The protein kinase Kic1 affects 1,6-β-glucan levels in the cell wall of *Saccharomyces cerevisiae*

Edwin Vink,¶† Jack H. Vossen,¶‡ Arthur F. J. Ram,§ Herman van den Ende, Stephan Brekelmans, Hans de Nobel∥ and Frans M. Klis

Author for correspondence: Frans M. Klis. Tel: +31 20 525 7834. Fax: +31 20 525 7056.
e-mail: klis@science.uva.nl

*KIC1* encodes a PAK kinase that is involved in morphogenesis and cell integrity. Both over- and underexpressing conditions of *KIC1* affected cell wall composition. Kic1-deficient cells were hypersensitive to the cell wall perturbing agent calcofluor white and had less 1,6-β-glucan. When Kic1-deficient cells were crossed with various kre mutants, which also have less 1,6-β-glucan in their wall, the double mutants displayed synthetic growth defects. However, when crossed with the 1,3-β-glucan-deficient strain *fks1Δ*, no synthetic growth defect was observed, supporting a specific role for *KIC1* in regulating 1,6-β-glucan levels. Kic1-deficient cells also became highly resistant to the cell wall-degrading enzyme mixture Zymolyase, and exhibited higher transcript levels of the cell wall protein-encoding genes *CWP2* and *SED1*. Conversely, overexpression of *KIC1* resulted in increased sensitivity to Zymolyase and in a higher level of 1,6-β-glucan. Multicopy suppressor analysis of a Kic1-deficient strain identified *RHO3*. Consistent with this, expression levels of *RHO3* correlated with 1,6-β-glucan levels in the cell wall. Interestingly, expression levels of *KIC1* and the MAP kinase kinase *PBS2* had opposite effects on Zymolyase sensitivity of the cells and on cell wall 1,6-β-glucan levels in the wall. It is proposed that Kic1 affects cell wall construction in multiple ways and in particular in regulating 1,6-β-glucan levels in the wall.

**Keywords:** *KIC1/CWH30, PBS2, RHO3, cell wall synthesis*

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**INTRODUCTION**

*Saccharomyces cerevisiae* is protected from extracellular challenges by its cell wall. These challenges can vary from hypo-osmotic stress to mechanical damage and toxic compounds from other organisms. The yeast cell wall consists of 1,3-β-glucan, 1,6-β-glucan, chitin and mannoproteins, which are interconnected in an ordered manner (Klis *et al.*, 2002). Cell wall construction and composition are highly dynamic: the composition and structure of the newly formed cell wall are continuously adjusted in response to extracellular conditions, and even to progress in the cell cycle. This indicates that cell wall construction is highly regulated.

The cell wall perturbing agent calcofluor white has been a valuable tool in identifying mutants with a defective cell wall (Roncero *et al.*, 1988; Ram *et al.*, 1994; Lussier *et al.*, 1997; De Groot *et al.*, 2001). Analysis of mutants hypersensitive to calcofluor white has resulted in the identification of numerous genes involved in different aspects of cell wall biogenesis (Ram *et al.*, 1995; Jiang *et al.*, 1995, 1996; Vossen *et al.*, 1995; Van Berkel *et al.*, 1999). In addition, screening for mutants resistant to this...
compound has led to the identification of several genes involved in chitin biosynthesis (Roncero et al., 1988).

The KIC1 gene encodes an essential protein kinase, which is involved in cell integrity and morphogenesis. This kinase was identified in a two-hybrid screen with the yeast centrin CDC31. The in vitro kinase activity of Kic1 was found to be dependent on CDC31. However, KIC1 did not share the CDC31 functions in spindle pole body (SPB) duplication, but rather revealed a novel function for CDC31 (Sullivan et al., 1998). This was further supported by a mutational analysis of CDC31, which resulted in disruption of the SPB-related functions and KIC1-related functions (Ivanovska & Rose, 2001).

The PKC mitogen-activated protein kinase (MAPK) pathway is commonly known as the cell (wall) integrity pathway, although this is certainly not the only MAPK pathway that has an effect on cell wall biosynthesis and composition (Klis et al., 2002). For instance, activation of the pheromone response pathway results in the formation of a mating projection and considerable alteration in the cell wall. In this case, at least part of this may be coordinated through the PKC MAPK pathway that has an effect on cell wall biosynthesis and assembly. Evidence is also further supported by a mutational analysis of CDC31, which resulted in disruption of the SPB-related functions and KIC1-related functions (Ivanovska & Rose, 2001).

METHODS

Strains and media. The yeast strains used in this study are listed in Table 1. The strains were grown in YPD [1% (w/v) yeast extract, 1% (w/v) Bacto Peptone, 3% (w/v) glucose], YPGal [1% (w/v) yeast extract, 1% (w/v) Bacto Peptone, 3% (w/v) galactose] or in SD [0-17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate, 2% (w/v) glucose, 0-5% (w/v) ammonium sulfate, buffered at pH 6-0 with 1% (w/v) MES] supported with the necessary amino acids, at 28°C or 37°C. For solid media, 2% (w/v) Bacto Agar was added. The K1 killer assays were performed in either YPD or SD media, which for this purpose were buffered at pH 4-7 using 3% (w/v) sodium citrate and supplemented with 0-03% methylene blue. Yeast genetics, sporulation and transformation followed established procedures (Sherman & Hicks, 1991). Escherichia coli strain DH5α was used for propagation of all plasmids and was grown in LB medium [1% (w/v) Bacto Tryptone, 1% (w/v) NaCl, 0-5% (w/v) yeast extract]. Yeast extract, Bacto Peptone, Bacto Tryptone, yeast nitrogen base and Bacto Agar were all from Difco Laboratories.

