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Functionally redundant protein phosphatase genes *PTP2* and *MSG5* co-regulate the calcium signaling pathway in *Saccharomyces cerevisiae* upon exposure to high extracellular calcium concentration

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Reversible phosphorylation is one of the key post-translational modifications for the regulation of many essential cellular processes. We have previously reported that the disruption of two protein phosphatase (PPase) genes, *PTP2* and *MSG5*, causes calcium sensitivity indicating that functional redundancy exists between the two PPases in response to high extracellular calcium. In this paper, we found that the inactivation of calcineurin by the disruption of the calcineurin regulatory subunit, *CNB1* or treatment with a calcineurin inhibitor, FK506, can suppress the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant. In the wake of a calcium-induced, calcineurin-driven signaling pathway activation, the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant can be suppressed by regulating the SLT2 pathway through the disruption of the major kinases in the SLT2 signal cascade that include *BCK1*, *MKK1* and *SLT2*. Also, we show that *PTP2* and *MSG5* are key regulatory PPases that prevent over-activation of the calcium-induced signaling cascade under the parallel control of the SLT2 and calcineurin pathways.

[**Key words:** *Saccharomyces cerevisiae*; *PTP2*; *MSG5*; Calcium sensitivity; Stress response]

Upon exposure to stress, *Saccharomyces cerevisiae* employs a variety of signaling cascades such as the mitogen-activated protein kinase (MAPK) pathways that control patterns of gene expression and protein activity to cope with the deleterious changes in the environment (1). The ability of the cells to correctly perceive and respond to the environment is critical to the organism's survival thus; inappropriate activation of cell signaling pathways often has dire consequences on the viability of the cell. Accordingly, regulation is an integral part of the signaling cascade architecture that ensures the maintenance of cellular homeostasis upon fluctuations in environmental conditions. Since over-activation of a particular signal can have damaging effects, cells possess a myriad of ways to combat improper activation of signaling pathways, one of which is regulation via protein phosphorylation and dephosphorylation catalyzed by protein kinases (PKases) and protein phosphatases (PPases) respectively.

In *S. cerevisiae*, high extracellular levels of calcium usually elicit the activation of calcineurin, a calcium/calmodulin-dependent serine/threonine PPase (2). Calcineurin is known to regulate Ca^{2+} pumps and exchangers responsible for calcium homeostasis in yeast to maintain the cytoplasmic concentration in the range of 100–300 nM (3,4). These include the vacuolar H^+ ATPases encoded by *VCX1* (5), vacuolar Ca^{2+} ATPase Pmc1p (3) and high affinity Ca^{2+}/Mn^{2+} P-type ATPase Pmr1p (6). A rigid control of the intracellular Ca^{2+} concentration is an important feature of calcium signaling in which transient alteration of the cytosolic calcium concentration leads to the activation of several signal transduction pathways (7). In the regulation of these signaling pathways, calcineurin plays various physiological roles including recovery from α-factor-induced growth arrest, salt and temperature tolerance, calcium and ion homeostasis, regulation of cell wall biogenesis and Mn^{2+} tolerance (8,9).

We have previously reported that the disruption of two PPases namely *PTP2* and *MSG5* has a detrimental effect on the growth of yeast in a calcium-rich medium. Furthermore, the fact that *ptp2Δ* and *msg5Δ* single disruptants were calcium tolerant implies that there is functional redundancy existing between the two PPases in response to high extracellular calcium (10). The calcium-sensitive *ptp2Δmsg5Δ* double disruptant was further described to exhibit G1 delay, reduced *CLN2* expression and vacuole fragmentation (11). In addition, we identified six PKases namely *BCK1*, *MKK1*, *SLT2*, *MCK1*, *YAK1* and *SSK2* whose additional disruption in the *ptp2Δmsg5Δ* double disruptant background confers calcium tolerance (12). Interestingly, these suppressors fall into two groups based on their capacity to restore cell cycle progression as shown by FACS analysis. Disruption of *BCK1*, *MKK1*, *SLT2* or *MCK1* in the *ptp2Δmsg5Δ* double disruptant was unable to alleviate the G1 transition defect while *SKK2* or *YAK1* disruption was able to initiate G1 to S transition, implying that there are at least two mechanisms governing the suppression of the calcium-sensitive phenotype of the *ptp2Δmsg5Δ*

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double disruptant. Although Msg5 and Ptp2 are known to be regulators of the *SLT2* pathway (13), their specific involvement in the calcium-induced signaling related to growth is not yet fully understood.

In the present study, we show the importance of the functionally redundant *PTP2* and *MSG5* in the growth of *S. cerevisiae* in high extracellular calcium conditions. Furthermore, we found another suppressor of the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant, *cnb1Δ*, which for the first time implicates the calcineurin pathway in the calcium-induced signaling pathway regulated by the functionally redundant *PTP2* and *MSG5*. We also describe how growth is inhibited in the calcium-exposed *ptp2Δmsg5Δ* double disruptant in relation to calcineurin and SLT2 pathways. Lastly, we explain the mechanism of suppression conferred by the previously discovered suppressors (*bck1Δ*, *mkk1Δ* and *slt2Δ*) of the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant in relation to the redundant regulatory function exhibited by SLT2 and calcineurin pathways in cell processes related to growth.

MATERIALS AND METHODS

Strains and media Yeast strains used in this study are listed in Table 1. FY833 or SH5209 (*MAT*a *ura3-52 his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63*) was used as the wild-type

TABLE 1. *S. cerevisiae* strains used in the study.

