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The PKC, HOG and Ca^{2+} signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*

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Summary

Chitin is an essential component of the fungal cell wall and its synthesis is under tight spatial and temporal regulation. The fungal human pathogen *Candida albicans* has a four member chitin synthase gene family comprising of *CHS1* (class II), *CHS2* (class I), *CHS3* (class IV) and *CHS8* (class I). LacZ reporters were fused to each *CHS* promoter to examine the transcriptional regulation of chitin synthesis. Each *CHS* promoter had a unique regulatory profile and responded to the addition of cell wall damaging agents, to mutations in specific *CHS* genes and exogenous Ca^{2+}. The regulation of both *CHS* gene expression and chitin synthesis was co-ordinated by the PKC, HOG MAP kinase and Ca^{2+}/calcineurin signalling pathways. Activation of these pathways also resulted in increased chitin synthase activity *in vitro* and elevated cell wall chitin content. Combinations of treatments that activated multiple pathways resulted in synergistic increases in *CHS* expression and in cell wall chitin content. Therefore, at least three pathways co-ordinately regulate chitin synthesis and activation of chitin synthesis operates at both transcriptional and post-transcriptional levels.

Introduction

The fungal cell wall is a dynamic structure whose composition and structural organization is regulated during the cell cycle and in response to changing environmental conditions, imposed stresses and mutations in cell wall biosynthetic processes (reviewed in Klis *et al*., 2006; Ruiz-Herrera *et al*., 2006). Chitin and β(1–3)-D-glucan, represent the main structural components of the fungal cell wall. These polysaccharides oppose the positive turgor pressure within the cell and ultimately determine the morphology of the cell (Munro and Gow, 2001; Klis *et al*., 2002; Roncero, 2002). Chitin and glucan synthesis therefore play fundamental roles in maintaining fungal cell integrity during growth and morphogenesis and in adaptation to stress (Cabib, 1987; Wessels, 1990; Shaw *et al*., 1991; Sietsma and Wessels, 1994; Gooday, 1995). Because these structural polysaccharides do not occur in mammals and are essential for fungi, there is considerable potential for cell wall synthesis as a target for antifungal drugs (Munro and Gow, 1995; Munro *et al*., 2001; Odds *et al*., 2003). New generation echinocandins that target the synthesis of cell wall β(1–3)-D-glucan are proving effective agents in the treatment of opportunistic fungal pathogens such as *Candida albicans* (Denning, 2003). Chitin synthase inhibitors have not yet been discovered that have clinical use in the treatment of fungal infections (Odds *et al*., 2003).

Regulation of chitin synthesis occurs both at the transcriptional and post-translational levels and is dependent on precise targeting and activation of chitin synthases to specific locations in the plasma membrane, and the provision of adequate substrate (Munro and Gow, 1995). All fungi examined to date have multiple genes encoding chitin synthase families (Munro and Gow, 2001; Roncero, 2002; Ruiz-Herrera and San-Blas, 2003). Individual chitin synthase enzymes perform distinct functions at specific stages of the cell cycle. *Saccharomyces cerevisiae* has three chitin synthase enzymes – Chs1p (Class I), Chs2p (Class II) and Chs3p (Class IV) while *C. albicans* has four chitin synthases – two class I enzymes – *Ca*Chs2p and *Ca*Chs8p, *Ca*Chs3p (Class IV) and *Ca*Chs1p (a class II enzyme which is the orthologue of *Sc*Chs2p). Relatively little is known about the transcriptional regulation of chitin synthase genes in fungi but considerable attention has been focused on post-transcriptional regulation by Chs4–7, which influences Chs3p chitin synthase activation and localization in *S. cerevisiae* and *C. albicans*. *Sc*Chs7p controls exit of *Sc*Chs3p from the ER, *Sc*Chs5p and *Sc*Chs6p regulate its exit from the *trans*-Golgi

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network (Ziman *et al*., 1996; Santos and Snyder, 1997; Santos *et al*., 1997; Ziman *et al*., 1998; Trilla *et al*., 1999). *Sc*Chs4p tethers *Sc*Chs3p to the septins at the mother-bud neck via *Sc*Bni4p (Demarini *et al*., 1997; Trilla *et al*., 1997). Chitin synthesis is therefore influenced by endogenous and exogenous factors that directly and indirectly regulate the chitin synthase catalytic proteins.

Disruption of genes in cell wall biosynthetic pathways of *S. cerevisiae* and *C. albicans* often results in alteration and redistribution of chitin and β(1–3)-D-glucan in the cell wall, the synthesis of new cell wall proteins and changes in their cross-linking to cell wall polysaccharides (reviewed in Popolo *et al*., 2001; Klis *et al*., 2002; Klis *et al*., 2006). Defects in cell wall integrity are sensed by the transmembrane proteins of the Mid2p and the Wscp family, which signal via the Rom2p guanine nucleotide exchanger leading to activation of the Rho1p GTPase. Rho1p has many downstream targets including protein kinase C and the β(1–3)-D-glucan synthase subunits Fks1p and Fks2p (Popolo *et al*., 2001). In *S. cerevisiae* this 'cell wall salvage' or 'cell wall compensatory' pathway is activated in response to cell wall perturbing agents such as Calcofluor white (CFW), Congo Red (CR), caffeine, β-glucanases and cell wall mutations and is mediated primarily through the PKC cell integrity MAP kinase cascade and its downstream target the transcription factor Rlm1p (Lagorce *et al*., 2003; Boorsma *et al*., 2004; Garcia *et al*., 2004). In *S. cerevisiae*, elevation of chitin levels in response to activation of the salvage pathway is largely dependent upon *Sc*Chs3p (Valdivieso *et al*., 2000; Carotti *et al*., 2002). Several studies have highlighted the importance of signalling systems in co-ordinating this regulation. A higher proportion of *Sc*Chs3p localized to the plasma membrane in heat-stressed cells (Valdivia and Schekman, 2003). This mobilization of *Sc*Chs3p was dependent upon activation of Rho1p and Pkc1p, and the phosphorylation of *Sc*Chs3p by Pkc1p.

A second MAP kinase cascade, the high osmolarity glycerol response (HOG) pathway, has also been suggested to play a role in regulating cell wall architecture in *S. cerevisiae* (Garcia-Rodriguez *et al*., 2000; Kapteyn *et al*., 2001) and in *C. albicans* (Eisman *et al*., 2006). In *S. cerevisiae*, the HOG pathway is required for the response to CFW and mutants in several components of the pathway are resistant to CFW (Garcia-Rodriguez *et al*., 2000). In addition, changes in osmotic pressure have been shown to regulate chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasii* suggesting the HOG pathway is involved in chitin regulation (Deshpande *et al*., 1997).

Transcript profiling studies have implicated Ca^{2+} in the regulation of *ScCHS1* (Yoshimoto *et al*., 2002). In addition, sequences recognized by the Ca^{2+}/calcineurin-dependent transcription factor Crz1p/Tcn1p have been identified upstream of a number of genes that are upregulated in cell wall mutants that activate the cell wall salvage pathway (Lagorce *et al*., 2003; Boorsma *et al*., 2004; Garcia *et al*., 2004; Karababa *et al*., 2006). These studies directed us towards examining the role of Ca^{2+} signalling in the regulation of chitin synthesis in *C. albicans*.

Each of the four *C. albicans* Chs enzymes plays a distinct role in cellular growth. *Ca*Chs1p synthesizes the septal chitin and contributes to chitin in the lateral cell wall and is essential for viability in both the yeast and hyphal forms (Munro *et al*., 2001). *Ca*Chs2p encodes the major chitin synthase activity *in vitro*, and *chs2*Δ null mutants have fractionally less chitin in hyphal cells (Gow *et al*., 1994; Munro *et al*., 1998). *Ca*Chs3p synthesizes the majority of the chitin in the lateral cell wall and the ring of chitin at the site where a new bud emerges (Bulawa *et al*., 1995; Mio *et al*., 1996). *Ca*Chs8p and *Ca*Chs2p account for almost all the measurable *in vitro* chitin synthase activity in membrane preparations but are non-essential for growth (Munro *et al*., 2003). In *C. albicans*, northern analyses suggested that *CaCHS2* and *CaCHS3* are upregulated shortly after induction of hyphal formation while *CaCHS1* is expressed at low but constant levels in both yeast and hyphae (Chen-Wu *et al*., 1992; Munro *et al*., 1998). Hyphal formation in *C. albicans* is accompanied by a three to fivefold increase in the chitin content of the cell wall (Chattaway *et al*., 1968; Sullivan *et al*., 1983; Munro *et al*., 1998).

Here we examine the regulation of chitin synthesis of *C. albicans* and describe the signalling pathways that co-ordinate this process. We used a *lacZ* reporter gene fused to the putative promoters of each of the *C. albicans CHS* genes to test hypotheses about the expression of *CHS* genes when cells are challenged with cell wall perturbing agents or subjected to environmental stresses. We show that transcriptional regulation of the *CHS* genes is stimulated via at least three pathways – the PKC and HOG MAP kinase cascades and the Ca^{2+}/calcineurin pathway. Each of the four chitin synthase promoters was regulated differentially, but all were activated by exogenous Ca^{2+} in a calcineurin and Crz1p-dependent manner. In addition, hyper-stimulation of *CHS* gene expression was observed when multiple signalling pathways were activated simultaneously and this resulted in greatly elevated cell wall chitin levels.

