Schizosaccharomyces pombe Pmr1p Is Essential for Cell Wall Integrity and Is Required for Polarized Cell Growth and Cytokinesis

Juan Carlos G. Cortés,1 Reiko Katoh-Fukui,2 Kanako Moto,2 Juan Carlos Ribas,1 and Junpei Ishiguro2*

Instituto de Microbiología Bioquímica and Departamento de Microbiología y Genética, Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Salamanca, Salamanca, Spain, 1 and Department of Biology, Faculty of Science and Engineering, Konan University, Okamoto, Kobe, Japan2

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The cps5-138 fission yeast mutant shows an abnormal lemon-like morphology at 28°C in minimal medium and a lethal thermosensitive phenotype at 37°C. Cell growth is completely inhibited at 28°C in a Ca2+-free medium, in which the wild type is capable of growing normally. Under these conditions, actin patches become randomly distributed throughout the cell, and defects in septum formation and subsequent cytokinesis appear. The mutant cell is hypersensitive to the cell wall-digesting enzymatic complex Novozym234 even under permissive conditions. The gene SPBC31E1.02c, which complements all the mutant phenotypes described above, was cloned and codes for the Ca2+-ATPase homologue Pmr1p. The gene is not essential under optimal growth conditions but is required under conditions of low Ca2+ (<0.1 mM) or high temperature (>35°C). The green fluorescent protein-tagged Cps5 proteins, which are expressed under physiological conditions (an integrated single copy with its own promoter in the cps5Δ strain), display a localization pattern typical of endoplasmic reticulum proteins. Biochemical analyses show that 1,3-β-D-glucan synthase activity in the mutant is decreased to nearly half that of the wild type and that the mutant cell wall contains no detectable galactomannan when the cells are exposed to a Ca2+-free medium. The mutant acid phosphatase has an increased electrophoretic mobility, suggesting that incomplete protein glycosylation takes place in the mutant cells. These results indicate that S. pombe Pmr1p is essential for the maintenance of cell wall integrity and cytokinesis, possibly by allowing protein glycosylation and the polarized actin distribution to take place normally. Disruption and complementation analyses suggest that Pmr1p shares its function with a vacuolar Ca2+-ATPase homologue, Pmc1p (SPAPB2B4.04c), to prevent lethal activation of calcineurin for cell growth.

A transient increase in the intracellular Ca2+ concentration ([Ca2+]) plays a key role in transmitting signals that regulate a variety of cellular functions in eukaryotes. A substantial body of knowledge has been accumulated concerning the roles of Ca2+ as a second messenger in various types of eukaryotic cells (5, 11). In yeasts, Ca2+ plays an essential role in the mating process (9, 26), and the Ca2+ calmodulin-dependent protein phosphatase calcineurin plays crucial roles in a variety of cellular functions, including ion homeostasis, cytokinesis, and transcriptional regulation (14, 55, 63). In the budding yeast Saccharomyces cerevisiae, FKS1, which codes for a putative catalytic subunit of 1,3-β-D-glucan synthase, is predominantly expressed under optimal growth conditions in a cell cycle-dependent manner, while the transcription of FKS2, an alternative gene for the putative glucon synthase, is completely dependent on calcineurin in the absence of a functional Fks1p and in the presence of mating pheromone or a high extracellular [Ca2+] (42, 74). In the fission yeast Schizosaccharomyces pombe, 1,3-β-D- and 1,3-α-D-glucan synthases, both of which contribute to the mechanical strength of the cell wall (12, 23, 29, 32), are presumed to be the downstream targets of Pck2p, a protein kinase C homologue (4, 8, 32). The overexpression of pck2+ (OP-pck2+) has been shown to increase 1,3-β-D-glucan synthase activity to a significant extent, as well as to induce an extremely high intracellular [Ca2+] (4, 8). The effects of OP-pck2+ were reported to be abolished in the absence of Ehls1p, a homologue of the calcium channel component Mid1p of S. cerevisiae (9, 25). The ehs1-I-1 mutant displays several cell wall-related defective phenotypes, and these defects are suppressed by moderate OP-pck2+ levels, suggesting that Pck2p contributes, along with the Ehls1p calcium channel, to the integrity of the cell wall (9). More recently, another component, Cta4p, a cation P2-type ATPase that is also required for calcium homeostasis, has been identified (45). The null mutant displayed pleiotropic phenotypes, including defects in cytokinesis and microtubule dynamics, similar to the calcineurin null mutant phenotypes (73). These findings suggest that the regulation of intracellular [Ca2+] is critical to cell wall integrity, cytokinesis, and cytoskeletal organization, all of which are essential for fungal-cell morphogenesis. Transient and spatial changes in intracellular [Ca2+] are thus crucial for generating signals that regulate cellular events or maintain ion homeostasis; the processes are mediated by Ca2+-channels, Ca2+ antiporters, and Ca2+-transporting ATPases, which are localized in the plasma or vesicle membranes to allow the transport of ions into or out of the cell or organelles via the membranes. However, at present, little is known concerning the molecular mechanisms of calcium signaling required for these cellular processes during the cell cycle. To address these issues, the molecular characterization of the mutants that show calcium-sensitive phenotypes might be useful.

* Corresponding author. Mailing address: Department of Biology, Faculty of Science and Engineering, Konan University, Okamoto, Kobe 658-8501, Japan. Phone: 81-78-435-2516. Fax: 81-78-435-2539. E-mail: ishiguro@konan-u.ac.jp.
Fourteen genes, named cps1 to cps14, were identified as mutant alleles that confer hypersensitivity to the mitotic poison isopropyl chlorophenyl carbamate (CIPC) (30). Among these, cps1" was found to code for a putative β-1,3-glucan synthase subunit, and the cps1-12 mutation causes cells to lose in the presence of cyclosporin A (CsaA), a potent inhibitor of calcineurin (29), cps8-188 encodes a mutated actin molecule (G273D), and the mutant shows a depolarized and multiteps- morphated morphology with a disorganized cell wall structure (28, 31, 35). These findings suggest that in fission yeast, both calcium signaling and the actin organization are crucial to the integrity of the cell wall. We have recently found that the cps1-128 mutant is incapable of growing in a medium depleted of calcium or at high temperature, under which conditions the cells show an abnormally rounded shape. In the present study, we demonstrate that cps5 is a mutant allele of pmr1" , a gene encoding a putative Ca2+/Mn2+-ATPase (SPBC31E1.02c) that plays a crucial role in the maintenance of cell wall integrity and cytokinesis. In addition, we show that the S. pombe Pmi1p is required for intracellular Ca2+-homeostasis, cooperating with a vacuolar Ca2+-ATPase homologue, Pmc1p (SPAPB2B4#6), to prevent the lethal activation of calcineurin for cell growth.