Strain	Alias	Genotype	Source/description
SH6314	BY4742	*MAT α his3 Δ1 leu2 Δ0 lys2 Δ0 ura3Δ0*	Invitrogen
SH5209	BY5209, FY833	*MAT* a *ura3-52 his3Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	NBRP, YGRC[a]
SH5210	BY5210	*MAT α ura3-52 his3Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	NBRP, YGRC[a]
SH5406	BY5210 *CRZ1-GFP*	*MAT α ura3-52 his3Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63 CRZ1-GFP::CgTRP1*	Trp^+ transformant of SH5210 with *CRZ1-GFP* cassette
SH6790	*ptp2Δ*	*MAT* a *Δptp2::CgHIS3 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	SH5209 disruptant
SH6791	*msg5Δ*	*MAT α Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	SH5210 disruptant
SH6792	*ptp2Δ msg5Δ*	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	(SH6790 x SH6791)-1B
SH6793	*ptp2Δ msg5Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	(SH6790 x SH6791)-2B
SH8966	*ptp2Δ msg5Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Leu^+ disruptant of SH6790 single disruptant using p1807 (*CgLEU2*)
SH8971	*ptp2Δ msg5Δ CRZ1-GFP*	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63 CRZ1-GFP::CgTRP1*	Trp^+ transformant of SH6792 with *CRZ1-GFP* cassette
SH8928	*cna1Δ*	*MAT α Δcna1::KanMX4 his3 Δ1 leu2 Δ0 lys2 Δ0 ura3Δ0*	Invitrogen
SH8933	*ptp2Δ msg5Δ cna1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcna1::KanMX4 ura3-52* (or *ura3Δ0*) *his3-Δ200* (or *his3Δ1*) *leu2Δ1* (*leu2Δ0*) *lys2Δ202* (*lys2Δ0*) *trp1Δ63*	(SH6793 x SH8928)-4C
SH8929	*cna2Δ*	*MAT α Δcna2::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8934	*ptp2Δ msg5Δ cna2Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcna2::KanMX4 ura3-52* (or *ura3Δ0*) *his3-Δ200* (or *his3Δ1*) *leu2Δ1* (*leu2Δ0*) *lys2Δ202* (*lys2Δ0*) *trp1Δ63*	(SH6793 x SH8929)-3C
SH8930	*cnb1Δ*	*MAT α Δcnb1::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8935	*ptp2Δ msg5Δ cnb1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcnb1::KanMX4 ura3-52* (or *ura3Δ0*) *his3-Δ200* (or *his3Δ1*) *leu2Δ1* (*leu2Δ0*) *lys2Δ202* (*lys2Δ0*) *trp1Δ63*	(SH6793 x SH8930)-13A
SH8967	*crz1Δ*	*MAT α Δcrz1::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8968	*ptp2Δ msg5Δ crz1Δ*	*MAT* a *Δptp2::CgHIS3 Δmsg5::CgLEU2 Δcrz1::KanMX4 ura3-52* (or *ura3Δ0*) *his3-Δ200* (or *his3Δ1*) *leu2Δ1* (*leu2Δ0*) *lys2Δ202* (*lys2Δ0*) *trp1Δ63*	(SH6793 x SH8967)-4B
SH8931	*slt2Δ*	*MAT α Δslt2::KanMX4 his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Invitrogen
SH8972	*ptp2Δ msg5Δ slt2Δ*	*MATα Δptp2::CgHIS3 Δmsg5::CgLEU2 Δslt2::KANMX4 ura3-52* (or *ura3Δ0*) *his3-Δ200* (or *his3Δ1*) *leu2Δ1* (*leu2Δ0*) *lys2Δ202* (*lys2Δ0*) *trp1Δ63*	(SH6793 x SH8931)-2B
SH8986	*ptp2Δ msg5Δ ste12Δ*	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δste12::CgTRP1 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Trp^+ disruptant of SH6792 double disruptant using p1805 (*CgTRP1*)
SH8987	*ptp2Δ msg5Δ sok2Δ*	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δsok2::ScURA3 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Ura^+ disruptant of SH6792 double disruptant using p3276 (*ScURA3*)
SH8988	*ptp2Δ msg5Δ ste12Δ sok2Δ*	*MAT α Δptp2::CgHIS3 Δmsg5::CgLEU2 Δste12::CgTRP1Δsok2::ScURA3 ura3-52 his3-Δ200 leu2 Δ1 lys2 Δ202 trp1 Δ63*	Ura^+ disruptant of SH8987 triple disruptant using p3276 (*ScURA3*)

[a] NBRP/YGRC, National BioResource Project/Yeast Genetic Research Center, Japan (http://yeast.lab.nig.ac.jp/nig/index_en.html).

strain. All experiments using yeast strains were conducted at 30°C using standard recipes for YPDA (Sigma–Aldrich Co., St. Louis, MO, USA), SC dropout and Sporulation medium (SPM). YPDA was supplemented with 0.6 M $CaCl_2$ for the calcium phenotype test while 0.3 M $CaCl_2$ was used for total RNA isolation. For the calcineurin phenotype test, YPDA was added with 1 μg/ml FK506 (a calcineurin inhibitor). *Escherichia coli* strains used as sources of plasmids namely BYP1804 for p1804 (*CgHIS3*), BYP1805 for p1805 (*CgTRP1*), BYP1419 for p1807 (*CgLEU2*), SHB3276 for p3276 (*ScURA3*), BYP2446 for p2446 (*GFP-CgTPRP1*) were acquired from NBRP, YGRC, Japan and cultivated in LB medium containing 100 μg/ml ampicillin at 37°C as previously described (14). *CRZ1* gene was tagged with GFP using the PCR-mediated gene tagging method described previously (15). To construct the *CRZ1-GFP-CgTRP1* tagging cassette for transformation of the wild type and *ptp2Δmsg5Δ* double disruptant strains, primers CRZ1-GFP F: 5′-GTACGAAGAAGCCAGACAGGAGAAATCGGGACAAGAGAGTTAA-3′ and CRZ1-GFP R: 5′-AAAAAAAATTCCTATTCAAAGCTTAAAAAAACAAAAATAAAAAACGACGGCCAGT-3′,corres ponding to 40 base pairs upstream and downstream of the *CRZ1* gene stop codon, were utilized for PCR using plasmid p2446 (containing the GFP-CgTRP1 fragment) from BYP2446 as template.

Genetic manipulations Construction of single, double and triple disruptants was carried out using the PCR-mediated gene disruption method (16) using *Candida glabrata HIS3*, *C. glabrata LEU2* or *S. cerevisiae* genes (*CgHIS3*, *CgLEU2* or *ScURA3*, respectively) or mating as previously described by Hermansyah (11,12). Confirmation PCR using specific primers was used to verify the correct disruption of genes. The primers for used in this study are listed in the Supplementary Table S1.

RNA isolation RNA was isolated using the hot phenol method (17) with some modifications. Briefly, cells grown to mid-log phase at 30°C in YPDA with or without 0.3 M $CaCl_2$ were harvested by centrifugation. Total RNA was extracted by sequential treatment of TES/distilled water saturated phenol, Trizol-LS (BRL), chloroform, and isopropanol before precipitation with Sodium Acetate (pH 5.2) – 99.9% EtOH (DEPC) mixture. The resulting RNA was dissolved in pre-chilled distilled water.

