INTRODUCTION

The yeast cell wall is composed of three major components: glucan, mannanproteins and chitin (Kollar et al., 1997; reviewed by Klis et al., 2002). While a 1,3-β-glucan–chitin complex constitutes the inner rigid structure, mannanproteins form the outer layer of the cell wall, and 1,6-β-glucan interconnects all cell-wall components. The main group of cell-wall mannanproteins are covalently attached to 1,3-β-glucan, either via a remnant of a GPI-anchor (GPI-proteins) or directly through an unidentified alkali-sensitive linkage (PIR-proteins). A second group of cell-wall proteins can be released from intact cells using reducing agents and/or from isolated cell walls using SDS. The majority of the latter are considered as S–S-linked or non-covalently bound cell-wall proteins (Cappellaro et al. 1998; reviewed by Klis et al., 2002).

Cell-wall compensatory mechanisms are known to be responsible for remodelling the cell wall to prevent cell lysis whenever its integrity is compromised by environmental stresses, by cell-wall perturbing compounds or in cell-wall mutants (Popolo et al., 2001; Lagorce et al., 2003; Garcia et al., 2004; Hagen et al., 2004). The PKC1-MAPK pathway is essential for cell-wall integrity (Heinisch et al., 1999). It is activated during vegetative growth in G1- to S-phase transition, during mating, and in response to cell-wall damage. Persistent activation of the PKC1 pathway was found in cells with cell walls altered due to treatment with Calcofluor White or zymolyase and in several cell-wall mutants (De Nobel et al., 2000; Lagorce et al., 2003; Garcia et al., 2004). However, the cell-wall compensation seems to also be activated via other signal-transduction pathways, including protein kinase A, HOG- and Ca2+/calcineurin-dependent pathways (Lagorce et al., 2003; Garcia et al., 2004).

Scw10p, a cell-wall glucanase/transglucosidase important for cell-wall stability in 

Saccharomyces cerevisiae

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Glycosyl hydrolases and transferases are crucial for the formation of a rigid but at the same time plastic cell wall in yeasts and fungi. The Saccharomyces cerevisiae glucan hydrolase family 17 (GH17) contains the soluble cell-wall proteins Scw4p, Scw10p, Scw11p and Bgl2p. For Bgl2p, endoglucanase/glucanosyltransferase activity has been demonstrated, and Scw11p has been shown to be involved in cell separation. Here, Scw4p and Scw10p, which show 63% amino acid identity, were characterized. scw4 and scw10 single mutants were sensitive towards cell-wall destabilizing agents, suggesting a role in cell-wall assembly or maintenance. Simultaneous deletion of SCW4 and SCW10 showed a synergistic effect, and activated the cell-wall compensatory mechanism in a PKC1-dependent manner. Both the amount of cell-wall chitin and the amount of mannanproteins attached to chitin were increased in mutant scw4Δscw10. Deletion of CHS3 proved the critical role of chitin in scw4Δscw10. However, the mannoprotein Sed1p and the glucan synthase Fks2p were also crucial for cell-wall stability in mutant scw4Δscw10. The exchange of two conserved glutamate residues localized in the putative catalytic domain of GH17 family members strongly suggests that Scw10p acts as a 1,3-β-glucanase or as a 1,3-β-glucanosyltransferase. In addition, the synthetic interactions between Bgl2p and Scw10p which support a functional cooperation in cell-wall assembly were analysed. The data suggest that Scw4p and Scw10p act as glucanases or transglucosidasases in concert with other cell-wall proteins to assure cell-wall integrity.
2004). The known PKC1-activated transcription factors are SBF (Swi4p/Swi6p; Madden et al., 1997) and Rlm1p (Watanabe et al., 1995; Dodou & Treisman, 1997). The expression of several cell-wall related genes was shown to be regulated by the PKC1 pathway through these transcription factors (Igual et al., 1996; Jung & Levin, 1999).

The yeast cell wall is a dynamic structure which has to be remodelled during growth and other morphogenetic processes by a continuous process of synthesis and degradation of cell-wall material (Klis et al., 2002). 1,3-β-Glucan is the main component of the yeast cell wall, and mainly responsible for its rigidity. Endogenous 1,3-β-glucanases and transglucosidases play an important role in the modification of cell-wall glucan (Cid et al., 1995; Smits et al., 2001). Glucosyl hydrolases (GH) and related enzymes have been classified on the basis of amino-acid-sequence similarities within the CAZy (Carbohydrate-Active enZYmes) database (Henrissat & Bairoch, 1996). Several of these families have been implicated to be important for the architecture of the cell wall in yeast.

The GH7 family of 1,3-β-glucanases comprises Gas1p and its homologues Gas2p, Gas3p, Gas4p and Gas5p (reviewed by Popolo & Vai, 1999). Gas1p is a GPI–protein: it acts in vivo as an assembly enzyme that transfers segments of 1,3-β-glucan on branching points of other glucans. The lack of Gas1p cross-linking activity causes aberrant cell morphology and activates the PKC1 pathway (reviewed by Popolo et al., 2001). Compared to wild-type cells, gas1 mutants show a series of phenotypes such as increased levels of cell-wall chitin, more mannoproteins linked to chitin, and resistance towards zymolase, all of which indicate that cell-wall compensatory mechanisms are crucial for survival of this mutant.

The exoglucanases Exg1p, Exg2p and Ssg1p/Spr1p are members of the GH5 family (reviewed by Cid et al., 1995). Exg1p has a possible function in cell-wall expansion during vegetative growth. The sporulation-specific exoglucanase Ssg1p/Spr1p is probably involved in the process of spore formation and in the morphogenesis of ascospore walls.

