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ORIGINAL PAPER

Thermosensitivity of the *Saccharomyces cerevisiae gpp1gpp2* double deletion strain can be reduced by overexpression of genes involved in cell wall maintenance

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Abstract A *Saccharomyces cerevisiae* strain in which the *GPP1* and *GPP2* genes, both encoding glycerol-3-phosphate phosphatase isoforms, are deleted, displays both osmo- and thermosensitive (ts) phenotypes. We isolated genes involved in cell wall maintenance as multicopy suppressors of the *gpp1gpp2* ts phenotype. We found that the *gpp1gpp2* strain is hypersensitive to cell wall stress such as treatment with β-1,3-glucanase containing cocktail Zymolyase and chitin-binding dye Calcofluor-white (CFW). Sensitivity to Zymolyase was rescued by overexpression of *SSD1*, while CFW sensitivity was rescued by *SSD1*, *FLO8* and *WSC3*—genes isolated as multicopy suppressors of the *gpp1gpp2* ts phenotype. Some of the isolated suppressor genes (*SSD1*, *FLO8*) also rescued the lytic phenotype of *slt2* deletion strain. Additionally, the sensitivity to CFW was reduced when the cells were supplied with glycerol. Both growth on glycerol-based medium and overexpression of *SSD1*, *FLO8* or *WSC3* had additive suppressing effect on CFW sensitivity of the *gpp1gpp2* mutant strain. We also confirmed that the internal glycerol level changed in cells exposed to cell wall perturbation.

Keywords *Saccharomyces cerevisiae* · Heat shock · PKC pathway · Cell wall stress · Glycerol

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Abbreviations

CFW	Calcofluor-white
FG	Filamentous/invasive growth
Glycerol-3-P phosphatase	glycerol-3-phosphate phosphatase
HOG	High osmolarity glycerol
MAP	Mitogen-activated protein
PKC	Protein kinase C
ts	Thermosensitivity
wt	Wild type

Introduction

Unicellular organisms, like yeast, must be able to adapt to changes in their natural environment such as temperature, osmolarity, pH or, e.g. the presence of reactive oxygen intermediates and the availability of nutrients (Hohmann and Mager 1997). These types of environmental conditions often lead to the loss of growth, need to be sensed and have to result in adaptive responses for cell survival. These responses may encompass metabolic changes, altered gene expression patterns and adaptation in cellular morphology (Botstein and Brown 2000; Causton et al. 2001; Gasch et al. 2000; Hohmann 2002; Siderius and Mager 2003).

Budding yeast, *Saccharomyces cerevisiae* utilises at least five different MAP kinase modules to respond to changes in its environment that evoke morphological adaptation.

One of these MAPK pathways is the cell integrity (protein kinase C) pathway (Gustin et al 1998). PKC pathway activation, induced by elevated temperature, morphogenetic events, hypo-osmotic shock or cell wall perturbation, is mediated by receptors (Wsc1-3p, Mid2p) through a small G-protein signalling system (Rom2p,

Sac7p and Rho1p) to the Pkc1p kinase (Philip and Levin 2001). The main target of activated Pkc1p is the MAPK cascade module consisting of Bck1p, Mkk1/Mkk2p and Slt2p MAP kinase, which is dually phosphorylated on threonine and tyrosine residues (Posas et al. 1998). The output of PKC pathway activity comprises enhanced transcription of several genes involved in cell wall maintenance (Garcia et al. 2004).

Other MAPK modules mediate the adaptive responses to increased osmolarity (HOG pathway), the absence of nutrients (filamentous/invasive growth—FG pathway and sporulation pathway) or the presence of mating pheromone (mating pathway; Gustin et al. 1998; Hohmann 2002). Interestingly, the functionality of these MAPK pathways can overlap, the HOG and FG MAPK pathways both mediate responses to high osmolarity (Davenport et al. 1999; de Nadal et al. 2003; O'Rourke and Herkowitz 2002; Siderius and Mager 2003). The PKC and FG pathways are implicated in cell wall adaptive responses (Cullen et al. 2000; Garcia et al. 2004; Heinisch et al. 1999; Lee and Elion 1999; de Nobel et al. 2000). Sometimes functionality of MAPK pathways is mutually exclusive, as was shown for the HOG and PKC pathways (Davenport et al. 1995; Wojda et al. 2003). Although morphological control has to be tightly regulated, *S. cerevisiae* displays remarkable flexibility in compensatory responses as is displayed by MAPK mutant strains. A *hog1* deletion strain displays an osmosensitive phenotype because it is unable to counteract the loss of turgor by accumulating glycerol, the osmolyte in yeast (Albertyn et al. 1994). Overexpression of *GPD1*, encoding glycerol-3-phosphate dehydrogenase responsible for the rate-limiting step in glycerol synthesis, suppresses the osmosensitive phenotype. Interestingly, suppression of the osmosensitive phenotype can also be achieved by culturing the cells at elevated temperatures resulting in a higher basal level of glycerol in the cell (Siderius et al. 2000). The molecular cause for the higher intracellular glycerol concentration at elevated temperatures could be the increased levels of *GPD1* and *GPP2* expression. These genes were shown to display enhanced transcription when the cells are exposed to cell wall perturbing compounds, conditions that, like increased temperature, trigger PKC MAPK pathway activity (Boorsma et al. 2004).