Microarray analysis Preparation of the cDNA targets, hybridization and washing of the DNA microarray, fluorescence intensity measurement and gene expression analyses were all carried out following the manufacturer's protocol (Ambion Inc., Austin, TX, USA). Labeled cDNA targets were prepared from 10 μg purified mRNA using fluorescent-dye-labeled nucleotides, Cy3 and Cy5 (Amersham Biosciences AB, Uppsala, Sweden). Microarrays were scanned with ScanArray Lite (PerkinElmer Inc., Waltham, MA, USA). Differential expression analysis of the microarray data was carried out using Genowiz™ 4.0 microarray data analysis software (Ocimum Biosolutions, Hyderabad, India). Replicated values for genes were merged and the median values of the expression ratios were considered for the dataset while empty spots were removed by filtering. Fold change analysis was done to detect highly expressed genes. Genes with 2-folds up/down-regulation were considered as differentially expressed at a p-value <0.05, Student's t-test. Functional classification of the genes was performed using gene ontology and pathway analysis. The list of calcium-regulated calcineurin-dependent genes considered was taken from the genome-wide gene expression studies conducted by Yoshimoto and colleagues (18).

mRNA level determination using quantitative PCR The differential expression data of selected genes were validated by quantitative RT-PCR. Using the total RNA isolated by the hot phenol extraction, first strand cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems AB, Uppsala, Sweden). The synthesized cDNA was used as template for real-time PCR. Quantitative PCR was performed using 20 μl reaction mixtures on an Applied Biosystems 7300 Real-Time PCR System with SYBR® Green PCR Master Mix (Applied Biosystems). PCR was performed using the following amplification program: initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing for 1 min at 60°C. *ACT1* was used as the reference gene for normalization of mRNA levels. The RT-PCR primers used in this study were designed using Primer Express version 2.0 (Applied Biosystems) and are listed in the Supplementary Table S2.

Phenotypic analysis Sensitivity of the strains to calcium and FK506 was assessed by spot plating on solid media. Ten-fold serial dilution of cell suspension (106 cells/5 μl) was spottted onto YPDA and YPDA with 0.6 M $CaCl_2$ and 1 μg/ml FK506. The plates were incubated at 30°C for 2–4 days.

Fluorescence microscopy Cells expressing GFP-tagged Crz1 proteins were cultured in YPDA medium with or without 0.3 M $CaCl_2$ at 30°C until the mid-log phase after which the intracellular localization of the GFP-tagged protein was observed using a fluorescence microscope (BX61, Olympus) and photographed using a CCD camera (CCD-EX1, Universal Imaging Co., NY, USA). DNA was stained for fluorescence using 4′,6-diamidino-2-phenylindole (DAPI).

RESULTS

An active calcineurin pathway is inhibitory to the growth of the calcium-exposed *ptp2Δmsg5Δ* double disruptant *S. cerevisiae* cells generally respond to high extracellular calcium exposure by eliciting the calcium/calmodulin-dependent signaling pathway to maintain cell viability (2). To determine whether activation of calcineurin occurs in the *ptp2Δmsg5Δ* double disruptant upon exposure to calcium, we examined the expression of 120 genes that are known to be regulated by calcineurin using microarray (see Materials and methods). Based on Gene Ontology (GO) categories, 46 out of 120 calcineurin-dependent genes belong to "molecular function" while 23 and 33 out of 120 belong to "cell component" and "biological process", respectively.

Microarray results showed up-regulation of calcineurin-regulated genes in both the *ptp2Δmsg5Δ* double disruptant (89.1%) and wild-type (87.7%) strains upon exposure to high extracellular calcium (Fig. 1, column 1 and column 3, respectively). Interestingly, the expression values corresponding to the up-regulated calcineurin genes in the calcium-exposed *ptp2Δmsg5Δ* double disruptant did not vary significantly to that of the calcium-treated wild-type strain although most exhibited slight decrease in expression (Fig. 1, column 2). To confirm these results, the transcription level of several representative genes known to be up-regulated in a calcineurin-dependent manner was validated using real-time RT-PCR (3). These include the β-1,3-glucan synthase gene *GSC2* (*FKS2*), *PMC1*, *PMR1* and *ENA1*(*PMR2*), in which the latter three encode for P-type ATPases required for ion homeostasis (18). Results of the RT-PCR analysis revealed a similar trend in the expression with the microarray data wherein exposure to calcium caused an increase in the expression level of *GSC2*, *PMC1* and *ENA1* in the *ptp2Δmsg5Δ* double disruptant. Interestingly, expression of *PMR1* was not induced either in the wild type or *ptp2Δmsg5Δ* double disruptant upon exposure to calcium probably because *PMR1* is controlled by other calcium-dependent mechanisms. These results indicate that the calcineurin pathway is active in the *ptp2Δmsg5Δ* double disruptant and the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant is not due to a defective calcineurin pathway (Fig. 2A).

To strengthen the above conclusion, we studied the localization of Crz1 in the wild type and the *ptp2Δmsg5Δ* double disruptant after calcium exposure. Since calcineurin activation is known to cause nuclear localization of Crz1 (19), we tagged Crz1 with GFP in both wild type and *ptp2Δmsg5Δ* double disruptant. As expected, Crz1 was localized in the nucleus of the calcium-exposed wild type. Similarly, nuclear localization was observed in the *ptp2Δmsg5Δ* double disruptant exposed to calcium thereby indicating an active calcineurin pathway in the *ptp2Δmsg5Δ* double disruptant upon calcium exposure (Fig. 2B).

We inhibited the calcineurin pathway either by FK506 (a calcineurin inhibitor) treatment or disruption of *cnb1Δ* (calcineurin regulatory subunit) to determine the effect of inactivation of the calcineurin pathway on the phenotype of the *ptp2Δmsg5Δ* double disruptant. As expected, disruption of either *cna1Δ* or *cna2Δ* in the *ptp2Δmsg5Δ* double disruptant did not cause calcium tolerance since *CNA1* and *CNA2* redundantly code for the calcineurin catalytic unit (Fig. 3B). However, addition of FK506 in the medium (Fig. 3A) or disruption of *CNB1* (Fig. 3B) was able to suppress the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant, indicating that deactivation of the calcineurin pathway in the *ptp2Δmsg5Δ* double disruptant can restore growth in a calcium-exposed environment. Taken together, we conclude that transcription of calcineurin genes is not impaired and calcineurin activation has a negative effect on the growth of the *ptp2Δmsg5Δ* double disruptant upon exposure to calcium.