Strain construction. Strains JV67 and JV68 were constructed by transforming strains HAB251-15B and AR835 with KIC1 disruption constructs, using the HIS3 and TRP1 markers, respectively. The disruption constructs were created according to Berben et al. (1991). Primers used for this purpose are listed in Table 2. Correct integration of the disruption constructs was confirmed by Southern analysis, using the 4 kb HindIII fragment from plasmid p14 as a probe (see below). JV80 and JV83 were haploid offsprings of JV68 and JV67, respectively. JV142 and JV143 were haploid offsprings of JV67 transformed with the Pgal1::KIC1 plasmid (see below). JV144 and JV145 were offsprings of JV68 transformed with the Pgal1::KIC1 plasmid. These four haploid strains propagated the Pgal1::KIC1 plasmid even without selective pressure.

JV202 was the progeny of the JV143×HAB813 diploid. JV215 was a haploid offspring of the JV143×TR95 diploid. JV220 was a haploid descendant from the JV143×HAB637-1A diploid. The JV168 strain was a haploid descendant from the JV145×AR100 diploid. JV264 was constructed in the FY833 background, using a PBS2 disruption construct with a HIS3 marker. This construct was created as described by Berben et al. (1991), and correct integration was confirmed by PCR. Primers are all listed in Table 2. JV268 resulted from the JV144×JV264 diploid.

Strains EV116 and EV077 were constructed in the FY834 background, using PCR-generated disruption constructs with the HIS3 marker (Berben et al., 1991). Primers are listed in Table 2. Correct integration was confirmed by PCR.

Plasmids, oligonucleotides and recombinant DNA techniques. For the cloning of the KIC1/CWH30 gene, a YCp50-based genomic library was used (Rose et al. 1987). The plasmid that could complement the cebh30-1 mutant was named p14. The 4 kb HindIII fragment containing the complete KIC1 ORF, was subcoloned into YEplac195 (Gietz & Sugino, 1988) and named p61. Plasmid p62 was isolated from a YEp13-based genomic library based on its ability to complement the cebh30-1 mutant.

DNA handling and manipulation were carried out according to Sambrook et al. (1989). DNA sequencing was performed as described by Sanger et al. (1977), using T7 DNA polymerase (Pharmacia). Restriction enzymes, nucleotides, Klenow fragment and alkaline phosphatase were all from Pharmacia. DNA ligase was purchased from Gibco-BRL, SuperTaq polymerase was from HT Biotechnology, Expand high fidelity

Here we describe the identification and characterization of CWH30, which is allelic to the previously described KIC1 gene. A Kic1-deficient strain is not only hyper-sensitive to calcfluor white, but is also resistant to Zymolyase, a cell wall degrading enzyme mixture, indicating that its cell wall is affected. We show that mutation of KIC1 results in K1 killer toxin resistance and decreased levels of 1,6-β-glucan. Furthermore, KIC1 expression levels were found to correlate with 1,6-β-glucan levels in the cell wall. Multicopy suppressor analysis of a Kic1-deficient strain identified RHO3 which itself was found to strongly affect 1,6-β-glucan levels. We propose that KIC1 is involved in regulating the 1,6-β-glucan levels in the cell.

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polymerase was from Boehringer Mannheim and oligonucleotides were from Eurogentec.

Cloning of \textit{KIC1} behind the \textit{GAL1} promoter. The Expand high fidelity polymerase was used to create a \textit{KIC1} fragment with a 5' XhoI restriction site, followed by three bases of the 5' untranslated region (UTR) of \textit{KIC1} and then the \textit{KIC1} ORF. The first 500 bases of the 3' UTR were included in this fragment, which was followed by an XhoI restriction site. The primers used for this are listed in Table 2. This PCR generated fragment was digested with XbaI and XhoI, and subsequently cloned into the corresponding sites of the pYEura3 plasmid (Clontech), resulting in the \textit{P}_{GAL1}; \textit{KIC1} plasmid.

\textbf{Multicopy suppressor screen}. A high-copy pMA3a-based genomic library [kindly provided by M. Crouzet (University of Bordeaux II, France) & M. F. Tuite (University of Kent, UK)] with a mean insert size of 5–10 kb was transformed to strain JV141, which contains \textit{kic1::TRP1} and carries the \textit{P}_{GAL1}; \textit{KIC1} plasmid. A total of 3500 transformants were replica-plated on selective SD medium containing 100 µg calcifluor white ml\(^{-1}\). Viable colonies were isolated, library plasmids were recovered and retransformed into the JV141 strain. Serial dilutions of transformants of these strains were spotted on selective SD medium containing 50 µg calcifluor white ml\(^{-1}\). The seven plasmids that showed the best suppression were selected for further analysis, and were found to contain five unique inserts (Table 3). Clone 2 contained amongst others the RH03 gene, which proved to be the gene responsible for the suppression. A 2.2 kb SalI–XhoI fragment containing the RH03 ORF was removed from clone 2 and cloned into a YEplac181 vector (Gietz & Sugino, 1998) resulting in the pEV021 plasmid. The remaining part of clone 2 was religated, and the resulting plasmid lost the ability to suppress the \textit{kic1} mutant, whereas plasmid pEV021 retained the ability to suppress the \textit{kic1} mutant. Clones 11 and 13 contained an identical insert, and clone 11 was subjected to further analysis. A 2.4 kb XhoI–SalI fragment (the \textit{SalI} restriction site was located in the pMA3a plasmid, 0.3 kb from

