Protein phosphatase CaPpz1 is involved in cation homeostasis, cell wall integrity and virulence of *Candida albicans*

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†With sadness we note that our colleague and co-author passed away on 22. 07. 2011.

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Abstract

The opportunistic pathogen *Candida albicans* has a single protein phosphatase Z candidate gene termed *CaPPZ1* which shows significant allele variability. We demonstrate here that bacterially expressed CaPpz1 protein exhibits phosphatase activity which can be inhibited by recombinant Hal3, a known inhibitor of *S. cerevisiae* Ppz1. Site-directed mutagenesis experiments based on natural polymorphisms allowed the identification of three amino acid residues affecting enzyme activity or stability. The expression of *CaPPZ1* in *ppz1 S. cerevisiae* and *pzh1 S. pombe* cells partially rescued the salt and caffeine phenotypes of the deletion mutants. CaPpz1 also complemented the *slt2 S. cerevisiae* mutant that is crippled in the MAP kinase mediating the cell wall integrity signalling pathway. Collectively, our results suggest that the orthologous PPZ enzymes have similar but not identical functions in different fungi. The deletion of the *CaPPZ1* gene in *C. albicans* resulted in a mutant that was sensitive to salts like LiCl and KCl, to caffeine, and to agents affecting cell wall biogenesis like Calcofluor White and Congo Red, but was tolerant against spermine and hygromycin B. Reintegration of the *CaPPZ1* gene into the deletion mutant alleviated all of the mutant phenotypes tested. Thus CaPpz1 is involved in cation homeostasis, cell wall integrity and the regulation of the membrane potential of *C. albicans*. In addition, the germ tube growth rate and the virulence in the BALB/c mice model was reduced in the null mutant, suggesting a novel function for CaPpz1 in the yeast to hypha transition that may have a medical relevance.

INTRODUCTION

The Ppz Ser/Thr protein phosphatases constitute a family of enzymes that are structurally related to the type-1 protein phosphatases, and are restricted to fungal species (Arino, 2002). In *Saccharomyces cerevisiae*, where these proteins were discovered (Lee *et al.*, 1993;Posas *et al.*, 1992;Posas *et al.*, 1993), there exist two genes, namely *PPZ1* and *PPZ2*. To avoid confusion, these two phosphatase genes will be referred to *ScPPZ1* and *ScPPZ2* in the present paper. The encoded proteins contain C-terminal catalytic domains, which are highly similar to each other, while their N-terminal moieties are much less related (Fig. 1a). Deletion of *ScPPZ2* does not result in a detectable phenotype. However, strains lacking ScPpz1 exhibit increased tolerance to NaCl and LiCl (Posas *et al.*, 1995), altered K^+ influx, and hypersensitivity to caffeine (Posas *et al.*, 1993;Yenush *et al.*, 2002). On the other hand, overexpression of *ScPPZ1* results in slow growth and cell cycle blockage at the G1-S transition (Clotet *et al.*, 1999). The effect of the *Scppz1* mutation on cation homeostasis is due to the elevated expression of the Na^+-ATPase *ENA1* gene (Posas *et al.*, 1995;Ruiz *et al.*, 2003) and to the increased influx of potassium ions mediated by the high-affinity K^+-transporter Trk1 (Yenush *et al.*, 2002). *ScPPZ1* exhibits genetic interactions with *SLT2*, encoding a MAP kinase required for signalling in the cell wall integrity (CWI) pathway (reviewed in (Levin, 2005)). *slt2* cells are prone to lysis and are very sensitive to high temperature, caffeine, or cell wall damaging compounds. Overexpression of *ScPPZ1* suppresses the lytic phenotype of a *slt2* mutant, whereas deletion of the *ScPPZ1* gene, or inhibition of its phosphatase activity results in a phenotype additive to that of the *slt2* strain (de Nadal *et al.*, 1998;Lee *et al.*, 1993). The functional interaction between ScPpz1 and the CWI pathway has been explained on the basis of the combination of increased internal turgor pressure in Ppz-deficient strains and cell wall instability observed in strains lacking Slt2 (Merchan *et al.*, 2004). Therefore, in budding yeast the Ppz proteins play key roles in cation homeostasis, which is likely to affect cell cycle regulation. The Hal3 and Vhs3 regulatory subunits bind to the catalytic domain of ScPpz1 and inhibit its activity (de Nadal *et al.*, 1998;Ruiz *et al.*, 2004). The overexpression of these inhibitors mimics the effects of the *scppz1* mutation.

The PPZ phosphatases have been only partially characterized in other yeasts or fungi. *Schizosaccharomyces pombe* contains the *pzh1* gene, encoding a protein that is similar to budding yeast Ppz (Fig. 1a), but has a shorter and rather divergent N-terminal domain (Balcells *et al.*, 1997). In *S. pombe,* the deletion of $pzh1^+$ results in cells hypertolerant to Na^+

and hypersensitive to K^+ (Balcells *et al.*, 1997). However, the mechanisms of action of Pzh1 in fission yeast are probably different from that observed for ScPpz1 in budding yeast, since cells lacking Pzh1 have no altered sodium or lithium efflux, rather they display decreased influx for these cations, together with a reduced K^+ efflux (Balcells *et al.*, 1999). A PPZ phosphatase gene from the filamentous fungus *Neurospora crassa*, named *Pzl-1*, has been identified by (Szoor *et al.*, 1998). The PZL-1 protein (Fig. 1a) was expressed in *S. cerevisiae* and was shown to fulfil every known function carried out by its *S. cerevisiae* counterpart, despite the marked sequence divergence within their N-terminal moieties (Vissi *et al.*, 2001). The expression of PZL-1 in the *pzh1* mutant *S. pombe* resulted in a less efficient complementation. Recently, a PPZ orthologous, termed DhPpz1, has been characterized in the extremely halotolerant yeast species *Debariomyces hansenii* (Minhas *et al.*, 2012). This phosphatase also has a disordered N-terminal segment that includes a short conserved Ser/Arg rich motif which is important in salt tolerance but not in CWI. Interestingly, this fungus utilises a Na^+/H^+ antiporter to evade the toxic effects of cations. The comparison of known PPZ enzymes suggests that although their major functions are retained across fungi, the underlying mechanisms can be different.

Candida albicans is an opportunistic pathogen with considerable medical significance. This organism contains a single PPZ candidate gene, termed *CaPPZ1*, that has at least four distinct alleles (Kovacs *et al.*, 2010). The allele combinations in the diploid organism together with individual point mutations result in a great genetic variability. The *CaPPZ1* gene codes for a protein whose primary structure is similar to the better characterized fungal counterparts (Figs. 1a and S1). Homologous modelling suggests that the three dimensional structure of the CaPpz1 catalytic domain is reminiscent to that of the protein phosphatase 1 catalytic subunit (Fig. 1b). The physiological significance of the *C. albicans* PPZ phosphatase has not been uncovered yet. The only available information extracted from large scale genetic screens tells that the disruption of either one (Xu *et al.*, 2007) or both (Hanaoka *et al.*, 2008) of the alleles is not detrimental. In the present work we characterize the function of this enzyme by biochemical assays of the recombinant protein, by expressing the *CaPPZ1* gene in Ppz-deficient *S. cerevisiae* and *S. pombe* strains, as well as by studying the relevant *C. albicans* mutant. Our work reveals that despite of structural similarities; CaPpz1 only partially complements the lack of its orthologs in other fungi and has a novel function in controlling the germ tube formation of *C. albicans*.

METHODS

DNA cloning. The cloning of *C. albicans CaPPZ1* gene was described before (Kovacs *et al.*, 2010). The *CaPPZ1-3* allele (accession number: GQ487308) was used to insert the *ORF* into a pET28a+ expression vector (Novagen) in two steps. The two halves of the coding region were amplified by PCR with the UPPZ-NdeI / LPPZ-NdeI and UPPZ-PstI- / LPPZ-XhoI primer pairs (Table S1a), and were ligated together resulting in the CaPPZ1-pET28a+ plasmid. In order to confirm the predicted gene structure, we also cloned the corresponding cDNA from the ATCC 10231 reference strain. Total RNA was prepared with SV Total RNA Isolation System (Promega), and the mRNA was reverse-transcribed with the 5'/3' RACE Kit (Roche). The cDNA was amplified by PCR using the 2U and 2L primers (Table S1a) and was placed into a pGEM-T Easy vector (Promega) to yield the CaPPZ1-pGEM-T plasmid whose sequence was deposited into the NCBI database under the accession number JF330253.