**MATERIALS AND METHODS**

**Strains and culture conditions.** The strains used in this study are listed in Table 1. Routine yeast extract (YE) medium and selected Edinburgh minimal medium (EMM) supplemented with the appropriate amino acids and sporulation medium have been described elsewhere (2, 20). Ca2+-free EMM is EMM from which CaCl2 was omitted and in which calcium panthothenate was replaced by sodium panthothenate. In some experiments, 0.1 and 10 mM EGTA was added to Ca2+-free liquid and solid EMM, respectively. The yeast cells were cultured at 28°C, unless otherwise specified, on a plate or in liquid medium with continuous shaking. The *E. coli* strain DH5α was used for the routine propagation of plasmids, as described elsewhere (56).

**Plasmids and DNA techniques.** DNA manipulations were carried out according to the standard methods described in Sambrook et al. (56). The plasmids pAL-KS and pREP1 are described elsewhere (29, 41, 65). The plasmids pDB248Xpkc1", pDB248Xpkc2", pREP3Xpkc1", and pREP3Xpkc2" were obtained from P. Pérez (4).

**Gene cloning and mutation site determination.** The mutant strain CP13-3-4D was transformed with an S. pombe genomic library constructed in the pAL-KS vector (pTN-L1; prepared by T. Nakamura). Four transformants capable of growing on both EMM and YE plates containing 280 μM isopropyl N-3-chlorophenyl carbamate (CIPC; Sigma) were isolated, and the plasmids were recovered. Nucleotide sequence determination revealed that all four plasmids contained only one complete open reading frame (ORF). SPBC31E1.02c. The plasmid called pCP5-15, which contained an 8.8-kb genomic DNA insert, was used for further analysis. PCR was carried out to detect the mutation site, using the cps5-138 mutant genome as a template. The primers used were 5'GGACT ATGCTATAAGAAACCGCGGAAA GC-3' (forward, with an Spel site at the 5' end) and 5'CCCCGGGGGTCTGTTTTGGTTGATGA-3' (reverse, with an Smal site at the 5' end). The amplified fragments were digested with Spel and EcoRI/Sacl, and the nucleotide sequence of each generated fragment (1.1, 1.1, and 0.7 kb, respectively) was determined by means of a Hitachi SQ5500E sequencer using the RPNI44 premixed cycle-sequencing kit (Amersham Pharmacia Biotech UK).

**Gene disruption and overexpression.** The one-step gene disruption method (53) was employed to construct the cps5Δ and pmr1Δ (SPAPB2B4#6) strains. The one-step gene, amplified from the wild-type genome by the PCR procedure described above, was digested with Spel/Smal and ligated into pBluescript II KS (+) plasmids (Stratagene). The ura4" gene was inserted into the BamHII site of the cps5" ORF, and the Spel/Smal disruption fragment from the propagated plasmid was used for diploid transformation. Gene disruption in the haploid cells was checked by tetrad analysis and PCR, as described above. For cps5" overexpression, the SPBC31E1.02c ORF (also called pgak2) was amplified by PCR from pCP5-15 using the primers 5'TCCCCGGGGGAATTTAGGAATCCTTITACACA-3' (forward, with an Smal site at the 5' end) and 5'TCCCCGGGGGAATTTAGGAATCCTTITACACA-3' (reverse, with a BglII site at the 5' end). The amplified fragment (3.3 kb), which lacked the stop codon, was digested with Smal/BglII and cloned into the pEGFP vector (BD Biosciences and Clontech). The resulting cps5"-GFP fragment with a Sacl site at the 3' end of the GFP ORF was inserted between the Smal and Sacl sites of the pAL-KS plasmid and used to transform the cps5Δ strain, CP13-8-4D. To integrate a copy of cps5"-GFP into the chromosome, the cps5"-GFP fragment was inserted between the Smal and Sacl sites of pki18 (12), and the plasmid was cut with XbaI at position −245 of the cps5" ORF. The linearized plasmid was used to isolate leucine-nonrequiring transformants from the A3-2D-2A strain. For green fluorescent protein (GFP) tagging of pmr1", essentially the same procedure was used. The primers used for the gene amplification were 5'TCCCCGGGGGAATTTAGGAGATCCTTITACACA-3' (forward, with an Smal site at the 5' end) and 5'GGCGAGATCCGAACTTCTGGTTT-3' (reverse, with a BamHII site at the 5' end). The amplified fragment (4.4 kb) was digested with Smal/BamHII and ligated in frame to the BamHII site just before the ATG codon of the GFP ORF that was generated by PCR from the pEGFP vector (BD Biosciences and Clontech). The resulting cps5"-GFP fragment with a Sacl site at the 3' end of the GFP ORF was inserted between the Smal and Sacl sites of the pAL-KS plasmid and used to transform the cps5Δ strain, CP13-8-4D. To integrate a copy of cps5"-GFP into the chromosome, the cps5"-GFP fragment was inserted between the Smal and Sacl sites of pki18 (12), and the plasmid was cut with XbaI at position −245 of the cps5" ORF. The linearized plasmid was used to isolate leucine-nonrequiring transformants from the A3-2D-2A strain. For green fluorescent protein (GFP) tagging of pmr1", essentially the same procedure was used. The primers used for the gene amplification were 5'TCCCCGGGGGAATTTAGGAGATCCTTITACACA-3' (forward, with an Smal site at the 5' end) and 5'GGCGAGATCCGAACTTCTGGTTT-3' (reverse, with a BamHII site at the 5' end). The amplified fragment (4.4 kb) was digested with Smal/BamHII and ligated in frame to the BamHII site just before the ATG codon of the GFP ORF. The resulting GFP-tagged fragment was inserted between the Smal and Sacl sites of the pAL-KS plasmid and used to transform the pmlc1Δ strain, B2B4#6. For GFP tagging of cps1" and used to transform the strains CP13-8-4D, B2B4#6, and 903. **GFP tagging.** Two methods were employed to express a cps5"-GFP fused gene in cps5Δ cells: one was a plasmid-borne expression, and the other was a physiological level of expression using an integrated copy of the fused gene in the chromosome. The cps5" gene bearing its own promoter region was amplified by PCR using the primers 5'TCCCCGGGGGAATTTAGGAGATCCTTITACACA-3' (forward, with an Smal site at the 5' end) and 5'TCCCCGGGGGAATTTAGGAGATCCTTITACACA-3' (reverse, with a BglII site at the 5' end). The amplified fragment (3.3 kb), which lacked the stop codon, was digested with Smal/BglII and ligated in frame to the Sacl site of the cps5"-GFP plasmid between the Sacl site at the 5' end and inserted into the pREP1 plasmid at the Smal site. The plasmids bearing the cps5" gene in the right orientation were selected and used to transform the strains CP13-8-4D, B2B4#6, and 903.
The mutant was found to be incapable of growing in the absence of Ca$^{2+}$ under low-extracellular-[Ca$^{2+}$] or high-temperature conditions. 