### Table 1. Yeast strains used in this study

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<th>Strain</th>
<th>Genotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td>SEY6210</td>
<td>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9</td>
<td>S. D. Emr (University of California, San Diego, CA, USA)</td>
</tr>
<tr>
<td>HAB251-15B</td>
<td>MATα/α SEY6210 autodiploid</td>
<td>Roemer &amp; Bussey (1991)</td>
</tr>
<tr>
<td>FY833</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63</td>
<td>Winston et al. (1995)</td>
</tr>
<tr>
<td>FY834</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63</td>
<td>Winston et al. (1995)</td>
</tr>
<tr>
<td>AR835</td>
<td>MATα/α FY833 × FY834</td>
<td>A. F. J. Ram</td>
</tr>
<tr>
<td>T158C/S14a</td>
<td>MATα ura3-52 cwb30-1</td>
<td>Bussey et al. (1979)</td>
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<td>JV67</td>
<td>HAB251-15B kic1α::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>JV68</td>
<td>AR835 kic1α::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>JV80</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1α::HIS3</td>
<td>This study</td>
</tr>
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<td>JV83</td>
<td>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1α::HIS3</td>
<td>This study</td>
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<td>JV141</td>
<td>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1α::TRP1 + \textit{P}_{GAL1}; \textit{KIC1}</td>
<td>This study</td>
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<tr>
<td>JV143</td>
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<td>JV144</td>
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<td>JV145</td>
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<td>AR100</td>
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<td>HAB637-1A</td>
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<td>This study</td>
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<td>HAB813</td>
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<td>TR95</td>
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<td>JV168</td>
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<td>This study</td>
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<td>JV202</td>
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<td>This study</td>
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<td>JV215</td>
<td>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1α::HIS3</td>
<td>This study</td>
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<tr>
<td>JV220</td>
<td>MATα leu2-3,112 ura3-52 his3Δ200 lys2-801 trp1-Δ901 suc2Δ9 kic1α::HIS3</td>
<td>This study</td>
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<td>JV264</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1α::TRP1 + \textit{P}_{GAL1}; \textit{KIC1}</td>
<td>This study</td>
</tr>
<tr>
<td>JV268</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 kic1α::TRP1 + \textit{P}_{GAL1}; \textit{KIC1}</td>
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<tr>
<td>EV116</td>
<td>MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63 rbo3::HIS3</td>
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<td>EV077</td>
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Table 2. Oligonucleotide primers used in this study

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<th>Name</th>
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<td>KIC1 forward disruption</td>
<td>CAGTAATGACGACGAAGCCACAAATAGTAAGCAGGGTTTAGCCGAAGGAGAATTCCCGGGGATCCG</td>
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<tr>
<td>KIC1 reverse disruption</td>
<td>CAGAACCTGGGCTTCTGGCAAGAAACGAAGCTAGCTTGGCTGCAG</td>
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<tr>
<td>P&lt;sub&gt;GAL&lt;/sub&gt;:KIC1 forward</td>
<td>ACTCTAGATATGACGACGAAGCCAC</td>
</tr>
<tr>
<td>P&lt;sub&gt;GAL&lt;/sub&gt;:KIC1 reverse</td>
<td>TTCTCGAGTCTAGCGCTGTGTTATAAG</td>
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<tr>
<td>PBS2 forward disruption</td>
<td>AAGATGGAAGACAAAGTGGCTAACCTAGCTTGGCTAAGAGAATTTCCCGGGGATCCG</td>
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<tr>
<td>PBS2 reverse disruption</td>
<td>ACCTAAGACACACATATGTAATGCGCTACATTGGATAGAATTCCCGGGGATCCG</td>
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<tr>
<td>PBS2 forward control</td>
<td>CAGATCGAGACGTTAATTTCTAAAA</td>
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<tr>
<td>PBS2 reverse control</td>
<td>TCAGTGCGCTTGGCTTTT</td>
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<td>RHO3 forward disruption</td>
<td>AACATGTCATTTCATATGACGCTACCGGCGGTCCGCAATAGTGCAAAGTCAGGATCCG</td>
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<td>RHO3 reverse disruption</td>
<td>ATATACATTACGTTAATATGAGCAGGCTACCGGCTTCGGGATGGCTCAG</td>
</tr>
<tr>
<td>RHO4 forward disruption</td>
<td>TTACATGAGTCTACTATTAATTGACGAAAAAGGGCTACGGGCAATGAGGCGGATCCG</td>
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<tr>
<td>RHO4 reverse disruption</td>
<td>TTCTACATTATATACTACCGTTCGCGGCTTCGGGACCGGCTGAGTGGCTCAG</td>
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Table 3. High copy suppressors of KIC1

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<th>Clone no.</th>
<th>Chromosome</th>
<th>Coordinates</th>
<th>Complete ORFs*</th>
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<td>2</td>
<td>9</td>
<td>135225–144766</td>
<td>YIL120w, RPII, RHO3, YIL117c, HIS5, NUP159</td>
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<tr>
<td>11/13†</td>
<td>14</td>
<td>523677–531475</td>
<td>VAC7, MSG5</td>
</tr>
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<td>23/44†</td>
<td>4</td>
<td>791592–798528</td>
<td>STB3</td>
</tr>
<tr>
<td>24</td>
<td>11</td>
<td>487450–497450†</td>
<td>GNC3, YKR027w, SAP190</td>
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<tr>
<td>43</td>
<td>2</td>
<td>243957–253522</td>
<td>YBR004c, YBR005w, UGA2, YBR007c</td>
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</tbody>
</table>

* Bold type is used in cases where the ORF responsible for the suppression has been determined by subcloning.  
† Two clones with identical inserts were identified.  
‡ This clone was only sequenced from the left flank. The right flank was estimated based on mean insert size and restriction analysis.

the BamHI site which was used for the insertion of the genomic fragments) was cloned into YEpLac181 resulting in plasmid pEV017, which contained the MSG5 ORF and could suppress the kic1 mutant. Clone 23 and 44 also showed an identical insert, in which the STB3 gene was the only complete ORF. In addition, 2.3 kb of the 3' end of the SEC7 coding sequence was present in this insert. This 2.3 kb fragment was removed by an XhoI/Sall digest of clone 44, using the XhoI site in the insert and the Sall site in the pMA3a vector, 0.3 kb from the insert. The resulting plasmid (pEV020) retained the ability to suppress the kic1 mutant.

**Phenotypic screens.** Calcofluor white sensitivity was analysed as described previously (Ram et al. 1998). Precultures were concentrated or diluted to OD<sub>600</sub> 10. Subsequently, ten-fold dilution series were made of which 4 µl of each dilution was spotted onto YPD or SD plates containing 0, 10 and 50 µg calcofluor white ml<sup>-1</sup>. Plates were incubated for 3 days at 28 °C.