Results

Endogenous CHS *promoter activity in wild-type cells and* chsΔ *mutants*

Transcriptional activity of the four chitin synthase genes of *C. albicans* was characterized using a *lacZ* reporter system. Plasmid placpoly 6 containing *URA3* and *RPS1*

Table 1. *Candida albicans* strains used in this study.

Strain	Parental strain	Genotype[a]	Source or reference
CAF2-1	SC5314	*URA3/ura3*Δ::*λimm434*	Fonzi and Irwin (1993)
CAI-4	CAF2-1	*ura3*Δ::*λimm434/ura3*Δ::*λimm434*	Fonzi and Irwin (1993)
NGY210	CAI-4	*RPS1/rps1*Δ::pCHS1plac	This study
NGY211	CAI-4	*RPS1/rps1*Δ::pCHS2plac	This study
NGY212	CAI-4	*RPS1/rps1*Δ::pCHS3plac	This study
NGY213	CAI-4	*RPS1/rps1*Δ::pCHS8plac	This study
C155	C154	*chs2*Δ::*hisG/chs2*Δ::*hisG*	Mio *et al.* (1996)
Myco3	Myco4	*chs3*Δ::*hisG/chs3*Δ::*hisG*	Bulawa *et al.* (1995)
NGY128	CAI-4	*chs8*Δ::*hisG/chs8*Δ::*hisG*	Munro *et al.* (2003)
C157	C155	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs3*Δ::*hisG/chs3*Δ::*hisG*	Mio *et al.* (1996)
NGY138	CAI-4	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs8*Δ::*hisG/chs8*Δ::*hisG*	Munro *et al.* (2003)
CM1613c	CAI-4	*mkc1*Δ::*hisG/mkc1*Δ::*hisG*	Navarro-Garcia *et al.* (1995)
DSY2842	CAI-4	*crz1*Δ::*hisG/crz1*Δ::*hisG*	Karababa *et al.* (2006)
DSY2101	CAI-4	*cna1*Δ::*hisG/cna1*Δ::*hisG*	Sanglard *et al.* (2003)
CNC15	RIM1000	*hog1*Δ::*hisG/hog1*Δ::*hisG*	Alonso-Monge *et al.* (1999)
NGY258	C155	*chs2*Δ::*hisG/chs2*Δ::*hisG; RPS1/rps1*Δ::pCHS1plac	This study
NGY260	C155	*chs2*Δ::*hisG/chs2*Δ::*hisG, RPS1/rps1*Δ::pCHS3plac	This study
NGY261	C155	*chs2*Δ::*hisG/chs2*Δ::*hisG, RPS1/rps1*Δ::pCHS8plac	This study
NGY262	Myco3	*chs3*Δ::*hisG/chs3*Δ::*hisG, RPS1/rps1*Δ::pCHS1plac	This study
NGY263	Myco3	*chs3*Δ::*hisG/chs3*Δ::*hisG, RPS1/rps1*Δ::pCHS2plac	This study
NGY265	Myco3	*chs3*Δ::*hisG/chs3*Δ::*hisG, RPS1/rps1*Δ::pCHS8plac	This study
NGY290	NGY128	*chs8*Δ::*hisG/chs8*Δ::*hisG, RPS1/rps1*Δ::pCHS1plac	This study
NGY291	NGY128	*chs8*Δ::*hisG/chs8*Δ::*hisG, RPS1/rps1*Δ::pCHS2plac	This study
NGY292	NGY128	*chs8*Δ::*hisG/chs8*Δ::*hisG, RPS1/rps1*Δ::pCHS3plac	This study
NGY270	NGY138	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs8*Δ::*hisG/chs8*Δ::*hisG*; *RPS1/rps1*Δ::pCHS1plac	This study
NGY272	NGY138	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs8*Δ::*hisG/chs8*Δ::*hisG*; *RPS1/rps1*Δ::pCHS3plac	This study
NGY266	C157	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs3*Δ::*hisG/chs3*Δ::*hisG; RPS1/rps1*Δ::pCHS1plac	This study
NGY269	C157	*chs2*Δ::*hisG/chs2*Δ::*hisG; chs3*Δ::*hisG/chs3*Δ::*hisG; RPS1/rps1*Δ::pCHS8plac	This study
NGY294	DSY2101	*cna1*Δ::*hisG/cna1*Δ::*hisG; RPS1/rps1*Δ::pCHS1plac	This study
NGY295	DSY2101	*cna1*Δ::*hisG/cna1*Δ::*hisG; RPS1/rps1*Δ::pCHS2plac	This study
NGY296	DSY2101	*cna1*Δ::*hisG/cna1*Δ::*hisG; RPS1/rps1*Δ::pCHS3plac	This study
NGY297	DSY2101	*cna1*Δ::*hisG/cna1*Δ::*hisG; RPS1/rps1*Δ::pCHS4plac	This study
NGY282	CM1613c	*mkc1*Δ::*hisG/mkc1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS1plac	This study
NGY283	CM1613c	*mkc1*Δ::*hisG/mkc1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS2plac	This study
NGY284	CM1613c	*mkc1*Δ::*hisG/mkc1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS3plac	This study
NGY285	CM1613c	*mkc1*Δ::*hisG/mkc1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS8plac	This study
NGY314	DSY2842	*crz1*Δ::*hisG/crz1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS1plac	This study
NGY315	DSY2842	*crz1*Δ::*hisG/crz1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS2plac	This study
NGY316	DSY2842	*crz1*Δ::*hisG/crz1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS3plac	This study
NGY317	DSY2842	*crz1*Δ::*hisG/crz1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS8plac	This study
NGY321	CNC15	*hog1*Δ::*hisG/hog1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS1plac	This study
NGY322	CNC15	*hog1*Δ::*hisG/hog1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS2plac	This study
NGY323	CNC15	*hog1*Δ::*hisG/hog1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS3plac	This study
NGY324	CNC15	*hog1*Δ::*hisG/hog1*Δ::*hisG*; *RPS1/rps1*Δ::pCHS8plac	This study

a. All strains apart from CAF2-1 are also *ura3*Δ::*λimm434/ura3*Δ::*λimm434*.

was used to create a fusion between the promoter of each *C. albicans* chitin synthase gene and the *Streptococcus thermophilus lacZ* open reading frame (ORF). A 1 kb region upstream from the ATG start codon of *CHS1*, *CHS2*, *CHS3* and *CHS8* was cloned into placpoly 6 generating, respectively, plasmids pCHS1plac, pCHS2plac, pCHS3plac and pCHS8plac. Ura$^-$ *C. albicans* cells were transformed with each linearized plasmid, homologous recombination resulted in integration of the plasmid at the *RPS1* locus and Ura$^+$ transformants were selected. Transformants were screened by Southern blot analysis and those with a single copy integration of pCHSplac (strains NGY210-NGY213, Table 1) were analysed further. The *CHS2* and *CHS3* promoters had the highest and lowest level of expression, respectively, for growth in YPD medium ($P < 0.05$) (Fig. 1). Real-time quantitative polymerase chain reaction (PCR) confirmed these results (data not shown).

The pCHSplac plasmids were transformed into isogenic mutant strains derived from CAI-4 with single or double

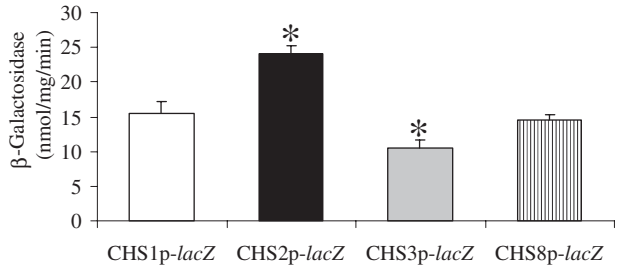

Fig. 1. Native *CHS* promoter activities. The β-galactosidase activity of cell-free protein extracts of parental *C. albicans* strain CAI-4 transformed with CHS1p-*lacZ*, CHS2p-*lacZ*, CHS3p-*lacZ* or CHS8p-*lacZ*, grown on YPD at 30°C. Data are from three independent experiments (average ± SD $n = 9$). Asterisks indicate significant differences from CHS1p-*lacZ* where $P \leq 0.05$.

CHS gene disruptions (Table 1) to test whether deletion of *CHS* genes results in a compensatory upregulation of other members of the *CHS* family. The *CHS1* promoter activity was significantly increased in the single mutants *chs3*Δ (strain Myco3) and *chs8*Δ (NGY138) and the double mutants *chs2*Δ *chs8*Δ (NGY128) and *chs2*Δ *chs3*Δ (C157) (Fig. 2). Chs1p may contribute to the maintenance of lateral wall integrity (Munro *et al*., 2001) and play a compensatory role when *CHS3* and *CHS8* gene functions are lost. Expression from the *CHS8* promoter was stimulated when either *CHS2* or *CHS3* were deleted and was increased further in the *chs2*Δ *chs3*Δ double null mutant. *CHS3* expression was slightly elevated in *chs8*Δ and *chs2*Δ *chs8*Δ mutants while the *CHS2* promoter did not show any significant changes in any of the mutants tested (Fig. 2). Therefore, the deletion of single *CHS* genes resulted in activation of the expression of others.