Interestingly, additional disruption of *CRZ1* in the *ptp2Δ msg5Δ* double disruptant background did not confer calcium tolerance. The fact that the *crz1Δ* single disruptant is also calcium sensitive implies that Crz1 is essential to the cell's viability under high extracellular calcium stress conditions. Our result also indicates that *CRZ1* is located downstream of the

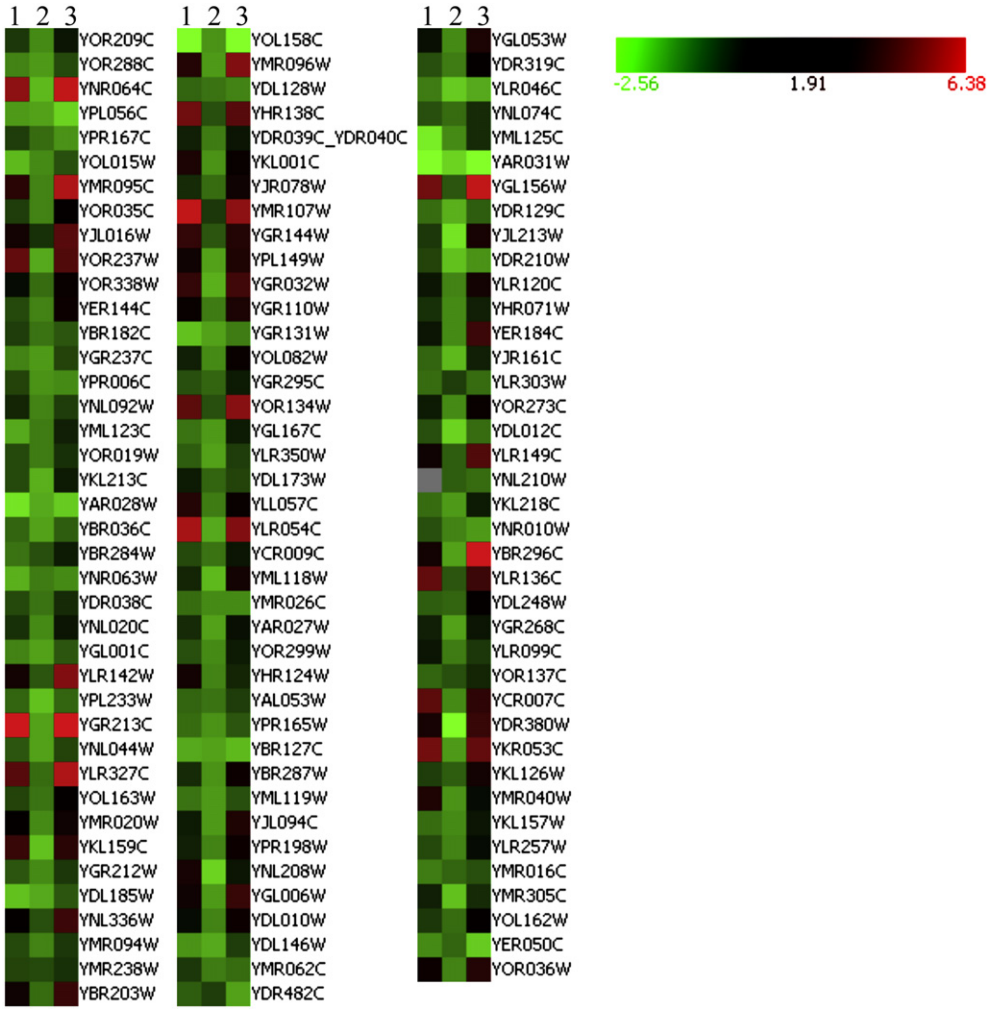

FIG. 1. Heatmap of the expression response of calcium-regulated calcineurin genes in the wild type and *ptp2Δmsg5Δ* with or without calcium exposure. The column number indicates the set-up as follows: (i) comparison between Ca^{2+}-exposed *ptp2Δmsg5Δ* with untreated *ptp2Δmsg5Δ*; (ii) comparison between Ca^{2+}-exposed *ptp2Δmsg5Δ* with Ca^{2+}-exposed wild type; (iii) comparison between Ca^{2+}-exposed wild type with untreated wild type. All strains were cultivated in YPDA media and calcium treatment was done by supplementing the YPDA medium with 0.3 M $CaCl_2$. Four independent DNA microarray data were analyzed using Genowiz (Ocimum Biosolutions). Genes showing more than 2-fold induction correspond to the rows and the columns represent the experimental set-ups. Red and green colors represent up-regulation and down-regulation of expression respectively. The color saturation represents the magnitude of the expression ratio, as indicated by the scale at the right side of the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

calcium-induced, growth-related redundant pathways mediated by Ptp2 and Msg5 where the parallel pathways converge (Fig. 3B).

Up-regulated cell wall genes in the *ptp2Δmsg5Δ* double disruptant share common transcription factors Sok2 and Ste12 Activation of the SLT2 pathway leads to the induction of transcription factors like Rlm1 and Swi4/Swi6 which in turn stimulates the expression of downstream or effector genes that play roles in the maintenance of cell wall integrity (20–22). Based on this, we inferred that hyper-activation of the SLT2 pathway essentially causes up-regulation of several downstream genes that eventually leads to cell toxicity (20,23). Since many of the effector genes downstream of the SLT2 pathway are involved in cell wall construction and cell wall-related processes, we used the microarray data to search for cell wall-related genes that are differentially regulated in the calcium-exposed *ptp2Δmsg5Δ* double disruptant. Out of the 31 cell wall-related genes that are reported to be differentially expressed during cell cycle, we found five genes namely *CWP1*, *CHS1*, *PST1*, *SCW11* and *EXG1* that are up-regulated while another gene, *CWP2*, was down-regulated in *ptp2Δmsg5Δ* double disruptant exposed to calcium (Supplementary Table S3) (22,24). To find a link between the cell wall genes that are differentially expressed, we looked for transcription factors that were common among the differentially regulated genes. YEASTRACT analysis identified two probable transcription factors shared by the altered genes as Sok2 and Ste12. However, compared to the wild type, *SOK2* and *STE12* were not differentially expressed in the *ptp2Δmsg5Δ* double disruptant; indicating that the mode of regulation involved is post-transcriptional (data not shown).

To further elucidate the roles of the transcription factors Sok2 and Ste12 in the calcium phenotype of the *ptp2Δmsg5Δ* double disruptant, we disrupted *SOK2* and *STE12* independently and compositely in the *ptp2Δmsg5Δ* double disruptant background. Results showed that neither disruption of *STE12* or *SOK2* conferred calcium tolerance to the *ptp2Δmsg5Δ* double disruptant. However, disruption of both *STE12* and *SOK2* was able to confer a slightly calcium tolerant phenotype as shown in the quadruple disruptant, *ptp2Δmsg5Δste12Δsok2Δ* (Fig. 4). This indicates that both *STE12* and *SOK2* play roles in the calcium phenotype of the *ptp2Δmsg5Δ* double disruptant and their synergistic relationship negatively affects growth in the presence of calcium.

