisk indicates a non-specific band. (B) Semi-quantitative RT-PCR analysis of the HOG pathway genes in *H. polymorpha* DL1-L and A16 strains under osmotic stress conditions. Yeast cells were grown to mid-logarithmic phase (OD_{600} = 1.0) in YPD, and then treated with 1 M NaCl or sorbitol for 2 h. Total RNA was isolated and analyzed by semi quantitative RT-PCR using the gene-specific primer pairs (Supplementary data Table S1) under the following thermocycler conditions: 95°C, 2 min; 26 cycles of [95°C, 30 sec; 62°C, 30 sec; 72°C, 30 sec]; 72°C, 5 min. The *ACT1* cDNA was amplified as a loading control.

treatment induced distinct patterns of regulation for downstream target genes (Fig. 4B). Most of the genes tested in the DL1-L strain, including genes involved in glycerol synthesis (*GPD1* and *GPP2*), carbohydrate metabolism (*STL1*, *TPS2*, and *GSY2*), and cell wall structure (*SED1*), were clearly up-regulated by 1 M NaCl, but not at all by 1 M sorbitol. In the case of the A16 strain, induction of *GSY2* and *SED1* was not observed. Interestingly, the expression of the gene encoding sorbitol dehydrogenase (*SOR1*), associated with sorbitol biosynthesis and utilization, apparently rather down-regulated between the two *H. polymorpha* strains with different extent. In contrast to a slightly decreased expression level of *SOR1* by NaCl, but not by sorbitol, in the DL1-L strain, the *SOR1* expression was clearly down-regulated in the presence of NaCl or sorbitol in the A16 strain. These data suggest that the HOG pathways in the two *H. polymorpha* strains might also have distinctive regulatory networks in responding to different osmotic stressors, which might then subsequently affect the ability of the *mpk1*Δ strains to cope with hostile environments that disrupt cell wall integrity.

Differential activation of Mpk1p by cell wall stressors in the *H. polymorpha* strains

The Mpk1p MAP kinase-mediated CWI pathway is triggered in response to various chemicals and environmental cues that lead to cell wall damage (Levin, 2011). Although the requirements and molecular functions of the CWI signaling pathway for adaptation to environmental stresses are highly conserved in several yeasts and fungi, species-dependent differential responses also exist (Kraus *et al.*, 2003; Levin, 2005; Monge *et al.*, 2006; Valiante *et al.*, 2015). Furthermore, it is possible that the roles of CWI signaling in responding to cell wall stressors may be different in the same, or very closely related, species. Thus, in order to understand the reasons for the different sensitivities to various cell wall disrupting agents seen upon *MPK1* deletion in the DL1-L and A16 *mpk1*Δ strains, we examined the phosphorylation and activation of Mpk1p in these strains following treatment with the cell wall stressors, caffeine, CFW, and CR. The phosphorylation of Mpk1p at Thr190 and Tyr192, which indicates the degree of Mpk1p activation, was determined by western blotting using an anti-phospho-p44/p42 antibody. In order to obtain information on the change of Mpk1p expression at the protein level, along with the phosphorylation status, we employed the FLAG-tagged version of Mpk1p. As a result, we found that the phosphorylation level of the Mpk1 protein was significantly enhanced only upon exposure to caffeine in the DL1-L strain, which might be ascribed in part to the increased expression of the protein, as indicated by western blotting using an anti-Flag antibody (Fig. 5A). In addition, CFW treatment slightly increased the phosphorylation of HpMpk1p in the DL1-L strain, whereas it decreased HpMpk1p phosphorylation in the A16 strain. Interestingly, CR treatment drastically reduced the levels of phosphorylated HpMpk1p in the A16 strain (Fig. 5A). The apparent lack of effect of CFW and CR on Mpk1p phosphorylation in *H. polymorpha* is quite different from what is observed in *S. cerevisiae* (Hawle *et al.*, 2007; Tatjer *et al.*, 2016), indicating there are differences in the CWI signaling pathway between the two yeast species.