CHS *promoter activity responds to wall perturbing agents*

Transcriptional regulation of the four *CHS* genes was determined in response to various environmental changes and perturbations (Fig. 3). Growth at 37°C stimulated *CHS1*, *CHS2* and *CHS3* promoters compared with growth at 25°C (not shown) and 30°C (control conditions). The addition of SDS, which perturbs membrane integrity, or CFW that interferes with cell wall assembly, induced expression from three of the four promoters (Fig. 3). CR

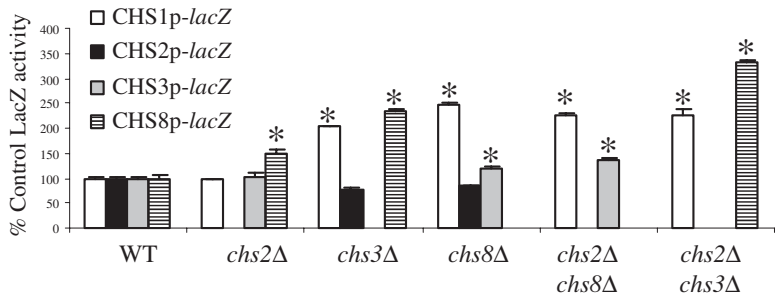

Fig. 2. Compensatory activation of *CHS* promoters in response to mutation in *CHS* genes. β-galactosidase activities are from cell-free protein extracts of *C. albicans* strain CAI-4 and chitin synthase mutants transformed with CHSp-*lacZ* constructs. Assays were performed on three independent YPD cultures grown at 30°C (average ± SD $n = 9$). Asterisks indicate differences where $P \leq 0.05$ compared with the wild-type parent strain transformed with the same promoter–*lacZ* fusion.

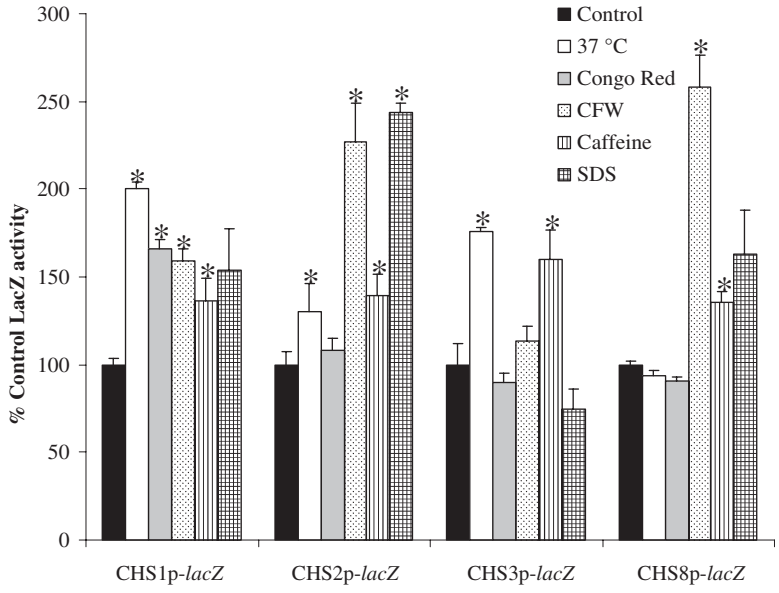

Fig. 3. *CHS* promoters are activated by cell wall and membrane perturbing agents. β-galactosidase activities of cell-free protein extracts of *C. albicans* strain CAI-4 transformed with the CHSp-*lacZ* constructs (strains NGY210 – NGY213, Table 2). Cells were grown on YPD supplemented with the compounds at 30°C or at 37°C and harvested at OD_{600} 0.8. Assays were performed in triplicate on three independent cultures (average ± SD $n = 9$). Asterisks denote significant differences to controls that were untreated strains, NGY210 – NGY213, grown on YPD at 30°C ($P \leq 0.05$).

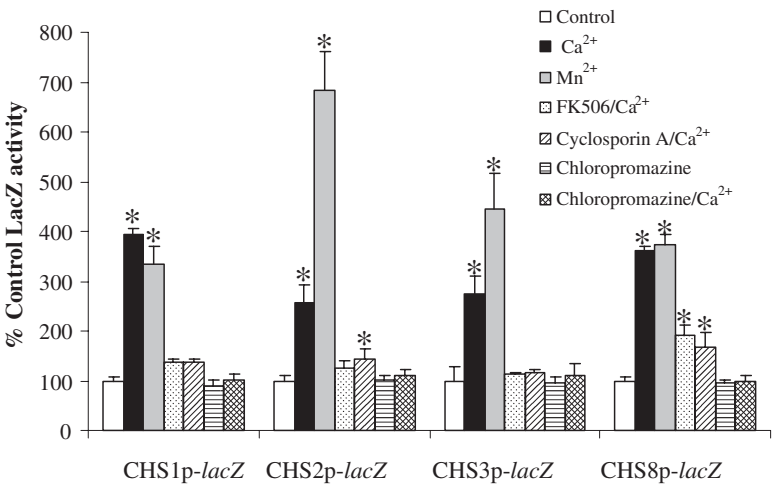

Fig. 4. *CHS* promoters response to Ca^{2+} and Mn^{2+}. β-galactosidase activities were measured in cell-free protein extracts of *C. albicans* strain CAI-4 transformed with the CHSp-*lacZ* constructs (strains NGY210 – NGY213, Table 2). Cells were grown at 30°C on YPD supplemented with Ca^{2+} and Mn^{2+} ± FK506, cyclosporin A or chloropromazine. Assays were performed in triplicate on three independent cultures (average ± SD $n = 9$). Asterisks indicate that there are significant differences to untreated control samples prepared in the same experiment from strains NGY210 – NGY213 ($P \leq 0.05$).

stimulated only *CHS1* expression. Caffeine, an inhibitor of cAMP phosphodiesterase, stimulates dual phosphorylation of *Sc*Slt2, the MAP kinase component of the PKC cell wall integrity signal transduction pathway (Martin *et al*., 2000). The addition of 12 mM caffeine to the growth medium resulted in significantly elevated expression from all four *CHS* promoters.

The *CHS* transcriptional response to cations and salts – 200 mM Ca^{2+}, Mn^{2+}, K^{2+}, Li^{2+}, Mg^{2+} or 800 mM NaCl was tested. Addition of K^{2+}, Li^{2+}, Mg^{2+} or Na^{2+} had no detectable effects (data not shown), however, exogenous Ca^{2+} and Mn^{2+} activated all four *CHS* promoters (Fig. 4). Some response was observed even with 5 mM Ca^{2+} (data not shown). Exogenously applied Ca^{2+} leads to activation of the calcineurin pathway, which induces dephosphorylation of the Crz1p transcription factor (Cyert, 2003). The calcineurin specific inhibitors FK506 and Cyclosporin A inhibited Ca^{2+} activation of *CHS* transcription (Fig. 4). Activation of *CHS* expression with Mn^{2+} was also reduced, but not totally blocked, by simultaneous addition of FK506 (data not shown) suggesting the Mn^{2+}-specific activation occurred in part through the calcineurin signalling pathway but also involved a calcineurin-independent mechanism. These results suggest that Ca^{2+} and Mn^{2+} activated *CHS* expression via both calcineurin/Crz1-dependent and independent mechanisms. The calmodulin inhibitor chloropromazine had no effect on *CHS* transcriptional activity but chloropromazine with 200 mM Ca^{2+} completely inhibited the Ca^{2+}-activation response. The calcium ionophore A23187 also inhibited the Ca^{2+}-activation response (data not shown). Inhibition by chloropromazine suggested that the observed Ca^{2+} stimulation involved the classical Ca^{2+} signalling pathway acting through calmodulin and calcineurin.

To corroborate these findings the four *CHS*-reporter constructs were transformed into null mutant strains lacking genes involved in the calcineurin pathway. The Crz1p transcription factor is dephosphorylated when the phosphatase calcineurin is activated by Ca^{2+}/calmodulin. It then enters the nucleus and induces expression of a number of genes, many of which encode proteins with cell wall-related functions (Yoshimoto *et al*., 2002; Lagorce *et al*., 2003; Garcia *et al*., 2004; Karababa *et al*., 2006; Pardini *et al*., 2006). Putative calcium-dependent response element (CDRE) motifs that are recognized by the Crz1p transcription factor were found in the promoter region of the *CHS* genes in *C. albicans* (Table 2). In the *cna1*Δ strain, which is mutated in one of the calcineurin subunits, the *CHS2* and *CHS8* promoters still responded to Ca^{2+}, but the response of the *CHS2* promoter was reduced (Fig. 5). The Ca^{2+} responses of the *CHS1* and *CHS3* promoters were not significantly different to the untreated *cna1*Δ control. Stimulation with Ca^{2+} was abrogated by simultaneous addition of FK506. In the *crz1*Δ mutant background, expression from the *CHS1* promoter was elevated when cells were grown in YPD, and addition of Ca^{2+} did not further stimulate *CHS1* expression (Fig. 5). The response of the *CHS2* and *CHS3* promoters to exogenous $Ca^{2+}$was significantly different in the *crz1*Δ mutant compared with wild-type cells. Therefore, *CHS2* and *CHS3* were activated in part via the Ca^{2+}/calcineurin/Crz1 pathway but Crz1p repressed the expression of *CHS1*. Transformation of the CHSp-*lacZ* constructs into the double calcium channel mutant *mid1*Δ *cch1*Δ had no significant effect on Ca^{2+}-stimulated activation of expression (data not shown).