On the other hand, yeast cells that display features of a weakened cell wall, e.g. PKC pathway mutants can be rescued by growth in high osmolarity media (Martin et al. 1996). Furthermore, osmosensitive mutant strains can be rescued by overexpression of genes known to mediate cell wall changes or trigger PKC MAPK pathway activity (Alonso-Monge et al. 2001; Wojda et al. 2003). Moreover, HOG pathway mutants display phenotypes such as sensitivity to cell wall perturbing agents (Alonso-Monge et al. 2001; Garcia-Rodrigues et al. 2000; de Nobel et al. 2000). The mutual exclusiveness of the activation of the HOG and PKC MAPK pathways apparently is not only the consequence of opposite stimuli but can also be controlled at the level of intracellular turgor and cell wall composition.

To further investigate the interconnection between glycerol synthesis, cell wall properties and activation of PKC MAPK pathway, we set out to characterise the thermosensitive (ts) phenotype that is displayed by the *gpp1gpp2* double deletion strain. As was shown previously, loss of both genes encoding the glycerol-3-P phosphatases, resulted in an osmosensitive phenotype (Pahlman et al. 2001; Siderius et al. 2000). This phenotype occurs despite the existence of an alternative metabolic route of glycerol synthesis or the possibility to take it up from the medium through transporters (Blomberg 1997; Lages and Lucas 1997). Apparently, the alternative means to produce/take up glycerol suffice for the need of glycerol as the precursor of lipids and for the role that glycerol synthesis plays in maintaining cellular redox balance (Ansell et al. 1997). However, at elevated temperatures, the *gpp1gpp2* strain cannot grow at all, a phenotype that is displayed already by *gpp2* single deletion strain (Siderius et al. 2000).

In order to understand the role of intracellular glycerol in *S. cerevisiae*, we aimed to find multicopy suppressors of the temperature-sensitive phenotype of the *gpp1gpp2* mutant strain. We will show that the nature and functionality of these multicopy suppressors further underscore the relation between intracellular glycerol level and susceptibility to cell wall stress.

Materials and methods

Yeast strains and growth conditions

Yeast strains and plasmids used in this study are listed in Table 1. Standard yeast media and growth conditions were used. YNB media (0.67% yeast nitrogen base, 2% glucose) containing the appropriate supplements were used whenever selective conditions were required. Thermosensitivity of yeast strains was tested on YPD solid medium (2% glucose, 2% bactopeptone, 1% yeast extract). Serially diluted (1:10, starting from OD_{600} of 0.1) cell suspension (5 µl) was spotted onto YPD media and cultured at 37°C (and at 28°C as a control). Sensitivity of strains to Calcofluor-white (CFW) was tested on YPD media supplemented with indicated amount of CFW. Where indicated, other carbon sources were utilised, e.g. 3% glycerol (YPG) or 1% ethanol (YPE).

Multicopy suppression screening

The *gpp1gpp2* double deletion strain was transformed with *S. cerevisiae* multicopy genomic library in the

Table 1 *Saccharomyces cerevisiae* strains and plasmids used in this study

Strain/plasmid	Genotype	Source/reference
Strain		
W 303-IA	*MAT a leu2-3/112 ura3-1 trp1-1 his 3-11/15 ade2-1 can 1-100 GAL SUC mal0*	S. Hohmann, Goteborg University, Goteborg, Sweden
YA 103	*W 303 (MATa) leu2-3/112 ura3-1 trp1-1 his 3-11/15 ade2-1can 1-100 GAL SUC mal0 gpp1::kanMX4, gpp2::HIS3MX6*	(Pahlman et al. 2001)
YSH 849	*W303 (MATa) leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC mal0 slt2::TRP1*	L. Adler, University of Goteborg, Sweden
Plasmids		
YE*plac*195	*URA3 2μ*	(Alonso-Monge et al. 2001)
YEp*lacWSC3*	*URA3 2μ WSC3*	
YEp*lacPKC1* (YE*plac*24)	*URA3 2μ PKC1*	H. Sakurai, Kanazawa University, Japan
pFL44L		(Bonneaud et al. 1991)
pFL*SSD1*	*URA3 2μ SSD1*	This study
pFL*FLO8*	*URA3 2μ FLO8*	This study
pFL*WSC2*	*URA3 2μ WSC2*	This study

pFL44L vector (Bonneaud et al. 1991) using the lithium-acetate method (Gietz et al. 1992). First, transformants (about 25,000) were selected on minimal medium. These transformants were then taken up in YPD medium, the OD_{600} was measured, and the cells were spread on 20 YPD plates (2,000 cells/12 cm × 12 cm plate) and cultured at 37°C. We obtained 180 clones capable of growing at 37°C and analysed plasmids of randomly picked transformants of this pool. Plasmid rescue from yeast and transformation to *Escherichia coli* DH5α was performed using standard techniques. Transformation of yeast strains with isolated plasmids was performed using the freeze–thaw method (Klebe et al. 1983). Nucleotide sequences were determined by the dideoxynucleotide method using the thermal sequenase kit (Amersham). The *WSC3* was recloned (3′*Hind* III and 5′*Hind* III site in the pFL44L polylinker) into YEp*lac*195. The *PKC1* gene cloned into *Sal*1 site of YEp*lac*24 was a kind gift from Dr Hiroshi Sakurai (Kanazawa University, Japan).