cps5 mutants show defects in cell growth, morphology, and cytokinesis under low-extracellular-[Ca$^{2+}$] or high-temperature conditions. 

FIG. 1. Defective cell growth of the cps5-138 mutant under restrictive culture conditions. Cell growth was monitored by measuring the turbidity at 640 to 700 nm (red filter) with a Klett-Summmerson colorimeter. Wild-type (WT) (○) and cps5-138 (●) cells in EMM at 28°C, wild-type (□) and cps5-138 (■) cells in EMM at 37°C, wild-type (▲) and cps5-138 (▲) cells in Ca$^{2+}$-free EMM at 28°C, and cps5-138 cells in EMM plus 50 mM CaCl$_2$ at 37°C (▼).

extract sugar minus phosphate) medium, which contains a reduced amount of inorganic phosphate, and further cultured for 8 h. Samples prepared from the cell lysate were subjected to electrophoresis on a 6% nondenaturing acrylamide gel, and then activity staining of acid phosphatase was carried out as described by Schweingruber et al. (58).

Cell wall analysis and 1,3-β-D-glucan synthase assay. For the monitoring of enzymatic cell lysis, log-phase cells, grown at 28°C in YE, were washed with 50 mM citrate-phosphate buffer (pH 5.6), suspended in the same buffer containing 30 μg of Novozym234 (Novo Industries)/ml, followed by incubation at 30°C with shaking. The residual absorbance at 600 nm was monitored at hourly intervals, assuming an absorbance of 100% at time zero. Cell extracts for preparation of the enzyme and the 1,3-β-D-glucan synthase assay were carried out as described elsewhere (12). [14C]glucose labeling and the fractionation of cell wall polysaccharides were carried out as described previously (29).

RESULTS

cps5 mutants show defects in cell growth, morphology, and cytokinesis under low-extracellular-[Ca$^{2+}$] or high-temperature conditions. cps5-138 was originally identified as a mutant allele that confers hypersensitivity to the mitotic poison CIPC (30). cps5-138 mutant cell growth was also found to be slower than wild-type cell growth under normal conditions and defective under low-[Ca$^{2+}$] or high-temperature conditions (Fig. 1). The mutant was found to be incapable of growing in the absence of Ca$^{2+}$ (Ca$^{2+}$-free EMM), in which the wild-type strain is capable of growing normally. The growth defect in EMM (0.1 mM CaCl$_2$) at 37°C was significantly recovered by the addition of CaCl$_2$ (50 mM) to the medium. These results indicate that the cps5-138 mutant requires a higher [Ca$^{2+}$] in the medium for normal growth than the wild type. The mutant grown in EMM or in YE at high temperature (37°C) became lemon-like in shape, showing abnormal staining patterns with the dye calcofluor, which has a specific affinity for septum glucans (Fig. 2a to h). The mutant septum was stained much more intensely and broadly than the wild type, suggestive of the excessive deposition of septum materials. A prolonged exposure to high temperature caused cell wall materials to disperse from the medial plane to the cell surface. The rounded shape and abnormal septum staining observed in EMM reverted to the wild-type morphology when the [Ca$^{2+}$] in the medium was increased to 10 mM (data not shown). The growth and morphology defects at 37°C can be restored to the wild-type phenotype in the presence of an osmotic stabilizer (1.2 M sorbitol), which suggests that the mutant has a cell wall defect (data not shown). Fluorescence-activated cell sorter (FACS) analysis showed that, when the mutant strain is exposed to Ca$^{2+}$-free EMM, 4C cells with an unseparated septum accumulate gradually (Fig. 3). These results indicate that cell separation becomes defective in the cps5-138 mutant under restrictive conditions, because in S. pombe, DNA synthesis.

FIG. 2. Abnormal cell morphology and septum formation of the cps5-138 mutant under restrictive culture conditions. Wild-type and cps5-138 mutant cells were stained with calcofluor. Log-phase cells of the wild type (a) and cps5-138 mutant (b) cultured in YE medium at 28°C and wild-type (c) and cps5-138 mutant (d) cells in EMM at 28°C. Log-phase cells of the wild type (e) and cps5-138 mutant (f) were transferred to Ca$^{2+}$-free EMM and cultured for 3 h at 28°C. Wild-type (g) and cps5-138 mutant (h) cells cultured in YE medium at 35°C for 15 h. Wild-type (i) and cps5-138 mutant (j) cells cultured in YE medium plus CsA (10 μg/ml) at 28°C for 15 h. Bar, 5 μm.
FIG. 3. Defective cytokinesis of the cps5-138 mutant under restrictive culture conditions. The cps5-138 mutant was cultured in YE medium at 28°C to the log phase (a), and the collected cells were transferred to Ca²⁺-free EMM and cultured for 3 (b) and 6 (c) h. Each cell culture was stained with propidium iodide (57) and analyzed with an LSR flow cytometer (BD Biosciences). The horizontal and vertical axes show relative DNA content and cell number, respectively.

cps5 mutants show a depolarized distribution of actin patches when exposed to low [Ca²⁺] or high temperature. The F-actin cytoskeleton is known to be important for cell wall integrity and cytokinesis. To determine whether F-actin is affected by cps5 mutations, the cytoskeleton was visualized with rhodamine-conjugated phalloidin and observed during the cell cycle by fluorescence microscopy. The wild-type cells showed a polarized F-actin organization during the cell cycle; in interphase, actin patches were specifically localized to the growing end(s), and during late anaphase, they moved to either side of the actin ring, associating with the sites of septum wall formation (Fig. 4a). In the mutant cells, a polarized distribution was also observed under permissive conditions, although actin patches at the cell poles appeared to be somewhat dispersed compared with those in the wild type (Fig. 4b). When the mutant cells were exposed to low-[Ca²⁺] or high-temperature (37°C) conditions, the actin patches became randomized throughout the cell (Fig. 4c, d, e, and f). Therefore, the altered morphology of the cps5-138 mutant can be attributed to an altered actin distribution.