For the expression in *S. cerevisiae* under the control of the *ScPPZ1* promoter, first the -525/-10 segment of the promoter region was amplified by PCR from *S. cerevisiae* FY1679 genomic DNA with ScPromSacI and ScPromXbaI primers (Table S1a) according to (Vissi *et al.*, 2001) and the amplicon was cloned into pGEM-T Easy plasmid. Next, the promoter region was released with SacI and XbaI and was cloned into the plasmids YCplac111 and YEplac181. Then the coding region of the *CaPPZ1-3* allele was amplified from the CaPPZ1-pGEM-T cDNA clone by PCR with C1XbaI and C2HindIII primers (Table S1a) and was placed after the promoter to generate the YCp-*CaPPZ1* and YEp-*CaPPZ1* plasmids. The construction of the control plasmid YCp111-*ScPPZ1* was described previously (Clotet *et al.*, 1996).

For the expression in *S. pombe,* the coding region of *CaPPZ1-3* was amplified from the CaPPZ1-pGEM-T construct by PCR with C3BamHI and C4BamHI primers (Table S1a) and was inserted into the expression vector pREP41 (Basi *et al.*, 1993;Maundrell, 1993) producing the pREP41-*CaPPZ1* plasmid. All of the vector constructs were confirmed by DNA sequencing.

In vitro mutagenesis. The positions of the mutated amino acids are shown in Figs. 1(b) and S1. The homologous model of the CaPpz1 catalytic domain was built on the crystal structure of rabbit muscle PP1 catalytic subunit fragment (amino acid residues 7-300; (Goldberg *et al.*, 1995); PDB accession number: 1FJM) using the Modeller7 program (Sali & Blundell, 1993). Sequence alignment was done by ClustalW (Thompson *et al.*, 1994). Mutations resulting in

single or double amino acid exchanges were introduced into the *CaPPZ1-3* sequence with the aid of the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). PCR of the CaPPZ1-pET28a+ plasmid target with the oligonucleotide primers described in Table S1(b) resulted in the R262L, D261N, G333E, and C337R mutations, while the double mutant encompassing both G333E and C337R replacements was generated from the G333E mutant by a second round of mutagenesis.

Expression of *CaPPZ1* in *E. coli.* Wild type and mutated CaPpz1-3 was expressed in *E. coli* BL21 (DE3)-RIL (Stratagene) after the addition of 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich) followed by 16 h incubation at 18 ºC (Fig. S2a). The recombinant proteins were purified from the supernatant of the bacterial extract with Ni-NTA Agarose (Qiagen) affinity chromatography. The fractions eluted from the columns were analyzed by SDS-PAGE (Fig. S2b). The protein concentration of the fractions was assayed with the Bradford method (Bradford, 1976). The CaPpz1 content in the peak fractions was estimated by scanning of the electrophoretograms. The phosphatase activity of the fractions was assayed in triplicate samples containing 1to 2 μg of recombinant phosphatase with p-nitrophenylphosphate (Sigma-Aldrich) substrate in the presence or in the absence of Hal3 as reported before (Munoz *et al.*, 2004). Recombinant *S. cerevisiae* Hal3 was expressed in *E. coli* and was purified according to (Garcia-Gimeno *et al.*, 2003).

Expression of *CaPPZ1* in *S. cerevisiae.* The genotypes and origin of the *S. cerevisiae* strains used in this work are listed in Table 1. Single *kanMX* deletion mutants in the BY4741 background were generated in the context of the *Saccharomyces* Genome Deletion Project (Winzeler *et al.*, 1999). The mutants were transformed with different rescue plasmids as in (Vissi *et al.*, 2001). *S. cerevisiae* cells were grown at 28 °C in YPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) or, when carrying plasmids, in synthetic complete drop-out medium (lacking leucine).-Sensitivity of yeast cells to LiCl, NaCl, caffeine (Merck) or Calcofluor White (Sigma) was evaluated by growth on agar plates (drop tests) as previously described (Posas *et al.*, 1995).

Expression of *CaPPZ1* in *S. pombe.* The genotypes and origin of the *S. pombe* strains are summarized in Table 1. The *pzh1*$^{+}$ control strain was isolated from a cross between the *S. pombe h*$^{+}$ wild type strain and *S. pombe* 117 strain (*h*$^{-}$ *ade6-M210 leu 1-32 ura4-D18*). For cultivation of *S. pombe* a standard complete medium: yeast extract agar (YEA), yeast extract

liquid (YEL) (Gutz *et al.*, 1974) and Edinburgh minimal medium 2 (EMM2, US Biological) plates or liquid cultures were used with appropriate supplements (100 mg/l adenine, uracil, leucine). Fission yeast strains were transformed by the lithium-acetate method (Ito *et al.*, 1983). Transformed cells grew on EMM2+ade medium which contained 400 μmol/l thiamine to repress the *nmt1* (no message in thiamine) promoter of the vectors. In our assays first the transformants were grown in EMM2+ade (without thiamine) overnight (for the expression of pREP construct) and these pre-cultures were used to inoculate EMM2+ade liquid media supplemented with different salts or caffeine in the following concentrations: 300, 350, and 400 mM KCl; 150, 200, and 250 mM NaCl, 5, 8, and 10 mM LiCl, as well as 5, 10, and 15 mM caffeine. The starting OD_{595} values were set to 0.2 and cell growth was characterized by measuring the optical density of the cultures after 24 h incubations.

Disruption of the *CaPPZ1* gene in *C. albicans*. The SN87 strain was used for gene disruption (Table 1). By cloning and sequencing of the *CaPPZ1* gene according to (Kovacs *et al.*, 2010) we proved that this strain was homozygous for the *CaPPZ1-1* allele. *C. albicans* strains were cultivated in YPD medium at 37 ºC, while the transformants were selected and grown on SD solid medium (6.7 g/l yeast nitrogen base with ammonium sulphate, 20 g/l glucose, 15 g/l agar with or without the amino acids 100 mg/l leucine and 20 mg/l histidine, as needed).

The *CaPPZ1-1* gene was deleted by using a PCR-based procedure with primers harbouring approximately 100-bp regions from the 5'- and 3'-flanking sequences of the target gene (Gola *et al.*, 2003). The *cappz1* strain was generated by successive transformations with two disruption cassettes containing the *HIS1* and *LEU2* auxotrophic markers. The cassettes were amplified from the pFA-CdHIS1 and pFA-CmLeu2 plasmids (Schaub *et al.*, 2006) with the primers described in Table S1(c), and were transformed into the SN87 strain by electroporation in two steps (Noble & Johnson, 2005).

Characterization of the *C. albicans* strains. For the validation of gene disruption, genomic DNA was extracted from *C. albicans* as described (Lee *et al.*, 1988). The integration of the disruption cassettes was tested by PCR using "diagnostic" primers (Table S1d, Figs. S3a and S3b). Southern hybridizations were performed with cassette specific probes generated by PCR (Table S1e) to verify the deletions and to exclude the possibility of the ectopic integration of disruption cassettes (Fig. S3c). The absence of *CaPPZ1* transcripts from the *cappz1* strain was confirmed by RT-PCR with the primers given in Table S1f (Fig. S3b).

One functional *CaPPZ1* gene copy was reintegrated into the *cappz1* mutant with the aid of the *SAT1* flipper containing pSFS2A vector (Reuss et al., 2004) as described in Fig. S3d. The presence of the *CaPPZ1* gene and either of the *HIS1* or *LEU2* cassettes in the heterozygous transformants was demonstrated by PCR. It was also shown that the *CaPPZ1-HIS1* strain exhibited His^+ and Leu^- phenotype, while the *CaPPZ1-LEU2* strain was His^- and Leu^+. The genotypes of the *C. albicans* strains investigated in the present study are described in Table 1.