K1 killer toxin sensitivity was measured using the halo assay (Brown et al. 1994), with some modifications. Precultures were concentrated or diluted to OD<sub>600</sub> 10 and 45 µl cells were seeded in 13 ml killer agar medium. On the surface, 5 µl of a dilution series of 10<sup>-5</sup>, 5 × 10<sup>-5</sup>, 10<sup>-5</sup>, 5 × 10<sup>-6</sup> and 10<sup>-6</sup> of isolated killer toxin was spotted. Killer toxin was isolated according to Brown et al. (1994). In short, the medium of the K1 killer toxin-producing strain was concentrated 1000-fold by ultrafiltration using a 10 kDa Amicon filter and was used as such in the halo assay. In all experiments, samples and controls were treated with toxin from the same isolation. Plates were incubated for 4–6 days at 20 °C. The diameters of the haloes were measured for each toxin dilution. Relative apparent sensitivities were calculated according to Reneke et al. (1988). In short, the diameter of the growth inhibition zone is proportional to the logarithm of the K1 killer toxin dose applied in the centre of the zone. Plotting these parameters against each other allows estimation of the dose required to produce an inhibition zone of a given diameter. The ratio of this dose estimated for wild-type cells divided by the dose found for mutant cells is termed the ‘relative apparent sensitivity’.

Zymolyase sensitivities were measured essentially as described by De Nobel et al. (1990). Yeast strains were grown to equivalent optical densities, and 1 OD<sub>590</sub> unit was taken for analysis. Cells were washed once and resuspended in 900 µl 10 mM Tris·HCl pH 7.5. The OD<sub>590</sub> was followed for 1 h after the addition of 100 µl Zymolyase 20T (10 µg ml<sup>-1</sup> in 10 mM Tris·HCl pH 7.5; Zymolyase 20T was from Kirin Brewery).

**Isolation of cell walls.** Cell walls from cells grown to early exponential phase were isolated according to Van Rinsum et al. (1991). Walls were extracted twice in 50 mM Tris·HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 2% (w/v) SDS, 0.3% (v/v) β-mercaptoethanol for 5 min at 100 °C. Walls were extensively washed in distilled water and subsequently freeze-dried.

**Determination of 1,6-β-glucan levels.** The levels of 1,6-β-glucan in the alkali-insoluble cell wall fraction were basically determined in accordance with Brown et al. (1994). Essentially, cells were grown for 24 h at 28 °C to stationary phase, harvested and washed twice in distilled water. Samples were split up into four aliquots and three fractions were each three times extracted in 3% (w/v) NaOH at 75 °C for 1 h. The remaining fraction was freeze-dried and used for the determination of the cell dry weight. The alkali-insoluble material was washed twice in 100 mM Tris·HCl, pH 7.5 and then washed in distilled water. The pellet was resuspended in 10 mM Tris·HCl, pH 7.5, 10% (v/v) glycerol, 1 mg Zymolyase 100T ml<sup>-1</sup> (Kirin Brewery) and incubated at 37 °C overnight. Following incubation, samples were centrifuged at 14000 g for 5 min, and the supernatant was dialysed against distilled water using a Spectra/POR 3 (6000–8000 molecular mass cut-off) dialysis membrane. The glucose content of the residue – 1,6-β-glucan and chitin – was determined by the phenol/sulfuric acid method (Dubois et al., 1956). In later experiments, the procedure was as follows. Cells were grown for 24 h in YPD to early stationary phase, washed twice in 30 mM Tris·HCl, 1 mM EDTA, pH 7.4 and collected in five aliquots. Two were freeze-dried to quantify the cell dry weight, whereas the three other samples were resuspended in 600 µl 50 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4 and broken with glass beads. Walls were collected and extracted twice in 50 mM Tris·HCl, 150 mM NaCl, 5 mM EDTA, 2% SDS, 40 mM β-mercaptoethanol, pH 7.4, at 100 °C for 5 min. Walls were extensively washed in distilled water and suspended in 10 mM Tris·HCl, pH 7.5, with 1 mg Zymolyase 100T ml<sup>-1</sup> (Kirin Brewery) and incubated at 37 °C for 16 h. Following incubation, solutions were centrifuged for 5 min at 15000 g and the supernatant was dialysed against distilled water using a Spectra/POR 3 (3500 molecular mass cut-off) dialysis membrane. The residue was hydrolysed in 2 M trifluoroacetic acid at 100 °C for 4 h, freeze-dried, and subsequently glucose levels were determined using the D-glucose oxidase assay.
Northern analysis. RNA was isolated from early exponential phase cells with hot acidic phenol (Ausubel et al., 1998). Fifteen microgrammes of RNA was loaded on a 1% agarose gel containing 24% formaldehyde. Following electrophoresis, the RNA was blotted onto Hybond-N+ (Amersham) through capillary paper transfer and UV cross-linked to the membrane.

RESULTS

CWH30 is allelic to KIC1

The cwh30-1 mutant was isolated in a general screen for cell wall mutants (Ram et al., 1994), based on their hypersensitivity to the cell wall perturbing agent calcofluor white. The gene mutated in cwh30-1 was identified by functional complementation of the hypersensitivity to calcofluor white, using a YCp50-based genomic library. Out of 40000 transformants, only 15 were able to grow in the presence of 50 µg calcofluor white ml−1. The plasmids from these transformants were recovered and upon retransformation only five were able to complement the calcofluor white hypersensitivity of the cwh30-1 mutant. All five complementing plasmids contained the same genomic insert (data not shown). The complementing activity of this insert resided in a 44 kb fragment that remained after HindIII truncation of the fragment, but was lost after further truncation to 42 kb with XbaI. The 44 kb fragment contained the YHR102w ORF, which previously has been named KIC1 for kinase interacting with Cdc31 (Sullivan et al., 1998). The XbaI truncation removed part of the 5′ upstream region of the KIC1 gene, which seemed essential for complementation of the mutant. Additional evidence that CWH30 was allelic to KIC1 came from the resulting diploid of the cwh30-1 mutant and the kic1 disruption strain JV143. The JV143 strain has been disrupted for kic1 but is supported by a plasmid-borne Pgal1::KIC1 fusion. When the diploid was cultured on glucose-containing medium, the calcofluor white hypersensitivity caused by the recessive cwh30-1 mutation was not complemented (not shown), indicating that CWH30 is allelic to KIC1.