cps5 encodes a homologue of the S. cerevisiae Pmr1 ATPase. The cps5 gene was cloned from a fission yeast genomic library by complementation of the CIPC-hypersensitive phenotype of the cps5-138 mutant strain. Four clones, which were capable of growing in EMM as well as in YE medium containing 280 μM CIPC, were isolated by the screening of 7 × 10⁴ transformants. A nucleotide sequence analysis of the DNA fragments from the clones revealed that only one complete ORF, SPBC31E1.02c (The S. pombe Genome Project, The Wellcome Trust Sanger Institute; http://www.sanger.ac.uk/Projects/S_pombe/), is common to all the obtained DNA clones. Plasmids bearing this single ORF complemented not only the hypersensitivity but the abnormal cell morphology and defective cell growth of the cps5-138 mutant as well under restrictive conditions (data not shown). To determine whether the cloned gene was cps5⁺ itself, the ORF was amplified by high-fidelity PCR from the cps5-138 genome as a template, and the nucleotide sequence was determined. Analysis of three clones independently obtained by PCR showed that the guanine at nucleotide 1200 of the coding sequence had been replaced by adenine, resulting in the substitution of a nonsense codon (UGA) for the Trp codon. These results indicate that SPBC31E1.02c is cps5⁺ and not an extragenic multicopy suppressor. The ORF was next disrupted in a diploid strain (JY741 × JY746) by replacing cps5⁺ with a copy containing the ura4⁺ gene inserted at the BamHI site that is situated near the middle of the ORF. The transformed diploid was sporulated, and the resulting tetrads were examined for viability. The ura4⁺ disruptants grew normally on a YE plate (data not shown), indicating that cps5⁺ is not essential for cell growth under optimal growth conditions. No phenotypic dif-

FIG. 4. Depolarized actin distribution of the cps5-138 mutant under restrictive culture conditions. Wild-type and cps5-138 mutant cells were stained with rhodamine-conjugated phalloidin. Log-phase cells of the wild type (a) and cps5-138 mutant (b) cultured in YE medium at 28°C. Log-phase cells of the wild type (c) and cps5-138 mutant (d) were transferred to Ca²⁺-free EMM and cultured at 28°C for 3 h. Log-phase cells of the wild type (e) and cps5-138 mutant (f) were transferred to YE medium and cultured at 37°C for 6 h. Bar, 5 μm.
ferences between \(\text{cps}5^{-138}\) and \(\text{cps}5\Delta\) were noted with respect to cell shape, actin distribution, and cell growth under restrictive conditions (data not shown). These results are consistent with the finding that the \(\text{cps}5^{-138}\) allele is a UGA opal mutation, predicted to encode only 399 amino acids instead of the 899 residues of SPBC31E1.02c. The effects of \(\text{cps}5^{-}\) overexpression in the wild-type background were examined using the pREP1 plasmid in the absence of thiamine (derepressed conditions), but no remarkable phenotype was observed (data not shown).

The predicted amino acid sequence of the SPBC31E1.02c protein reveals significant homology to proteins of the Pmr1 ATPase family (overall identity with \textit{S. cerevisiae} Pmr1p, 53\%) (46). Because the SPBC31E1.02c protein was identified very recently as \textit{S. pombe} Pmr1p (37), we use the name Pmr1p in the following description instead of Cps5p. The \textit{S. pombe} Pmr1p protein was shown to play an important role in cell morphology, cooperating with an \textit{Nramp}-related metal transporter via the control of \(\text{Mn}^{2+}\) homeostasis (37). In this study, the effects of \(\text{Mn}^{2+}\) on the defective cell growth and morphology of the \(\text{cps}5^{-138}\) and \(\text{cps}5\Delta\) cells under \(\text{Ca}^{2+}\)-free conditions were also examined. Supplying 0.5 mM \(\text{MnCl}_2\) to the medium rescued the defective cell growth to a significant extent in both mutants (Fig. 5A) but did not suppress the aberrant cell morphology caused by the \(\text{Ca}^{2+}\)-free medium (Fig. 5B).

**Pmr1p shows a localization pattern typical of endoplasmic reticulum (ER) proteins.** The intracellular localization of \textit{S. pombe} Pmr1p was examined by fluorescence microscopy using GFP-tagged Pmr1p. The proteins were observed under two different conditions, plasmid-borne and chromosomal (an integrated single copy with its own promoter) expression of the fused gene in a \(\text{cps}5\Delta\) strain. Both transformants are capable of growing in the absence of calcium with an almost normal morphology (data not shown), suggesting that the GFP-tagged Pmr1p is functional with respect to its pump activity. The typical localization pattern is shown in Fig. 6 (top panel). Most of the Pmr1p-GFP was observed in the nuclear and plasma membranes throughout the cell cycle. This localization pattern is typical of the ER proteins, since in \textit{S. pombe}, the ER is associated with the nuclear membrane and extends longitudinally to the cell ends, reaching the entire plasma membrane (6, 49, 50). To confirm this, the ER was also stained with a GFP-tagged Alg3 mannosyltransferase homologue encoded by \textit{cps}11\+ (SPAC7D4.06c), because the protein is well established as localizing in the ER membrane. The Alg3p-GFP localization pattern was essentially the same as the case of Pmr1p-GFP (Fig. 6, middle panel). These results suggest that the fission yeast Pmr1p resides predominantly in the ER.

**\(\text{cps}5\) mutants show decreased 1,3-β-D-glucan synthase activity with weakened cell walls.** In yeast, the cell wall plays an essential role in determining cell shape, and therefore, a large number of morphological mutants have defects in cell wall integrity and cytokinesis (27). In order to examine whether this is the case for the \(\text{cps}5\) mutant, physiological and biochemical analyses were carried out. Sensitivities to Novozym234, a cell wall-digesting enzyme complex, in the mutant and wild-type strains were initially compared. The results are shown in Fig. 7. The \(\text{cps}5^{-138}\) mutant grown in YE medium at 28°C was much more sensitive to Novozym234 than the wild-type strain, suggesting that the mutant cell wall is altered, even under permissive conditions. The \(\text{cps}5\Delta\) mutant showed the same sensitivity as \(\text{cps}5^{-138}\) (data not shown). The \(\text{cps}1{\text{-12}}\) \(\text{cps}5^{-138}\) double mutant was found to be more sensitive to Novozym234 than any of the single mutants, suggesting that Pmr1p and Cps1p/ Bgs1p regulate cell wall integrity by different mechanisms.