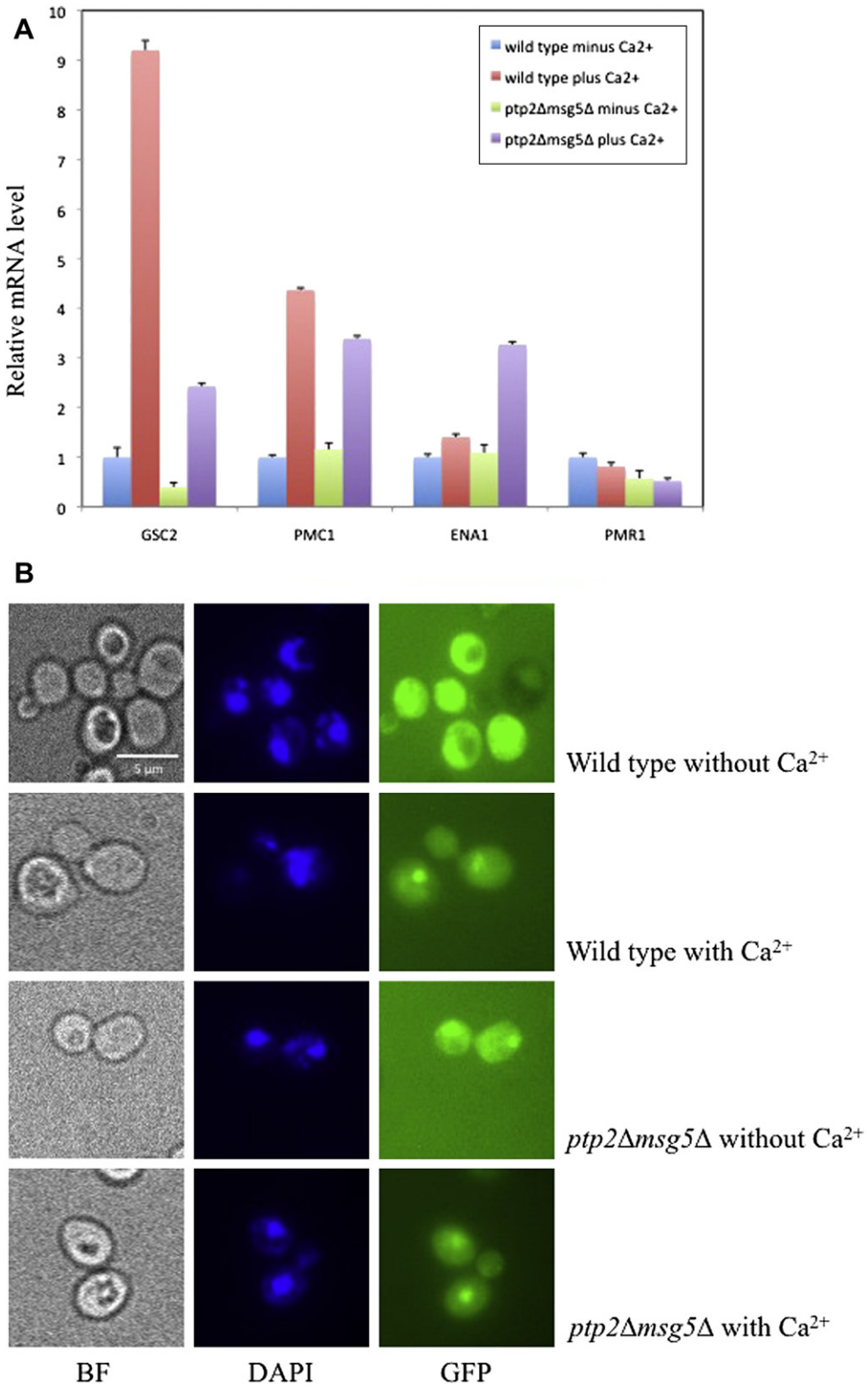

FIG. 2. Calcineurin activation occurs in the *ptp2Δmsg5Δ* double disruptant. (A) Representative calcineurin genes in the *ptp2Δmsg5Δ* double disruptant are up-regulated upon calcium exposure. cDNA synthesized from total RNA isolated from the sample strains grown with (0.3 M $CaCl_2$) or without calcium were used as templates for RT-PCR using SYBR Green and specific primers for *GSC2*, *PMC1*, *ENA1* and *PMR1*. *ACT1* was used as control. *GSC2*, *PMC1* and *ENA1* showed increased expression while *PMR1* expression remained the same in the *ptp2Δmsg5Δ* double disruptant upon calcium exposure. The results are means of three experiments from three independent preparations. (B) Crz1p localization in the nucleus occurs in the *ptp2Δmsg5Δ* double disruptant. Wild type and *ptp2Δmsg5Δ* double disruptant cells expressing GFP-tagged Crz1p were grown in medium with or without calcium until mid-log phase. Subcellular localization of GFP-Crz1p (GFP) and DAPI-stained nucleus (DAPI) was observed by fluorescence microscopy. Unstained cells were visualized by bright field microscopy (BF). Approximately 200 individual cells for each strain and treatment were observed for Crz1p localization.