The sensitivity of the *C. albicans* cells to salts (LiCl, NaCl, and KCl), toxic cations (spermine and hygromycin B), cell wall damaging agents (Calcofluor White, Congo Red) and caffeine, was evaluated in YPD medium. The relative growth of the liquid cultures was measured in 96 well plates using a Multiskan RC ELISA reader (Thermo Labsystems) at OD_{620} after 18 h incubations at 37 ºC. The starting OD_{620} was 0.0005 in all experiments.

Hyphal growth was induced by the addition of 90% sheep serum and the germination capacity of the cells was determined microscopically (Gyetvai *et al.*, 2007). More than 100 cells were counted in each experiment. For the better visualisation of hyphae the fungal samples were stained with Calcofluor White (CFW) according to the manufacturer's recommendation (http://www.sigmaaldrich.com/etc/medialib/docs/Fluka/Datasheet/18909dat.Par.0001.File.tmp/18909dat.pdf), and were analysed under an Olympus BX40 immersion microscope.

The virulence of the *C. albicans* strains was analyzed in immunocompetent female BALB/c mice essentially as in (Noble & Johnson, 2005) with the exception that less pathogen was used for inoculation. Higher doses of fungi led to 80-90 % lethality within 2-3 days. To prevent bacterial infections all mice received ceftazidime (5 mg/day subcutaneously) every day after infection. Ten mice/*Candida* strains were inoculated through the lateral tail vein with 1.8-2.4 x 10^5 CFU/mouse in three independent experiments. Mice were followed up for 14 days and the survival rate was analyzed by Kaplan-Meyer test (GraphPad Prism v4.03 software). The animal experiments were approved by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary (permission no. 12/2008).

RESULTS

Biochemical characterization of the CaPpz1 protein

First we tested the phosphatase activity of the *CaPPZ1* gene product. We demonstrated that the bacterially expressed and purified recombinant CaPpz1-3

dephosphorylated p-nitrophenylphosphate, and that this phosphatase activity was inhibited by recombinant *S. cerevisiae* Hal3 (Fig. 2). Thus the enzymatic properties of the wild type CaPpz1 are similar to those of ScPpz1.

Next the functional significance of four selected amino acid residues in the catalytic domain of CaPpz1 (Fig. 1b) was analyzed by site directed mutagenesis. Expression in *E. coli* and purification of the mutated proteins were accomplished with the same efficiency as the control wild type enzyme (Fig. S1), suggesting that the mutations did not affect either the production or the solubility of the phosphatase. As a proof of the principle we replaced an essential amino acid in the catalytic cleft and found that the R262L mutation resulted in an inactive protein (Fig. 2), as expected from a previous publication (Clotet *et al.*, 1996), reporting that an analogous point mutation in *S. cerevisiae* ScPpz1 eliminated phosphatase activity. Then the effects of naturally occurring polymorphisms (Kovacs *et al.*, 2010) were investigated in the same way. The D261N mutation caused a moderate reduction in the phosphatase activity. When G333 was modified to E the phosphatase reaction rate doubled. On the other hand, the C337R replacement completely eradicated the catalytic reaction. Likewise, the G333E/C337R double mutation rendered the recombinant phosphatase inactive.

Complementation of the phenotypes of mutant yeasts by the expression of CaPPZ1

Based on the structural and biochemical similarities between CaPpz1 and the ScPpz1 (Figs. 1a, 2, and S1), we examined if the *C. albicans* protein was able to complement the phenotypes of the *S. cerevisiae scppz1* deletion mutant. CaPpz1-3 was expressed in the mutant budding yeast cells under the control of the *ScPPZ1* promoter from either a low copy number centromeric YCp-*CaPPZ1,* or a high copy number episomal YEp-*CaPPZ1* plasmid (Fig. 3a). The heterologous *C. albicans* phosphatase partially normalized the tolerance to LiCl of the *S. cerevisiae scppz1* mutant, but was rather ineffective when cells were challenged with 1 M NaCl. In contrast, the *CaPPZ1* markedly rescued the growth defect of the *ppz1* mutant in the presence of caffeine and, when in high copy, resulted in a near wild type phenotype.

A strain lacking the *SLT2* MAP kinase gene shows strong hypersensitivity to compounds that affect cell wall synthesis, such as caffeine or CFW. Overexpression of *ScPPZ1* in the *S. cerevisiae slt2* mutant is known to attenuate these defects. As shown in Fig. 3b, high-copy number expression of *CaPPZ1* was able to markedly improve tolerance of the *slt2* mutant when cells were grown in the presence of caffeine or CFW. Therefore, CaPpz1 is able to reproduce the genetic interaction with the *SLT2* MAP kinase.

Since the primary structures of CaPpz1 and Pzh1 proteins are similar to each other (Figs. 1a and S1), the functional competence of CaPpz1-3 was analyzed in the genetically distant fission yeast. The *C. albicans* phosphatase was expressed from the pREP41 plasmids in the *S. pombe pzh1* deletion mutant cells (Fig. 4). The *C. albicans* protein rescued, at least in part, the salt and caffeine sensitivity of the mutant. Similar results were obtained at three different LiCl, NaCl, KCl, and caffeine concentrations (data not shown). Thus, CaPpz1 can perform similar but not completely identical physiological functions as its *S. cerevisiae* and *S. pombe* counterparts.

Dissection of CaPpz1 functions in *C. albicans* by gene inactivation

In order to identify the physiological functions of CaPpz1 in *C. albicans,* we disrupted both copies of the corresponding gene in the diploid organism. The efficiency and specificity of the gene deletion were proven by PCR, RT-PCR, and Southern blot experiments (Figs. S3a-c). The mutant *C. albicans* did not exhibit any easily recognizable phenotype when cultivated in YPD medium. Therefore, the viability of the *ΔCaPPZ1* strain was compared to that of the parental SN87 strain under different stress conditions. There was practically no difference between the two strains in the presence of NaCl, however, the disrupted strain was more tolerant against LiCl, spermine and hygromycin B and was more sensitive to KCl, caffeine, CFW and Congo Red treatments (Fig. 5). In order to verify that these phenotypes were indeed related to the absence of the target gene, we reintegrated one functional *CaPPZ1* copy into its original locus. Both of the heterozygous transformants that carried a single copy of *CaPPZ1* in a different genetic background behaved more similarly to the parental SN87 strain under selected stress conditions (Fig. 5e-h). Reintegration partially compensated sensitivity to CFW and Congo Red, and complemented spermine as well as hygromycin B tolerance. Our biochemical and genetic data collectively suggest that CaPpz1 has similar but not identical functions as the well characterized ScPpz1.

Beside the typical stress treatments, we also analyzed the germ tube formation, a biological process that is supposed to be related to the virulence of this pathogenic fungus. The germination frequency was determined for the parental as well as for the homozygous and heterozygous mutant strains (Table 2). At 30 min after the addition of sheep serum a significant delay was detected in the germ tube formation of the *C. albicans* that had no functional *CaPPZ1*. The difference between the null mutant and the other strains gradually diminished with the time of incubation and disappeared after 90 minutes. CFW staining of the

cells demonstrated that all of the tested strains produced regular hyphae (Sudbery *et al.*, 2004) during the incubation period.

The virulence of the disrupted strain was directly tested in BALB/c mice (Fig. 6). It turned out that the *cappz1* mutant was a less effective pathogen than the parental SN87 strain, while both of the heterozygous strains were more virulent than the null mutant. The Kaplan-Meyer analysis of the combined survival data proved that the virulence of the four strains analyzed was significantly different (p=0.0115). Pair-wise comparisons revealed that the SN87 and the *CaPPZ1-LEU2* strains were significantly more pathogenic than the null mutant (p=0.078 and p=0.0343, respectively), but the difference between the *CaPPZ1-HIS1* and *cappz1* cells did not reach the level of significance. In fact, *CaPPZ1-LEU2* was even more pathogenic than SN87, probably because it grew somewhat faster than the other strains. The reversion to the more pathogenic phenotype by gene reintegration suggests that the disruption of the *CaPPZ1* was responsible for the reduced virulence.