Crh1p, Crh2p/Utr2p and Crr1p show homology with bacterial β-glucanases and eukaryotic endotransglycosidases, and belong to the GH16 family. Crh1p and Crh2p are localized at the cell surface in chitin-rich areas (Rodriguez-Peña et al., 2000). They play a putative role in cell-wall assembly at the sites of polarized cell growth, whereas Crr1p seems to be involved in sporulation. Deletion of CRH1 in combination with CRH2 shows additive effects in hypersensitivity to Congo Red and an increased amount of alkali-soluble glucan.

The endoglucanases Eng1p and Acf2p/Eng2p belong to the GH81 family. Eng1p, an extracellular endo-1,3-β-glucanase, is cell-cycle regulated and localizes asymmetrically to the daughter site of the septum (Baladron et al., 2002). This indicates that Eng1p might be involved in the dissolution of the septum together with chitinase. In Schizosaccharomyces pombe, Eng1p is required for the dissolution of the primary septum during cell separation (Martin-Cuadrado et al., 2003).

Biotinylation of yeast cell walls has identified the soluble cell-wall proteins Scw3p/Scw10p and Scw15p and Scw1lp (Cappello et al., 1998). Scw3p shows sequence homology to β-glucosidase of Candida wickerhamii (Skory & Freer, 1995). It is a member of the Saccharomyces cerevisiae SUN gene family, and plays a role in the final steps of cell-wall septation (Mouasse et al., 2000).

Scw4p, Scw10p and Scw11p, together with Bgl2p, belong to the GH17 family. Scw11p has been characterized as a daughter-specific G1-regulated protein that is important for cell separation (Colman-Lerner et al., 2001). Bgl2p is one of the most abundant cell-wall proteins (Mraša et al., 1993). It was characterized as an endo-1,3-β-glucanase and glucanosyltransferase that introduces β-1,6-linkages in 1,3-β-glucan chains (Mraša et al., 1993; Goldman et al., 1995). Deletion of BGL2 does not obviously affect cell morphology, whereas overexpression is harmful for cell viability (Mraša et al., 1993). Scw4p and Scw10p share 63% amino acid identity and an apparent molecular mass of 66 kDa (Cappello et al., 1998). Both proteins contain an ER-translocation sequence and a Kex2p processing site. Expression of the SCW10 gene is cell-cycle regulated, with a maximum of expression in the G1-phase (Spellman et al., 1998). Transcription analyses suggest an important role for Scw10p in cell-wall assembly and/or stability, since SCW10 expression is highly induced in several cell-wall mutants (Lagorce et al., 2003; Hagen et al., 2004). A homologue of Scw10p in Candida albicans CaMP65 was characterized as a major immunodominant antigen. This 65 kDa mannoprotein is particularly observed in extracellular fractions of hyphae (La Valle et al., 2000).

Here, we analyse the role of Scw10p in cell-wall stability in combination with other members of the GH17 family. Mutagenesis of two conserved glutamate residues indicated that Scw10p functions as a glucosyl hydrolase/transglycosidase. Analyses of scw mutants showed that the PKC1 pathway is activated and cell-wall structure altered in the absence of SCW4 and SCW10. Our data suggest that Scw10p acts in concert with Scw4p and Bgl2p to assure cell-wall integrity.

**METHODS**

**Strains, media and growth conditions.** The *S. cerevisiae* strains used are listed in Table 1. Yeast stains were grown in YPD or in synthetic minimal medium with auxotrophic supplements and 2% glucose (SD) (Kaiser et al., 1994). Deletion mutants were selected on either SD or YPD medium containing 200 µg geneticin ml⁻¹ (G418; Gibco).

**Deletion constructs.** Standard procedures were used for all DNA manipulations (Sambrook et al., 1989). All cloning and transformations were made in Escherichia coli strain DH5α. Oligonucleotide
Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEY6210</td>
<td>MATα ura3-52 leu2-3,12 his3-A100 trp1-A901 lys2-801 suc2-A9</td>
<td>Robinson et al., 1988</td>
</tr>
<tr>
<td>scw4</td>
<td>SEY6210 except scw4::LEU2</td>
<td>Cappellaro et al., 1998</td>
</tr>
<tr>
<td>scw10</td>
<td>SEY6210 except scw10::HIS3</td>
<td>Cappellaro et al., 1998</td>
</tr>
<tr>
<td>bg2</td>
<td>SEY6210 except bg2::URA3</td>
<td>Cappellaro et al., 1998</td>
</tr>
<tr>
<td>scw4scw10</td>
<td>SEY6210 except scw4::LEU2, scw10::HIS3</td>
<td>Cappellaro et al., 1998</td>
</tr>
<tr>
<td>SES101</td>
<td>SEY6210 except sed1::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>SES102</td>
<td>SEY6210 except sed2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>SES103</td>
<td>SEY6210 except chs3::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>SES104</td>
<td>SEY6210 except chs2::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>SES105</td>
<td>SEY6210 except fs32::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>SES106</td>
<td>SEY6210 except scw4::LEU2, scw10::HIS3, fs32::kanMX4</td>
<td>This study</td>
</tr>
</tbody>
</table>

Phenotypic tests. Yeast cells were grown on YPD or SD medium. Portions of 10^5 cells were harvested, washed and resuspended in 1 ml sterile water. A series of fivefold dilutions was prepared in sterile water and 3 μl of each dilution was spotted on YPD plates containing Calcofluor White (CFW), Congo Red (CR) or caffeine at the required concentration.