Synergistic stimulation of CHS *promoters by combined* Ca^{2+} *and CFW treatment*

Addition of Ca^{2+} or CFW stimulated the *CHS* promoters – therefore we tested the effects of combinations of Ca^{2+} and CFW treatments. All four promoters were hyper-stimulated by combined treatment with Ca^{2+} and CFW

Table 2. Putative transcription factor binding sites.

Gene	Position[a]/strand	CDRE[b]	Position[a]/strand	Sko1 binding site[c]
CHS1	−351/+	GGGCTTC	−121/+	TACGT
	−380/−	TGGCTTG	−851/−	TACGT
	−744/+	AGGCTCC		
	−810/+	TGGCTCT		
CHS2	−539/−	TGGCTTT		
	−879/+	GGGCGTG		
	−918/+	AGGCTGA		
CHS3	−498/−	AGGCGGG	−130/+	TACGT
	−904/−	AGGCTCA	−665/−	TACGT
CHS8	−847/−	TGGCTTC	−782/+	TACGT
	−893/+	AGGCTTA		

a. Start codon A taken as position 1.
b. Crz1p consensus NGGC(G/T)CA.
c. Sko1p binding site consensus TACGT.

(Table 3). The pCHSplac plasmids were transformed into mutant strains lacking MAP kinase genes of the PKC and HOG signal transduction pathways (Table 1). The first mutant tested had a disrupted *MKC1* gene that encodes the MAP kinase of the PKC pathway (Navarro-Garcia *et al*., 1998). In the *mkc1*Δ strain background the *CHS8* promoter, and to a lesser extent, the *CHS2* promoter had reduced activity. All four *CHS* promoters were still stimulated by Ca^{2+} in the *mkc1*Δ mutant (Table 3), therefore the Ca^{2+}-induced upregulation of *CHS* promoters can be independent of the PKC pathway. However, the ability of the *CHS2* and *CHS8* promoters to respond to CFW was impaired in the *mkc1*Δ mutant. All four promoters were stimulated by the combined Ca^{2+}/CFW treatment but the level of stimulation of *CHS2* and *CHS8* promoters was significantly less in *mkc1*Δ cells compared with wild-type cells. The response of the *CHS* promoters to CFW and combined Ca^{2+}/CFW was also examined in the *crz1*Δ mutant (Table 3). Again the response of *CHS2* and *CHS8* promoters to CFW was significantly reduced in the *crz1*Δ mutant and the Ca^{2+}/CFW induction of all four promoters was dramatically reduced (three- to five-fold).

Three of the four *CHS* promoter sequences contained ATF/CREB elements – potential binding sites for the Sko1p transcription factor that is regulated by Hog1p (Table 2) (Proft *et al*., 2005). Expression from the *CHS1*, *CHS2* and *CHS8* promoters was reduced in the *hog1*Δ mutant compared with wild-type cells in YPD and in the presence of Ca^{2+} or CFW (Table 3). In contrast, expression from the *CHS3* promoter was increased in the *hog1*Δ mutant, suggesting Hog1p normally repressed *CHS3* transcription. In the *hog1*Δ mutant the *CHS3* promoter still responded to exogenous Ca^{2+}, but not to CFW and combinations of Ca^{2+} and CFW stimulated the *CHS3* promoter in both the *hog1*Δ and wild-type backgrounds. The Ca^{2+}/CFW-induced stimulation of the *CHS1* and *CHS2* promoters was not significantly altered in the *hog1*Δ strain but the level of expression from the *CHS8* promoters was significantly less than in wild-type cells. Together these data suggest that Mkc1p, Crz1p and Hog1p play significant roles in the Ca^{2+}/CFW hyper-stimulation of *CHS* promoters.

Exogenous Ca^{2+} stimulates chitin synthase activity

We tested whether the Ca^{2+}-activated *CHS* gene expression translated into measurably higher chitin synthase enzyme activity. *C. albicans y*east cells were cultured in YPD or YPD plus 100 mM Ca^{2+} for 5 h and membrane fractions of wild type (CAI-4), *chs*Δ and signalling mutant strains were prepared and assayed for chitin synthase activity. The specific chitin synthase activity of wild-type mixed membrane fractions (MMF) increased slightly when exogenous Ca^{2+} was added to the growth medium (Fig. 6). In the *mkc1*Δ mutant, Chs activity was comparable to the control strain and did not increase upon addition of Ca^{2+}. The *hog1*Δ mutant had markedly elevated Chs activity compared with the control and addition of Ca^{2+} did not further stimulate chitin synthase activity. Chitin synthase activity of the *crz1*Δ mutant was comparable to wild type and decreased in Ca^{2+}-treated cells. As shown previously (Munro *et al*., 2003), the Chs activity of the *chs2*Δ *chs8*Δ double mutant was only around 5% of wild-type levels and no further stimulation was observed when cells were grown in the presence of Ca^{2+}. The *chs3*Δ mutant had reduced Chs activity but this was elevated in response to Ca^{2+}. Therefore, Chs2p and Chs8p are mainly responsible for the elevated Chs activity in response to Ca^{2+} and this was mediated via Crz1p, Mkc1p and Hog1p. Attempts were made to measure chitin synthase activity from membranes prepared from cells grown in the presence of CFW and Ca^{2+}/CFW. No detectable Chs activity was found (not shown). CFW inhibition of *in vitro* chitin synthase activity has been reported previously (Roncero and Duran, 1985) and was shown to be dependent upon pH of the growth medium (Roncero *et al*., 1988).

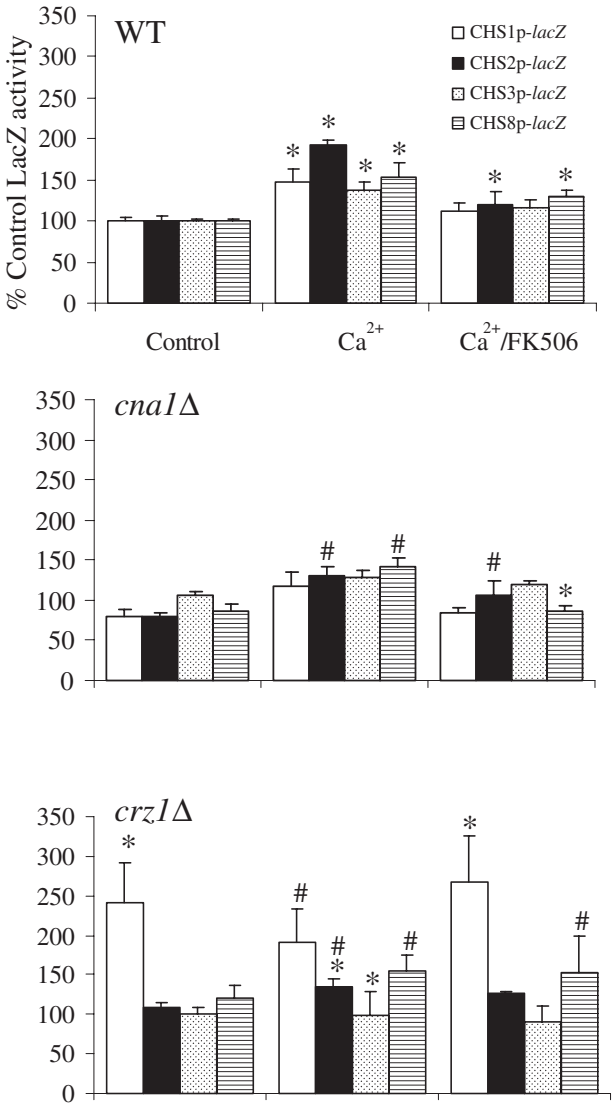

Fig. 5. Stimulation of the *CHS* promoters by Ca^{2+} is dependent upon calcineurin and Crz1p. β-galactosidase activities of protein extracts of CAI-4 (control), *cna1*Δ and *crz1*Δ transformed with the CHSp-*lacZ* constructs were measured. Cells were grown at 30°C on YPD, YPD supplemented with Ca^{2+} ± FK506. Triplicate assays were performed on three independent cultures (average ± SD, $n = 9$). In WT asterisks indicate significantly different to untreated control of that promoter under the same conditions ($P \leq 0.05$). For mutants, an asterisk indicates a significant difference relative to wild type under the same conditions, a number sign indicates a significant difference to the untreated control in the same mutant background ($P \leq 0.05$).