Western blotting and antibody staining

Total protein samples were isolated as described previously (Alonso-Monge et al 2001; Davenport et al. 1995) and protein concentration was determined according to Bradford (1976). Protein samples (20 μg of total protein) were separated on 10% polyacrylamide gel and blotted onto Immobilon filters. Dually phosphorylated Slt2p was detected using an anti-phospho-p42/44 MAPK antibody (New England, Biolabs). The level of actin measured by an anti-actin antibody (Sigma) was used as a loading control. Secondary goat anti-rabbit antibody conjugated with horseradish peroxidase was used to detect antibody binding using ECL system (New England, Biolabs).

Glycerol measurements

Cell cultures of 10 ml in YNB medium were cooled down, centrifuged and quickly washed with ice-cold YNB to remove extra-cellular glycerol and centrifuged again. Pellets were re-suspended in 1 ml 20 mM Tris–HCl pH 7.5, boiled for 15 min and centrifuged for 15 min, 14,000 rpm. The supernatant cleared from cellular debris, was used for glycerol measurement according to manufacture's instructions (Glycerol measurement kit, Boehringer, Mannheim). The results are presented as the amount of glycerol per protein units as described earlier (Wojda et al. 2003).

β-1,3-Glucanase (Zymolyase) sensitivity assay

Sensitivity of yeast cells to the β-1,3-glucanase cocktail Zymolyase (100T, Seikagaku) was estimated using the growth inhibition assay as described in Alonso-Monge et al. (2001). Yeast cell culture of OD_{600} 0.025 was supplemented with the indicated amount of Zymolyase. OD_{600} was measured after overnight culture at 30°C. Zymolyase sensitivity is expressed as a percentage of growth compared to the growth in YPD without Zymolyase.

Results

The thermosensitive phenotype of the *gpp1gpp2* double deletion strain is suppressed by overexpression of genes involved in cell wall maintenance and by the growth on glycerol-based medium

In order to determine the relation between intracellular glycerol level, cell wall adaptations and MAPK pathway activation, we set out to search for multicopy suppressors of the ts phenotype of the *gpp1gpp2* double deletion strain (Siderius et al. 2000). As expected, the *GPP2* gene was isolated a few times. The *GPP1* gene was not isolated in this screen as multicopy suppressor, which is in agreement with the observation that the *gpp2* single deletion strain already displays a ts phenotype (Siderius et al. 2000). In addition, we isolated *SSD1*, *FLO8*, *WSC3* and *WSC2* as genes suppressing the ts phenotype of the *gpp1gpp2* strain (Fig. 1a). The *WSC2*, *SSD1* and *FLO8* genes were isolated as the only complete ORFs present on the respective inserts of the multicopy suppressor plasmids (co-ordinates 104624-107517; 1044214-1050085; 374111-378974, on chromosomes 14, 4 and 5, respectively). Deleting parts of the *WSC2, SSD1* and *FLO8* genes resulted in loss of suppression of the ts phenotype of the *gpp1gpp2* deletion strain (not shown). The *WSC3* gene was isolated together with *NDJ1* (co-ordinates 113909-119411, on chromosome 15) and verification of the *WSC3* as a suppressor gene was done by recloning of *WSC3* into YEp*lac*195 as described in Section 'Materials and methods'.

Ssd1p is a protein with a role in the maintenance of cellular integrity (Kaeberlein and Guarante 2002; Reinke et al. 2004). The *FLO8* gene encodes a transcription factor required for flocculation, diploid filamentous growth and haploid invasive growth (Kobayashi et al. 1996; Liu et al. 1996; Rupp et al. 1999). Wsc2p and Wsc3p are upstream sensors of PKC pathway signalling (Verna et al. 1997). We tested whether other sensors of this pathway or Pkc1p, the upstream kinase of the PKC MAPK pathway, could suppress the phenotype that is displayed by the *gpp1gpp2* deletion strain. Overexpression of *PKC1* (Fig. 1a) but not *WSC1* and *MID2* (data not shown) rescued the growth of the *gpp1gpp2* mutant at elevated temperature. Interestingly, we also found that the ts phenotype of the *gpp1gpp2* strain