Transcriptome profiling of *H. polymorpha* DL1-L treated with caffeine

To investigate which genes are regulated in response to caffeine and led to the increased expression and phosphorylation of Mpk1p in the DL1-L strain, we carried out a transcriptome analysis of the DL1-L strain treated with caffeine. Total RNA isolated from cells cultivated with and without caffeine treatment were subjected to RNA-Seq analysis. Following treatment of the DL1-L strain with caffeine, a total of 2,434 genes showed a more than 1.5-fold change in gene expression level, including 1,311 induced genes and 1,123 re-

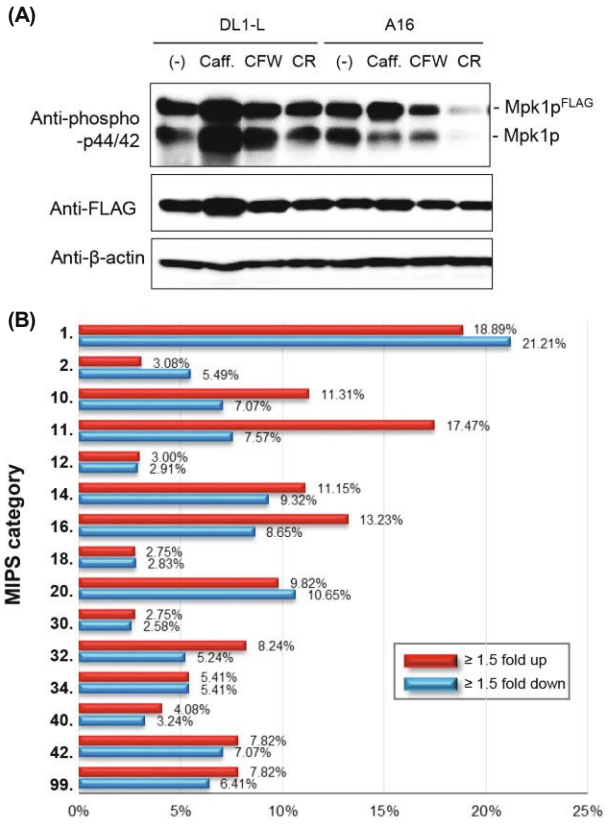

Fig. 5. Analysis of the Mpk1p-mediated signaling pathway in *H. polymorpha* in the presence of cell wall disruptors. (A) Analysis of Mpk1 kinase activation in response to cell wall disrupting agents in the DL1-L and A16 strains. Cell extracts from wild type *H. polymorpha* DL1-L and A16 strains expressing HpMpk1^{FLAG}, which had been grown to early exponential phase, and then treated with 20 mM caffeine, 0.2 mg/ml CFW, or 10 mg/ml CR for 2 h. The expression levels of HpMpk1p and phosphorylated HpMpk1p were analyzed by western blotting using the anti-FLAG and anti-phospho-p44/42 MAPK antibodies, respectively. Protein loading was normalized by measuring β-actin levels using an anti-β-actin antibody. (B) Transcriptome analysis of the *H. polymorpha* DL1-L strain with caffeine treatment. Genes showing changes in expression levels of 1.5-fold or more upon caffeine treatment were analyzed by GO enrichment, and grouped by the following MIPS category: 1, Metabolism; 2, Energy; 10, Cell cycle and DNA processing; 11, Transcription; 12, Protein synthesis; 14, Protein fate (folding, modification, destination); 16, Protein with binding function/cofactor requirement; 18, Regulation of metabolism and protein function; 20, Cellular transport, transport facilities and transport routes; 30, Cellular communication/signal transduction mechanism; 32, Cell rescue; defense and virulence; 34, Interaction with the environment; 40, Cell fate; 42, Biogenesis of cellular components; 99, Unclassified proteins.