Disruption of KIC1

To generate a KIC1 knock-out mutant, the KIC1 gene was replaced by the TRPI marker in the diploid AR835 wild-type strain, and by the HIS3 marker in the diploid HAB251-15B wild-type strain. For both heterozygous diploids (JV68 and JV67), tetrad analysis resulted in two wild-type colonies and two very poorly growing mutant colonies (data not shown). The poor growth of the kic1Δ strain could not be suppressed by osmotic support of the medium (data not shown). In addition our results show that mutant spores failed to germinate on medium with galactose as the sole carbon source (data not shown). Microscopic analysis showed that kic1Δ cells were enlarged and round, forming large clumps, indicating a cell separation defect which is in accordance with previously described phenotypes of kic1 mutants (data not shown). In addition, the cells were very sensitive to pipetting and centrifugation. Fig. 1 shows that the kic1Δ mutant displayed calcofluor white hypersensitivity. This was similar to that of the cwh30-1 mutant (Ram et al., 1994; data not shown). The morphological defects, the hypersensitivity to calcofluor white and the fragility of the cells are all in agreement with a role in cell wall integrity for KIC1.

Construction of Pgal1::KIC1

The kic1Δ cells were not only very fragile, but they also had low transformation efficiencies, and even heterozygous diploids sporulated very poorly. In addition, kic1Δ cells occasionally developed second-site suppressor mutations. To circumvent the technical problems of working with a kic1Δ strain, a repressible KIC1 allele was constructed by placing it under the control of the GALT promoter in the pYEp43 plasmid. The activity of the Pgal1::KIC1 construct was confirmed by its ability to complement the kic1Δ growth defect (data not shown). This plasmid was transformed into a diploid heterozygous for kic1 and this strain was sporulated. When germinated on YPGal medium, spores lacking the endogenous KIC1 gene grew indistinguishably from spores with the endogenous KIC1 gene (data not shown). However, when germinated on YEPD medium, the spores lacking the endogenous KIC1 gene had a noticeable growth defect, albeit not as severe as a kic1Δ strain without the Pgal1::KIC1 plasmid (data not shown). This suggests that under glucose repression conditions there still is some expression of the KIC1 gene. Under these conditions however, cells still display calcofluor white hypersensitivity and other defects in cell wall integrity (see Figs 1, 2 and 4). The kic1Δ mutant carrying the Pgal1::KIC1 plasmid will be referred to as the Pgal1::KIC1 mutant. Interestingly, when grown on nonselective media (e.g. YEPD), the kic1Δ strain was not cured of the Pgal1::KIC1 plasmid. In addition, no viable colonies were found when Pgal1::KIC1 cells were put on media containing 5′-FOA (data not shown).

Pgal1::KIC1 mutant cells are resistant to Zymolyase

Zymolyase is a commercial enzyme preparation with both 1,3-β-glucanase and protease activities, which can
KIC1 affects 1,6-β-glucan synthesis

Fig. 2. KIC1 gene dosage affects cell wall composition. (a) Cells from wild-type (□), kic1Δ (●), P_GAL1::KIC1 (■), and wild-type with 2µ KIC1 (▲) were precultured, washed and incubated in the presence of Zymolyase 20T. (b) Isolated cell walls of wild-type (□), kic1Δ (●), P_GAL1::KIC1 (■), and wild-type with 2µ KIC1 (▲) were incubated in the presence of Zymolyase 20T. (c) KIC1 and PBS2 have opposing effects on Zymolyase sensitivity. Wild-type (□), P_GAL1::KIC1 (●), pbs2Δ (■) and P_GAL1::KIC1 pbs2Δ (▲) cells were precultured, washed and incubated in the presence of Zymolyase 20T. (d) mRNA levels of cell wall protein encoding genes SED1, CWP2 and CWP1 in wild-type and the P_GAL1::KIC1 mutant. Actin mRNA levels are shown as a loading reference.

be used to assay differences in cell wall structure and composition (De Nobel et al., 1990; Ram et al., 1994; De Groot et al., 2001). Wild-type cells were sensitive to treatment with Zymolyase 20T, whereas Kic1-deficient cells were resistant (Fig. 2a). Conversely, KIC1 expressed from a high-copy plasmid resulted in hypersensitivity to
Zymolyase (Fig. 2a). The major substrate for Zymolyase, i.e. 1,3-β-glucan, forms the inner layer of the cell wall. The outer layer consists of mannoproteins, which in intact cells limits the permeability to macromolecules (Zlotnik et al., 1984; De Nobel et al., 1990). It is conceivable that (at least part) of the Zymolyase sensitivity of intact cells can be attributed to changes in the protein outer layer and thus in cell wall permeability. One way to investigate this is to compare the Zymolyase sensitivity of intact cells and isolated walls, in which the inner layer is now exposed to Zymolyase. The increase in Zymolyase resistance of cell walls from the $P_{GAL}::KIC1$ mutant was much less dramatic compared to intact cells (Fig. 2b), but was still significant. Cell walls derived from a strain with high copy numbers of KIC1 now were more resistant to Zymolyase than wild-type (Fig. 2b). Taken together, these data suggest that KIC1 affects cell wall permeability. This might be caused by altered mannoprotein levels (see below) and possibly to some extent altered glucan levels.