Next, 1,3-β-D-glucan synthase activities were determined for the wild-type, \(\text{cps}5^{-138}\) mutant, and \(\text{cps}5\Delta\) mutant strains grown under permissive and restrictive conditions (37°C in YE medium). As shown in Table 2, the specific activities of the mutant strains grown under permissive conditions in YE medium at 28°C were dramatically reduced by almost half for \(\text{cps}5^{-138}\) (56\%) or even more for \(\text{cps}5\Delta\) (45\%) compared to the

![FIG. 5. Effects of \(\text{MnCl}_2\) on cell growth and morphology of the wild-type (WT), \(\text{cps}5^{-138}\), and \(\text{cps}5\Delta\) strains. A: Each strain was cultured in \(\text{Ca}^{2+}\)-free EMM and \(\text{Ca}^{2+}\)-free EMM containing 0.5 or 5.0 mM \(\text{MnCl}_2\). After 48 h of cultivation, the optical density (O.D.) at 600 nm was measured to quantify the cell growth. B: The \(\text{cps}5^{-138}\) cells were grown in \(\text{Ca}^{2+}\)-free EMM or \(\text{Ca}^{2+}\)-free EMM containing 0.5 mM \(\text{MnCl}_2\) to the log phase and observed with a Nomarski interference microscope. w/o, without.](image-url)
wild-type strain. Similar results were obtained when the mutant strains were grown in YE medium plus 1.2 M sorbitol at 28°C. Both cps5-138 and cps5Δ present a thermosensitive phenotype at 37°C that can be prevented by osmotic stabilization (1.2 M sorbitol), suggesting that it is due to a cell wall defect. The activity of 1,3-β-D-glucan synthase was assayed in both mutants grown at 37°C, either in the presence or in the absence of sorbitol. In the presence of sorbitol, the cells were normal in shape and growth, and the activity rose to almost 90% of that of the wild type. However, in the absence of sorbitol, in which the cells have a strong oval or rounded phenotype and grow slowly but are still viable (for 24 h cultivation), the activity increased considerably to 120% (cps5-138) and 140% (cps5Δ) that of the wild type (Table 2). Therefore, the thermosensitive morphological phenotype of the cps5 mutants is not due to a defect in 1,3-β-D-glucan synthase activity but rather to other cps5-related defects.

The cps5 mutants are unable to grow at 28°C in the absence of Ca²⁺ and grow normally but show an altered morphology in YE medium and EMM (0.1 mM Ca²⁺). The rounded shape is suppressed when both media contain 10 mM Ca²⁺. The cps1-12 mutant also displays hypersensitivity to high tempera-

| Growth temp (°C) | Strain         | Sp act (mean ± SD) |
|------------------|----------------|--------------------|
|                  | Wild type      | 10.1 ± 0.1 (100)   |
| 28               | cps5-138       | 5.7 ± 0.2 (56)     |
|                  | cps5Δ          | 4.5 ± 0.2 (45)     |
| 28 + sorbitol    | Wild type      | 5.8 ± 0.2 (100)    |
|                  | cps5-138       | 3.3 ± 0.4 (57)     |
|                  | cps5Δ          | 2.3 ± 0.2 (40)     |
| 37               | Wild type      | 4.6 ± 0.1 (100)    |
|                  | cps5-138       | 5.7 ± 0.0 (124)    |
|                  | cps5Δ          | 6.8 ± 0.7 (148)    |
| 37 + sorbitol    | Wild type      | 6.1 ± 0.1 (100)    |
|                  | cps5-138       | 5.5 ± 0.1 (90)     |
|                  | cps5Δ          | 5.2 ± 0.1 (85)     |

a Specific activity is expressed as milliunits per milligram of protein. The reaction mixture contained 150 μM GTP. The values were obtained from three independent experiments. Values in parentheses are percentages of specific activity compared to that of the wild type under the same conditions.

b The cells were grown for 24 h at 37°C. Under these conditions, the thermosensitive morphological phenotype is completely expressed in the absence of sorbitol, and the cells are still viable and able to grow.
Table 3. Characterization of (1,3)-β-D-glucan synthase activities from S. pombe wild-type, cps1-12, cps5-138, and cps5Δ mutant; and cps1-12 cps5-138 double mutant strains grown at 28°C in EMM in the presence or absence of calcium

| CaCl₂ concn (mM) | Strain   | Sp act (mean ± SD)* |
|------------------|----------|---------------------|
| 10               | Wild type| 7.4 ± 0.4 (100)     |
|                  | cps1-12  | 8.7 ± 0.7 (118)     |
|                  | cps5-138 | 6.0 ± 0.6 (89)      |
|                  | cps5Δ    | 6.1 ± 0.4 (82)      |
|                  | cps1-12 cps5-138 | 6.2 ± 0.6 (84)    |
| 0.1              | Wild type| 9.8 ± 0.1 (100)     |
|                  | cps1-12  | 12.4 ± 1.2 (127)    |
|                  | cps5-138 | 8.7 ± 1.2 (89)      |
|                  | cps5Δ    | 6.2 ± 0.8 (63)      |
|                  | cps1-12 cps5-138 | 8.0 ± 0.5 (82)    |
| 0*               | Wild type| 15.0 ± 0.4 (100)    |
|                  | cps1-12  | 12.3 ± 0.9 (82)     |
|                  | cps5-138 | 8.0 ± 0.7 (53)      |
|                  | cps5Δ    | 7.6 ± 1.3 (51)      |
|                  | cps1-12 cps5-138 | 12.6 ± 1.1 (84)  |

* Values were obtained from three independent experiments as for Table 2. Values in parenthesis are percentages of the corresponding polysaccharide in the cell wall composition.