Treatment with Ca^{2+} and CFW elevates cell wall chitin levels

The chitin content of cells was measured under conditions where Ca^{2+} stimulated *CHS* gene expression and *in vitro* Chs activity. Addition of Ca^{2+}, CFW and Ca^{2+}/CFW resulted in elevated cell wall chitin levels in wild-type CAI-4 cells (Fig. 7) with the combination of Ca^{2+}/CFW giving the greatest stimulation. Chs3p is responsible for the synthesis of the majority of the chitin in the *C. albicans* cell wall (Bulawa *et al.*, 1995; Mio *et al.*, 1996). In the *chs3*Δ mutant, chitin levels were dramatically reduced and Ca^{2+}-treatment had only a slight effect on chitin content. The *chs2*Δ *chs8*Δ mutant behaved similarly to wild type. In the *mkc1*Δ mutant, chitin levels were lower than in parental controls, but were elevated after combined Ca^{2+}/CFW treatment. However, addition of Ca^{2+} or CFW alone had little effect on chitin content in the *mkc1*Δ mutant. Chitin levels of untreated *crz1*Δ cells were significantly higher than wild-type cells again suggesting that under some conditions Crz1p represses chitin synthesis. Chitin levels of *crz1*Δ were elevated by CFW or Ca^{2+}/CFW treatments but did not respond, or were repressed, when treated with Ca^{2+} alone. Untreated *hog1*Δ cells had wild-type chitin levels that were increased marginally when cells were grown with Ca^{2+} but the activation with CFW or Ca^{2+} plus CFW was significantly reduced compared with wild-type cells. These results suggest that elevated cell wall chitin content in response to combined treatments with Ca^{2+} and CFW is due mainly to Chs3p and that the PKC and HOG and to a lesser extent the Ca^{2+}/Crz1 pathways are involved in this Chs3p-dependent stimulation of chitin synthesis.

Ca^{2+}- and CFW-dependent phosphorylation of Mkc1p and Cek1p

The phosphorylation status of the Mkc1p and Cek1p MAP kinases was examined in order to assess the status of the PKC and SVG (STE vegetative growth) pathways in the strains and treatments described above (Navarro-Garcia

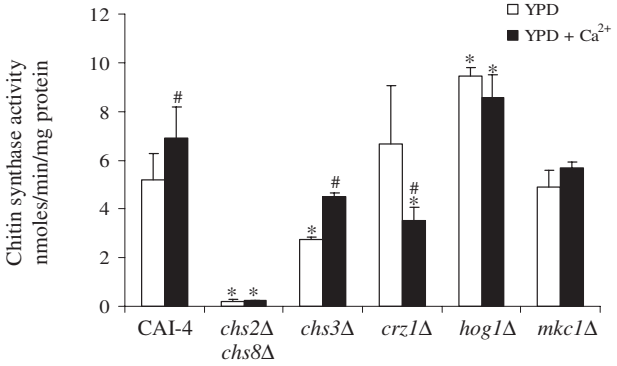

Fig. 6. Exogenous Ca^{2+} elevates *in vitro* chitin synthase activity of yeasts cells of *C. albicans* strains. Activities are from mixed membrane fractions after trypsin-treatment isolated from mid-exponential cells grown at 30°C in YPD (open bars) or YPD + 100 mM Ca^{2+} (black bars). Triplicate assays were performed (average ± SD, $n = 3$). Asterisks indicate significant differences from CAI-4 under the same conditions, a number sign indicates significant differences to untreated samples in the same strain background ($P \leq 0.05$).

Table 3. The *CHS* promoters are hyper-stimulated by combined Ca^{2+}/CFW treatment.

Strain	Treatment	Mean % *lacZ* activity	Fold change[a]	*P*-value	Fold change[b]	*P*-value
CHS1p-*lacZ*						
wt	Control	100 ± 7				
	+Ca^{2+}	174 ± 38	**1.74**	1.60E-05		
	+CFW	190 ± 34	**1.90**	3.71E-02		
	+Ca/CFW	888 ± 59	**8.88**	1.30E-06		
mkc1Δ	Control	91 ± 9			0.91	7.06E-01
	+Ca^{2+}	189 ± 25	**2.09**	4.00E-04	1.20	1.00E+00
	+CFW	141 ± 41	1.55	2.27E-01	0.82	7.08E-01
	+Ca^{2+}/CFW	735 ± 40	**8.11**	4.40E-10	0.91	3.08E-01
crz1Δ	Control	209 ± 11			**2.09**	3.10E-10
	+Ca^{2+}	202 ± 22	0.97	1.00E+00	0.55	7.81E-01
	+CFW	154 ± 19	**0.74**	3.70E-04	0.39	7.91E-01
	+Ca^{2+}/CFW	411 ± 174	1.96	2.78E-01	**0.22**	1.43E-03
hog1Δ	Control	39 ± 12			**0.39**	2.00E-06
	+Ca^{2+}	48 ± 9	1.22	9.94E-01	**0.70**	1.44E-09
	+CFW	47 ± 13	1.18	1.00E+00	**0.62**	2.80E-03
	+Ca^{2+}/CFW	592 ± 219	**15.00**	8.79E-03	1.69	3.85E-01
CHS2p-*lacZ*						
wt	Control	100 ± 3				
	+Ca^{2+}	226 ± 43	**2.26**	1.52E-07		
	+CFW	221 ± 23	**2.21**	7.67E-08		
	+Ca^{2+}/CFW	889 ± 121	**8.89**	3.05E-06		
mkc1Δ	Control	68 ± 3			**0.68**	9.98E-11
	+Ca^{2+}	196 ± 13	**2.88**	1.77E-08	1.27	6.02E-01
	+CFW	76 ± 3	**1.12**	7.00E-03	**0.51**	1.08E-08
	+Ca^{2+}/CFW	379 ± 18	**5.56**	5.96E-10	**0.63**	5.20E-11
crz1Δ	Control	142 ± 10			**1.42**	3.80E-05
	+Ca^{2+}	153 ± 12	1.08	8.91E-01	**0.48**	2.13E-04
	+CFW	147 ± 6	1.04	1.00E+00	**0.47**	9.56E-06
	+Ca^{2+}/CFW	402 ± 43	**2.84**	2.13E-06	**0.32**	1.06E-05
hog1Δ	Control	43 ± 3			**0.43**	2.30E-14
	+Ca^{2+}	54 ± 2	**1.24**	8.95E-06	**0.55**	1.58E-09
	+CFW	38 ± 1	**0.88**	8.63E-03	**0.40**	1.40E-09
	+Ca^{2+}/CFW	354 ± 113	**8.15**	2.00E-03	0.92	2.67E-01
CHS3p-*lacZ*						
wt	Control	100 ± 7				
	+Ca^{2+}	154 ± 26	**1.54**	1.69E-04		
	+CFW	118 ± 10	**1.18**	1.36E-02		
	+Ca^{2+}/CFW	693 ± 33	**6.93**	2.94E-10		
mkc1Δ	Control	101 ± 8			1.01	1.00E+00
	+Ca^{2+}	184 ± 23	**1.82**	1.20E-04	1.18	4.27E-01
	+CFW	104 ± 25	1.03	1.00E+00	0.87	9.94E-01
	+Ca^{2+}/CFW	732 ± 93	**7.23**	2.08E-06	1.04	1.00E+00
crz1Δ	Control	110 ± 8			1.10	4.45E-01
	+Ca^{2+}	89 ± 6	0.81	8.87E-02	**0.52**	1.20E-04
	+CFW	91 ± 4	0.83	2.32E-01	0.70	3.00E-01
	+Ca^{2+}/CFW	288 ± 41	**2.61**	1.66E-04	**0.38**	6.10E-10
hog1Δ	Control	156 ± 14			**1.56**	1.29E-05
	+Ca^{2+}	251 ± 21	**1.61**	2.06E-07	**1.04**	6.24E-07
	+CFW	135 ± 9	**0.86**	2.12E-05	0.73	5.15E-02
	+Ca^{2+}/CFW	988 ± 237	6.32	2.16E-04	0.91	2.12E-01
CHS8p-*lacZ*						
wt	Control	100 ± 9				
	+Ca^{2+}	158 ± 16	1.58	2.28E-11		
	+CFW	263 ± 14	2.63	8.27E-07		
	+Ca^{2+}/CFW	1237 ± 421	12.37	9.01E-09		
mkc1Δ	Control	27 ± 5			**0.27**	1.75E-09
	+Ca^{2+}	76 ± 17	**2.86**	7.52E-04	1.81	1.61E-01
	+CFW	16 ± 2	**0.59**	6.41E-03	**0.22**	4.38E-08
	+Ca^{2+}/CFW	378 ± 47	**14.12**	8.50E-07	**1.14**	3.81E-06
crz1Δ	Control	141 ± 8			**1.41**	1.99E-06
	+Ca^{2+}	160 ± 12	1.13	1.13E-01	**0.72**	3.95E-07
	+CFW	233 ± 16	**1.65**	4.08E-07	**0.63**	4.13E-09
	+Ca^{2+}/CFW	529 ± 94	**3.74**	9.74E-05	**0.30**	4.42E-05
hog1Δ	Control	43 ± 8			**0.43**	2.03E-08
	+Ca^{2+}	48 ± 5	1.11	9.99E-01	**0.70**	1.09E-07
	+CFW	34 ± 6	0.79	6.00E-01	**0.30**	3.70E-09
	+Ca^{2+}/CFW	315 ± 69	**7.29**	1.23E-04	**0.59**	7.10E-04

a. Fold change with respect to the control in the same genetic background.
b. Ratio of fold change in the mutant compared with fold change in wild-type cells under the same conditions.
Statistically significant changes are highlighted in bold.