Expression of some cell wall proteins is altered in the kic1 mutant

The mRNA expression levels of some known cell wall proteins in the $P_{GAL}::KIC1$ mutant showed increased levels of both SED1 and CWP2 in comparison to wild-type levels (Fig. 2d). CWP1 mRNA levels, however, remained unaffected (Fig. 2d). Interestingly, both sed1Δ and cwp2Δ mutants are more sensitive to Zymolyase than wild-type (Van der Vaart et al., 1995; Shimoi et al., 1998). Overexpression of SED1 resulted in Zymolyase resistance (Shimoi et al., 1998). This strongly suggests that at least part of the Zymolyase resistance of the $P_{GAL}::KIC1$ mutant is caused by increased levels of SED1 and CWP2.

The $P_{GAL}::KIC1$ mutant is more resistant to K1 killer toxin

K1 killer toxin has been a powerful tool for identifying genes involved in 1,6-β-glucan synthesis. Lower sensitivity to this toxin is generally associated with decreased levels of 1,6-β-glucan in the cell wall, which is a receptor for the toxin (Boone et al., 1990; Brown et al., 1993). K1 killer toxin sensitivities were compared using the halo assay. Fig. 3(a) shows the halo assays of some strains tested, to exemplify the difference in sensitivity. The K1 killer toxin sensitivity of various strains compared to their corresponding wild-type, is depicted graphically in Fig. 3(b). Note that the overexpression studies were performed on supplemented SD-based media, as opposed to the other strains which were tested on YEPD media. The halo assays performed on SD-

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**Fig. 3.** KIC1 expression levels affect K1 killer toxin sensitivity. Relative apparent sensitivity to the K1 killer toxin was determined as described in Methods. (a) Some examples of the halo plate assay. Tenfold dilution series of isolated K1 killer toxin were spotted onto seeded plates. After 4 days at 20 °C, halo diameters were measured and relative apparent sensitivities were calculated. (b) Relative apparent sensitivities of several strains. Strains displayed in the upper panel were grown on SD-based medium with selective amino acid mix. Strains displayed in the lower panel were grown in YEPD-based medium.
Table 4. KIC1 gene dosage affects 1,6-β-glucan levels

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alkali-insoluble 1,6-β-glucan (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>100</td>
</tr>
<tr>
<td>kic1A†</td>
<td>72 ± 7</td>
</tr>
<tr>
<td>Wild-type†</td>
<td>100</td>
</tr>
<tr>
<td>kic1A†‡</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>Wild-type + 2µ KIC1†</td>
<td>112 ± 1</td>
</tr>
</tbody>
</table>

*Measured as µg alkali-insoluble glucan (mg dry weight cell wall)⁻¹ and expressed as percentages relative to wild-type as described in Methods. Values represent mean ± SEM (n = 3).
† These strains were grown on SD medium.
‡ Strain with improved growth rate, probably resulting from a second-site suppressor mutation.

based media commonly showed larger haloes than on YEPD media, but the sensitivities compared to wild-type remained consistent.

The sensitivity of the kic1A mutant (JV83) to the K1 killer toxin was very low (not shown). The P\textsubscript{GAL1}:KIC1 mutant (JV142) retained a low sensitivity to the toxin, although not to the same extent as the deletion mutant. Conversely, overexpression of KIC1 in wild-type (JV39 with plasmid p62) resulted in an increased sensitivity to the K1 killer toxin (see Fig. 3b). The KIC1 gene dosage effect on K1 killer toxin sensitivity might reflect altered 1,6-β-glucan levels.

KIC1 affects cell wall 1,6-β-glucan levels

To determine if the changes in K1 killer toxin sensitivity can be attributed to changes in 1,6-β-glucan levels, the alkali-insoluble cell wall fraction was analysed. As expected, the kic1A strain showed a marked decrease in 1,6-β-glucan levels (Table 4). Consistently, overexpression of KIC1 resulted in a slight increase in 1,6-β-glucan levels (Table 4). These data are in agreement with the data from the K1 killer toxin assay. The gene dosage effect of KIC1 on 1,6-β-glucan levels suggests a role for KIC1 in 1,6-β-glucan deposition.

The P\textsubscript{GAL1}:KIC1 mutant displays synthetic growth defects with kre mutants

The proposed role of KIC1 in 1,6-β-glucan deposition in the cell wall is further supported by the strong genetic interaction occurring between a P\textsubscript{GAL1}:KIC1 mutant and kre mutants, which have lowered 1,6-β-glucan levels in the cell wall. The P\textsubscript{GAL1}:KIC1 mutant was crossed with kre6Δ, kre9Δ and kre1Δ mutants, and the resulting diploids were sporulated. Double mutants were selected on galactose-containing media and the growth phenotypes were analysed on glucose-containing media. kic1 showed a strongly enhanced growth defect with both kre6Δ and kre9Δ. Interestingly, when the P\textsubscript{GAL1}:KIC1 mutant was crossed with kre1Δ, enhancement of the growth defect was minor (Fig. 4). In contrast, KIC1 did not show an enhanced growth defect with FKS1, a mutant impaired in 1,3-β-glucan synthesis (Fig. 4). This suggests that the growth defects of Kre1-deficient cells are to a large extent related to the biogenesis of 1,6-β-glucan, and offers further support for the notion that KIC1 is involved in the regulation of 1,6-β-glucan biogenesis.