Zymolyase sensitivity. Yeast cells were grown on YPD to exponential phase. Cells equivalent to 1 OD₆₀₀ unit were harvested, washed and resuspended in 50 mM Tris/HCl, pH 7-5, to OD₆₀₀ 0-7-0-8. Zymolyase 100T was added to a final concentration of 10 μg ml⁻¹, and OD₆₀₀ was monitored at room temperature.

Cell wall preparation. Yeast cells were grown on YPD to late exponential phase. Cells equivalent to 1 OD₆₀₀ units were harvested, washed with 25 ml ice-cold water, 1 ml 50 mM Tris/HCl, pH 7-5, and resuspended in 200 μl of the same buffer. An equal volume of glass beads was added and cells were lysed by vortexing four times (with 1 min intervals on ice) for 1 min. After puncturing the bottom of the tube, the cell lysate was collected. Cell walls were collected by centrifugation for 5 min at 3000 r.p.m. at 4°C. Cell walls were washed five times with 1 ml ice-cold water and freeze-dried overnight.

Cell wall analysis

Enzymic hydrolysis. Freeze-dried cell walls equivalent to 50 OD₆₀₀ units (~4 mg) of cells were resuspended either in 0-5 ml of 50 mM Tris/HCl, pH 7-5, containing 20 U of recombinant 1,3-β-glucanase (Quantzyme; Qiogene) or in 0-5 ml 50 mM potassium acetate, pH 5-5, containing 1 U of recombinant 1,6-β-glucanase (Glyko) and digested at 37°C for 20 h. Sodium azide (0-02%) was used as a preservative. The insoluble material was pelleted (20000 g for 10 min) and the supernatant stored at 4°C. Pellets were washed two times with 0-5 ml water. Subsequently, the 1,3-β-glucanase-resistant material was digested with 1,6-β-glucanase. The 1,6-β-glucanase-resistant material, on the other hand, was digested with 1,3-β-glucanase using the same conditions as above. Supernatants were collected after centrifugation. Total carbohydrate content of the soluble material was measured using the phenol-sulphuric acid method (Dubois et al., 1956). The soluble material was freeze-dried and hydrolysed in TFA (see TFA-hydrolysis). Glucose and mannose contents were quantified by HPAEC PAD (see below).

Trifluoroacetic acid (TFA) hydrolysis. Hydrolysis of total cell walls was carried out in 0-5 ml 4 M TFA in sealed tubes at 100°C for 4 h. Tubes were cooled on ice and centrifuged (20000 g for 1 min). TFA from 200 μl of the supernatants was evaporated under nitrogen. Sequences are available upon request. Yeast transformations were carried out according to the method of Gietz et al. (1995). Deletions of SCW4, SCW10 and BGL2 were performed as described by Cappellaro et al. (1998). For deletion of CHS3, the plasmid P4160 (chs3::KanMX4; EUROSCARF) was used. For disruption of SED1, the sed1::KanMX4 cassette was amplified from genomic DNA of strain Y04012 (EUROSCARF) using oligonucleotides SED1A and SED1B. For disruption of FKS2, fs32::KanMX4 was amplified from genomic DNA of strain Y06979 (EUROSCARF) using oligonucleotides FKS2A and FKS2B. Purified PCR products were transformed directly into yeast. Correct integrations were tested by PCR analysis. Plasmid construction

Plasmid pSE10GAL (GAL-SCW10::kanMX4). A BamHI site was introduced immediately upstream of the SCW10 start codon, and an endorokinase cleavage site followed by an XbaI site were introduced immediately upstream of the SCW10 stop codon by PCR using oligonucleotides BAM-F, XBA and genomic DNA of strain SEY6210 as a template. The PCR product was subcloned into vector pYES2/CT (Invitrogen) digested with BamHI and XbaI. In the resulting construct, SCW10::kanMX4 is under the control of the inducible GALI promoter.

Plasmid pSE10 (SCW10::kanMX4). A 1292 bp HindIII–Pndl fragment of pSE10GAL was subcloned into pRS416HA (Lommel et al., 2004) digested with HindIII and SmaI, resulting in pRS416SCW. An 880 bp fragment of the 5'-untranslated region of SCW10 was amplified using oligonucleotides HIND and BAM-R and yeast genomic DNA as a template. The PCR product was subcloned into vector pYES2/CT (Invitrogen) digested with BamHI and XbaI. In the resulting construct, SCW10::kanMX4 is under the control of the inducible GALI promoter.

Plasmids pSE10-1 (SCW10E326Q) and pSE10-2 (SCW10E380Q). Glu-326 and Glu-380 respectively, were exchanged for glutamine by PCR. The primer pairs E326-F/E326-R and E380-F/E380-R, respectively, were used. pSE10 served as template. DNA sequence analysis confirmed the basepair exchanges.

Plasmids pRS424SCW and pRS425SCW (SCW10::kanMX4). A 3550 bp PvuII–PnuI fragment from plasmid pSE10 was subcloned into pRS424 and pRS425 (Christianson et al., 1992), respectively.

Plasmid NEV-BGL (BGL2::kanMX4). A 960 bp DNA fragment was amplified on genomic DNA using oligonucleotides BGL-E and BGL-HAE. An EcoRI site was thereby introduced immediately upstream of the BGL2 start codon, as well as one copy of the haemagglutinin (HA) epitope followed by an EcoRI site. The PCR product was subcloned into NEV-E cut with EcoRI (gift of Dr J. Stolz).
The dry residue was washed with 0.5 ml methanol two times and resuspended in 200 μl Milli-Q water. Carbohydrates were separated and quantified by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC PAD) using a Dionex Bio-LC system.