DISCUSSION

The heterogeneity of the *CaPPZ1* gene is an inherent property of *C. albicans* (Kovacs *et al*., 2010) that has some impact on the structure-function investigations. The natural alleles used in the present study (*CaPPZ1-1* and *CaPPZ1-3*) encode the same amino acid sequence in the catalytic domain (Fig. S1) and are expected to have the same catalytic properties. In addition, due to the different codon usage of *C. albicans* vs. other organisms (Omaha et al., 1993) five CUG triplets were translated as Leu instead of Ser in *S. cerevisiae, S. pombe*, and *E. coli* (Fig. S1). Only one of these (L452) is in the catalytic domain, where it is found in an external loop (Fig. 1b). We suppose that these surplus mutations (that are present in all of the recombinant enzymes tested) have no significant effect on the enzyme activity. We proved in biochemical assays that, in agreement with the structural conservation of its catalytic domain the CaPpz1 protein exhibits phosphatase activity. This activity was inhibited by the ScHal3 protein that is a specific inhibitor of PPZ phosphatases and, at the same time, one of the putative subunits of the phosphopantothenoylcysteine decarboxylase (Ruiz *et al.*, 2009). It should be noted that in *C. albicans* there are two distantly related Hal3 orthologs (orf19.7378 and orf19.3260). Interestingly, in orf19.7378, all of the known amino acid residues required for Ppz1 binding and phosphatase inhibition (Munoz *et al.*, 2004) have been conserved. Therefore, it is likely that orf19.7378 encodes the inhibitory component of the *C. albicans* Ppz1/Hal3 system.

With the help of the inactivating R262L mutagenesis we proved the validity of the phosphatase assay. According to our structural model, the conserved R262 residue is essential for activity because it is coordinating the metal ions which are indispensable for the catalytic reaction (Fig. 1b). After proving the principle we investigated the effects of allele-specific amino acid polymorphisms on the enzymatic activity (Figs. 1b and 2). Despite of its proximity to the essential R262, the D261N replacement, characteristic to the *CaPPZ1-4* allele of the *C. albicans* WO-1 strain, had only a moderate effect. Obviously, N fits well into the place of D and the loss of a negative charge has no dramatic effect as the D side chain is pointing outwards from the active site. Unexpectedly, the G333E exchange (that is present in the *CaPPZ1-2* allele) significantly activated the phosphatase. The homologous modelling of the catalytic domain predicts that G333 is at the surface of the protein and is not supposed to influence the structure of the catalytic cleft. The C337R polymorphism (that is also found in the *CaPPZ1-2* allele) caused the inactivation of the enzyme. C337 is in the middle of the central beta sheet and may have important functions in the stability of the catalytic domain. Its replacement with a bulky charged residue can interfere with the proper folding of the tertiary structure. Indeed, the C337R mutation eliminated the activating effect of the G333E exchange and resulted in an inactive double mutant (Fig. 2). Previously we identified these two amino acid exchanges together in the heterozygous ATCC 10231 strain harbouring alleles *CaPPZ1-2* and *CaPPZ1-3* (Kovacs *et al.*, 2010). In addition, we isolated several clinical samples that were homozygous for the *CaPPZ1-2* allele according to the RFLP of the PCR fragment encompassing the hypervariable 3'-noncoding region (Kovacs *et al.*, 2010). We tested one of them (number 10934) under several stress conditions and found that it did not show the characteristic phenotypes of the null mutant. In order to reveal the molecular bases of this unexpected behaviour we amplified and sequenced the *CaPPZ1* gene from the clinical sample. It turned out that both copies of the gene exhibited the typical characteristic DNA sequence of the *CaPPZ1-2* allele, but in both alleles the triplets GAG and TGT coding for E333 and R337, respectively, reverted to GGG and CGU coding for the G333 and C337, that is for the amino acids of the active CaPpz1 isoenzymes! Our finding indicates that the destabilizing C337R replacement is not tolerated in a homozygous organism, and indirectly supports the notion that the phosphatase activity is important for the survival of this pathogenic fungus in its natural habitat.

The functions of CaPpz1 were first tested in complementation experiments. If CaPpz1 was expressed in *S. cerevisiae ppz1* cells it partially complemented the salt sensitivity and rescued the caffeine sensitivity of the mutant. In addition, the caffeine and CFW sensitivity of

a strain lacking the Slt2 MAP kinase were also alleviated. The observation that the presence of the CaPpz1 protein can, at least in part, reverse the effects of the absence of ScPpz1 or mimic its overexpression in both cation homeostasis and cell wall integrity is coherent, since it was shown that these two phenotypes are interrelated (Merchan *et al.*, 2004). The partial complementation of the *S. pombe pzh1* deletion mutant by the expression of *CaPPZ1* revealed that, albeit the heterologous protein can replace the authentic *S. pombe* enzyme, it is clearly less efficient. Similar conclusions were previously drawn from the functional study of *N. crassa* Pzl-1 (Vissi *et al.*, 2001).

The functions of CaPpz1 were directly assessed by the disruption of the gene in the *C. albicans* SN87 strain. In accord with earlier reports (Hanaoka *et al.*, 2008;Xu *et al.*, 2007) the deletion of the gene was not lethal, thus it has no essential roles under the common cultivation conditions. However, when the *C. albicans* cells were challenged by various stress treatments, the lack of the phosphatase became detectable. We also demonstrated that the reintegration of *CaPPZ1* into the deletion mutant alleviated the typical mutant phenotypes. We found that the null mutant was tolerant against LiCl, and was sensitive to KCl. According to these properties it behaves like the *S. cerevisiae ppz1* mutant (Posas *et al.*, 1995;Ruiz *et al.*, 2003;Yenush *et al.*, 2002). However, there was a clear-cut difference between the two mutants when the sensitivity against NaCl was tested. It is known that *S. cerevisiae ppz1* tolerates this saline treatment, but we found no significant differences between the parental and mutated *C. albicans* strains in the presence of NaCl. It was previously suggested that the tolerance against NaCl (and LiCl) of the *S. cerevisiae* mutant can be explained by the overexpression of the *ENA1* sodium transporter (Posas *et al.*, 1995;Ruiz *et al.*, 2003). Sequence comparisons revealed that in *C. albicans* the orf19.6070 protein is an *ENA1* ortholog, however its function and regulation is not known at the moment. On the other hand, in the highly salt-tolerant fungus, *D. hansenii* the hypertolerance to toxic cations caused by deletion of *DhPPZ1* is not due to the increased expression of the *DhENA1* Na^+-ATPase, but of the *DhEHA1* Na^+/H^+-antiporter, which appears as the likely functional target for the phosphatase in this organism (Minhas *et al.*, 2012). Consequently, different fungi may utilize distinct molecular mechanisms to elicit similar physiological responses.

As the *cappz1* mutant was sensitive against cell wall damaging agents like caffeine, CFW, and Congo Red, we suggest that CaPpz1 interacts with the CWI pathway, like its *S. cerevisiae* counterpart. Furthermore, the *C. albicans* mutant was tolerant against toxic cations (spermine and hygromycin B) indicating that the absence of CaPpz1 resulted in cell membrane potential depolarization that decreased uptake of the toxic agents, as previously

found for budding yeast *ppz* mutants (Yenush *et al.*, 2002). These experiments underline again the similarity between the *C. albicans* and *S. cerevisiae* PPZ phosphatases. In conclusion, our data collectively indicate that PPZ phosphatases have similar but not identical functions in different yeasts.

In addition, we identified a novel function for this enzyme that operates in the filamentous form of *C. albicans*. We observed that after serum stimulation the onset of germ tube growth was significantly reduced in the *cappz1* mutant relative to the parental SN87 strain or to the heterozygous mutants that had one reintegrated *CaPPZ1* copy. The size and the shape of the tubes were similar in all of the strains tested (Fig. S4), suggesting that the phosphatase affected the initiation and not the rate of hyphal outgrowth in liquid cultures. Previously, we reported that two phosphatase inhibitors, cantharidin and calyculin A, hindered the hyphal growth in the filamentous fungus *N. crassa* (Yatzkan *et al.*, 1998). Genetic evidence indicated that protein phosphatase 2A (pph-1) was involved in the process; however the role of additional phosphatases was not excluded. Now we found that 220 μM cantharidin completely blocked, while 250 nM calyculin A significantly reduced the phosphatase activity of recombinant CaPpz1 (data not shown). The genetic and biochemical data together support the hypothesis that (besides PP2A) CaPpz1 can also contribute to the regulation of hypha formation. Since filament formation is considered as a critical element of *C. albicans* pathogenesis we tested the virulence of the mutated *C. albicans* in BALB/c mice, and we found that the *cappz1* mutant was somewhat less virulent than its parental strain. This result differs from those reported in a previous paper (Hanaoka *et al.*, 2008) indicating that a *cappz1* strain displays wild type virulence in a silkworm infection model. It is conceivable that the difference in the model employed could explain the contradictory results. The rescue of the less virulent phenotype with the reintegration of a single copy of the *CaPPZ1* gene indicates that the phosphatase gene is involved in the virulence of the pathogen. The finding that the absence of CaPpz1 function moderately reduces virulence in a mammalian model may be of importance since Ppz phosphatases are fungi-specific. Therefore, CaPpz1 could be considered as a possible target for antifungal treatments and CaPpz1-specific inhibitors may act as antifungal drugs.