Multicopy suppressor screen of the calcofluor white sensitivity of P\textsubscript{GAL1}:KIC1 mutant

To further elucidate the regulatory function of KIC1 in cell wall biosynthesis, we introduced a high-copy pMA3a-based genomic library into the P\textsubscript{GAL1}:KIC1 mutant strain JV141. Three thousand five hundred transformants were replica-plated on selective SD medium containing 100 µg calcofluor white ml⁻¹. Plasmids isolated from the surviving colonies were retransformed into JV141 and the original cwh30-1 point mutant to ensure plasmid-dependent suppression of the calcofluor white phenotype. Serial dilutions of cells were spotted
Fig. 5. Multicopy suppressors of the calcofluor white hypersensitivity of the $P_{GAL1}$:KIC1 mutant. (a) Tenfold serial dilutions of wild-type (FY69) + YEplac181, $P_{GAL1}$:KIC1 (JV141) + YEplac181, $P_{GAL1}$:KIC1 (JV141) + 2µ RHO3 (pEV021), $P_{GAL1}$:KIC1 (JV141) + 2µ MSG5 (pEV017), $P_{GAL1}$:KIC1 (JV141) + pMA3a and $P_{GAL1}$:KIC1 (JV141) + pMA3a-STB3 (pEV020) were spotted onto selective SD media with or without 50 µg calcofluor white ml$^{-1}$. (b) Tenfold dilution series of wild-type (FY834), rho3Δ (EV116), rho4Δ (EV077), kic1Δ + $P_{GAL1}$:KIC1 (JV144) and kic1Δ (JV80) were spotted onto YPD with or without 50 µg calcofluor white ml$^{-1}$. Cells were grown for 2–3 days at 30 °C.
KIC1 affects 1,6-β-glucan synthesis

Overexpression of the RHO3 gene in the P_{GAL1}:KIC1 mutant produced an increase in sensitivity to the K1 killer toxin, in contrast to overexpression of the RHO4 gene (Fig. 6a). Deletion of RHO3 resulted in a decrease in K1 killer toxin sensitivity (Fig. 6b), and a decrease of about 40% in cell wall 1,6-β-glucan (3-3% of cell dry weight in wild-type cells compared to 1.9%). In contrast, deletion of RHO4 had no effect on K1 killer toxin sensitivity (Fig. 6b). Overexpression of the RHO3 gene in wild-type also resulted in an increase in K1 killer toxin sensitivity (Fig. 6c). However, when RHO4 was overexpressed in wild-type, cells displayed a decrease in killer sensitivity (Fig. 6c). A possible explanation for this is that high levels of Rho4 might compete with Rho3. Taken together, the observations suggest that the levels of RHO3 influence the level of cell wall 1,6-β-glucan and thus the sensitivity of the cells to the K1 killer toxin. This further implicates KIC1 in 1,6-β-glucan biogenesis, evidently for a part through RHO3.

**KIC1 antagonizes the PBS2–HOG1 pathway in cell wall biogenesis**

Cell wall phenotypes caused by overexpression of the MAP kinase kinase PBS2 from the HOG pathway show a remarkable resemblance to some of the phenotypes shown by P_{GAL1}:KIC1 mutant cells. Similar to cells that overexpress PBS2 (Jiang et al., 1995; Lai et al., 1997), P_{GAL1}:KIC1 mutant cells were less sensitive to K1 killer toxin, and showed a modest decrease in 1,6-β-glucan levels (Fig. 3b; Table 4). The reverse phenotypes were found in the pbs2Δ mutant and cells overexpressing KIC1, i.e., hypersensitivity to the K1 killer toxin (Jiang et al., 1995; Lai et al., 1997; Fig. 3b) and an increase in 1,6-β-glucan levels (Jiang et al., 1995; Table 4). A P_{GAL1}:KIC1 pbs2Δ double mutant was generated and this strain displayed an intermediate sensitivity to K1 killer toxin (Fig. 3b). KIC1 deficiency and deletion of PBS2 also reversely affected the sensitivity of the cells to cell wall degrading enzymes (Fig. 2c), whereas the double mutant displayed an intermediate phenotype. In summary, our observations in combination with data from the literature indicate that KIC1 and PBS2 have opposing roles in cell wall biogenesis.

**DISCUSSION**

The KIC1 gene was originally identified in a two-hybrid screen interacting with CDC31, which encodes yeast centrin. KIC1, member of the PAK1/Ste20 kinase family, encodes a 116 kDa protein which interacts in vivo with CDC31 and has in vitro kinase activity dependent on CDC31. However, it was shown that KIC1 did not play

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**Fig. 6. RHO3 gene dosage affects K1 killer toxin sensitivity.** Relative apparent sensitivity to the K1 killer toxin was determined as described in Methods. (a) kic1Δ + P_{GAL1}:KIC1 (JV144) + YEplac181, kic1Δ + P_{GAL1}:KIC1 (JV144) + 2μ RHO3 and kic1Δ + P_{GAL1}:KIC1 (JV144) + RHO4 are compared. (b) Wild-type (FY834), rho3Δ (EV116), rho4Δ (EV077) and kic1Δ + P_{GAL1}:KIC1 (JV144) was compared. (c) Wild-type (FY834) + YEplac181, wild-type (FY834) + 2μ RHO3 and wild-type (FY834) + 2μ RHO4 were compared.

multicopy suppressor plasmids allowed the loss of the P_{GAL1}:KIC1 plasmid. No viable colonies were found, indicating that none of these plasmids could restore (all of) the essential function(s) of KIC1 (data not shown). RHO3 was chosen for further analysis.

**The rho3 del tant is impaired in cell wall biogenesis**

RHO3 was disrupted and the cells were tested for their sensitivity to calcofluor white. Although not to the same extent as Kic1-deficient cells, rho3Δ cells showed increased sensitivity to calcofluor white, indicating a defect in cell wall biogenesis (Fig. 5b). In the absence of RHO3, the functionally related RHO4 can suppress the growth defect when overexpressed (Matsui & Toh-e, 1992a), whereas the rho3 rho4 double mutant is inviable. The double mutant also proved inviable in our genetic background (data not shown). Cells disrupted for RHO4 did not show an increased sensitivity to calcofluor white (Fig. 5b). In addition, overexpression of RHO4 did not restore the calcofluor white hypersensitivity of the P_{GAL1}:KIC mutant (data not shown). This suggests that the function that is suppressed by overexpression of RHO3 in the P_{GAL1}:KIC1 mutant, is not shared by RHO4.