**Glucan analysis.** To remove mannoproteins, cell walls were treated with 1 mg trypsin (Boehringer Mannheim) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C for 2 h. After washing three times with 1 ml water, trypsinated cell walls were extracted in 0.5 ml 1 M NaOH at 75 °C for 1 h. Alkali-soluble glucans were estimated from soluble NaOH-extracted material by precipitating carbohydrates with two volumes of ethanol at −20 °C. After NaOH extraction, the insoluble material was washed to neutrality and digested for 15 h with 1 mg of Zymolyase 100T (Seikagaku) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C. Alkali-insoluble glucans were estimated from zymolyase supernatants precipitated by ethanol. The total carbohydrate content of Zymolyase 100T (Seikagaku) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 40 °C for 1 h. Alkali-soluble fractions were spotted onto nitrocellulose, and 1,6-glucan values were expressed in μg per mg dry cell wall.

**Measurement of chitin.** Cell walls were digested for 15 h with 1 mg of Zymolyase 100T (Seikagaku) in 0.5 ml 50 mM Tris/HCl, pH 7.5, at 37 °C. The insoluble material was pelleted, washed with 1 ml water, resuspended in 0.5 ml 4M HCl and hydrolysed at 100 °C for 10 h. After cooling on ice and neutralization with saturated Na2CO3, the N-acetylglucosamine (GlcNAc) content was measured according to the spectrophotometric method of Davidson (1966). Total chitin was measured in the HCl-hydrolysates of the mock-treated cell walls. GlcNAc values were expressed in μg per mg dry cell wall.

**Western blot analysis.** Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. The monoclonal antibody anti-HA (16B12; Babco) was used at 1:8000 dilution, anti-c-myc (9E10; Babco) at 1:5000 dilution, and His-probe (H-3; Santa Cruz Biotechnology) at 1:2000 dilution. The polyclonal antibody anti-Bgl2p (Mrsˇa et al., 1993) was used at 1:2000, and anti-1,6-β-glucan (Lussier et al., 1998) at 1:2000 dilution. Protein–antibody complexes were visualized by enhanced chemiluminescence using the Amersham ECL system.

**RESULTS**

**Cell-wall architecture is altered in mutant scw4scw10**

To analyse the function of Scw4p and Scw10p in cell-wall assembly, scw4 and scw10 mutants were analysed. Growth rates and general cell morphology of both mutants were similar to wild-type (data not shown; Cappellaro et al., 1998). However, both mutants showed sensitivity to CFW and CR (Fig. 1a), and cell-wall chitin levels were slightly increased compared to wild-type (data not shown). These phenotypes were more pronounced in a scw4scw10 double mutant. When compared to wild-type yeast, scw4scw10 mutant cells displayed slower growth rates, morphological abnormalities and hypersensitivity towards CFW, CR and caffeine at concentrations which do not affect single mutants (Fig. 1a, b; Cappellaro et al., 1998).

To study the cell-wall defects in more detail, we analysed the cell-wall composition of mutant scw4scw10. The chemical cell-wall analysis showed that levels of chitin (Table 2) and alkali-soluble glucan (Table 3) were significantly increased in mutant scw4scw10. Moreover, scw4scw10 mutant cells
showed higher resistance to zymolyase (Fig. 2a) compared to wild-type. Increased chitin levels and changes in zymolyase sensitivity indicated that the cell-wall composition was changed, probably due to cell-wall compensatory mechanisms. Similar observations have been made for gas1Δ and fks1Δ mutants (Popolo et al., 2001). To analyze whether the PKC1 pathway is activated in mutant scw4scw10, a PKC1 pathway-dependent β-galactosidase reporter Rlm1–lacZ (Kirchrath et al., 2000) was used.

Under normal growth conditions, β-galactosidase activity was at least three times higher in mutant scw4scw10 relative to wild-type (Table 4). Further activation of the PKC1 pathway by 5 mM caffeine was possible (Table 4). These data suggested that the PKC1 pathway is important for cell-wall stability in the absence of Scw4p and Scw10p.

Since chitin synthase 3 (CHS3) is a major target gene of the PKC1–pathway, CHS3 was disrupted in mutant scw4scw10. A 90% reduction of cell-wall chitin in mutant strain scw4scw10chs3 compared to wild-type yeast was observed (Table 2). Mutant scw4scw10chs3 cells grew in big clumps, showed irregular cell shapes and were hypersensitive towards caffeine and SDS (data not shown),

### Table 2. Chitin levels in Saccharomyces cerevisiae scwΔ mutants

Chitin content is expressed as μg N-acetylglucosamine per mg cell-wall dry weight (μg GlcNAc mg⁻¹). Values are mean ± SD of at least three independent determinations. WT, Wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chitin content (μg mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEY6210 (WT)</td>
<td>14.6 ± 0.8</td>
</tr>
<tr>
<td>scw4scw10</td>
<td>27.3 ± 2.9</td>
</tr>
<tr>
<td>scw4scw10chs3</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>scw4scw10bgl2</td>
<td>19.8 ± 2.1</td>
</tr>
<tr>
<td>scw4scw10ed1</td>
<td>38.0 ± 2.7</td>
</tr>
<tr>
<td>scw4scw10ks2</td>
<td>40.3 ± 3.5</td>
</tr>
</tbody>
</table>