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593 **FIGURE LEGENDS**

594

595 **Fig. 1. The structure of the CaPpz1 protein. (a)** The scheme represents the amino acid
596 sequence of CaPpz1 as compared to that of *S. cerevisiae, N. crassa,* and *S. pombe* orthologs.
597 The N-terminal moiety including a myristoylation site (asterisk) is green, the conserved
598 catalytic domain is red and the variable, disordered segments are represented by white boxes.
599 **(b)** The homologous model of the CaPpz1 catalytic domain. Alpha helices are red, beta sheets
600 are blue, and loops are yellow. Two essential metal ions in the catalytic centre are gray and
601 the amino acid side chains involved in catalysis are in violet. Three amino acid residues that
602 are affected by natural polymorphisms are highlighted in light brown and one replacement
603 caused by the specific codon usage of *C. albicans* is yellow. The amino acid exchanges
604 analyzed in the present study are labelled with arrows.

605

606 **Fig. 2. Biochemical properties of *C. albicans* CaPpz1.** The specific activity of wild-type and
607 mutated CaPpz1 phosphatases was determined with p-nitrophenylphosphate. *S. cerevisiae*
608 Hal3 protein was added to the wild-type enzyme in a 1:1 molar ratio (+Hal3). The means and
609 standard deviations of 3 to 5 independent experiments performed with two independent
610 preparations are shown.

611

612 **Fig. 3. Expression of *C. albicans* CaPpz1 protein in *S. cerevisiae***. **(a)** Wild-type BY4741
613 (*ScPPZ1*) strain and its isogenic derivative BY4741 *Scppz1* were transformed with the
614 indicated plasmids. Cells (two dilutions of approx. 3×10^3 and 3×10^2 cells) were spotted on
615 YPD plates containing the indicated concentrations of lithium chloride, sodium chloride or
616 caffeine and growth was monitored after 48 h of incubation at 28 ºC (except for cells growing
617 in 15 mM caffeine which were scored for growth after 60 h). **(b)** The strains BY4741 (*SLT2*)
618 and BY4741 *slt2* were transformed with the indicated plasmids and cultures were spotted on
619 YPD plates containing the indicated concentrations of the drugs. Growth was monitored after
620 72 h while the plate containing 20 mM caffeine was incubated for 96 h.

621

622 **Fig. 4. Expression of *C. albicans* CaPpz1 protein in *S. pombe***. The relative growth rate of
623 the *pzh1*$^+$ control cells transformed with the empty pREP1 vector (filled bars), the *pzh1*
624 disrupted LB2 cells transformed with the empty pREP1 vector (open bars), and with the
625 CaPPZ1-pREP41 (horizontally stripped bars) was compared in the presence of **(a)** 8 mM
626 LiCl, **(b)** 150 mM NaCl, **(c)** 300 mM KCl, and **(d)** 5 mM caffeine. The relative growth rate of

627 the cells was determined after 24 h incubations in the liquid cultures, and was expressed as the
628 percentage to the growth of the same strain without additions. The means and SD of 9
629 experiments are shown. The significances of the changes relative to the LB2 control cell are
630 given according to the T-test analysis (***, $p < 0.0005$).
631
632 **Fig. 5. Comparison of relative growth of the parental SN87 (●), the homozygous null**
633 **mutant *cappz1* (Δ), as well as the heterozygous *CaPPZ1-LEU2* (■) and *CaPPZ1-HIS1***
634 **(◇) reintegrant *C. albicans* strains under various stress conditions.** The effects of **(a)**
635 LiCl, **(b)** NaCl, **(c)** KCl, **(d)** caffeine, **(e)** Calcofluor White (CFW), **(f)** Congo Red (CR), **(g)**
636 spermine, and **(h)** hygromycin B (Hyg. B) were tested after 18 h incubations at 37 ºC. The
637 means and standard deviations of three independent experiments are shown.
638
639 **Fig. 6. The role of CaPpz1 in the virulence of *C. albicans.*** The survival of BALB/c mice
640 was tested after the administration of 1.8-2.0x10^5 SN87 (●), *cappz1* (Δ), *CaPPZ1-LEU2* (■)
641 and *CaPPZ1-HIS1* (◇) *C. albicans* cells. Representative results of one out of three
642 experiments are shown.

Table 1. Fungal strains used in the present study

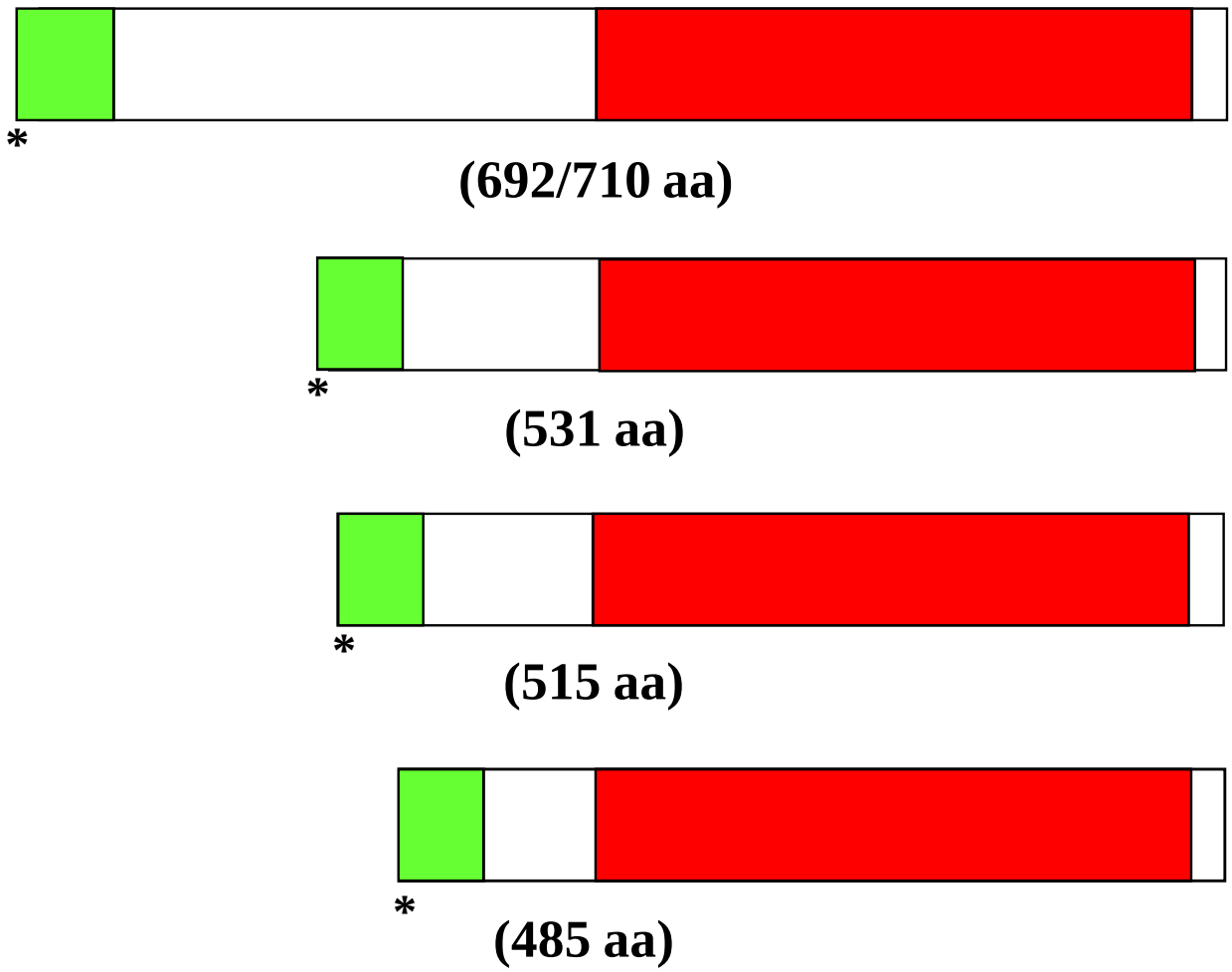
Strain	Genotype	Origin/reference
Saccharomyces cerevisiae FY1679	*MAT*a/α *ura3-52/ ura3-52 trp1*Δ63*/TRP1 leu2*Δ1*/LEU2 his3*Δ200*/HIS3 GAL2/GAL2*	(Winston *et al.*, 1995)
Saccharomyces cerevisiae BY4741	MATa *his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ	(Winzeler *et al.*, 1999)
Saccharomyces cerevisiae BY4741 *ppz1::KanMX4*	MATa *his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ *ppz1::kanMX4*	(Winzeler *et al.*, 1999)
Saccharomyces cerevisiae BY4741 *slt2::KanMX4*	MATa *his3*Δ1 *leu2*Δ *met15*Δ *ura3*Δ *slt2::kanMX4*	(Winzeler *et al.*, 1999)
Schizosaccharomyces pombe LB2	h^{-} *ade6-M210 leu1-32 pzh1::ura4*$^{+}$ *ura-D18*	(Balcells *et al.*, 1997)
Schizosaccharomyces pombe pzh1^{+}	h^{-} *ade6-M210 leu1-32*	This study
Candida albicans SN87	*ura3Δ-iro1Δ::imm*^{434}*/URA3-IRO1, his1Δ /his1Δ, leu2Δ /leu2Δ*	(Noble & Johnson, 2005)
Candida albicans cappz1	*ura3Δ-iro1Δ::imm*^{434}*/URA3-IRO1, his1Δ /his1Δ, leu2Δ /leu2Δ ppz1Δ::HIS1/ppz1Δ::LEU2*	This study
Candida albicans CaPPZ1-HIS1	*ura3Δ-iro1Δ::imm*^{434}*/URA3-IRO1, his1Δ /his1Δ, leu2Δ /leu2Δ ppz1Δ::HIS1/ppz1Δ::LEU2::PPZ1*	This study
Candida albicans CaPPZ1-LEU2	*ura3Δ-iro1Δ::imm*^{434}*/URA3-IRO1, his1Δ /his1Δ, leu2Δ /leu2Δ ppz1Δ::LEU2/ppz1Δ::HIS1::PPZ1*	This study