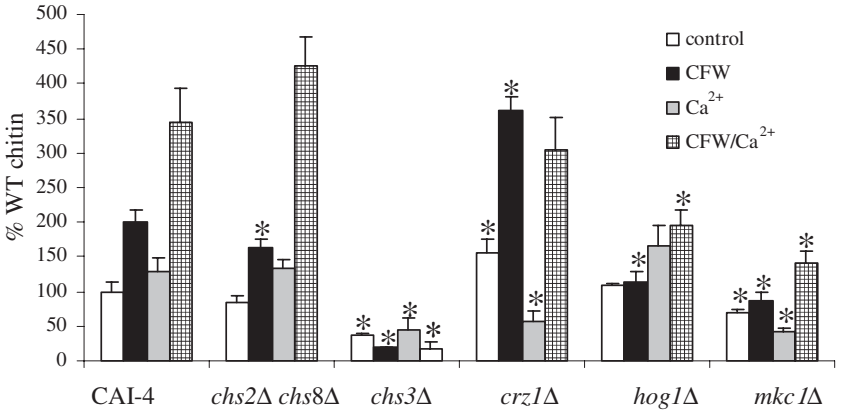

Fig. 7. Hyper-stimulation of cell wall chitin in response to Ca^{2+}/CFW. *C. albicans* strains were grown at 30°C on YPD and YPD supplemented with Ca^{2+}, CFW or both Ca^{2+} and CFW. Cell wall chitin assays were performed five times on three biologically independent samples (average ± SD $n = 15$). Asterisks indicate significant differences from CAI-4 cells grown under the same conditions.

et al., 2005; Eisman *et al*., 2006). Cek1p is the *C. albicans* orthologue of *Sc*Kss1p, which is the *S. cerevisiae* MAP kinase component of pathways that regulate filamentous growth, the pheromone response and promote vegetative growth (the latter via the SVG pathway) (Lee and Elion, 1999; Eisman *et al*., 2006). The SVG pathway is constitutively activated in the *och1*Δ *N*-glycosylation mutants of *S. cerevisiae* and *C. albicans* (Lee and Elion, 1999; Bates *et al*., 2006) and in the *hog1*Δ mutant and has been implicated in the response to cell wall perturbing agents (Eisman *et al*., 2006). Phospho-Mkc1p and phospho-Cek1p were identified by western analysis as 59 kDa and 48 kDa bands, respectively, using phospho-specific antibodies (Fig. 8). Phosphorylation status was assessed 10, 30, 60 and 120 min after treatment addition, however, only 10 and 120 min time points are presented here. In non-stressed conditions, no phospho-Mkc1p was detected in wild type or *hog1*Δ yeast cells however, Mkc1p was phosphorylated in the *crz1*Δ mutant.

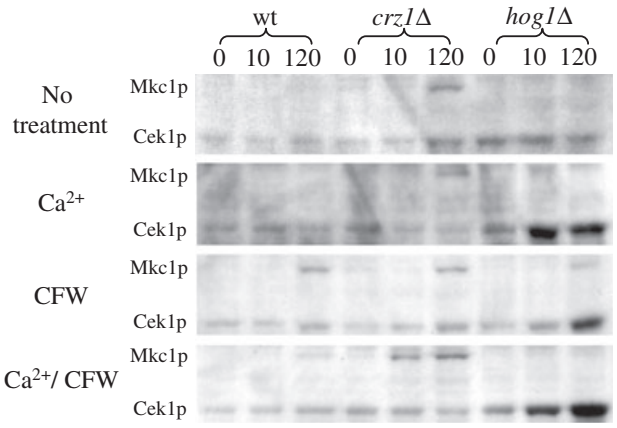

Fig. 8. Western blot analysis of Mkc1 phosphorylation status. *C. albicans* strains were grown at 30°C on YPD and YPD supplemented with Ca^{2+}, CFW or both Ca^{2+} and CFW. Total protein was extracted and phosphorylated Mkc1 and Cek1 detected using phospho-specific anti-p44/42 MAP kinase antibody.

Activation of Mkc1p was observed in the *crz1*Δ strain after treatment with Ca^{2+}. CFW stimulated strong activation of Mkc1p in the wild type, weaker activation in *hog1*Δ and activation comparable to untreated controls in *crz1*Δ. CFW-stimulated phosphorylation of Mkc1p was observed at 2 h in wild type and *hog1*Δ and after 1 h in *crz1*Δ. Combined treatment with Ca^{2+} and CFW had a synergistic effect on activation of Mkc1p in the *crz1*Δ strain where phospho-Mkc1p was detected after 10 min. In wild-type cells, Ca^{2+}/CFW did not give as strong a response in terms of Mkc1p phosphorylation as CFW alone. In agreement with Navarro-Garcia *et al*. (2005); Roman *et al*. (2005) and Eisman *et al*. (2006), phospho-Mkc1p was only detected in extracts prepared from *hog1*Δ cells when cells were treated with CFW. Under these conditions Cek1p appeared to be constitutively activated in the *hog1*Δ mutant. Treatment of the *hog1*Δ strain with Ca^{2+}, CFW and Ca^{2+}/CFW increased the level of phospho-Cek1p significantly. We conclude that the PKC pathway is activated when cells are treated with CFW and Ca^{2+}/CFW but Ca^{2+} alone could not stimulate phosphorylation of Mkc1p in a wild-type background.

Discussion

This study has shown that at least three signalling systems are involved in chitin synthesis regulation: (i) Ca^{2+}/calcineurin/Crz1p, (ii) PKC-Mkc1p and (iii) HOG pathways. At the transcriptional level *CHS* expression was monitored using a *lacZ* reporter gene fused to each of the four *C. albicans CHS* promoters. Each promoter was regulated differentially – the *CHS2* promoter was the most active under control conditions (YPD at 30°C) and the *CHS3* promoter was the least active. Real-time quantitative PCR confirmed these observations (data not shown). The *CHS* promoters responded to deletion of other *CHS* genes with a twofold increase in expression levels from *CHS1, CHS3* and *CHS8* promoters in several *chs*Δ mutants. In *Wangiella dermatitidis*, a melanized fungal

pathogen of humans, a compensatory increase in *WdCHS* expression has also been described in response to chitin synthase gene disruptions (Wang *et al*., 2002). Although there is no evidence of true functional redundancy within the chitin synthases examined to date, fungi appear to upregulate certain *CHS* in compensation for loss of others perhaps to maintain a robust cell wall.

The *C. albicans CHS* promoters were found to respond to a number of environmental stimuli notably when cells were treated with cell wall perturbing drugs and when growth medium was supplemented with Ca^{2+}. Addition of exogenous Ca^{2+}, stimulated *CHS* gene expression; stimulated *in vitro* chitin synthase activity, and resulted in increased cell wall chitin mediated through Chs3p. In addition, simultaneous treatment of cells with CFW and Ca^{2+} resulted in synergistically enhanced expression from all four *CHS* promoters and a threefold increase in the amount of chitin in the cell wall.

The *CHS* promoters were activated by exogenous Ca^{2+} and Mn^{2+} but not by equivalent concentrations of Mg^{2+} or Na^{+}. In *S. cerevisiae*, Ca^{2+} activates calcineurin via calmodulin, which induces gene expression by regulating the Crz1p/Tcn1p transcription factor. This plays a role in regulating cell wall structure including the induction of *ScCHS1* in response to Ca^{2+} (Yoshimoto *et al*., 2002) and tolerance of fungi to a wide range of antifungal agents (Edlind *et al*., 2002; Sanglard *et al*., 2003; Onyewu *et al*., 2004; Karababa *et al*., 2006). Ca^{2+}-activation of *CaCHS* expression was blocked by inhibitors of both calmodulin and calcineurin confirming the role of this pathway in the regulation of *CHS* genes. In addition, transcription from all *CHS* promoters was reduced in the *cna1*Δ mutant in response to exogenous Ca^{2+}. In the *crz1*Δ mutant, basal activity of the *CHS2*, *CHS3* and *CHS8* promoters was not altered but the *CHS1* promoter was de-repressed. In addition, *crz1*Δ cells were attenuated, but not completely blocked, in their ability to activate *CHS* expression in response to exogenous Ca^{2+} and the hyper-stimulation of *CHS* expression caused by cross-activation with Ca^{2+} and CFW was reduced dramatically in the *crz1*Δ mutant background. *In silico* analysis of the *CHS* promoter sequences also identified potential CDREs, motifs recognized by Crz1p. Therefore, activation of the *CHS* promoters due to Ca^{2+} was mainly regulated by the classical Cna1/Crz1 pathway; however, some of the Ca^{2+} stimulation was Cna1p and/or Crz1p-independent indicating that calcineurin and Crz1p may have roles that are distinct from their role in this signalling pathway. Our results suggest that in *C. albicans* the Ca^{2+} signalling pathway plays a major role in regulating chitin synthesis. This pathway may be vital to the co-ordination of responses to a variety of conditions that compromise cell wall integrity because it also regulates the expression of genes encoding cell wall proteins Utr2p and Crh11p (Pardini *et al*., 2006) and the glucan synthase catalytic subunit Fks1p/Gsc1p (Sanglard *et al*., 2003).

