KNR4, a suppressor of *Saccharomyces cerevisiae cwh* mutants, is involved in the transcriptional control of chitin synthase genes

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The *KNR4* gene, originally isolated by complementation of a K9 killer-toxin-resistant mutant displaying reduced levels of both 1,3-β-glucan and 1,3-β-glucan synthase activity, was recloned from a YCp50 genomic library as a suppressor of *Saccharomyces cerevisiae* calcofluor-white-hypersensitive (*cwh*) mutants. In these mutants, which were characterized by increased chitin levels, the suppressor effect of *KNR4* resulted, for some of them, in a lowering of polymer content to close to wild-type level, with no effect on the contents of β-glucan and mannan. In all cases, this effect was accompanied by a strong reduction in mRNA levels corresponding to *CHS1*, *CHS2* and *CHS3*, encoding chitin synthases, without affecting expression of *FKS1* and *RHO1*, two genes encoding the catalytic subunit and a regulatory component of 1,3-β-glucan synthase, respectively. Overexpression of *KNR4* also inhibited expression of *CHS* genes in wild-type strains and in two other *cwh* mutants, whose sensitivity to calcofluor white was not suppressed by this gene. The physiological relevance of the *KNR4* transcriptional effect was addressed in two different ways. In a wild-type strain exposed to α-factor, overexpression of this gene inhibited *CHS1* induction and delayed shmoo formation, two events which are triggered in response to the pheromone, whereas it did not affect bud formation and cell growth in a *chs1 chs2* double mutant. A chimeric protein made by fusing green fluorescent protein to the C terminus of Knr4p which fully complemented a *knr4Δ* mutation was found to localize in patches at presumptive bud sites in unbudded cells and at the incipient bud site during bud emergence. Taken together, these results demonstrate that *KNR4* has a regulatory role in chitin deposition and in cell wall assembly. A mechanism by which this gene affects expression of *CHS* genes is proposed.

Keywords: *Saccharomyces cerevisiae*, cell wall, chitin, *KNR4*, localization

INTRODUCTION

Yeasts and fungi are surrounded by a thick cell wall which accounts for 25 % of the dry mass (Valentin *et al.*, 1987; Fleet, 1991; Dallies *et al.*, 1998). The cell wall is essential for maintenance of cell shape and offers protection against harmful environmental conditions. It is a dynamic structure which can adapt to different physiological states (conjugation, sporulation, station-ary phase, etc.) and morphological changes (i.e. pseudo-hyphal and agar-invasive growth; Gimeno *et al.*, 1992; Roberts & Fink, 1994). It is composed of β-glucans and mannoproteins, which account for about 80–95 % of wall dry mass, and of smaller amounts of chitin (2 %) and lipids (3–5 %) (for a review, see Klis, 1994).

Recently, genes involved in spatial and temporal cell wall construction in *Saccharomyces cerevisiae* have been the subject of intensive genetic research based on screening EMS-mutagenized (Ram *et al.*, 1994) and transposon-mutagenized (Lussier *et al.*, 1997) cells for increased sensitivity to calcofluor white, a cationic fluorescent dye known to interfere with cell wall