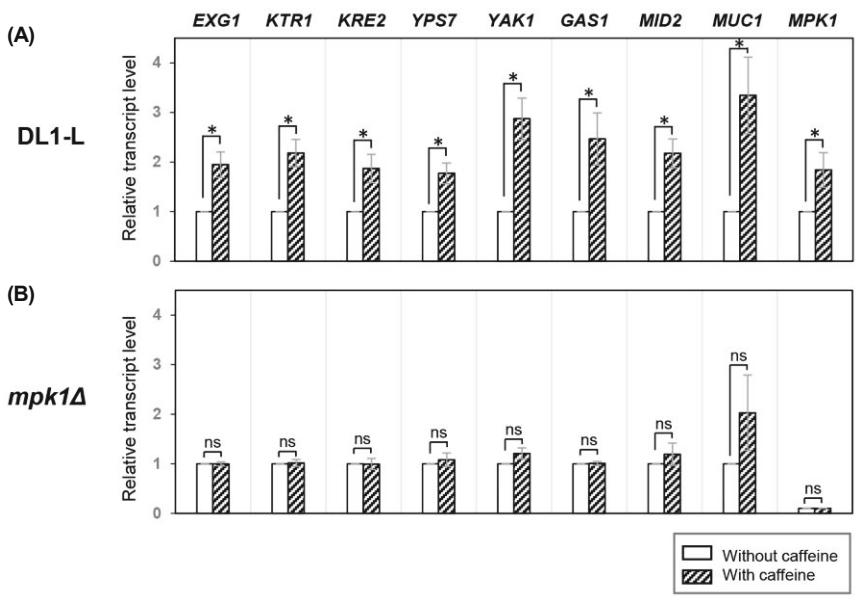

Fig. 6. Quantitative RT-PCR analysis of cell wall related genes in the wild-type (A) and *mpk1*Δ (B) strains of *H. polymorpha* DL1-L. Yeast cells were cultured to the mid-logarithmic phase, and then one-half of the culture was treated with 20 mM caffeine for 2 h. Total RNA was isolated and subjected to qRT-PCR analysis. The experiments were carried out in triple independent experiments with duplicated samples. The error bars with p values represent the standard deviation of triplicate measurements. The transcript levels, relative to *ACT1*, were calculated by the modified formula $2^{\text{mean Ct}}_{ACT1}/2^{\text{mean Ct target}}$. *$P < 0.05$, ns = not significant ($P > 0.05$).

pressed genes (Supplementary data Table S2). Initial functional classification of these differentially expressed genes, using gene ontology (GO) term assignments, revealed that the expression of several subsets of genes involved in the 'cell cycle and DNA processing', 'transcription', 'protein fate', 'protein with binding function', and 'cell rescue, defense and virulence', were apparently up-regulated under caffeine-treated conditions. On the other hand, the genes down-regulated by caffeine treatment were mostly involved in 'metabolism' and 'cellular transport' (Fig. 5B).

Among the genes up-regulated under caffeine-treated conditions, a subset of genes associated with cell wall biogenesis, such as the genes encoding β-1,3-glucanosyltransferase (*GAS1* and *GAS3*), β-1,3-glucanase (*BGL1* and *BGL2*), α-1,2-mannosyltransferase (*KTR1*, *KRE1*, and *KRE2*), GPI-anchored aspartic protease (*YPS1* and *YPS7*), chitin deacetylase (*CDA1* and *CDA2*), chitin synthase *CHS1*, and α-1,6-mannosyltransferase *HOC1*, were shown to increase their expression upon caffeine treatment. To validate the RNA-Seq data, we carried out an RT-PCR analysis of the cell wall-related genes *GAS1*, *EXG1*, *KTR1*, *KRE2*, *YPS7*, *MID2*, *MPK1*, the membrane peptidase gene *MUC1*, and the Ras/cAMP signal pathway gene *YAK1*. All of the genes examined were found to be up-regulated following caffeine treatment. In addition, with the exception of *MUC1* and *YAK1*, the expression levels of these genes in the DL1-L *mpk1*Δ mutant strain were similar regardless of caffeine treatment (Fig. 6). The RNA-Seq analysis strongly suggests that HpMpk1p has a role in controlling a number of cell-wall related genes. It is noteworthy that most of the cell wall-related genes induced in a Mpk1p-dependent manner in *H. polymorpha* following caffeine treatment are not the target genes for Rlm1, which has been reported to be the downstream transcription factor regulated by Mpk1p in the CWI signaling pathway in *S. cerevisiae* (Jung and Levin, 1999). This lack of induction of the putative Rlm1-regulated genes, such as the GPI proteins (*SED1* and *SSR1*), the PIR family proteins (*PIR1* and *PIR2*), and the cell wall biogenesis proteins (*GSC2*), was also confirmed by qRT-PCR analysis (Supplementary data Fig. S3).

Discussion

The yeast cell wall must be appropriately remodeled in order for the cell to divide and grow, especially under stress conditions. A weakening or a local loss of cell wall integrity can cause a deterioration in cell physiology, and even jeopardize cell survival. From a practical point of view, controlling and maintaining cell wall strength is often required during the long-term cultures used for the production of recombinant proteins and metabolites (Gellissen, 2006; Chou, 2007). On the other hand, thick, rigid cell walls are a major problem in the efficiency of downstream processes, such as protein isolation and purification. In such cases, the expression of genes involved in the CWI pathway, such as *PKC1* that initiates the kinase cascade in CWI signal transduction, has been down-regulated in order to develop a cell-wall mutant strain that is useful for recombinant protein production (Omara *et al.*, 2010).