Many of the conditions that stimulated the *CHS* promoters including growth at 37°C, treatment with cell wall perturbing agents CFW, CR and SDS and the cAMP-phosphodiesterase inhibitor caffeine lead to hyperphosphorylation of Slt2p/Mkc1p (De Nobel *et al*., 2000; Martin *et al*., 2000; Navarro-Garcia *et al*., 2005). We used the *mkc1*Δ MAP kinase null mutant to test the role of the PKC pathway in chitin synthesis regulation. In the *mkc1*Δ mutant background the *CHS2* and *CHS8* promoters were less responsive to CFW but were still stimulated by Ca^{2+} and compared with wild-type cells only the *CHS2* promoter had a significantly reduced response to Ca^{2+}/CFW. However, there was a dramatic decrease in chitin levels in the *mkc1*Δ mutant under all conditions tested suggesting post-transcriptional regulation of Chs3p occurs via the PKC pathway. This pathway has been shown to regulate Chs3p in *S. cerevisiae* (Valdivia and Schekman, 2003).

The HOG signalling pathway was the third pathway implicated in *CHS* transcriptional regulation. Promoter sequences recognized by the HOG-regulated transcription factor Sko1p were identified in the *CHS1*, *CHS3* and *CHS8* promoters. Loss of the HOG pathway in non-stressed conditions resulted in reduced expression of *CHS1*, *CHS2* and *CHS8* but increased expression of *CHS3*. Therefore, as with Crz1p, blocking a particular signalling pathway had both positive and negative effects on *CHS* expression. Although the *CHS1*, *CHS2* and *CHS8* promoters had attenuated responses to either CFW or Ca^{2+} in the *hog1*Δ mutant, only the *CHS8* promoter had markedly reduced activity in response to the combined Ca^{2+}/CFW treatment compared with wild-type cells. Despite the apparently low basal level of expression of *CHS2* and *CHS8* in the *hog1*Δ mutant, the levels of chitin synthase enzyme activity were greater than in wild-type cells. This suggests the presence of a compensatory mechanism that is activated in response to loss of Hog1p that acts post-transcriptionally and results in enhanced Chs enzyme activity. Nevertheless, the amount of chitin in the wall of the *hog1*Δ mutant synthesized in response to co-stimulation with Ca^{2+} and CFW was significantly (40%) lower than wild type implying that the HOG pathway is involved in activation of chitin synthesis via Chs3p.

The Ca^{2+}/calcineurin, PKC and HOG pathways contribute to the hyper-stimulation of chitin synthesis in response to Ca^{2+}/CFW treatment. The role of the Ca^{2+}-signalling pathway appears to be mainly in regulating *CHS* transcription, whereas the PKC and HOG pathways also contribute to regulation of chitin synthase enzyme activity and total cell wall chitin content. The Mkc1 pathway positively regulates *CHS* expression, chitin synthase activity and chitin levels in the cell wall while the Ca^{2+}/Crz1 and HOG pathways have both positive and negative regulatory

effects on different *CHS* genes and Chs isoenzymes (Fig. 9).

Interpretation of experiments studying *CHS* expression is complicated by cross-talk between signalling pathways and by compensatory mechanisms that are triggered in mutants defective in single signalling pathways. For example, mutants blocked in the HOG pathway have a constitutively active Cek1 MAP kinase, which contributes to a CR resistance phenotype (Roman *et al*., 2005; Eisman *et al*., 2006). We examined the phosphorylation status of Mkc1p and Cek1p in cells treated with Ca^{2+}, CFW and Ca^{2+}/CFW. We confirmed phosphorylation of Cek1p in the *hog1*Δ mutant and enhanced phosphorylation of Cek1p when *hog1*Δ was treated with Ca^{2+}, CFW and Ca^{2+}/CFW. Despite activation of Cek1p, chitin levels are reduced in the *hog1*Δ mutant suggesting Cek1p may not make a major contribution to chitin regulation. Our findings also corroborate the observations of Navarro-Garcia *et al*. (2005) that Hog1p was required for phosphorylation of Mkc1p under a variety of conditions, but not with CFW treatment. In addition, our Western analyses suggested that Mkc1p was phosphorylated in the *crz1*Δ mutant. These data suggest that these pathways do not operate in isolation and that mutations in one pathway results in activation of others as in the case of Cek1p activation in the *hog1*Δ mutant and Mkc1p phosphorylation in the *crz1*Δmutant. Hence, the activation of Mkc1p is not solely responsible for the elevated chitin synthesis under the conditions tested. Instead, the PKC, HOG and Ca^{2+} signalling pathways all contribute to the regulation of chitin synthesis.

In conclusion, the Ca^{2+}/Crz1p, PKC-Mkc1p and HOG signalling pathways co-ordinate the regulation of chitin synthesis in *C. albicans*. The use of multiple pathways may enable the fungus to fine-tune the co-ordinated assembly of cell wall chitin to exogenous stresses by modulating chitin synthesis. *CHS* gene expression responded to a wide range of environmental conditions and individual *CHS* genes and Chs enzymes responded differently to these stresses. This regulation is vital for the maintenance of a robust cell wall during growth and morphogenesis but also under conditions where the integrity of the cell wall is compromised by treatments with antifungal drugs that target fungal cell wall synthesis.

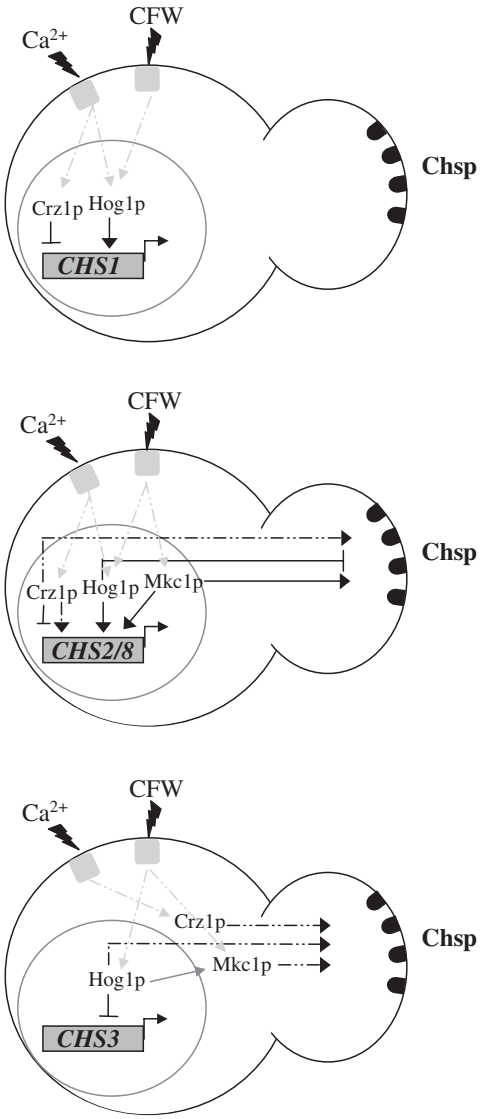

Fig. 9. Summary model of the co-ordinated regulation of chitin synthesis. Solid lines indicate regulation under non-stress conditions and dotted lines indicate regulation in response to various stresses. Under normal growth conditions Hog1 is required for basal levels of *CHS1* transcription and Crz1p acts as a repressor. $CaCl_2$ or CFW-stimulated *CHS1* expression is dependent upon Crz1p and Hog1p, while the PKC pathway does not play a major role. The contribution of these three pathways to post-transcriptional regulation of Chs1p cannot be determined by measurement of *in vitro* chitin synthase activity and chitin content because Chs1p is only a minor contributor to these. *CHS2* and *CHS8* are regulated by the Crz1p, Hog1p and Mkc1p pathways under normal growth conditions. Although Crz1p represses *CHS2* expression it is required for $CaCl_2$ stimulation. Hog1p and Mkc1p are both required for normal control levels of *CHS2* and *CHS8* mRNA and CFW-activated transcription is dependent upon both pathways. In addition, Hog1p is required for $CaCl_2$ activation of *CHS2* and *CHS8* expression. In Chs activity assays, Hog1p negatively regulates Chs2p and Chs8p and Crz1p is responsible for their activation due to the presence of exogenous $CaCl_2$. Hog1p had a significant negative contribution to *CHS3* promoter activity but all three pathways were involved in elevation of chitin levels due to treatment with $CaCl_2$ and CFW – a measure of Chs3p post-transcriptional regulation.

Experimental procedures

Strains, media and growth conditions

Candida albicans strains used in this study are listed in Table 1. *C. albicans* cultures were maintained on solid YPD medium comprising 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar. Yeast cells of *C. albicans* were grown at 30°C in YPD with shaking at 200 r.p.m.

Transformation of C. albicans

Ura⁻*C. albicans* strains were cultured in 10 ml of YPD supplemented with 25 μg ml^{-1} uridine at 30°C for 36–72 h. After centrifugation, the cell pellets from 200 μl of cells were resuspended in 100 μl of OSB (200 mM LiAc pH 7.5, 100 mM DTT, 50% v/w PEG 6000, 10 mg ml^{-1} Clontech herring testis carrier DNA) and then transforming DNA was added. Samples were incubated at 43.5°C for 60 min and spread over SD agar plates (2% (w/v) D-glucose, 0.67% (w/v) yeast nitrogen base (YNB) (Bio 101, Carlsbad), 1.5% (w/v) purified agar, Oxoid) and incubated at 30°C. Single colonies were picked and grown in 5 ml of SD medium, and genomic DNA was extracted for Southern analysis.