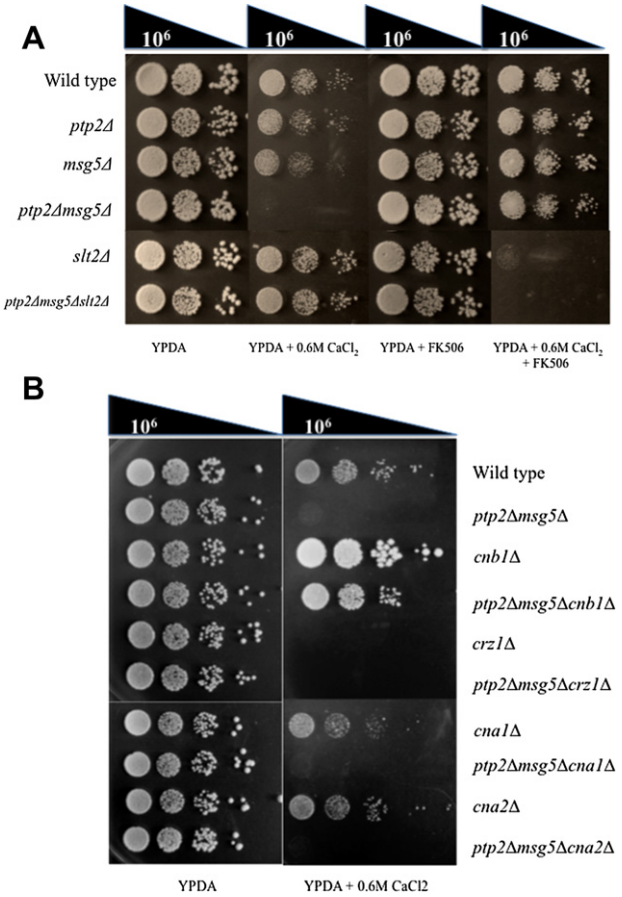

FIG. 3. Inhibition of calcineurin by *CNB1* disruption or FK506 treatment suppresses the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant. (A) Wild-type (SH5209), *ptp2Δ*, *msg5Δ*, *slt2Δ*, *ptp2Δmsg5Δ* and *ptp2Δmsg5Δslt2Δ* were grown in YPDA to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA, YPDA supplemented with 0.6 M $CaCl_2$, YPDA added with FK506 (calcineurin inhibitor) and YPDA with both $CaCl_2$ and FK506. The plates were incubated at 30°C for 2–4 days. (B) The subunits of calcineurin were individually disrupted in the *ptp2Δmsg5Δ* background to produce triple disruptants. *CNA1* and *CNA2* are catalytic units while *CNB1* is the regulatory subunit of calcineurin. Wild-type (SH5209), *cna1Δ*, *cna2Δ*, *cnb1Δ*, *crz1Δ*, *ptp2Δmsg5Δ*, *ptp2Δmsg5Δcna1Δ*, *ptp2Δmsg5Δcna2Δ*, *ptp2Δmsg5Δcnb1Δ* and *ptp2Δmsg5Δcrz1Δ* were grown in YPDA to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA with and without 0.6 M $CaCl_2$. The plates were incubated at 30°C for 2–4 days.

SLT2 pathway is hyper-activated in the *ptp2Δmsg5Δ* double disruptant upon exposure to high extracellular calcium MAPK signaling cascades are activated by the process of phosphorylation of cascades of protein kinases (25). Furthermore, regulation of these pathways is via dephosphorylation of MAPK by protein phosphatases like Ptc1, Ptc2, Ptc3, Ptp2, Ptp3 and Msg5 to name a few (13). Since Ptp2p and Msg5p are known to negatively regulate Slt2p phosphorylation, we confirmed if Slt2p was indeed hyper-phosphorylated in the *ptp2Δmsg5Δ* double disruptant. As expected, Western blot analysis revealed that Slt2p was hyper-phosphorylated in the *ptp2Δmsg5Δ* double disruptant compared to the wild type (Fig. 5). Our result is consistent with the earlier findings that disruption of both *PTP2* and *MSG5* PPase genes results in a hyper-phosphorylated state of Slt2p (11).

Calcium sensitivity in the *ptp2Δmsg5Δ* double disruptant results from a hyper-activated, calcium-induced signaling pathway caused by the improper activation of the SLT2 pathway and an active calcineurin We presumed that the simultaneous activation of both SLT2 and calcineurin pathways might be toxic to cells as genetic evidence indicates a functionally redundant relationship between SLT2 and calcineurin pathways in growth-related functions (26). Thus, we tried to inactivate either one of the redundant pathways (calcineurin or SLT2 pathways) to determine if the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant can be suppressed. Indeed, *slt2Δ* disruption was able to confer calcium tolerance in the *ptp2Δmsg5Δ* double disruptant (12). Moreover, when calcineurin was inactivated in the *ptp2Δmsg5Δ* double disruptant by either disrupting *CNB1* (calcineurin regulatory subunit gene) or by treatment with a calcineurin inhibitor, FK506, we found that inactivation of calcineurin by either means was also able to suppress the calcium sensitivity of the *ptp2Δmsg5Δ* double disruptant (Fig. 3). Thus, calcineurin seems to have an inhibitory role in the growth of the calcium-exposed *ptp2Δmsg5Δ* double disruptant under a hyper-phosphorylated SLT2 state (Fig. 3).

Inactivation of both calcineurin (FK506 treatment) and SLT2 pathways (disruption of *slt2Δ* in *ptp2Δmsg5Δ*) led to a calcium-sensitive phenotype, indicating that the presence of at least one of the redundant pathways is essential for viability under high calcium conditions (Fig. 3A). Taken together, these findings indicate that a hyper-activated signaling state caused by a simultaneously over-activated SLT2 pathway and an active calcineurin pathway led to a calcium-sensitive phenotype in the *ptp2Δmsg5Δ* double disruptant upon exposure to high extracellular calcium.

DISCUSSION

We previously reported a PPase double disruptant, *ptp2Δmsg5Δ*, exhibiting sensitivity at very high concentrations of calcium (0.6 M). This calcium-sensitive phenotype was suppressed by an additional disruption of either one of the six PKases namely *BCK1*, *MKK1*, *SLT2*, *MCK1*, *SSK2* and *YAK1* (12). Although we have reported in our earlier study that the SLT2 pathway is over-activated in the *ptp2Δmsg5Δ* double disruptant, the detailed mechanism on how a hyper-activated SLT2 pathway causes calcium sensitivity remained unclear. In this work, we clarified the conditions that lead to the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant in terms of functional redundancy at the protein and signal cascade levels.

At the protein level, Ptp2p and Msg5p, which are protein tyrosine (PTP) and dual specificity (DSP) PPases respectively, are known to co-regulate Slt2p (Mpk1p) of the SLT2 (Cell Wall Integrity or Mpk1) pathway (13,21). Based on genetic analysis, we established the redundant roles of these two PPases in the calcium-mediated signaling process since single disruption of either *ptp2Δ* or *msg5Δ* is calcium tolerant (10). In general, the MAPK cascade is comprised of a sequential activation of protein kinases. Specifically, MEK kinase (MEKK or MAPKKK) activates the MAPK kinase (MEK or MAPKK) which in turn activates the MAP kinase by dual phosphorylation of its TXY motif (25). This cascade event will eventually result in the activation of various transcription factors or induction of specific sets of genes based on the environmental stimuli. Thus, we presumed that hyper-activation of the SLT2 pathway arises when there is accumulation of hyper-phosphorylated Slt2p in the absence of the negative regulators, Ptp2p and Msg5p (Fig. 6). In this case, a hyper-phosphorylated state of Slt2p is indicative of a hyper-activated SLT2 pathway since the activation of yeast MAPK cascades is through phosphorylation (25). This hyper-activated state of the SLT2 pathway induces G1 delay by possibly regulating the expression of effector genes such as *CLN2* via several transcription factors as we previously reported (11). In connection with this, we reported the down-regulation of *CLN2* in the *ptp2Δmsg5Δ* double disruptant upon exposure to high extracellular calcium (12). The *CLN2* down-regulation can be explained based on the model for regulation of Swi4 by Slt2 (Mpk1) (21). In this model, Slt2 is