### Table 3. Recovery of alkali-soluble and insoluble glucan from deproteinized cell walls of S. cerevisiae mutants

Values shown represent total hexoses (in μg per mg cell-wall dry weight) determined in ethanol precipitates of alkali extracts of trypsinized cell walls (1 M NaOH at 75 °C for 1 h) and in zymolyase hydrolysates of alkali-resistant material (1 mg ml⁻¹ Zymolyase 100T). Values are mean ± SD of at least three independent determinations. WT, Wild-type.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucan recovery (μg mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble</td>
</tr>
<tr>
<td>SEY6210 (WT)</td>
<td>225 ± 10</td>
</tr>
<tr>
<td>scw4scw10</td>
<td>270 ± 14</td>
</tr>
<tr>
<td>bgl2</td>
<td>295 ± 14</td>
</tr>
<tr>
<td>scw4scw10bgl2</td>
<td>310 ± 17</td>
</tr>
</tbody>
</table>

### Table 4. Indirect assessment of Mpk1p activity

Specific β-galactosidase activities were determined from cell lysates prepared from five independent transformants each. The activity is expressed in β-galactosidase units (U), where 1 U = 1000 × A₄₂₀/OD₆₀₀ × t, where t is in min. WT, Wild-type; −Caffeine, caffeine absent; + Caffeine, caffeine present.

<table>
<thead>
<tr>
<th>Strain</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Caffeine</td>
</tr>
<tr>
<td>SEY6210 (WT)</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>scw4scw10</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>scw4scw10bgl2</td>
<td>29 ± 4</td>
</tr>
</tbody>
</table>
pointing to an important role for CHS3 in the cell-wall integrity of mutant scw4scw10. However, deletion of CHS3 in mutant scw4scw10 did not change the zymolyase-resistant phenotype of the parental strain (Fig. 2a). Thus, zymolyase resistance is not caused by elevated cell-wall chitin levels in the mutant scw4scw10.

To further analyse cell-wall composition, the amounts of mannose, glucose and N-acetylglucosamine (GlcNAc) were determined in cell walls of wild-type and mutant scw4scw10 after total wall hydrolysis with TFA. Despite an approximately twofold increase in GlcNAc, due to increased chitin levels (Table 2), no significant change in mannose and glucose content could be detected. The mannose/glucose/GlcNAc ratio in the wild-type was 52/47/1 and in mutant scw4scw10 54/44/2:3. However, the amount of alkali-soluble glucan was increased in mutant scw4scw10 (Table 3), suggesting that cross-linking between cell-wall components, rather than total levels of glucan and mannan, might be affected in mutant scw4scw10.

The glucan structure of mutant scw4scw10 was further analysed. Cell walls were sequentially digested with recombinant 1,3-β-glucanase (Quantazyme) and 1,6-β-glucanase, and vice versa. The total hexoses released were determined in all hydrolytic fractions and, in addition, glucose and mannose were determined by HPAEC on Dionex in TFA hydrolysates of freeze-dried enzymic fractions (described in Methods). First, after digestion with 1,6-β-glucanase, the residual insoluble material was treated with 1,3-β-glucanase (Table 5). The 1,6-β-glucanase should release all mannoproteins attached to either 1,3-β-glucan or to chitin via 1,6-β-glucan. Subsequently, 1,3-β-glucanase degrades 1,3-β-glucan. Second, cell walls were first treated with 1,3-β-glucanase followed by 1,6-β-glucanase digestion (Table 5). In this case, the 1,3-β-glucanase fraction contains degraded 1,3-β-glucan and mannoproteins attached to 1,3-β-glucan. Subsequently, with 1,6-β-glucanase only mannoproteins attached to chitin should be released. Similar amounts of cell-wall sugars were released with 1,6-β-glucanase from wild-type and mutant scw4scw10, which indicates that the same amount of mannoproteins was attached to the cell wall via 1,6-β-glucan. In contrast, the amount of total hexoses released by 1,3-β-glucanase from cell walls of mutant scw4scw10 was reduced by approximately 25% compared to wild-type (Table 5). Subsequent hydrolysis of the 1,3-β-glucanase-resistant material with 1,6-β-glucanase released more than twice the amount of hexoses (Table 5). The increase of both glucose and mannose indicated an alteration in the cross-linking of mannoproteins to the cell wall via 1,6-β-glucan. Our data suggest that in mutant scw4scw10 mannoproteins are preferentially attached to chitin rather than to 1,3-β-glucan. This is further substantiated by the observation that the amount of 1,6-β-glucan (detected with 1,6-β-glucan antibodies) was reduced in the alkali-soluble fraction of mutant scw4scw10 (data not shown).

In summary, these results suggest that defects in the 1,3-β-glucan structure of the scw4scw10 double mutant might be the reason for an altered cross-linking of cell-wall polymers, specifically GPI-mannoproteins and chitin. Similar results have been reported for the gas1Δ and the fks1Δ mutants (Kapteyn et al., 1997).