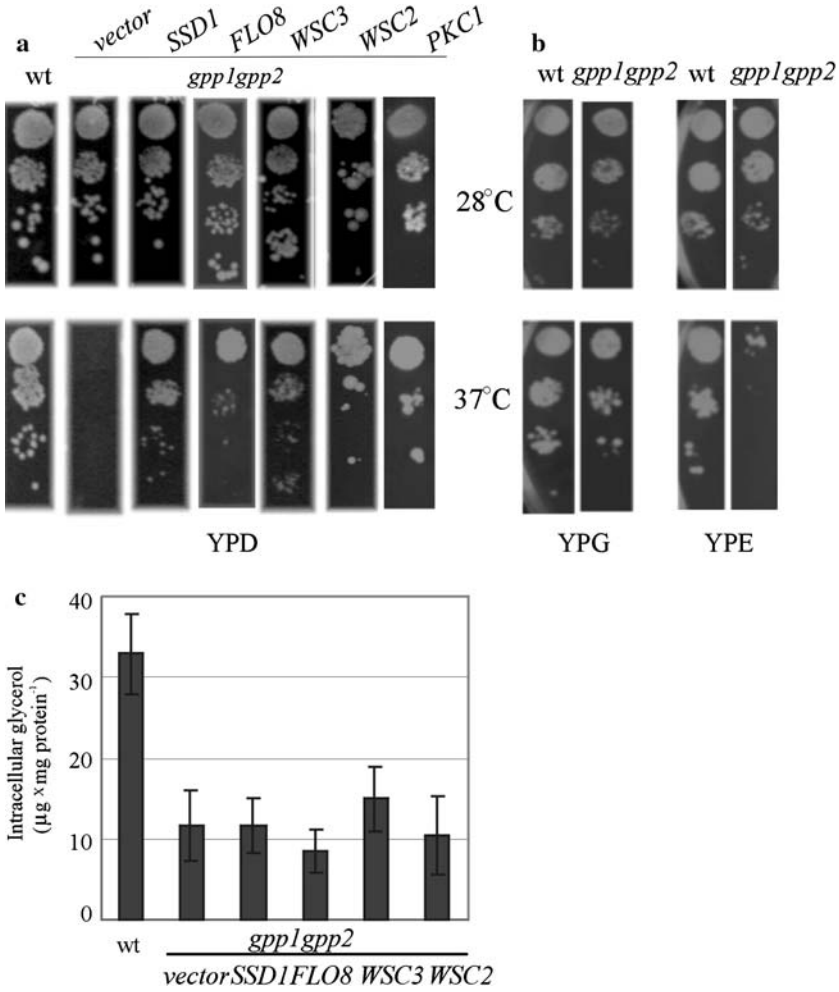

Fig. 1 **a** Thermo-sensitivity of the *gpp1gpp2* strain is suppressed by overexpression of *SSD1, FLO8, WSC3*, *WSC2* and *PKC1*. Serial dilutions of the wild type and the *gpp1gpp2* strains transformed with pFL44L vector alone, pFL*SSD1*, pFL*FLO8*, pFL*WSC2*, and YEp*WSC3*, YEp*PKC1* were spotted on YPD plates and cultured for 3 days at the indicated temperature. **b** Suppression of the *gpp1gpp2* ts phenotype on glycerol-based medium. Serial dilutions of the wild type and the *gpp1gpp2* were spotted on YPG (3% glycerol) and YPE (1% ethanol) plates and cultured at the indicated temperatures. **c** Overexpression of suppressor genes does not increase internal glycerol level of the *gpp1gpp2* strain. The wild type cells and the *gpp1gpp2* strain transformed with pFL44L vector alone, pFL*SSD1*, pFL*FLO8*, pFL*WSC2* and YEp*WSC3* grown overnight in YNB medium were switched to 37°C 2 h before harvesting. Intracellular glycerol level was measured as described in Sect. 'Materials and methods'. Means of three experiments are shown ±SD

could be suppressed by the growth on glycerol-based medium, whereas growth on ethanol-based medium did not rescue the cells, indicating that this may be a specific effect of the glycerol rather than the consequence of growth on a non-fermentable carbon source (Fig. 1b).

As was shown in Fig. 1c, the intracellular glycerol level of the *gpp1gpp2* strain was about three times lower than that of wild type (wt). However, overexpression of genes isolated in the suppression screen did not significantly increase the internal glycerol level in *gpp1gpp2* at 37°C.

PKC pathway is activated by elevated temperature in the *gpp1gpp2* double mutant strain

Since we found *WSC2*, *WSC3* and *PKC1*, components of the cell integrity pathway, as multicopy suppressors of the *gpp1gpp2* ts phenotype, we examined whether the temperature-induced phosphorylation of Slt2p MAP kinase is disturbed in the *gpp1gpp2* strain. As shown in Fig. 2, shifting the wt strain from 25 to 37°C resulted in the induction of Slt2p phosphorylation as described before (Kamada et al. 1995). Likewise, in the *gpp1gpp2* strain Slt2p was phosphorylated after the temperature was increased.

As expected (Martin et al. 2000; Wojda et al. 2003) overexpression of *WSC3* and *PKC1*, yielded a higher level of Slt2p phosphorylation, that still could be induced further exposing cells to elevated temperature (data not shown). Nevertheless, no differences concerning the heat-induced phosphorylation of Slt2p between the wt and the *gpp1gpp2* strain overexpressing *WSC3* or *PKC1* were detected (data not shown).

The results presented in Fig. 2 indicate that in spite of the isolation of upstream components of the PKC MAPK pathway, growth defect of the *gpp1gpp2* double deletion strain at 37°C was not due to the lack of Slt2p phosphorylation under these conditions.

The *gpp1gpp2* double deletion strain displays phenotypes of cell wall defects

We analysed phenotypes indicative of cell wall defects in the *gpp1gpp2* deletion strain. We examined the sensitivity of the *gpp1gpp2* to Zymolyase, a β-1,3-glucanase enzyme cocktail that reduces cellular integrity in yeast. As was shown in Fig. 3, the wt strain is relatively resistant to Zymolyase, whereas the *gpp1gpp2* strain showed a 50% decrease in viability already at 1 U ml^{-1} of Zymolyase. The decreased viability is in the same range as the sensitivity of the *hog1* deletion strain (Alonso-Monge et al. 2001). Overexpression of *SSD1* (Fig. 3) suppressed the Zymolyase sensitivity of the *gpp1gpp2* strain, whereas overexpression of the other genes analysed in this study was without effect (data not shown).