assembly (Elorza *et al.*, 1983). This work has led to the identification of more than 100 genes that can be roughly classified into four categories. The first class comprises structural genes for cell wall polymer biosynthesis. Chitin synthesis is governed by *CHS1*, *CHS2* and *CHS3*, encoding the three membrane-bound chitin synthases, each of which is involved in a specific function in septum and cell wall construction in vegetative and conjugating cells (Cabib *et al.*, 1996), and by regulatory components of these enzymes encoded by *CHS4*, *CHS5* and *CHS6* (Santos & Snyder, 1997; Trilla *et al.*, 1997; De Marini *et al.*, 1997). Knowledge of β-glucan synthesis is less advanced but *FKS1* and *FKS2*, encoding catalytic sub-units of 1,3-β-glucan synthases have been characterized (Douglas *et al.*, 1995; Inoue *et al.*, 1995; Mazur *et al.*, 1995), and a dozen genes involved in 1,6-β-glucan have been isolated on the basis of selection of mutants resistant to the K1 killer toxin (Klis, 1994; Cid *et al.*, 1995). This category also includes *MNN* and *ALG* genes involved in the biosynthetic pathway leading to the formation of mannoproteins (Herscovics & Orlean, 1993; Cid *et al.*, 1995). Enzymes catalysing linkages between chitin and β-glucan, and those required for cell wall remodelling by association of cell wall proteins to 1,6-β-glucan through a glycosylphosphatidylinositol remnant (Cid *et al.*, 1995; Kapteyn *et al.*, 1997; Kollar *et al.*, 1997) fall into a second category. Most of the genes in this category have not yet been identified. The third class contains genes involved in the signalling and morphogenetic pathways (budding, mating, polarized secretion, morphogenesis, sporulation; for a review, see Cid *et al.*, 1995) and those which participate in the temporal and spatial control of cell wall formation needed for cell integrity/cell proliferation (Paravicini *et al.*, 1992; Brown *et al.*, 1993; Igual *et al.*, 1997). The product of *RHO1*, controlling both the PKC1-MAP kinase pathway and 1,3-β-glucan synthesis represents one typical example of this class (Drgonova *et al.*, 1997; Qadota *et al.*, 1997). The fourth category comprises genes that cannot yet be included in any of the three previous classes, either because they encode proteins with other (vital) functions (ranging from involvement in metabolism, mitochondrial function, transcription, translation and DNA repair; Lussier *et al.*, 1997) that undoubtedly have indirect effects on cell wall construction, or because they have been poorly characterized. This is notably the case with at least three genes, *HKR1* (Kasahara *et al.*, 1994), *KNR4* (Hong *et al.*, 1994a) and *GNS1* (El-Sherbeini & Clemas, 1995). Truncation of the 3′ part of the *HKR1* coding region or deletion of the *KNR4* or *GNS1* genes causes a reduction in both 1,3-β-glucan synthase activity and 1,3-β-glucan content. These findings have led to the conclusion that they are part of the regulatory mechanism for β-glucan synthesis. However, the great diversity in their localization [Gns1p is predicted to be an integral membrane protein (El-Sherbeini & Clemas, 1995), Hkr1p, a surface protein (Yabe *et al.*, 1996) and Knr4p a nuclear protein (Fishel *et al.*, 1993)] makes a direct effect on β-glucan synthase activity very unlikely, and suggests a role in remodelling and assembly of cell wall polymers.

In this paper, we show that *KNR4* was able to suppress several *S. cerevisiae* calcofluor-white-hypersensitive (*cwh*) mutants, and report upon its effects on the expression of genes encoding chitin synthases and on its cellular localization.

METHODS

Strains and growth conditions. Yeast strains used in this study are listed in Table 1. Yeast media contained either 2 % (w/v) Bactopeptone, 1 % (w/v) yeast extract and 2 % (w/v) glucose (YEPD) or 0·17 % (w/v) yeast nitrogen base without amino acids and ammonium, 0·5 % (w/v) ammonium sulphate and 2 % (w/v) glucose (SD), supplemented with auxotrophic requirements. For solid media, agar (Difco) was added at 2 % (w/v) final concentration. Yeast cultures were grown at 30 °C. Calcofluor white M2R (Sigma F6259) was freshly prepared as a stock solution of 10 mg ml⁻¹ in water and filter-sterilized. The calcofluor-white-containing plates were made as follows: calcofluor white solution was added either to SD agar (buffered at pH 6·0 with MES, 50 mM) or to YEPD agar media (both melted and kept at 70 °C) to a final concentration ranging from 0·05 to 1 mg ml⁻¹. The K9-killer-toxin-containing plates were made according to the procedure of Hong *et al.* (1994a). Briefly, the strain *Hansenula mrakii* IFO0895 (a gift from Dr Didomenico, Shering-Plough Research Institute, NJ, USA) was grown for 72 h at 19 °C in liquid YEPD medium, buffered with sodium citrate 0·1 M, pH 4·7. Cells were collected by centrifugation and the culture medium was sterilized by filtration. Aliquots (5 ml) of filtered culture medium were added to 20 ml YEPD agar (buffered with sodium citrate, 0·1 M, pH 4·7; melted and kept at 55 °C).