Table 4. Incorporation of radioactivity from [14C]glucose into cell wall polysaccharides of S. pombe wild-type, cps5-138, and cps5Δ strains grown at 28°C in EMM in the presence or absence of calcium

| CaCl₂ Conc (mM) | Strain         | % Incorporation of [14C]glucose (mean ± SD)* |
|-----------------|----------------|---------------------------------------------|
|                 |                | Cell wall | Galactomannan | α-Glucan | β-Glucan |
| 10              | Wild type      | 34.3 ± 0.7 | 4.8 ± 0.2 (14.0) | 9.0 ± 0.4 (26.2) | 20.5 ± 0.1 (59.8) |
|                 | cps5-138       | 34.0 ± 1.2 | 3.4 ± 0.1 (10.0) | 8.0 ± 0.5 (23.5) | 22.6 ± 0.6 (66.5) |
|                 | cps5Δ          | 34.1 ± 0.7 | 3.4 ± 0.2 (10.0) | 8.2 ± 0.0 (24.0) | 22.5 ± 0.9 (66.0) |
| 0*              | Wild type      | 34.3 ± 0.8 | 3.7 ± 0.0 (10.8) | 9.1 ± 0.3 (26.5) | 21.5 ± 0.9 (62.7) |
|                 | cps5-138       | 37.4 ± 1.2 | 3.0 ± 0.0 (0) | 12.8 ± 0.6 (34.2) | 24.6 ± 0.6 (65.8) |
|                 | cps5Δ          | 40.8 ± 0.8 | 3.0 ± 0.0 (0) | 13.7 ± 0.4 (33.6) | 27.1 ± 0.4 (66.4) |

* Percent incorporation of [14C]glucose is the counts per minute incorporated per fraction × 100/total counts per minute incorporated. The values were obtained from three independent experiments. Values in parentheses are percentages of the corresponding polysaccharide in the cell wall composition.

The cells were grown for 4 h in Ca²⁺-EMM plus 0.1 mM EGTA.
pletely glycosylated (58). The acid phosphatase from the cps5 mutants exhibited increased electrophoretic mobility compared with that from the wild type (Fig. 8), indicating that the mutants synthesized an incompletely glycosylated acid phosphatase, even under optimal growth conditions. The electrophoretic patterns are similar to those from gmh3/H9004 and gms1/H9004 strains, the genes of which encode a galactosyltransferase and UDP-galactose transporter, respectively (66, 72), consistent with the finding that cps5 mutants contain no detectable galactomannan in the cell walls.

Pmr1p shares its function with the Pmc1p homologue to maintain intracellular Ca\(^{2+}\) homeostasis. In S. cerevisiae, it was shown that Pmr1p acts together with Pmc1p to deplete cytosolic Ca\(^{2+}\), by which a lethal activation of calcineurin is prevented (15). To examine whether this is also true in S. pombe, the PMC1 homologue, SPAPB2B4.04c, was disrupted, and the phenotypes were examined. The pmc1\(/\)H9004 strain B2B4#6 was found to grow almost normally in YE medium but was incapable of growing in the presence of high \([\text{Ca}^{2+}]\) in medium (>100 mM), conditions under which wild-type cells are able to grow normally (Fig. 9B). This result suggests that Pmc1p is not essential under normal growth conditions but is required for high-Ca\(^{2+}\) conditions. The high-Ca\(^{2+}\)-sensitive phenotypes of pmc1\(/\)A were significantly suppressed either in the presence of CsA, a potent inhibitor of calcineurin, or overexpression of the cps5\(/\) gene in pmc1Δ cells (Fig. 9A and B). These results suggest that the growth defect is due to an inappropriate activation of calcineurin, which is caused by an elevated intracellular [Ca\(^{2+}\)], and that Pmr1p also acts to deplete cytosolic Ca\(^{2+}\), cooperating with the Pmc1p homologue. In S. cerevisiae, the double mutation of PMR1 and PMC1 is synthetic lethal (14). To examine whether this is also the case in S. pombe, a tetrady analysis of the heterozygous diploids (CP13-8-4D × B2B4#6) was carried out. Five sets of four meiotic progeny (PD type), 9 sets of two progeny (NPD), and 23 sets of three progeny (T) were obtained. None of the double mutants grew on YE plates. These results strongly suggest that the two Ca\(^{2+}\)-ATPase homologues, Pmr1p and Pmc1p, in S. pombe share an essential role in depleting cytosolic Ca\(^{2+}\) to maintain a proper level of calcineurin activity for cell growth, even under standard culture conditions.

In S. cerevisiae, Pmc1p, which is 40% identical to the plasma membrane Ca\(^{2+}\)-ATPase of mammalian cells, was shown to be localized to the vacuole membrane (15). To identify the intracellular localization of the Pmc1p homologue in S. pombe, GFP-tagged Pmc1p was observed by fluorescence microscopy. The Pmc1p-GFP fusion protein was capable of complementing high-Ca\(^{2+}\)-sensitive phenotypes (Fig. 9A), suggesting that the fusion protein is functional with respect to its pump activity. As shown in Fig. 6 (bottom panel), Pmc1p-GFP was essentially localized to the vacuole membrane, which is similar to the case of budding yeast.

**DISCUSSION**

In the fission yeast S. pombe, 14 putative P-type ATPase genes were identified by a homology search of ORFs deduced from the S. pombe genome project (46, 71). At least three putative calcium ATPases have been assigned, namely, Cta4p (45), Pmc1p (SPAPB2B4.04c), and Pmr1p/Cps5p (SPBC31E1.02c) (37). At
present, however, knowledge concerning the roles of the pump activity in cellular functions and how the activities are coordinately regulated in response to changes in intra- and extracellular $[\text{Ca}^{2+}]$ is very limited. The SPBC31E1.02c protein shows significant sequence similarity to the Pmr1 family of ATPases, which are present in a wide range of fungi and animal tissues (the fission yeast Pmr1p protein is most closely related to that of \textit{Yarrowia lipolytica} [48], with a CLUSTALW score of 52.8). It has been well established in \textit{S. cerevisiae} that Pmr1p is required for a variety of cellular functions, including N-linked and O-linked protein glycosylation, protein sorting in secretory pathways, and ER-associated protein degradation via its $\text{Ca}^{2+}$-$\text{Mn}^{2+}$ transporting activity, and that the pump is localized primarily in the medial Golgi (3, 19, 36, 44, 54, 60). In \textit{S. pombe}, however, GFP-tagged Pmr1p appears to be localized predominantly in the ER rather than in the Golgi apparatus. It has been shown that the budding yeast Pmr1p is required for ER functions, such as $\text{Ca}^{2+}$ homeostasis in the ER lumen, oligosaccharide trimming, and ER quality control, despite its Golgi localization (19, 62, 70) and that Cod1p/Spf1p, a putative P-type ATPase, is involved in processing the outer chain of carbohydrates in the Golgi, despite its localization in the ER (13, 64). We have recently found that a mutation in the \textit{cps11} gene, which codes for the Alg3p homologue of \textit{S. cerevisiae}, a Dol-P-Man-dependent mannosyltransferase residing in the ER (1, 59), causes defects of cell wall integrity and cell growth under the same restrictive conditions as in the case of \textit{cps5} mutations (unpublished result). As expected, the GFP-tagged \textit{Cps11}p/Alg3p showed essentially the same localization pattern as Pmr1p. These findings support the view that the fission yeast Pmr1p resides predominantly in the ER membrane. This notion does not exclude the possibility that the Pmr1p pump activity is required for the Golgi functions, because it is quite possible to supply $\text{Ca}^{2+}$ predominantly in the ER membrane. This notion does not exclude, however, knowledge concerning the roles of the pump activity in cellular functions and how the activities are coordinately regulated in response to changes in intra- and extracellular $[\text{Ca}^{2+}]$.