To gain further insight into the cell integrity defects of the *gpp1gpp2* strain, we examined the sensitivity to CFW, a compound that interferes with cell wall biosynthesis by binding to nascent chitin chains (Garcia-Rodrigues et al. 2000). Again, we found that growth of the *gpp1gpp2* was diminished in the presence of this compound in comparison to the wt cells (Fig. 4a). No detectable (morphological) differences were observed between the wt and the *gpp1gpp2* double deletion strains stained with CFW and microscopically visualised using UV filter (data not shown). Addition of CFW to liquid medium, triggered

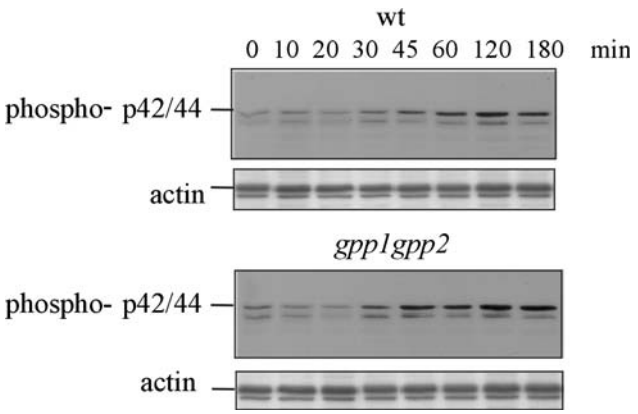

Fig. 2 Elevated temperature triggers PKC pathway signalling in the *gpp1gpp2* strain**.** The wild type and the *gpp1gpp2* strains were grown overnight at 25°C. The cultures were diluted to OD_{600} of 0.2 and grown to OD_{600} 0.6, and then they were transferred to 37°C for the times indicated. Total protein was isolated, separated by SDS-PAGE and blotted onto nitrocellulose membrane. Dual phosphorylation of Slt2p was detected as described in Sect. 'Material and methods'. Anti-actin blots stand for the loading control. Representative experiment is shown

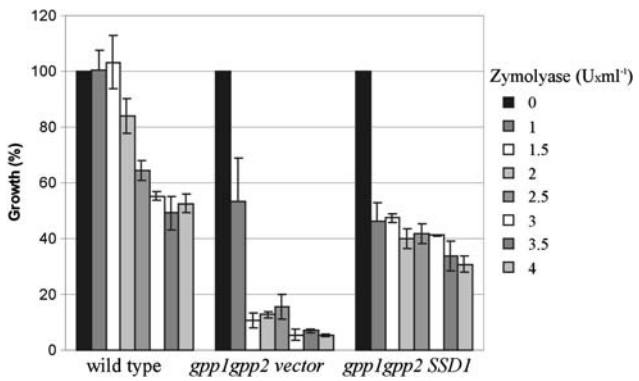

Fig. 3 The *gpp1gpp2* double mutant is hyper-sensitive to β-1,3-glucanase-containing enzyme cocktail Zymolyase, a phenotype that is suppressed by overexpression of *SSD1*. The wild type and the *gpp1gpp2* strain transformed with pFL44L plasmid alone or bearing *SSD1* grown overnight at 30°C in the presence of the indicated amounts of Zymolyase starting with OD_{600} of 0.025. Growth of a particular strain in the presence of Zymolyase is depicted as the percentages of its growth in YPD. Means of three experiments are shown ±SD

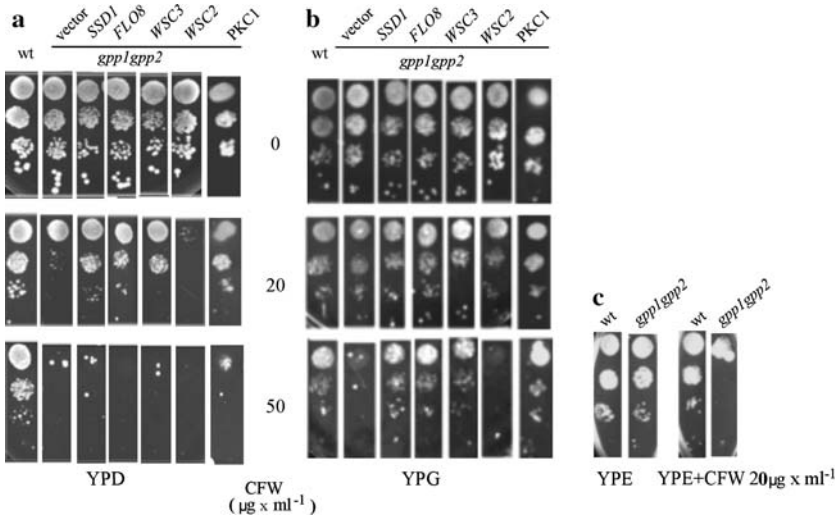

Fig. 4 The *gpp1gpp2* strain is hypersensitive to cell wall perturbing agent CFW and this phenotype is suppressed by overexpression of *SSD1*, *FLO8*, *WSC3* and *PKC1* (**a**) or by growth on glycerol-based medium (**b**). Serial dilutions of the wild type and the *gpp1gpp2* strain transformed with pFL44L plasmid alone or bearing *SSD1*, *FLO8*, *WSC2* and YEp*WSC3*, YEp*PKC1* were spotted on YPD (**a**) and YPG (**b**) plates containing the indicated amounts of CFW and grown at 28°C. In **c**, the wild type and the *gpp1gpp2* were spotted on YPE (1% ethanol) containing indicated amount of CFW