Transformation of calcofluor-white-hypersensitive mutants with a YCp50 library. Transformation of calcofluor-white hypersensitive-mutants (*cwh* mutants) was carried out by the standard LiAc method (Schietsl & Gietz, 1989), using a genomic library of *S. cerevisiae* constructed in YCp50 (Rose *et al.*, 1987) and purchased from ATCC (accession number 37415). Transformed cells were directly plated on SD medium without uracil (SD – uracil) containing 0·1 mg calcofluor white ml⁻¹, since preliminary assays indicated that the *cwh43-2* mutant cells displayed almost no growth on these plates after 2 d at 30 °C whereas the wild-type control AR27 grew very well. Colonies (about 20 out of 90000 expected URA⁺ transformants) were further screened on plates containing a higher calcofluor white concentration before and after the loss of URA⁺-containing plasmids [on media containing 0·1 % 5-fluoroorotic acid (5-FOA; Boeke *et al.*, 1987)]. Plasmids conferring resistance to calcofluor white were isolated according to Robzyk & Kassir (1992) and sent for partial sequencing (Genome Express, Grenoble, France). From the DNA sequences, homology searches in the yeast genomic sequence databases [SGD (Standford Genomic Database, USA) or MIPS (Martinsried Institute for Protein Sequences, Germany); Mewes *et al.*, 1997] were conducted to identify the chromosomal location of the DNA fragments and the ORFs present on these fragments. Putative ORFs with about 1 kb of 5′ and 3′ flanking regions were subcloned in centromeric pRS316 and episomic pRS426 derivative plasmids (Sikorski & Hieter, 1989; Christianson *et al.*, 1992) and checked for their ability to complement the calcofluor white sensitivity phenotype of the mutants.

Plasmid and strain construction. A 2·95 kb *Xba*I–*Spe*I fragment containing *KNR4* was cut from one of the genomic library inserts and cloned into the *Spe*I site of the episomic pRS426 and centromeric pRS316 shuttle vectors to yield

Table 1. *S. cerevisiae* strains used in this work

Strain	Genotype	Reference or source
AR27	*MATα ura3-52*	
AR27-2N	*MATα/MATa ura3-52/ura3-52*	
cwh3	AR27 *cwh3*	
cwh4	AR27 *cwh4*	
cwh5	AR27 *cwh5*	
cwh9	AR27 *cwh9*	Ram *et al.* (1994)
cwh39	AR27 *cwh39*	
cwh43-1	AR27 *cwh43-1*	
cwh43-2	AR27 *cwh43-2*	
cwh53-1	AR27 *cwh53-1*	
HM10	AR27 *knr4::kan*[R]	This study
HM20	AR27-2N *KNR4::knr4::kan*[R]	This study
ECY36-3C	*MATα-chs1-23 chs2::LEU2 leu2-2 ura3-52 trp1-1*	Choi *et al.* (1994)

pHM37 and pHM38, respectively. To construct a gene fusion encoding the full-length Knr4p fused to the green fluorescent protein (GFP) (Chalfie *et al.*, 1994), the ORF of *KNR4* was amplified by PCR using Vent DNA polymerase (New England Biolabs) with the primers 5′ CGCGCG<u>ACTAGT</u>ATGG-ATCTATTCAAAAGAAAAGTT 3′ and 5′ GCGCGC-<u>AAGCTT</u>TAAAGCTATATTTTCAAATTCTTC 3′, con-taining *Spe*I and *Hin*dIII sites (underlined). The PCR product was digested with these restriction endonucleases and ligated into the *Spe*I/*Hin*dIII-digested centromeric pGFP-C-FUS plas-mid (Niedenthal *et al.*, 1996) resulting in pGFP-C-KNR4. A disruption cassette for *KNR4* was made by insertion of a blunt-end fragment of the *kan*[R] gene into the *Ehe*1 site of *KNR4* in pHM37. The cassette was excised with *Spe*1 and *Nde*1, and used to transform strain AR27. Verification of the correct integration at the *KNR4* locus was carried out by PCR amplification using primers 5′ GAGTCATCTTGGATAA-TGAAGC 3′ and 5′ CCCCATCACTCCACTCCCCATC 3′. A plasmid with the *KNR4* gene under the control of the *GAL10/CYC1* promoter was constructed by cloning a PCR-amplified *KNR4* ORF into the *Bam*HI and *Eco*RI sites of pYEDP60-2 (Pompon *et al.*, 1996) using the two primers with sequence identical to those used for making the *GFP-C-KNR4* fusion gene, except that the *Spe*I and *Hin*dIII sites were replaced by *Bam*HI and *Eco*RI sites, respectively.