It has been well established in \textit{S. cerevisiae} that Pmr1p is required for a variety of cellular functions, including N-linked and O-linked protein glycosylation, protein sorting in secretory pathways, and ER-associated protein degradation via its $\text{Ca}^{2+}$-$\text{Mn}^{2+}$ transporting activity, and that the pump is localized primarily in the medial Golgi (3, 19, 36, 44, 54, 60). In \textit{S. pombe}, however, GFP-tagged Pmr1p appears to be localized predominantly in the ER rather than in the Golgi apparatus. It has been shown that the budding yeast Pmr1p is required for ER functions, such as $\text{Ca}^{2+}$ homeostasis in the ER lumen, oligosaccharide trimming, and ER quality control, despite its Golgi localization (19, 62, 70) and that Cod1p/Spf1p, a putative P-type ATPase, is involved in processing the outer chain of carbohydrates in the Golgi, despite its localization in the ER (13, 64). We have recently found that a mutation in the \textit{cps11} gene, which codes for the Alg3p homologue of \textit{S. cerevisiae}, a Dol-P-Man-dependent mannosyltransferase residing in the ER (1, 59), causes defects of cell wall integrity and cell growth under the same restrictive conditions as in the case of \textit{cps5} mutations (unpublished result). As expected, the GFP-tagged \textit{Cps11}p/Alg3p showed essentially the same localization pattern as Pmr1p. These findings support the view that the fission yeast Pmr1p resides predominantly in the ER membrane. This notion does not exclude the possibility that the Pmr1p pump activity is required for the Golgi functions, because it is quite possible to supply $\text{Ca}^{2+}$ and $\text{Mn}^{2+}$ ions to the Golgi apparatus despite the ER localization or by a residual pump activity from a small number of Golgi-resident Pmr1 molecules (19). In \textit{S. cerevisiae}, GFP-tagged Pmr1p was reported to show a typical Golgi localization pattern, even when expressed from multicopy plasmids (38). However, it has also been observed that hemagglutinin-tagged Pmr1p is localized in the Golgi but the GFP-tagged version is localized in the ER owing to GFP tagging, although the fusion protein is able to rescue the \textit{cps5} mutant phenotypes.

A phenotypic relationship between loss of Pmr1p function and an abnormal cell wall morphology was first reported in \textit{Kluuyveromyces lactis} (67). \textit{Klpmr1} cells revealed several defective phenotypes, including incomplete protein glycosylation, aberrant chitin deposition, and a thickened cell wall with an unbalanced ratio of insoluble to soluble glucans. These results suggest that the cell wall and wall-related glycoproteins, including the 1,3-$\beta$-D-glucan synthases, are affected by \textit{pmr1} mutations, resulting in changes in their enzymatic activities and/or in their subcellular distribution. Consistent with this hypothesis, we found that the in vitro 1,3-$\beta$-D-glucan synthase activity decreased by nearly 50% in \textit{cps5} mutant cells, even those grown in a standard YE medium. In the wild type, specific activity levels were higher under calcium-free conditions than in the presence of 0.1 or 10 mM calcium, and the level of $\alpha$- and $\beta$-glucans increased to some extent under $\text{Ca}^{2+}$-free conditions, particularly in the mutant cell walls. The reason for this is unclear, but it could be the result of a cell wall stress response, as described in Results. The 1,3-$\beta$-D-glucan synthase of the cell surface could be hyperactivated during the early stages of starvation as a survival mechanism and could be gradually inactivated during longer periods of starvation. Alternatively, the 1,3-$\beta$-D-glucan synthase activity measured in vitro may be different from the activity responsible for the synthesis of 1,3-$\beta$-D-glucan in vivo, as is the case in \textit{S. cerevisiae} for differences between in vivo chitin synthesis and in vitro chitin synthase activities measured for Chs1p, Chs2p, and Chs3p (69). In \textit{S. pombe}, four putative 1,3-$\beta$-D-glucan synthases have been identified, and at least three of them (Bgs1p/Cps1p, Bgs3p, and Bgs4p) are responsible for cell wall synthesis during the vegetative cell cycle (12, 18, 39; J. C. Ribas, unpublished results). We do not know yet which enzyme or enzymes are responsible for the activity detected in vitro and how they are regulated in response to changes in environmental conditions. However, it is clear that the specific activity levels of 1,3-$\beta$-D-glucan synthase were always much lower in the mutants than in the wild type under the same experimental conditions. An important finding is that there was almost no detectable galactomannan in the mutant cell walls, with an unbalanced ratio of $\alpha$- and $\beta$-glucans, when the mutant cells were grown in the absence of calcium; the former could be caused by a defect in protein glycosylation, and the latter could be induced by changes in 1,3-$\beta$-D-glucan synthase activity. Cell wall weakness thus can be attributed to the dramatic changes in the composition of the mutant cell wall. It should be noted that deletion of \textit{S. cerevisiae PMR1} does not result in a detectable change in cell wall composition, in spite of the glycosylation defects (67).