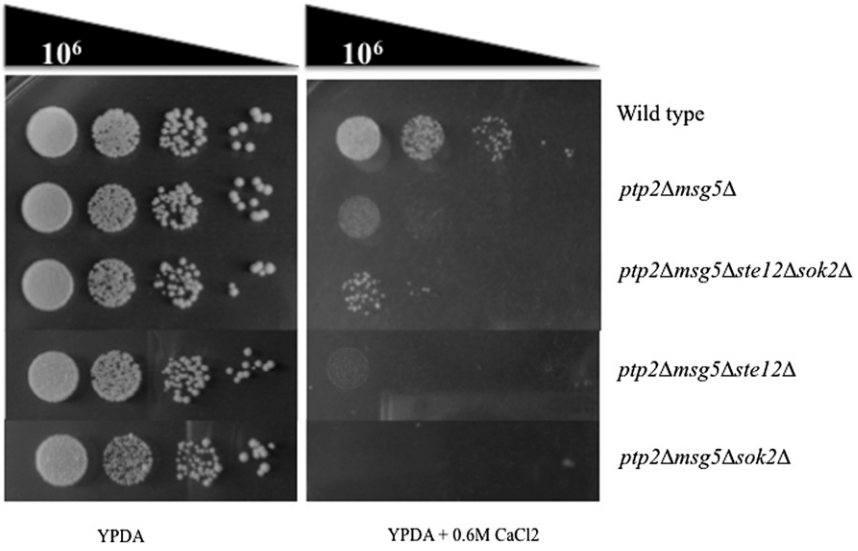

FIG. 4. Deletion of both transcription factors Ste12 and Sok2 confers partial calcium tolerance to *ptp2Δmsg5Δ* double disruptant. Wild-type (SH5209), *ptp2Δmsg5Δ*, *ptp2Δmsg5Δste12Δ*, *ptp2Δmsg5Δsok2Δ* and *ptp2Δmsg5Δste12Δ sok2Δ* were grown in YPD to mid-log phase. Ten-fold serial dilutions of the cell suspensions were prepared and spot plated on to YPDA with and without 0.6 M $CaCl_2$. The plates were incubated at 30°C for 2–4 days.

activated by a calcium-induced cell wall stress and is likely to phosphorylate Swi6, causing its exclusion to the nucleus. A non-nuclear localized Swi6 therefore cannot form the SBF complex, resulting in reduction or abolishment of transcription of late G1 genes including *CLN1* and *CLN2*.

The fact that the calcium-sensitive phenotype of the *ptp2Δmsg5Δ* double disruptant exhibits growth arrest (G1 arrest) similar to that of calcineurin-deficient mutants suggested the possible involvement of calcineurin in this signaling pathway. Although essential for survival during stressed conditions, we propose that an active calcineurin coupled with a hyper-activated SLT2 pathway becomes toxic to yeast cells upon exposure to high extracellular calcium (Fig. 3). To our knowledge, this is the first report of the existence of a functionally redundant relationship between the SLT2 and calcineurin pathways in response to high levels of calcium. In this case where the SLT2 pathway is hyper-activated due to the absence of the negative regulators Ptp2p and Msg5p, one way to alleviate the harmful effects of an over-active calcium-induced signaling pathway is to inactivate calcineurin by *cnb1Δ* disruption or treatment with a calcineurin inhibitor, FK506 (Fig. 3). Therefore, the redundant pathways related to growth serve as safeguards for unwanted effects of an inactive signaling pathway. In this system, a signal cascade assumes the function of another cascade when it is inactivated or impaired. However, improper activation of both signaling cascades becomes harmful to the cells due to signal hyper-activation that eventually leads to cessation of growth. In this study, we have demonstrated that such is the case for strains lacking signaling cascade regulators such as PPases Ptp2 and Msg5 when exposed to high extracellular calcium (Fig. 6).

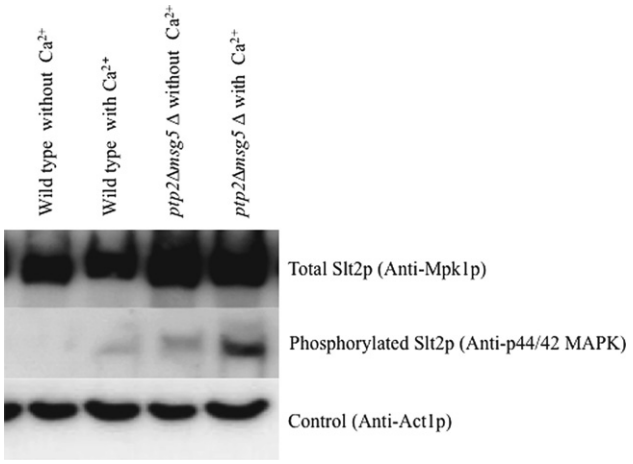

FIG. 5. Slt2p is hyper-phosphorylated in the calcium-exposed *ptp2Δmsg5Δ* double disruptant. Representative Western blot showing the phosphorylation level of Slt2p. Soluble protein extracts were prepared from cells grown in YPDA with (0.3 M CaCl2) or without calcium at mid-log phase. Total Slt2p and the phosphorylated form of Slt2p were detected using anti-Mpk1p and anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibodies, respectively. Anti-Act1p was used as control.

Similar to a published report (27), our result showed that expression of *GSC2* (*FKS2*) is low under normal growth conditions but immediately increases upon exposure to calcium. In the *ptp2Δmsg5Δ* double disruptant exposed to calcium, there was also an increase in *GSC2* expression (2-fold) although at a much lower increment compared to the wild type (6-fold) (data not shown). This can be an indication that the calcineurin pathway is operational but its activity is slightly impaired in the *ptp2Δmsg5Δ* double disruptant. There was an earlier report of a dual control of *GSC2* expression by PKC and calcineurin pathways induced by heat stress (28). If calcineurin activity is impaired, it is expected that calcium-induced expression of *GSC2* is also reduced because calcium induction of *GSC2* is not cell integrity pathway-dependent (28). Moreover, *GSC2* expression in response to extracellular calcium is strictly calcineurin-dependent. Our results imply that hyper-activation of the SLT2 pathway possibly has an inhibitory effect on calcineurin activity.