Table 2. The role of CaPpz1 in the germination of *C. albicans*

Strain/Time	30 min	60 min	90 min
SN87	23.5 ± 3.1	63.3 ± 4.4	96.1 ± 3.2
cappz1	6.0 ± 2.1***	42.6 ± 3.6**	95.7 ± 2.5
CaPPZ1-LEU2	25.6 ± 6.2	64.0 ± 2.1	97.9 ± 1.2
CaPPZ1-HIS1	23.8 ± 5.4	59.2 ± 5.6	97.2 ± 1.7

The ratio of cells producing germ tubes in sheep serum is given as % of all cells. The averages of three independent experiments ± standard deviations are shown. The significance of the changes relative to SN87 were calculated by T-test (**, $p < 0.01$; ***, $p < 0.001$).

Figure 1(a)



S. cerevisiae
Ppz1/Ppz2

N. crassa
PZL-1

S. pombe
Pzh1

C. albicans
CaPpz1

Figure 1(b)

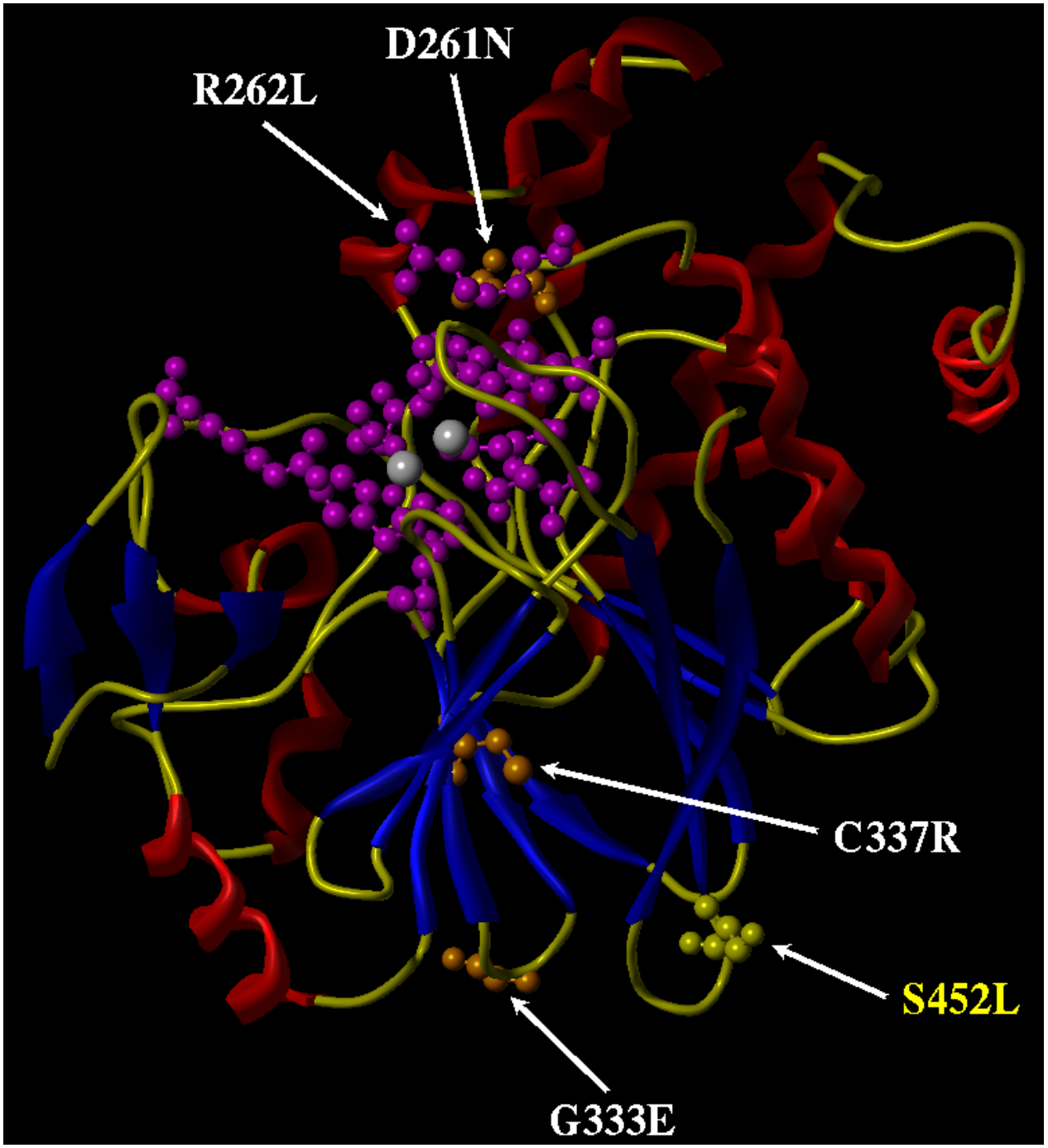


Figure 2

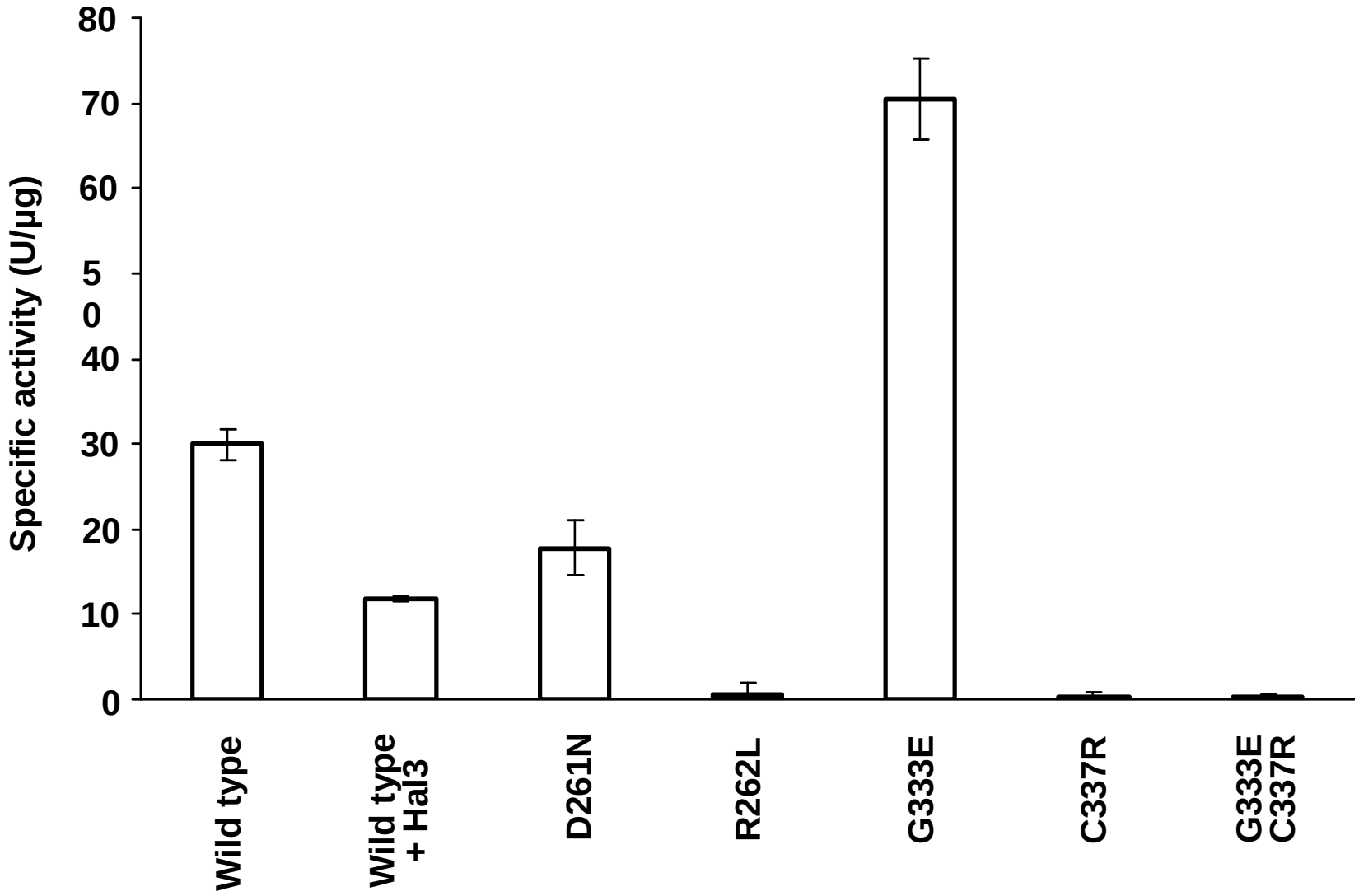

Specific activity (U/µg)
80
70
60
5
0
40
30
20
10
0
Wild type
Wild type + Hal3
D261N
R262L
G333E
C337R
G333E C337R

Figure 3

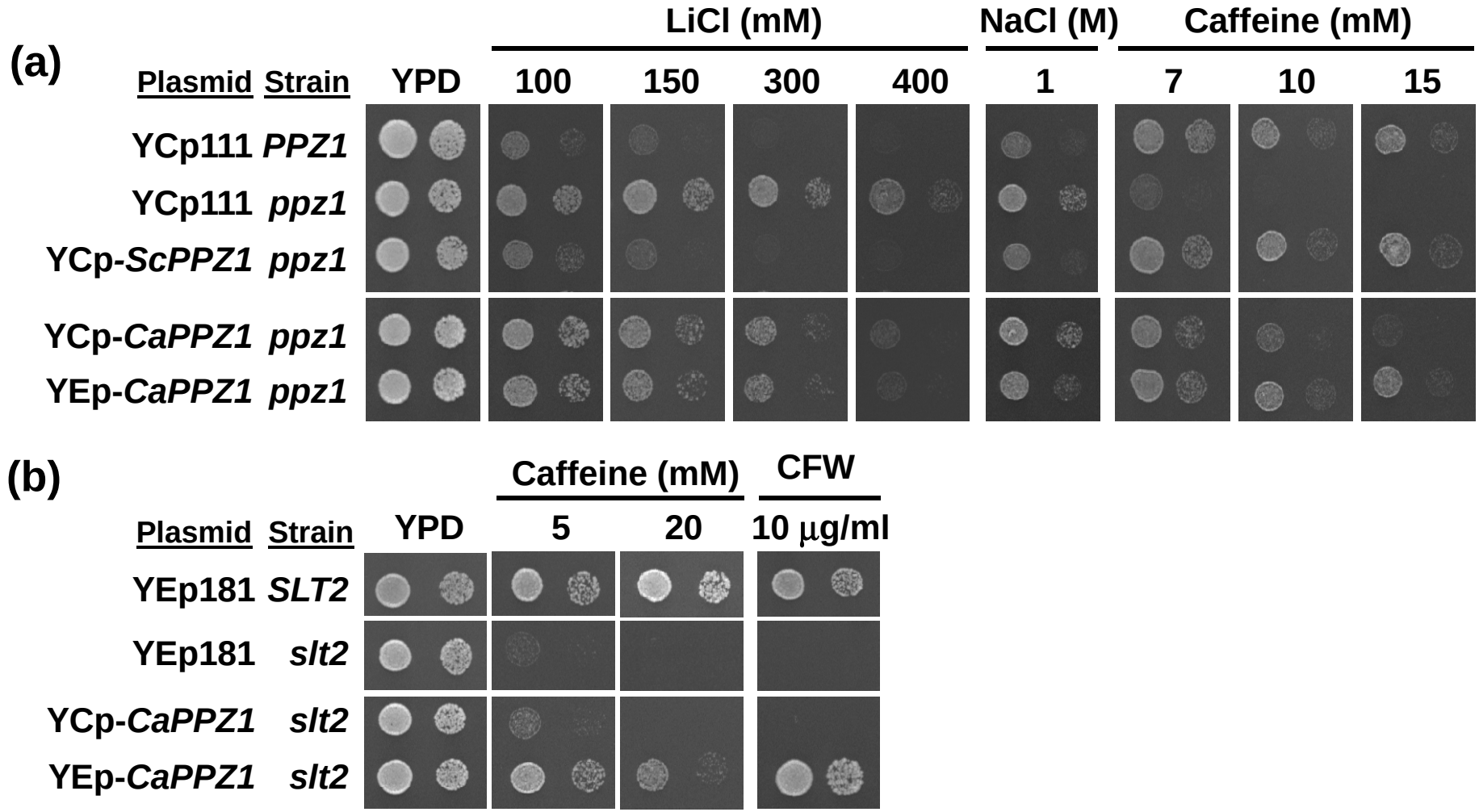

(a)
LiCl (mM)
NaCl (M)
Caffeine (mM)
Plasmid
Strain
YPD
100
150
300
400
1
7
10
15
YCp111 PPZ1
YCp111 ppz1
YCp-ScPPZ1 ppz1
YCp-CaPPZ1 ppz1
YEp-CaPPZ1 ppz1
(b)
Caffeine (mM)
CFW
Plasmid
Strain
YPD
5
20
10 μg/ml
YEp181 SLT2
YEp181 slt2
YCp-CaPPZ1 slt2
YEp-CaPPZ1 slt2