Construction of plasmids and C. albicans *strains*

The placpoly-6 vector was used for the promoter-fusion reporter system and was based on a plasmid previously described by Uhl and Johnson (2001). This contains the *CaURA*3 and the *CaRPS1* genes and was used to create fusion between the promoter of each *CaCHS* gene and the *S. thermophilus lacZ* ORF. A 1 kb upstream region from the ATG start codon of each *CHS1*, *CHS2*, *CHS3* and *CHS8* ORF was cloned into the *Pst*I–*Xho*I sites of placpoly-6 generating pCHS1plac, pCHS2plac, pCHS3plac, pCHS8plac respectively. Ura⁻*C. albicans* cells were transformed with these plasmids previously cut within the *RPS1* gene with StuI to target homologous recombination at the neutral chromosomal *RPS1* locus and the *URA*3 gene was the selectable marker (Murad *et al*., 2000). Southern analysis was used to screen transformants and only those with single integrations of each pCHSplac plasmid were selected. Genomic DNA from each transformant was digested with *Xho*I/*Bam*HI and hybridized to 693 bp *RPS1* specific probe.

Measurement of β-galactosidase activity

The expression of each *CHS* gene in *lacZ* promoter fusions was measured using a modified version of the assays described previously (Rose and Botstein, 1983). *C. albicans* cells were grown with shaking at 200 r.p.m. at the chosen growth condition and harvested at $OD_{600} < 1$. Yeast cells were centrifuged at 3000 *g* for 5 min at 4°C and the pellet was resuspended in 0.5 ml of ice–cold water and transferred to microcentrifuge tubes. The cells were then centrifuged at 13 000 *g* for 5 min and resuspended in 0.5 ml of breaking buffer [100 mM TRIS-HCl pH 7.5, 0.01% (w/v) SDS, 1 mM dithiothreitol (DTT), 10% (v/v) glycerol, pepstatin 4 μg ml^{-1}, 1 × proteinase cocktail tablets EDTA-free (Roche)]. Approximately equal volumes of glass beads (Sigma, Poole, UK G9268) and cell pellet were used and the cells were disrupted using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK) using six cycles of 30 s with chilling on ice for 1 min in between each cycle. The extract was centrifuged at 13 000 r.p.m. for 10 min and the protein concentration of the supernatant was measured using Coomassie® Protein Assay Reagent Kit (Pierce Biotechnology, Perbio, Rockford, UK). Varying quantities of protein extract, 30–300 μl, were added to Z-buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$), in a total volume of 800 μl. The reaction was initiated by adding 200 μl of *o*-nitrophenyl-*β*-D-galactopyranoside (ONPG) stock solution (4 mg ml^{-1} in phosphate buffer) and incubated until the yellow *o*-nitrophenol product was produced. The reaction was stopped by addition of 400 μl of 1 M Na_2CO_3. The specific *β*-galactosidase activity was measured in terms of the yield of product *o*-nitrophenol at the absorbance of 420 nm.

Measurement of chitin synthase activity

Mixed membrane fractions were prepared from exponential phase yeast cells and their chitin synthase activities measured as described previously (Munro *et al*., 1998). MMF proteins were activated by limited incubation with 100 ng trypsin μl^{-1} MMF at 30°C and the reactions were stopped by addition of 150 ng soybean trypsin inhibitor μl^{-1} MMF. Briefly, standard reactions for measuring chitin synthase activity were carried out in a 50 μl volume and were composed of; 50 μg MMF protein, 25 mM *N*-acetylglucosamine, 1 mM UDP-*N*-acetylglucosamine which included 25 nCi UDP-[U-^{14}C] *N*-acetylglucosamine, 50 mM Tris-HCl pH 7.5 and 10 mM $MgCl_2$. Incubations were carried out at 30°C for 30 min and the reaction was stopped by addition of 1 ml of 66% (v/v) ethanol. The reaction mixture was then filtered through GF/C filter discs (Whatman), which had been presoaked in 10% (v/v) trichloroacetic acid. The reactions tubes were rinsed out with 2 × 1 ml of 1% (v/v) Triton X-100 and each filter was then washed with 4 × 2 ml of 66% (v/v) ethanol. The radiolabelled chitin synthesized in the reaction was trapped on the filters and unincorporated substrate was removed by washing. Filters were dried at 80°C and their radioactivity counted in a scintillation counter.

Measurement of cell wall chitin content

Cell walls were prepared from 10 ml of *C. albicans* stationary phase yeast cultures grown in YPD and the chitin content was measured as described previously (Munro *et al*., 2003). Cells were disrupted with glass beads (Sigma, G9268) using a Fastprep cell breakage machine (Thermo Savant, Middlesex, UK) until at least 95% of cells were disrupted. They were then washed five times with 1 M NaCl and extracted in SDS-MerOH buffer (50 mM Tris, 2% sodium dodecyl sulphate (SDS), 0.3 M β-mercaptoethanol, 1 mM EDTA; pH 8.0) at 100°C for 10 min, then washed in dH_2O. Cell wall pellets were resuspended in sterile dH_2O, freeze dried, and the dry weight of recovered cell walls was measured. Chitin contents were determined by measuring the glucosamine released by acid hydrolysis of purified cell walls (Kapteyn *et al*., 2000).

Culture conditions

Cells were grown in normal laboratory media and under conditions of various environmental stresses. Cells were grown overnight in YPD then transferred to YPD supplemented with different agents: 1 M sorbitol, 0.8 M NaCl, 0.2 M $CaCl_2$, 100 μg ml^{-1} CFW, 200 μg ml^{-1} CR, 0.05% SDS, 12 mM caffeine, 25 mM DTT, 23 mM glucosamine, 50 μg ml^{-1}

cyclosporin A, 1 μg ml^{-1} FK506, 1 mM chloropromazine, 4 μM A23187. Cells were harvested at OD_{600} 0.8.

Western analysis

Western analysis was performed using the method of Millar *et al*. (1995) with some modifications. Overnight cultures of the wild type, *crz1*Δ and *hog1*Δ strains were diluted 1:50 into 25 ml of YPD supplemented with uridine and incubated shaking for 4 h at 30°C. The mid-log phase cultures were then treated with a final concentration of 100 mM $CaCl_2$, 100 μg ml^{-1} CFW, or both for 0, 10, 30, 60 or 120 min. No-treatment controls were also performed. After treatment, cells were harvested by centrifugation (1500 *g*, 2 min, 4°C) and washed in 1 ml of cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 2 μg ml^{-1} Leupeptin, 2 μg ml^{-1} Pepstatin, 1 mM PMSF, 2 mM Na_3VO_4, 50 mM NaF). Cells were collected by centrifugation (800 *g*, 5 min, 4°C) and resuspended in 250 μl of cold lysis buffer. Cells were broken using a FastPrep machine in the presence of acid-washed glass beads (4 × 15 s bursts at speed 6.5 with 1 min on ice between bursts). The extracts were clarified by centrifugation (16 000 *g*, 5 min, 4°C). Protein concentration in the cleared lysate was estimated using the method described by Bradford (1976) with BSA as a standard.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the XCell *SureLock*™ Mini-Cell system (Invitrogen) with NuPAGE®Novex Bis-Tris 4–12% precast gels (Invitrogen) in NuPAGE® MOPS-SDS Running Buffer (Invitrogen) containing NuPAGE® Antioxidant (Invitrogen) as per the manufacturer's instructions. Approximately 15 μg of protein was loaded in each lane. The proteins were transferred to Invitrolon™ PVDF Membranes (Invitrogen) in NuPAGE® Transfer Buffer containing methanol using the XCell II™ Blot Module (Invitrogen) following the manufacturer's instructions.

Following transfer, the membranes were rinsed in PBS and blocked in PBS-T + 10% BSA (PBS, 0.1% Tween-20, 10% (w/v) BSA, 50 mM (NaF) for 30 min at room temperature. The membranes were then incubated overnight at 4°C in PBS-T + 5% BSA (PBS, 0.1% Tween-20, 5% (w/v) BSA, 50 mM (NaF) containing a 1:1000 dilution of Phospho-p44/42 Map Kinase (Thr202/Tyr204) Antibody (Cell Signaling Technology). The membranes were washed five times for 5 min in PBS-T (PBS, 0.1% Tween-20) and then incubated for 1 h at room temperature in PBS-T + 5% BSA containing a 1:2000 dilution of Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology). The membranes were washed three times for 5 min in PBS-T and the signal was detected using LumiGLO™ Reagent and Peroxide (Cell Signaling Technology) as per the manufacturer's instructions.

Statistical analyses

Statistical significant differences in the assay results were determined with SPSS software using ANOVA and Post Hoc Dunnett's *T*-test, $P < 0.05$. When the results displayed unequal variance the Kruskal–Wallis non-parametric test or Dunnett's T3 test were applied.

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