The fact that in mutant scw4scw10 more mannoproteins were linked to chitin but zymolyase resistance was not affected when CHS3 was deleted suggested that further factors are involved in the compensation of cell-wall defects in mutant scw4scw10. To study this, we focused on two proteins which play an important role in cell-wall stability during stress and in stationary phase, but also when the amount of GPI-anchored cell-wall mannoproteins is decreased: Sed1p, a mannoprotein identified to be responsible for zymolyase resistance in stationary phase; and Fks2p, the alternative subunit of glucan synthase, which is upregulated in stationary phase and stress conditions (Shimoi et al., 1998; Mazur et al., 1995; Jung & Levin, 1999; De Nobel et al., 2000; Hagen et al., 2004). The single disruption of SED1 or FKS2 genes had no effect on growth or CFW and zymolyase sensitivity of exponential-phase cells (data not shown). However, the same deletions in

### Table 5. Sequential enzymic hydrolysis of cell walls isolated from the wild-type and from scw4scw10

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucanase</th>
<th>1,6-β-</th>
<th>1,3-β-</th>
<th>1,3-β-</th>
<th>1,6-β-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Step 1</td>
<td>Step 2</td>
<td>Step 1</td>
<td>Step 2</td>
</tr>
<tr>
<td>WT</td>
<td>Total hexoses</td>
<td>620 ± 60</td>
<td>350 ± 30</td>
<td>830 ± 60</td>
<td>170 ± 35</td>
</tr>
<tr>
<td></td>
<td>Glucose, mannose</td>
<td>28-7, 71-3</td>
<td>94-7, 5-3</td>
<td>57-9, 42-0</td>
<td>50-8, 49-1</td>
</tr>
<tr>
<td>scw4scw10</td>
<td>Total hexoses</td>
<td>630 ± 45</td>
<td>325 ± 20</td>
<td>610 ± 50</td>
<td>370 ± 40</td>
</tr>
<tr>
<td></td>
<td>Glucose, mannose</td>
<td>25-6, 74-4</td>
<td>92-5, 7-5</td>
<td>54-4, 45-5</td>
<td>47-7, 52-3</td>
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</table>
mutant scw4scw10 decreased growth rates and enhanced cell-morphology aberrations (data not shown). In addition, in mutants scw4scw10sed1 and scw4scw10fks2, sensitivity towards CFW (Fig. 3) and chitin levels (Table 2) were increased, whereas cells were less resistant to zymolyase (Fig. 2b). These results indicated that, in addition to Chs3p, Sed1p and Fks2p are also crucial for cell-wall integrity in the absence of Scw4p and Scw10p.

Taken together, our results suggest that Scw4p and Scw10p play a crucial role in the assembly of a stable cell wall, and that the concerted action of several compensatory effects is necessary to mitigate cell-wall defects due to the loss of Scw4p and Scw10p function.

Glu-326 and Glu-380 are essential for Scw10p activity

Our cell-wall analyses suggested that the glucan/chitin/ mannan meshwork is altered in the absence of SCW4 and/or SCW10. Scw4p and Scw10p belong to the GH17 family of endo-1,3-β-glucanases and 1,3-β-glucan tranglucosidases (CAZy database; http://afmb.cnrs-mrs.fr/CAZY/), suggesting that Scw10p and/or Scw4p might act as glucan hydrolases or tranoglucosidases. To support this assumption, we focused our further analyses on Scw10p since it is strongly upregulated in several cell-wall mutants, such as gas1Δ, kresΔ and mnn9Δ. To purify Scw10p, a HIS-tag was fused to the C-terminus of SCW10 and the tagged protein (Scw10pHis) was expressed from a 2 µ-plasmid under the control of the GAL1 promoter. The tagged protein was functional in vivo, since it fully complemented the growth defect of mutant scw4scw10 in the presence of cell-wall-perturbing agents (data not shown). Scw10pHis was purified to homogeneity and tested for hydrolytic as well as transglycosylase activity using laminarin, pustulan, chitin, yeast glucan, p-nitrophenylglucoside and isolated intact cell walls as substrates. However, no enzymic activity could be detected (data not shown).

To obtain evidence for Scw10p being a hydrolase/transglucosidase, a mutational analysis was performed. The three-dimensional structures of two plant enzymes of the GH17 family, endo-1,3-β-glucanase and lichenase of barley, have been resolved, and two glutamate residues identified as being essential for enzyme activity (Varghese et al., 1994). Both glutamate residues are conserved in all members of the GH17 family (Fig. 4a). To analyse whether these residues are also essential for Scw10p function, Glu-326 and Glu-380 were replaced with glutamine. In contrast to wild-type Scw10p, neither of the mutant proteins Scw10pE326Q and Scw10pE380Q complemented the sensitivity of scw4scw10 to cell-wall-perturbing compounds (Fig. 4b). Protein amounts were similar to wild-type Scw10p (data not shown). These data strongly support the assumption that Scw10p acts as a glucan hydrolase or transglycosidase on yeast cell-wall glucan.