Figure 4

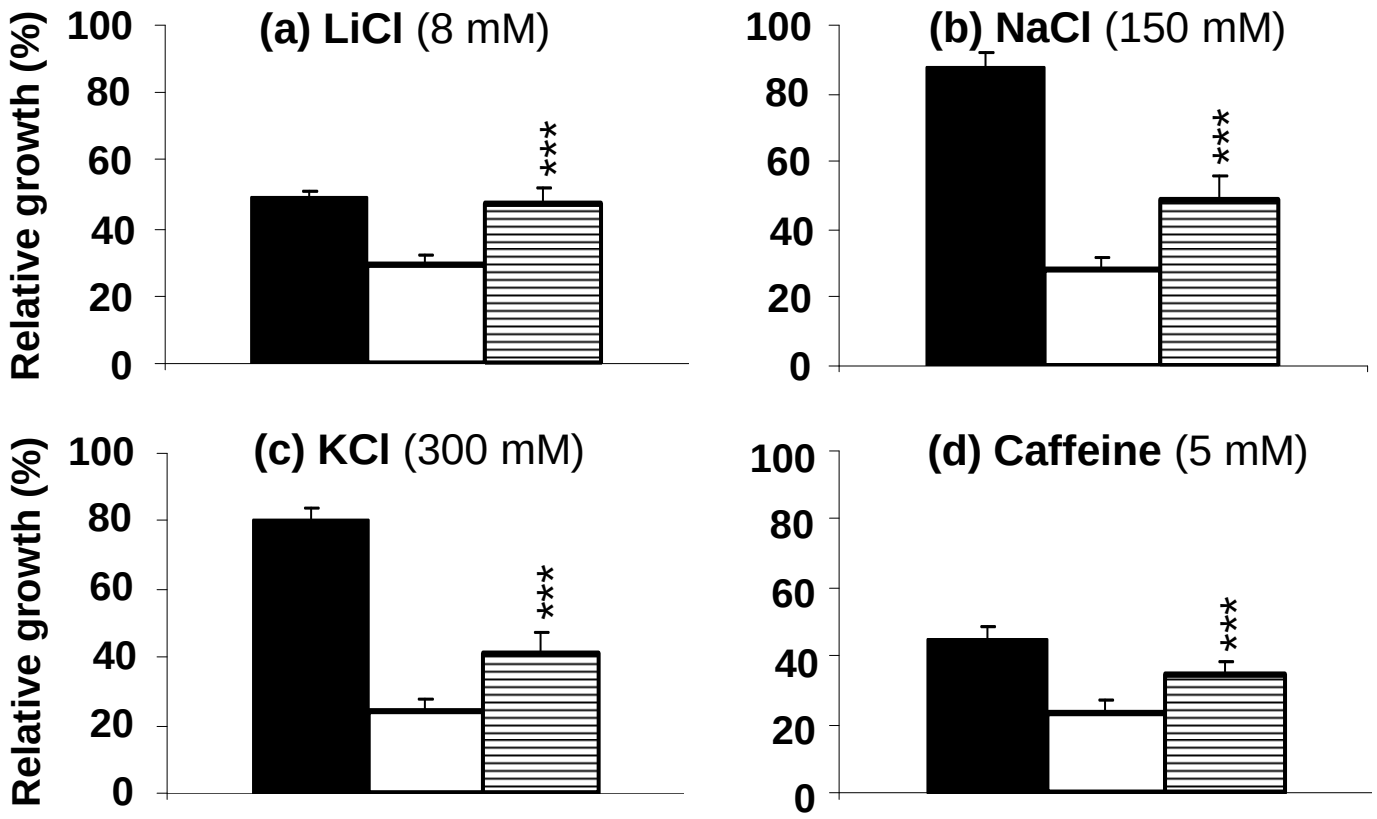

Relative growth (%)
100
80
60
40
20
0
(a) LiCl (8 mM)
***
(b) NaCl (150 mM)
***
(c) KCl (300 mM)
***
(d) Caffeine (5 mM)
***

Figure 5

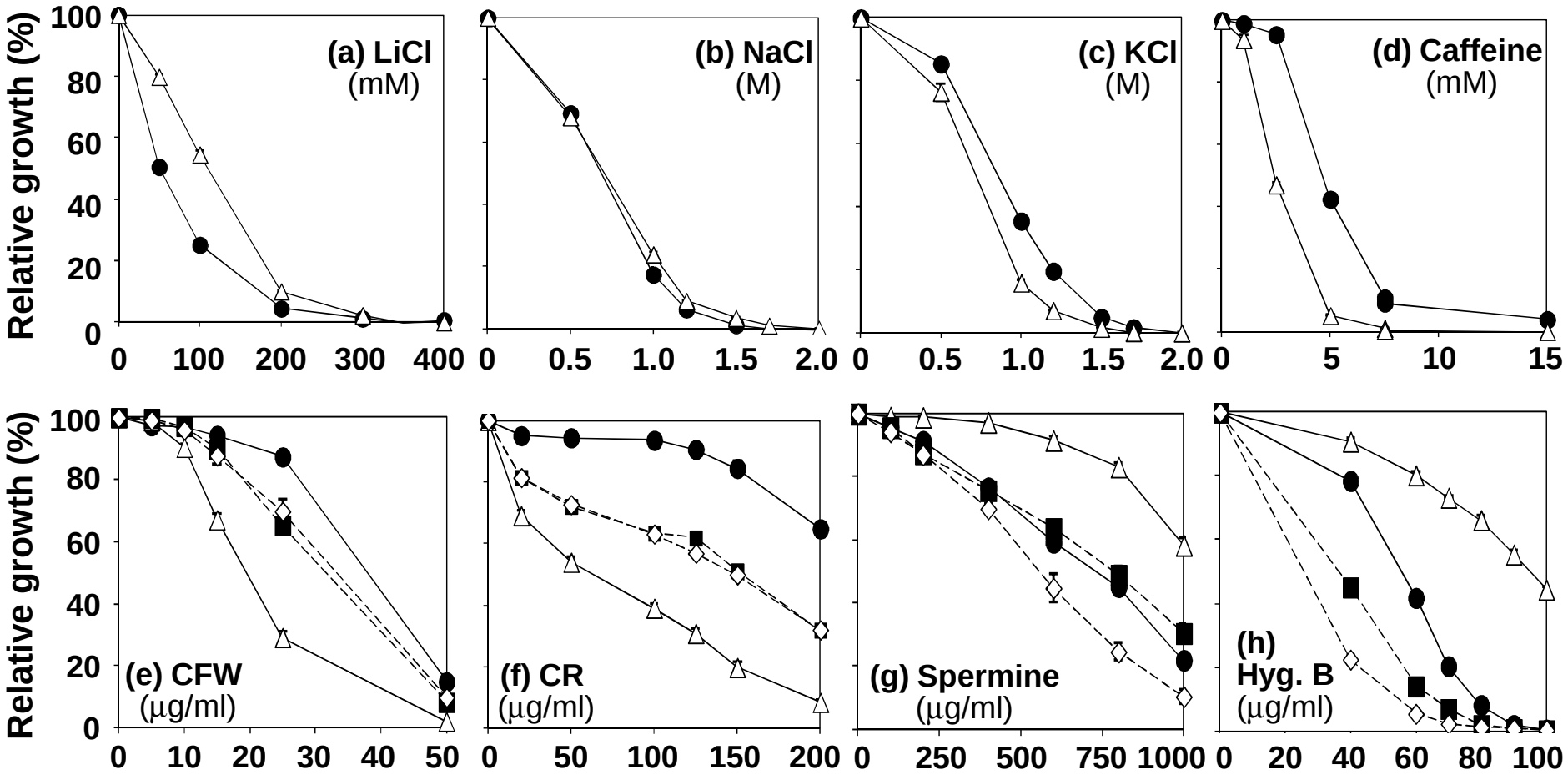

Relative growth (%)
100
80
60
40
20
0
(a) LiCl
(mM)
0 100 200 300 400
(b) NaCl
(M)
0 0.5 1.0 1.5 2.0
(c) KCl
(M)
0 0.5 1.0 1.5 2.0
(d) Caffeine
(mM)
0 5 10 15
Relative growth (%)
100
80
60
40
20
0
(e) CFW
(μg/ml)
0 10 20 30 40 50
(f) CR
(μg/ml)
0 50 100 150 200
(g) Spermine
(μg/ml)
0 250 500 750 1000
(h)
Hyg. B
(μg/ml)
0 20 40 60 80 100

Figure 6