Slt2p phosphorylation in the wt and in the *gpp1gpp2* mutant cells (de Nobel et al. 2000; data not shown). The CFW sensitivity of the *gpp1gpp2* strain was partially rescued by the overexpression of *SSD1*, *FLO8*, *WSC3*, *PKC1*, but not *WSC2*, all genes found as multicopy suppressors of the ts phenotype of the *gpp1gpp2* mutant (Fig. 4a). Interestingly the *ssd1* mutant in BY4741 background also displayed sensitivity to CFW comparable to that of the *gpp1gpp2* deletion strain (data not shown).

We examined the growth of the *gpp1gpp2* strain in the presence of CFW in medium containing glycerol as the sole carbon source. We observed that the sensitivity of this strain to 20 μg ml^{-1} of CFW was suppressed to the level comparable to that of wt. Increasing the concentration of CFW to 50 μg ml^{-1} diminished the suppressive effect of glycerol (Fig. 4b). Additionally, we noticed that overexpression of *SSD1*, *FLO8*, *WSC3* and *PKC1* combined with growth on glycerol-based medium suppressed the sensitivity of the *gpp1gpp2* mutant strain to 50 μg ml^{-1} CFW to a much higher level than that observed on YPD (Fig. 4).

These observations indicate that supplying yeast with glycerol and overexpression of *SSD1*, *FLO8*, *WSC3* and *PKC1* in the *gpp1gpp2* mutant had an additive effect on the ability to grow in the presence of CFW (compare Fig. 4a, 4b). In contrast, hypersensitivity of the *gpp1gpp2* to CFW was not rescued on ethanol-based medium (Fig. 4c).

The *gpp1gpp2* ts phenotype and the *slt2* lytic phenotypes have common multicopy suppressors

Loss of function of the PKC MAPK pathway components results in a lytic phenotype due to a weakened cell wall (Alonso-Monge et al. 2001). This phenotype can be rescued by the addition of an osmotic stabiliser like sorbitol in the growth medium. Some genes suppressing the ts phenotype of the *gpp1gpp2* strain, like *SSD1* and *FLO8* also rescued the lytic phenotype of the *slt2* deletion strain at 28°C. At elevated temperature (37°C), a condition in which the lytic phenotype of the *slt2* is more pronounced, suppression by *SSD1* still was evident while suppression by *FLO8* was very weak (Fig. 5).

Intracellular glycerol level changes upon cell wall damaging conditions

In order to examine the relation between cell wall stress and glycerol production, we measured the intracellular glycerol level in the wt cells exposed to cell wall perturbing agents. Cells pre-grown in the same culture in YNB medium were split into three parts early in the exponential growth phase: the first part was exposed to 100 μg ml^{-1} of CFW, the second to 1.5 U $ml^{-1}$of Zymolyase, the third remained untreated. The concentrations of Zymolyase and CFW used in this experiment did not inhibit the growth of the wild type cells in liquid medium as judged from OD_{600} measurements and microscopical observation (data not shown). At the indicated time-points, the cells were harvested and the intracellular glycerol level was measured. As shown in Fig. 6, CFW treatment resulted in an increase in the intracellular glycerol level as was shown before for cells exposed to increased temperature (Siderius et al. 2000). This increase was detectable at 4 h time-point (1.3-fold) and reached 2.3-fold at 18 h in comparison with control cells growing in the same conditions. Zymolyase treatment

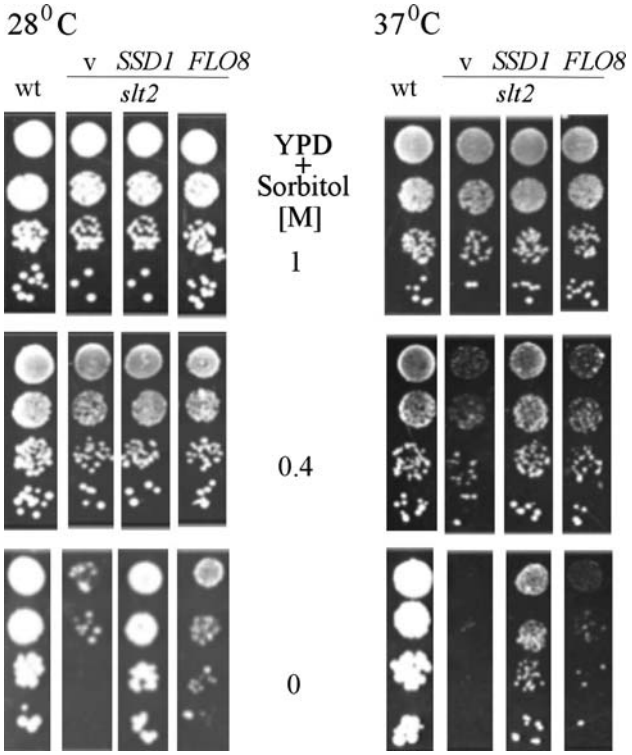

Fig. 5 The lytic phenotype of *slt2* strain is suppressed by overexpression of *SSD1* and *FLO8* both at 28 and 37°C. Serially diluted cell suspensions of the wt and the *slt2* deletion strains transformed with pFL44L vector alone or bearing *SSD1* and *FLO8* were spotted on YPD medium containing various amounts of sorbitol and grown at the indicated temperature