Interconnection of Bgl2p with Scw4p and Scw10p

We wished to analyse whether or not there is an interconnection between Scw4p/Scw10p and Bgl2p, a further member of the GH17 family. Deletion of BGL2 does not result in a dramatic change of the growth phenotype, although Bgl2p is one of the most abundant cell-wall proteins (Mrša et al., 1993). In a similar manner to the scw4 and scw10 single mutants, the bgl2 mutant showed increased sensitivity towards CFW and CR (Fig. 1a). In addition, the amount of alkali-soluble glucan was increased in mutant bgl2 (Table 3). However, in contrast to the scw4scw10 mutant, bgl2 cells showed increased zymolyase sensitivity (data not shown). To analyse the genetic interconnection between BGL2 and SCW4/SCW10, the BGL2 gene was deleted in mutant scw4scw10. The triple scw4scw10bgl2 mutant showed a minor increase in sensitivity towards CFW compared to mutant scw4scw10 (Fig. 1b). However, all other phenotypes, such as growth rate (data not shown) and sensitivity towards caffeine and zymolyase (Figs 1b and 2a), were much less pronounced compared to those of scw4scw10 and resembled wild-type features. Also, total cell-wall chitin content was similar to that of wild-type (Table 2). On the other hand, activation of the PKC1 pathway was less pronounced in scw4scw10bgl2 compared to scw4scw10 (Table 4). These data suggested that Scw4p/Scw10p and Bgl2p might have antagonistic functions.
Therefore, the mutual influence of Scw10p and Bgl2p was further analysed. When SCW10 was overexpressed in a bgl2 mutant, a dramatic increase in sensitivity towards CFW was observed, whereas overexpression in mutant scw4 had no effect (Fig. 5a). When Scw10p protein levels were checked in wild-type and bgl2 cells by Western blot analysis, the amount of Scw10p was reduced in mutant bgl2 (Fig. 5b) and Scw10p levels were found to be restored by introducing BGL2 on a plasmid. In addition, moderately enhanced expression of Bgl2p increased Scw10p levels in wild-type cells (Fig. 5c). Summarizing, our data indicate that levels of Bgl2p and Scw10p have to be balanced for the formation of an intact cell wall.

**DISCUSSION**

Cell-wall analyses and the phenotypes of scw4 and scw10 deletion mutants have shown that Scw10p and Scw4p are crucial for yeast cell-wall stability. Scw10p in particular seems to play a specific role in cell-wall integrity, as concluded from the mRNA expression pattern (Spellman et al., 1998) and genome-wide transcriptome analysis (Lagorce et al., 2003; Hagen et al., 2004).

Scw10p is a member of GH17, a family of mainly plant 1,3-β-glucanases (EC 3.2.1.39) and (1,3-1,4)-β-glucanases (EC 3.2.1.73). Catalytic amino-acid residues of 1,3-β-glucanase isoenzyme GII and the (1,3-1,4)-β-glucanase isoenzyme EII from barley have been investigated (Chen et al., 1993), and amino acids Glu-231 and Glu-232 have been identified as the likely catalytic nucleophiles in GII and EII, respectively. Amino acid Glu-288 represents the putative catalytic acid at the active site of both enzymes. The three-dimensional structures of both enzymes show potential catalytic glutamate residues located in a deep substrate-binding cleft at a distance which enables their function in the catalytic reaction (Varghese et al., 1994). Finally, site-directed mutagenesis of 1,3-β-glucanase demonstrates a 20 000-fold and 350-fold decrease in activity, respectively, when Glu-231 or Glu-288 is changed for glutamine (Chen et al., 1995). In all yeast members of the GH17 family, the catalytic glutamate residues of both barley enzymes are conserved (Fig. 4a). In the case of Scw10p, these are Glu-326 and Glu-380 at the C-terminus of the protein. The exchange of either of them for glutamine caused the loss of Scw10p function. Thus, Scw10p most likely acts as a glucanase. The reason that our biochemical analyses did not reveal any glucanase or

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**Fig. 4.** Glu-326 and Glu-380 are crucial for Scw10p activity. (a) Amino acid alignment of Bgl2p, Scw4p, Scw10p and barley 1,3-β-glucanase. Identical amino acids are shown in black, conserved amino acids in grey. Arrows indicate catalytic glutamate residues of barley 1,3-β-glucanase. (b) Sensitivity towards CFW, CR and caffeine of mutant scw4scw10 transformed with plasmid pRS416 (scw4scw10) or the same plasmid harbouring either SCW10 (pSE10) or mutated SCW10E326Q (pSE10-1) and SCW10E380Q (pSE10-2) (two independent transformants).
glucosyltransferase activity could be due to very specific substrate requirements.

Synergistic effects of the double deletion of \textit{SCW4} and \textit{SCW10} concerning the sensitivity to cell-wall-perturbing agents indicated a similar function for both proteins. On the one hand, this was supported by the finding that the amount of Scw10p was increased in mutant \textit{scw4} (data not shown). On the other hand, however, we observed a clear functional difference of both proteins. The phenotypes of the single deletion mutants were slightly different, and overproduction of Scw10p did not complement the sensitivity of mutant \textit{scw4} towards CFW. Moreover, \textit{SCW4} and \textit{SCW10} are differently regulated. The expression of \textit{SCW4} does not change during the cell cycle, whereas \textit{SCW10} is cell-cycle regulated, reaching a maximum of expression during G1-phase (Spellman \textit{et al}., 1998). It seems that Scw10p has a similar, but still distinct, function compared to Scw4p. Neither protein can fully complement the other, but the presence of one of them is sufficient for normal cell growth. Similar observations have been made with Crh1p and Crh2p (Rodriguez-Peña \textit{et al}., 2000), which also show significant homology. Deletion of the corresponding genes causes additive sensitivity towards CFW and CR. Both genes show a different pattern of transcriptional regulation: \textit{CRH1} is cell-cycle regulated, whereas \textit{CRH2} expression does not vary during the cell cycle. Consistent with the observed expression pattern, Crh1p-GFP is found to localize at the incipient bud site and in later stages around the septum area, whereas Crh2p is found mainly at a bud neck throughout the whole cell cycle.