Very recently, the \textit{S. pombe} Pmr1p was reported to play an important role in Mn$^{2+}$ homeostasis, cooperating with Pdt1p (an \textit{Ntrp}-related metal transporter) to regulate cell morphogenesis (37). The \textit{pmr1} \textit{pdt1} double mutants displayed more severe morphological defects than each single mutant when they were grown in EMM. Supplying Mn$^{2+}$ to the medium suppressed defects of the double mutant in both cell morphology and the glycosylation of acid phosphatase to a great extent (37), consistent with the fact that Mn$^{2+}$ is an important cofactor for protein glycosylation. In this study, we also confirmed that Mn$^{2+}$ has a rescuing effect on the defective growth of \textit{cps5}-138 and \textit{cps5} \textit{Δ} cells in Ca$^{2+}$-depleted medium, but it was incapable of restoring their cell morphology in the absence of calcium. As already mentioned, the growth defect in the absence of calcium was not rescued even in the presence of sorbitol. These findings suggest that, in addition to protein glycosylation, which requires Mn$^{2+}$ homeostasis in the secretory pathway, Pmr1p plays an important role in cell growth and cell-wall morphogenesis via control of cytosolic and/or organelle Ca$^{2+}$ concentrations.

In \textit{S. cerevisiae}, it has been shown that cell wall synthesis requires the coordination of a variety of cellular events, including the recruiting of cell wall materials and the glucan synthases themselves to the plasma membrane, enzyme activation by the GTP-binding protein Rho1p, and glycosylation of the glycosyl phosphatidylinositol anchor and stress sensor proteins (9, 10, 17, 33, 47). Another important component is the actin cytoskeleton (27, 52). F-actin is required for maintenance of cell wall structure and development (21, 34), as well as the polarized localization of Fks1p (16). Actin patch motility is
also required for Fks1p movement, by which the structure and function of the cell wall are maintained (68). In \textit{S. pombe}, the actin cytoskeleton was suggested to be involved in the secretion of cell wall materials, such as 1,6-β-glucan, α-glucan, and α-galactomannan (35). Although the molecular mechanisms for such interdependence of the actin cytoskeleton and cell wall synthesis remain unclear, the actin cytoskeleton is presumed to act as transporting machinery in the secretory pathway. Since Ca\(^{2+}\) is an important regulatory factor in the organization of F-actin, as well as in vesicle transport in mammalian cells, similar mechanisms could also function in fungal systems (43).

In most cases, cell wall mutants display a simultaneous defect in the formation of the septum (27). In \textit{cps5} mutants, the early steps in septum formation may not be impaired, because an F-actin ring appears to form normally. The late steps require enzymes responsible for the synthesis of glucans, as well as septum materials, to be properly transferred to the site where the septum is formed. It is easy to imagine that the unbalanced synthesis of polysaccharides observed in the mutant cell walls and/or the incomplete or missing glycosylation of wall proteins also impairs septum synthesis, resulting in a defect in cytokinesis followed by cell separation. Although an excessive deposition of septum materials was observed, the defective mechanism remains unclear. It may be worth noting that septated cells that accumulated in Ca\(^{2+}\)-free medium for 3 h were reduced in number by almost one-third when shifted back to permissive conditions within 3 h. This implies that ill-formed septa are reactivated to divide under permissive conditions when the cells are exposed for a short period to restrictive conditions.

In \textit{S. cerevisiae}, it has been shown that Ca\(^{2+}\) signals generated by mating pheromones or a high-Ca\(^{2+}\) environment induce the expression of certain genes, including \textit{PMR1} and \textit{PMC1}, which encode the Golgi and vacuolar Ca\(^{2+}\) ATPases, respectively, and \textit{FKS2}, which encodes a putative 1,3-β-glucan synthase catalytic subunit, via a calcineurin-dependent transcription factor, Tcn1p/Crz1p (40, 42, 61, 74). It was also reported that in \textit{S. pombe} the expression of \textit{pmr1} and \textit{pmc1} is controlled through a calcineurin-dependent transcription factor, Prz1p (22, 37). In budding yeast, \textit{Pmr1p} and \textit{Pmc1p} cooperatively regulate cytosolic [Ca\(^{2+}\)] to a level appropriate for the activation of calcineurin, implying the existence of a feedback mechanism for the calcineurin pathway via modulation of cytosolic [Ca\(^{2+}\)] (15). In this study, we also observed the same functional interdependence between \textit{S. pombe} \textit{Pmr1p} and \textit{Pmc1p} in preventing lethal activation of calcineurin. The reason why \textit{pmr1Δ} causes cells to be low-calcium sensitive and \textit{pmc1Δ} causes cells to be high-calcium sensitive is that \textit{Pmr1p} is essential for the supply of Ca\(^{2+}\) and Mn\(^{2+}\) to the organelles but is not necessarily required for degrading excess cytosolic [Ca\(^{2+}\)], because Pmc1p is fully active in the latter process, although simultaneous loss of \textit{Pmr1p} and \textit{Pmc1p} functions causes fatal damage to the cell. We have observed that \textit{pmc1Δ} cells become somewhat resistant to the cell wall-digesting agent Novozym234 (unpublished results), suggesting that cytosolic [Ca\(^{2+}\)] may also affect cell wall integrity. Indeed, Ehs1p, a homologue of the Ca\(^{2+}\) channel component Mid1p of \textit{S. cerevisiae}, is reported to be involved in the cell wall integrity pathway, functioning with Pck2p, a protein kinase C homologue (9). To determine whether \textit{Pmr1p} pump activity is involved in the Pck2 pathway, pck2\(^{-}\) was overexpressed in the \textit{cps5} mutants. As a result, neither the pck2\(^{-}\) overexpression-related phenotype nor the \textit{cps5} mutant phenotypes were suppressed, consistent with the finding that \textit{cps5}-- failed to rescue the \textit{ehs1-1} mutant phenotypes and vice versa (unpublished result). These results suggest that \textit{Pmr1p} is not involved in the Ehs1p-Pck2p pathway.

cps1-12 mutants display hypersensitivity to CsA, a potent inhibitor of calcineurin, as well as to Ca\(^{2+}\)-free EMM, and an increase of exogenous [Ca\(^{2+}\)] (to 10 mM) is able to partially suppress the thermosensitive phenotype (reference 29 and unpublished results). CsA appears to aggravate \textit{cps5-138} mutant phenotypes, and the \textit{cps5-138} \textit{cps1-12} double mutant is more hypersensitive to Novozym234 than either of the single mutants. From these findings, it is tempting to speculate that the gene expression of a 1,3-β-glucan synthase homologue(s) other than Bgs1p/Cps1p could be controlled by a calcineurin-dependent mechanism and that \textit{Pmr1p} is involved in the regulation of this pathway via the control of cytosolic and/or organelle [Ca\(^{2+}\)].

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