The double mutant also proved inviable in our genetic background (data not shown). Cells disrupted for RHO4 did not show an increased sensitivity to calcofluor white (Fig. 5b). In addition, overexpression of RHO4 did not restore the calcofluor white hypersensitivity of the P_{GAL1}:KIC mutant (data not shown). This suggests that the function that is suppressed by overexpression of RHO3 in the P_{GAL1}:KIC1 mutant, is not shared by RHO4.

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a role in the SPB duplication function of CDC31. KIC1 rather contributes to its function in cell integrity and morphogenesis, since several kic1 mutants showed aberrant cell wall morphology, wide bud necks, failure in cell separation and cell lysis (Sullivan et al., 1998). Previously, cells deleted for KIC1 were found to be inviable (Sullivan et al., 1998). However, differences in genetic backgrounds might explain the (albeit very poor) viability of the kic1Δ mutant in our backgrounds.

The cwb30/kic1 mutant was originally discovered because it was hypersensitive to the cell wall perturbing agent calcofluor white (Ram et al., 1994). Interestingly, mutant cells were also resistant to the K1 killer toxin, indicating that their walls contained less 1,6-β-glucan. As KIC1 encodes a protein kinase, this marked it as a potential regulator of 1,6-β-glucan biogenesis. The following evidence supports this. KIC1 deficiency resulted in decreased sensitivity to the K1 killer toxin and lower levels of 1,6-β-glucan in its walls. Conversely, overexpression of KIC1 resulted in increased sensitivity to K1 killer toxin and elevated levels of 1,6-β-glucan. In addition, the P₆₃₆₄::KIC1 mutant crossed with various kre mutants resulted in double mutants with a synthetic growth defect, whereas a combination of the P₆₃₆₄::KIC1 mutant with the 1,3-β-glucan impaired mutant /ks1Δ did not result in a synthetic growth defect. Taken together, these results imply a role for KIC1 in the regulation of 1,6-β-glucan biogenesis. Finally, the expression levels of RHO3 correlated with the sensitivity to K1 killer toxin and thus probably with 1,6-β-glucan levels. In addition, the mutant phenotypes of Kic1-deficient cells were partially suppressed by overexpression of RHO3. This is consistent with the postulated role for KIC1 in regulating 1,6-β-glucan biogenesis.

Besides the defects in 1,6-β-glucan deposition, the Kic1-deficient cells also displayed resistance to the cell wall-degrading enzyme mixture Zymolyase, whereas overexpression of KIC1 resulted in hypersensitivity to Zymolyase. This effect may partly be caused by changes in the cell wall mannoprotein composition, since the external layer of mannoproteins in the cell wall determines the porosity and needs to be removed for efficient cell wall degradation (Zlotnik et al., 1984; De Nobel et al., 1990). There are two lines of evidence that confirm this notion. First, when cell walls were isolated prior to Zymolyase treatment, walls from Kic1-deficient cells showed a much less pronounced resistance to Zymolyase compared to wild-type cell walls. Second, two known cell wall protein encoding genes, SED1 and CWP2, were found to be upregulated in the P₆₃₆₄::KIC1 mutant. Interestingly, overexpression of SED1 leads to Zymolyase resistance (Shimoi et al., 1998), and, reversely, deletion of both SED1 and CWP2 results in increased sensitivity to Zymolyase (Van der Vaart et al., 1995; Shimoi et al., 1998). Increased expression of these genes in the Kic1-deficient mutants might thus at least in part explain the resistance to Zymolyase. By which mechanism the expression of these cell wall proteins is induced is unknown. It might reflect the induction of a cell wall repair mechanism as the result of the decrease in 1,6-β-glucan. (Popolo & Vai, 1999; Kapteyn et al., 1999; Klis et al., 2002), but this normally includes induction of CWPl expression (Terashima et al., 2000; Kapteyn et al., 2001). However, CWPl expression was not induced in the P₆₃₆₄::KIC1 mutant. Alternatively, Kic1 might have a regulatory role in multiple cell wall biosynthetic steps and not only in 1,6-β-glucan biogenesis.

The identification of RHO3 as a multicopy suppressor of the P₆₃₆₄::KIC1 mutant suggests that RHO3 might be a downstream target of KIC1 in cell wall biogenesis. This is supported by the RHO3 gene dosage relationship with K1 killer toxin sensitivity and the reduction of 1,6-β-glucan levels in the rho3Δ mutant. Whereas RHO4 contributes to some of the known functions of RHO3 (Matsui & Toh-e, 1992a; Imai et al., 1996), the effects on cell wall biogenesis are not shared by RHO4.

Evidence is accumulating that suggests a role for the PBS2–HOG1 pathway in cell wall construction. Overexpression of PBS2 causes resistance to laminarinase, a cell wall degrading enzyme mixture (Lai et al., 1997), and deletion results in hypersensitivity (Fig. 2b; Alonso-Monge et al., 2001). In addition, PBS2 overexpression results in resistance to the K1 killer toxin and a decrease in cell wall 1,6-β-glucan levels (Jiang et al., 1995). Also, under noninducing conditions the HOG pathway contributes to the maintenance of cell wall architecture (García-Rodríguez et al., 2000). Furthermore, overexpression of some cell wall related genes suppress the hyperosmosensitive phenotype of a ste11 ssk2 ssk22 mutant. These include LRE1 and HLR1, which can also suppress the osmosensitivity and the glucanase sensitivity of both pbs2Δ and hog1Δ mutants (Alonso-Monge et al., 2001). Our report further supports a role for PBS2 in cell wall biogenesis. The P₆₃₆₄::KIC1 mutant and the pbs2Δ single mutant cells had reverse phenotypes in both K1 killer toxin and Zymolyase sensitivities. In the P₆₃₆₄::KIC1 pbs2Δ double mutant an intermediate phenotype was observed (Fig. 3b). These results suggest that KIC1 and PBS2 play opposing roles in cell wall biogenesis. The mechanism by which KIC1 and PBS2 counteract each other remains obscure. In summary, the protein kinase Kic1 is involved in regulating cell wall construction in multiple ways and seems to have a specific role in controlling 1,6-β-glucan levels.

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