Mutants defective in the synthesis of cell-wall components show altered cell-wall composition and architecture. This is due to mechanisms that compensate for the loss in strength of the cell wall (Popolo \textit{et al}., 2001; Smits \textit{et al}., 2001). An increased level of chitin is one of the typical compensatory mechanisms of cell-wall mutants (Popolo \textit{et al}., 1997; Garcia-Rodriguez \textit{et al}., 2000). Chitin is glycosidically linked to non-reducing branches of both 1,3-\textit{b}-glucan and 1,6-\textit{b}-glucan (Kollar \textit{et al}., 1995, 1997). It is essential to the insolubility of the wall material, and chitin incorporation results in transfer of the wall material from the alkali-soluble to the alkali-insoluble fraction (Hartland \textit{et al}., 1994). Activation of the PKC1 pathway and increased cell-wall chitin indicate a compensatory mechanism in mutant \textit{scw4scw10}. The chitin participating in linkages to both 1,3-\textit{b}-glucan and 1,6-\textit{b}-glucan is synthesized by Chs3p (Kollar \textit{et al}., 1995). Chs3p has been shown to be responsible for chitin accumulation in \textit{gas1A} and \textit{fks1A} mutants (Valdivieso \textit{et al}., 2000; Garcia-Rodriguez \textit{et al}., 2000). Deletion of \textit{CHS3} in these mutants causes severe growth defects. Similarly, in mutant \textit{scw4scw10}, deletion of \textit{CHS3} stressed its role for cell-wall integrity. Interestingly, in spite of the fact that there is more chitin in the cell wall of mutant \textit{scw4scw10}, the alkali-soluble fraction increased as well. Thus it seems that cross-linking of glucan and chitin is impaired in this mutant. These results, and the absence of significant

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5.png}
\caption{(a) Overexpression of \textit{SCW10} negatively affects cell-wall stability of mutant \textit{bg/2}. Mutants \textit{scw4} and \textit{bg/2} transformed with pRS424SCW and pRS425SCW, respectively, were grown on YPD containing CFW, as indicated. (b) and (c): Western blot analysis of cell-wall extracts probed with anti-HIS (b) and anti-MYC (c) antibody. The reduced expression level of \textit{SCW10} in mutant \textit{bg/2} (b) can be restored to the level of the wild-type (wt) by simultaneous expression of \textit{BGL2}. Mutant \textit{bg/2} and the wild-type strain expressing \textit{SCW10}\textsuperscript{HIS} (pRS424SCW) and \textit{BGL2} (NEV-BGL) are indicated. Overexpression of \textit{BGL2}\textsuperscript{HA} increases the expression level of \textit{SCW10} (c). The wild-type strain with \textit{SCW10}\textsuperscript{MYC} integrated at the genomic \textit{SCW10} locus transformed with \textit{BGL2}\textsuperscript{HA} is shown.}
\end{figure}
differences between wild-type and mutant scw4scw10 in the amounts of glucose and mannose, indicate that cross-linking of cell-wall polymers, rather than overall composition, is changed in the mutant. Alternative cross-linking of cell-wall mannanproteins to chitin has also been shown for 1,3-β-glucan mutants gas1Δ and fks1Δ (Kapteyn et al., 1997).

Another feature in common between the scw4scw10 and fks1Δ and gas1Δ mutants is resistance to zymolyase (Popolo et al., 2001). The resistance has been attributed to changes in the mannanprotein layer (Vink et al., 2002), with a specific function for some of the cell-wall proteins (Van Der Vaart et al., 1995). In mutant scw4scw10, zymolyase resistance is not lost after deletion of CHS3. Increased chitin levels and altered cell-wall structure in mutants scw4scw10sed1 and scw4scw10fks2 suggest an important role for both Sed1p and Fks2p in the cell-wall compensation of the scw4scw10 mutant. The reduced compensation is also evident from reduced zymolyase resistance. This indicates that an additional survival mechanism can be activated for yeast to cope with specific cell-wall stresses. In the case of multiple GPI-mutants, the overexpression of specific cell-wall-related genes has been shown to be responsible for specific compensation (Hagen et al., 2004).

Deletion mutant bgl2 presented as a mutant without any specific phenotype. On the other hand, the overexpression of BGL2 seems to be lethal (Mrsá et al., 1993). We could demonstrate in this study that cells carrying the BGL2 deletion behave as cells with a slightly impaired glucan structure. However, this defect does not affect normal cell growth and does not activate compensatory mechanisms. An unexpected phenotype was found when BGL2 was deleted in mutant scw4scw10. Most of the growth and phenotype parameters were much less pronounced compared to scw4scw10 and resembled wild-type features, including reduced activation of the PKC1 pathway. The deletion of BGL2 in mutant scw4scw10 does not result in a wild-type phenotype. The sensitivity of scw4scw10bgl2 towards CFW, as well as an increased alkali-soluble fraction, indicates a weakened cell wall. On the other hand, deletion of BGL2 stabilizes the cell-wall structure of the scw4scw10 mutant and reduces compensatory mechanisms. The effect of the BGL2 deletion in scw4scw10 was shown to be specific. The deletion of any of the other cell-wall-related genes tested, namely SCW11, CRH1, CRH2 and EXG1, in mutant scw4scw10 could not restore the phenotype in a similar manner to the scw4scw10bgl2 mutant (data not shown).

Thus, we conclude that the Scw4/10 proteins and Bgl2p might have antagonistic functions, and that levels of Bgl2p and Scw10p need to be in balance for stable cell-wall formation.

In summary, the resemblance of the scw4scw10 mutant to the gas1Δ and fks1Δ mutants indicates that Scw4p and Scw10p play an important role in the correct assembly of glucan structure and the cross-linking of glucan with other cell-wall polymers. The evidence that the glutamate residues essential for 1,3-β-glucanase activity in the GH17 family are essential for the function of Scw10p strongly supports this indication.

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