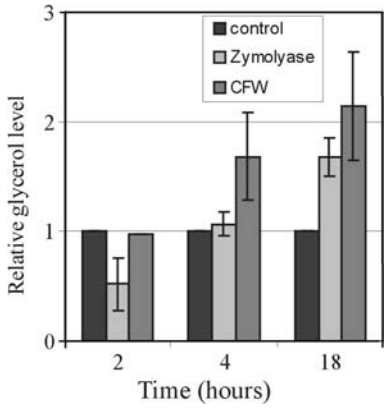

Fig. 6 The intracellular glycerol level changes when cells are exposed to cell wall damaging conditions. An overnight culture of wild type cells was diluted to an OD_{600} of 0.2 and grown to 0.3 at 28°C. Then it was divided into three parts. One was treated with 1.5 U ml^{-1} of Zymolyase, the second with 100 µg ml^{-1} of Calcofluor-white and the third one was untreated (control). At the indicated time-points, the cells were harvested and the intracellular glycerol concentration was measured as described in Sect. 'Materials and methods'. The results are shown as the relative glycerol level in comparison with untreated cells, ±SD was calculated from three experiments

initially resulted in a drop in the internal glycerol, at 2 h, followed by an increase, reaching 1.63-fold at 18 h timepoint.

Discussion

The *GPP1* and *GPP2* genes from *S. cerevisiae* encode isoforms of the glycerol-3-P phosphatase, an enzyme involved in the synthesis of glycerol (Pahlman et al. 2001). Deleting both *GPP* genes reduces the concentration of intracellular glycerol in cells. However, the *gpp1gpp2* double deletion strain is able to grow under optimal growth conditions, probably by utilising other biosynthetic pathways to ensure that sufficient glycerol is produced to survive (Pahlman et al. 2001). The *GPP*-deficient strains display osmo- and thermo- sensitive phenotypes (Siderius et al. 2000). We isolated *SSD1*, *FLO8*, *WSC3* and *WSC2* as multicopy suppressors of the *gpp1gpp2* ts phenotype. We further described that overexpression of *PKC1* also suppressed the ts phenotype displayed by the *gpp1gpp2* deletion strain. A phenotypic trait shared by the isolated genes is their involvement in cell wall maintenance/remodelling.

SSD1 encodes a protein implicated in the regulation of cell polarity and cell integrity (Jorgensen et al. 2002; Kaeberlein and Guarente 2002). Two allele classes designated *SSD1-v* (dominant) and *ssd1-d* (recessive) have been identified (Sutton et al. 1991). *SSD1-v* confers viability in the absence of Sit4 protein phosphatase and code for functional Ssd1 protein, while strain carrying *ssd1-d* alleles are inviable in the absence of Sit4p. Interestingly, Sit4p was shown to be involved in the downregulation of the PKC pathway (Angeles de la Torre-Ruiz et al. 2002). Also, *SSD1-v* supresses ts phenotype and hypersensitivity to CFW and SDS caused by mutation in *MPT5* (Kaeberlein and Guarente 2002). In strains lacking *SSD1-v*, deletion of *MPT5*, is synthetically lethal in combination with loss of function in either of the SBF or CCR4 transcriptional complexes (Kaeberlein and Guarante 2002), both of which function downstream of protein kinase C (Pkc1p) to promote cell-wall biosynthesis (Igual et al. 1996; Madden et al. 1997). This suggests that Mpt5p, Ssd1p and Pkc1p define three parallel pathways that function to ensure cell integrity (Kaeberlein and Guarante 2002). Additionally, Ssd1p physically interacts with Cbk1p, a protein kinase involved in the cell wall biosynthesis, which regulates the expression of *CTS1* encoding chitinase *via* Ace2p transcription factor (Bidlingmaier et al. 2001; Jorgensen et al. 2002). Interestingly, disruption of *ACE2* in *flo8* mutant results in the production of pseudohyphae (King and Butler 1998). *FLO8* encodes transcriptional activator involved in pseudohyphal differentiation of diploid- and invasive growth of haploid yeast cells, processes that require accurate cell wall remodelling (Kobayasi et al. 1996; Liu et al. 1996).

Wsc2p and Wsc3p are integral membrane proteins that together with Wsc1p (Slg1p) and Mid2p may register changes in external environment and trigger the cell integrity (PKC) pathway involved in cell wall maintenance (Verna et al. 1997).

We showed that in the *gpp1gpp2* strain, Slt2p, the MAP kinase in PKC pathway, is phosphorylated at elevated temperatures like in the wt strain. Therefore it seems unlikely that the ts phenotype as displayed by the *gpp1gpp2* deletion strain, is the consequence of a loss of Slt2p activation. The loss of Pkc1p functionality results in a more severe phenotype than loss of *BCK1* or other genes encoding MAP kinases downstream of Pkc1p, suggesting that Pkc1p may have additional target proteins (Lee and Levin 1992). For example it was shown that PKC pathway activity is needed for glucose-mediated derepression of *GUT1* gene encoding glycerol kinase, necessary to utilise glycerol as a carbon source. This effect was independent of the downstream MAP kinase module and shows the relation between glycerol metabolism and PKC pathway signalling (Gomes et al. 2005; Salgado et al. 2002). Nevertheless, none of our suppressors tested had a significant influence on the internal glycerol level in the *gpp1gpp2* mutant strain.