In the present study, we carried out a comparative analysis of cell wall integrity using two representative strains of industrially important yeast namely, *H. polymorpha*, DL1-L (*O. parapolymorpha*) and A16 (*O. polymorpha*), focusing in particular on analyzing the null mutant strains deficient in Mpk1p. We found that the DL1-L wild type strain and its *mpk1*Δ mutant strain were generally more sensitive to cell wall disrupting agents than the A16 strain, which appeared to be due to differences in cell wall structure and the CWI signaling pathway. The DL1-L strain has a slightly thinner β-glucan and chitin layer decorated with very short mannan fibers, compared to the A16 strain (Fig. 1). It is possible that these structural differences in the cell wall result in the differing sensitivity to heat and cell wall disrupting agents, such as CAS, CFW, and CR, in the *mpk1*Δ mutant cells from these

two strains (Figs. 2 and 3). This is supported by several reports demonstrating that defects in the biosynthesis of cell surface mannoproteins, such as the glycosylphosphatidylinositol (GPI) biosynthetic pathway or protein *N*-/*O*-glycosylation, affect the CWI and HOG pathways (Martinez-Lopez *et al*., 2004; Kim *et al*., 2013, 2015; Singh *et al*., 2016). It could be speculated that the long mannan brush-like fibers of the A16 strain could help to alleviate a variety of cell wall stresses (Fig. 1D).

Previous studies have demonstrated that the HOG and CWI pathways coordinate with each other, although no component seems to be directly shared by both pathways. The phosphorylation of Mkc1p is Pbs2- and Hog1-dependent in response to oxidative stress, strongly indicating cross-talk between the CWI and HOG pathways (Arana *et al*., 2005). The Sho1 branch of the HOG pathway is required for proper activation of the CWI pathway following zymolyase-induced cell stress in *S. cerevisiae* (Bermejo *et al*., 2008; Garcia *et al*., 2009). In the fungal pathogen *C. albicans*, the phosphorylation of Mkc1, a SLT2/MPK1 homolog, is either Pkc1-dependent under oxidative stress, or Hog1-dependent in the presence of calcium (Navarro-Garcia *et al*., 1995). Our data on the phosphorylation status of Hog1p, and the expression of downstream target genes also indicated that differential activation of HOG pathway genes might partially contribute to the differences of whole cell wall integrity between the two *H. polymorpha* strains, supporting a link between the CWI and HOG pathways in maintaining the integrity of cell wall.

In *S. cerevisiae,* the phosphorylation of Mpk1p has been reported to be enhanced by several cell wall-disrupting agents, including treatment of cells with glucanases, exposure to chitin-binding agents, exposure to oxidative stress, depolarization of the actin cytoskeleton, and pheromone-induced morphogenesis (Chen and Thorner, 2007). In contrast, the phosphorylation level of HpMpk1p was shown to be clearly enhanced only upon exposure to caffeine and TM in our previous study in the *H. polymorph* DL1-L strain (Kim *et al*., 2015). With the exception of caffeine, no apparent change, or rather decreased HpMpk1p phosphorylation, was also observed upon treatment of the *H. polymorpha* A16 strain with cell wall-disrupting agents (Fig. 5A). Reflecting the increased sensitivity to cell wall stressors, the phosphorylation of Mpk1 protein following caffeine treatment was also more apparent in the DL1-L strain than in the A16 strain. The transcriptome analysis, using RNA-Seq, revealed that a subset of genes related to cell wall integrity are induced by caffeine in a HpMpk1-dependent fashion in the DL1-L strain (Supplementary data Table S2). However, caffeine treatment of the DL1-L strain induced the expression of numerous genes involved in several other cellular processes, particularly genes associated with RNA processing and modification. Caffeine also preferentially activated genes involved in the detoxification process. A previous study in *S. cerevisiae* has proposed that caffeine inhibits the Tor1 kinase, which incidentally promotes Mpk1p phosphorylation, and affects the Ras/cAMP signaling pathway through Rom2p (Kuranda *et al*., 2006). Another study has suggested that the DNA damage check point kinase Rad53 is responsible for the caffeine-induced atypical phosphorylation of Mpk1p in *S. cerevisiae*, which does not lead to the activation of Rlm1 and Swi4, two transcription factors that lie downstream of Mpk1p (Truman *et al*., 2009). The transcriptome profile of the *H. polymorpha* DL1-L strain showed that there was no induction of Rlm1-target genes following caffeine treatment (Supplementary data Fig. S3), supporting that the phosphorylation of Mpk1p by caffeine treatment is independent of transcriptional activation of Rlm1p-regulated genes.

In conclusion, we have shown that the *H. polymorpha* Mpk1p homolog is a functional MAP kinase essential for maintaining cell wall integrity under several stress conditions, although the requirement of Mpk1p is evidently strain-specific. Our understanding of cell wall organization and its regulatory mechanisms in *H. polymorpha* can be now be applied to the development of *H. polymorpha* strains, through regulating the structural integrity of the cell wall, that have enhanced recovery yields for recombinant proteins and metabolites.

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