In the advent of sudden exposure to high extracellular calcium, induction of stress response plays an integral role in the cell's survival. It is therefore imperative that *S. cerevisiae* possess parallel, alternative mechanisms to control calcium signaling in cases where the major pathway is impaired. However, stringent regulation of these redundant pathways is essential to avoid over-activation. Our results show that the SLT2 and calcineurin pathways co-mediate cellular processes related to growth upon exposure to high extracellular calcium. It is highly probable that upon exposure to calcium, the calcineurin pathway is the primary signaling response that the yeast employs while the SLT2 pathway, being secondary, is only activated in conditions where the calcineurin pathway is

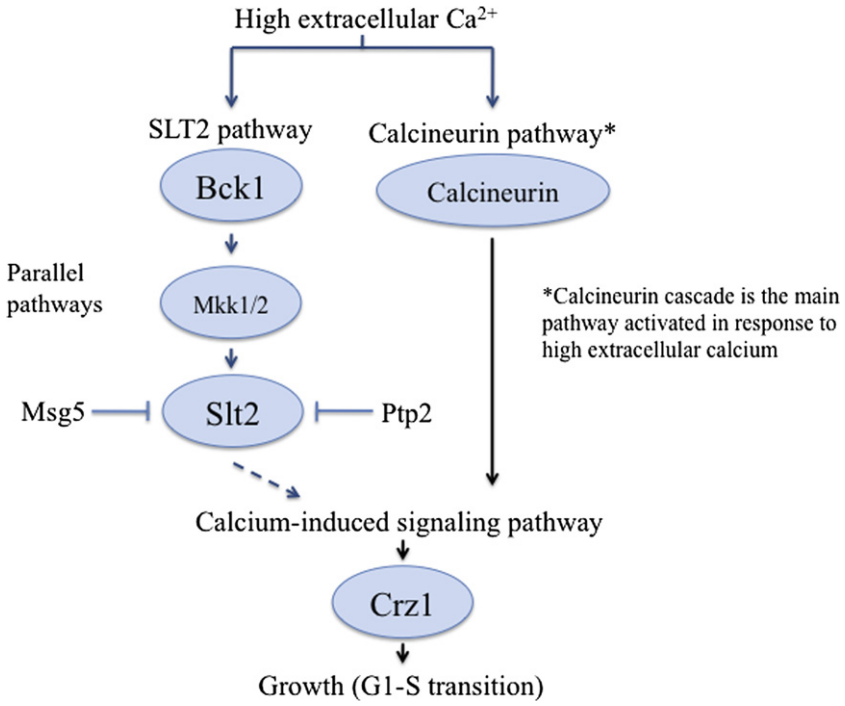

FIG. 6. Over-activation of the calcium-induced signaling pathway in the *ptp2Δmsg5Δ* double disruptant causes calcium sensitivity. Calcineurin and SLT2 pathways share redundant functions in cell processes related to growth. Upon exposure to high extracellular calcium, calcineurin is the preferred route while SLT2 pathway is inactivated through dephosphorylation of Slt2p by the functionally redundant Ptp2p and Msg5p. In the absence of both Ptp2 and Msg5 PPases, the SLT2 pathway is hyper-activated and causes growth delay due to a hyper-activated state of the calcium-induced signaling cascade when coupled with an active calcineurin pathway. The two parallel and redundant pathways that are induced by calcium converge at Crz1.

completely impaired. In the wild type, this is accomplished by inhibition of the SLT2 pathway via Slt2p dephosphorylation with the functionally redundant PPases, Ptp2 or Msg5 (Fig. 6). However, over-activation of the calcium-induced signaling pathway can occur if both pathways are active and this state promotes growth inhibition. Our results signify that in the event of SLT2 pathway hyper-activation in the presence of high extracellular calcium, calcineurin function can be abrogated to prevent growth inhibition. However, we found that complete inhibition of both pathways by the disruption of *SLT2* pathway genes and treatment of FK506 led to a calcium-sensitive phenotype (Fig. 3A), indicating that the presence of at least one of the redundant pathways is essential for viability in high calcium conditions (Fig. 6).

Our results revealed that Crz1 is an essential part of the calcium-induced, growth-related signaling pathway manifested in the *ptp2Δmsg5Δ* double disruptant. Genetic analysis suggested that Crz1 is located downstream of the redundant SLT2 and calcineurin pathway and is possibly the point of convergence between the parallel pathways. We also found that common calcineurin and Crz1-induced genes like *PMR1*, *GSC2*, *PMC1* and *ENA1* were not the transcriptional targets of the calcium-induced signaling pathway in the *ptp2Δmsg5Δ* double disruptant (Fig. 2A) thus pointing out that the parallel pathways might induce the expression of other genes related to growth and stress response. This assumption is reinforced by the fact that representative calcineurin-regulated genes *PMC1* and *ENA1* were induced in the *ptp2Δmsg5Δslt2Δ* but not in *ptp2Δmsg5Δcnb1Δ* upon exposure to high extracellular calcium conditions (Supplementary Fig. S1). The identity of the transcriptional targets of the parallel SLT2 and calcineurin pathways mediated by Ptp2 and Msg5 is still unknown.

Interestingly, Sdp1 or Yil113p has been implicated with SLT2 pathway regulation via dephosphorylation of Slt2, which is similar to the function of Ptp2 and Msg5 in the SLT2 pathway (23,29–31). In the *ptp2Δmsg5Δ* double disruptant, the presence of a functional Sdp1p could not compensate for the absence of both Ptp2 and Msg5, resulting in the over-activation of the calcium-induced signaling pathway and eventually calcium sensitivity. This fact rules out the possibility that Sdp1 is functionally redundant to Ptp2 and Msg5 in relation to the calcium-induced signaling pathway in the *ptp2Δmsg5Δ* double disruptant. In accordance with our previous statement, our unpublished data showed that the *sdp1Δ* single disruptant (BY4742 background) was calcium tolerant (0.6 M $CaCl_2$) which is an indication that Sdp1 activity is stress specific and has no relation to calcium stress. This conclusion is supported by reports showing that *YIL113W* disruptant (*sdp1Δ*) exhibited normal response to mating pheromone and has no effect on the phosphorylation of Hog1p MAPK thus pointing out that Sdp1 is not involved in regulating the response to pheromone and osmotic stress in *S. cerevisiae* (31). Results of these studies support the claim that Yil113p phosphatase is a specific regulator of signaling through the Slt2/Mpk1 MAPK pathway and it is highly probable that calcium-induced signaling mediated by the functionally redundant Ptp2 and Msg5 is independent of the Sdp1 function.

This paper discussed the mechanism of calcium sensitivity in the *ptp2Δmsg5Δ* double disruptant and the suppression mechanism conferred by either disrupting SLT2 pathway genes or inactivating calcineurin. The disparity in the mechanism in which calcium tolerance is conferred by *ssk2Δ* or *yak1Δ* disruption remains to be elucidated. Also, the extent and cause of calcineurin impairment is not presently known and is the subject of future study. In this study, we found proof of the functional redundancy of Ptp2 and Msg5 at the protein level as well as the SLT2 and calcineurin pathways at the cascade level in response to high extracellular calcium conditions. Furthermore, our results imply the important roles of PPases Ptp2 and Msg5 in preventing hyper-activation of SLT2 pathway upon exposure to high calcium concentrations. Lastly, strict modulation of the calcium-induced signaling pathway mediated by the functionally redundant SLT2

and calcineurin pathways is imperative for cell growth under high calcium environments.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2012.08.022.

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