We investigated the possibility that the ts phenotype of the *gpp1gpp2* strain may, at least partially, be a consequence of decreased resistance to cell wall stress conditions. Indeed, the *gpp1gpp2* double deletion strain also displayed cell wall phenotypes such as increased sensitivity to Zymolyase and CFW. Among the suppressors of the ts phenotype of the *gpp1gpp2* mutant strain that were analysed, only *SSD1* was able to improve growth in the presence of Zymolyase. As it was mentioned before Ssd1p interacts with and modulates kinase activity of Cbk1p (Bidlingmaier et al. 2001; Jorgensen et al. 2002; Racki et al. 2000; Versele et al. 2001). This kinase plays a role in the RAM complex of proteins that regulate the transition from mitosis to the G1 phase of a following cell division cycle. Therefore it may be rewarding to investigate sensitivity to cell wall perturbing agents in specific phases of the cell cycle.

Sensitivity of the *gpp1gpp2* strain to CFW was rescued by overexpression of all the isolated suppressor genes except *WSC2*. Suppression of the CFW hypersensitivity by overexpression of *WSC3* (but not *WSC2*) may indicate different specific functions of Wsc proteins in the activation of downstream components. The specific functionality of the upstream components of the PKC pathway has been discussed earlier (Reinoso-Martin et al. 2003). The authors indicated that Wsc1p, but not Wsc2-4p and Mid2p, were required for sensing anti-fungal drug caspofungin.

Some suppressors of the *gpp1gpp2* ts phenotype: *SSD1* and *FLO8* also suppressed the lytic phenotype of the *slt2* deletion strain caused by the weakened cell wall. Also, the cell wall phenotype of HOG-pathway triple mutant *ste11ssk2ssk22*, resulting in the hyperosmosensitivity at elevated temperature was suppressed by *WSC3* (Alonso-Monge et al. 2001) and *SSD1* (I. Wojda, unpublished result). These observations that ts phenotype of the *gpp1gpp2* strain share common multicopy suppressors with other mutants in which cellular integrity is affected is another indication that inability to sythetise glycerol results in altered cell wall properties. Likely, suppression of the ts phenotype, as displayed by the *gpp1gpp2* strain, is brought about *via* different mechanisms, however, rearrangements of the cell wall may be a common theme.

We showed that yeast cells with a reduced intracellular level of glycerol were more sensitive to cell wall perturbing agents, which may explain their inability to grow at elevated temperature. Indeed, when the cells were grown on glycerol-based medium sensitivity to CFW was significantly reduced. Interestingly, overexpression of *SSD1*, *FLO8* and *WSC3* together with the growth on glycerol additively effected the resistance to CFW. This effect was not observed on ethanol-based medium, suggesting that the phenotype is glycerol-specific rather than the consequence of growth on a non-fermentable carbon source.

These observations underscore the possible role (direct or undirect) of glycerol in the maintenance of cell wall flexibility and resistance to cell wall perturbing conditions. On the other hand cell wall mutants display ts phenotypes that can be suppressed by growth in the presence of sorbitol (osmo-remedial growth; Turchini et al. 2000). The molecular basis for the osmo-remedial phenotype has not yet been reported. It is likely that the increase in internal glycerol may play a role in osmo-remedial growth by its effects on the cellular turgor and perhaps by affecting enzymatic activities involved in cellular integrity.

Previously, several groups have shown that mutant strains defective in producing glycerol in conditions of high osmolarity displayed sensitivity to cell wall perturbing agents. Notably, the HOG-pathway mutants (*pbs2*, *hog1*) that have reduced intracellular glycerol levels displayed sensitivity to the β-1,3-glucan degrading enzyme cocktail, Zymolyase (Alonso-Monge et al. 2001; Garcia-Rodrigues 2000). The hypersensitivity of HOG pathway mutants was suppressed by overexpression of genes involved in cell wall rearrangement such as *LRE1* and *HLR1* and by growth at 37°C, the temperature at which they increased their glycerol level (Alonso-Monge et al. 2001; Siderius et al. 2000; Wojda et al. 2003). Nevertheless the cell wall phenotypes of the *hog1* mutant is probably not only the consequence of reduced glycerol levels but also due to the absence of Hog1p-mediated signalling.

The cell wall phenotype of the *gpp1gpp2* strain prompted us to measure the intracellular glycerol level in yeast growing in the presence of cell wall perturbing agents. Indeed, we could show that exposure to CFW and

Zymolyase, increased the intracellular glycerol concentration, as was shown earlier for cells grown at elevated temperature (Siderius et al. 2000). We have investigated cell wall composition of the wild type and the *gpp1gpp2* double deletion strain; however, no significant differences in glucan, mannan, chitin and total protein content were detected (data not shown). The observed phenotypes could be a result of differences in particular cell wall protein(s) as was shown in cells exposed to low pH (Kapteyn et al. 2001). The particular role of glycerol in the maintenance of cellular integrity remains to be further investigated.

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