RNA analysis. Total RNA was extracted from exponentially growing cells (OD$_{600}$ 1·5), separated in formaldehyde agarose gels as described by Siderius *et al.* (1997) and transferred to Hybond-N$^+$ filters (Amersham). Hybridization and washings were performed as recommended by the manufacturer. Blots were probed with the following restriction fragments: a 1·5 kb *Bam*H1–*Hin*dIII fragment of *ACT1* from plasmid pJJ16 (a gift from Dr C. Gancedo, CSIC, Madrid, Spain), which was used as a loading control; a 2 kb *Eco*RV–*Stu*1 fragment of *CHS1* from pAS1; a 1·9 kb *Stu*1 fragment of *CHS2* from pEC2; a 2·4 kb *Xho*I–*Hin*dIII fragment of *CHS3* from pHV8 (these three latter plasmids were a gift from E. Cabib, NIH, Bethesda, MD, USA); a 2·1 kb *Xba*I–*Cla*I fragment of *FKS1* (a gift from F. M. Klis, University of Amsterdam, The Netherlands); a 1·3 kb fragment of *RHO1* (obtained from the YCp50 genomic library as one of the suppressors of the *cwh43-2* mutation, unpublished results). The fragments were radiolabelled with ^{32}P using the Megaprime DNA labelling system (Amersham).

Phenotypic tests. Yeast cells were grown overnight in liquid SD – uracil or YEPD to OD$_{530}$ ∼ 4·0, concentrated by centri-

fugation and resuspended in sterile water to OD$_{530}$ 8·0. Serial dilutions of 1/1, 1/10, 1/100 and 1/1000 were spotted on solid media containing either calcofluor white or K9 killer toxin. Growth was scored after 2 d incubation at 30 °C.

Treatment with α-factor. *MAT*a cells were grown to early exponential phase (OD$_{600}$ 0·2) in liquid YEPD medium to which α-factor (Sigma T6901; 1 mM in methanol) was added to a final concentration of 2×10^{-6} M. Samples for RNA isolation and for counting shmoos were taken at different times as indicated in Fig. 4.

Quantitative cell wall carbohydrate analysis. The sugar composition of cell walls was determined from exponential cultures of yeast cells grown in SD – uracil and harvested at OD$_{600}$ 1·0. Isolation of cell walls, hydrolysis of cell wall polymers in H$_2$SO$_4$ and quantification of the liberated sugars (glucose, mannose, glucosamine) were carried out as described by Dallies *et al.* (1998).

Observation by fluorescence microscopy. Localization of the Knr4–GFP fusion protein was visualized in living cells from strain AR27 deleted for *KNR4* and transformed with the centromeric plasmid pKnr4-C-GFP. Cells were grown ex-ponentially in liquid SD medium supplemented with auxo-trophic requirements. Cells were viewed with a Zeiss Axio-phot 2 microscope using the filter combination 450-490, FT510, BP 515-565 and to prevent movement during visual-ization, yeast cells were mounted in 0·05 % (w/v) agarose. For staining nuclei, living cells were incubated for 1 h in DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; 2·5 mg ml^{-1}).

RESULTS

KNR4 is a suppressor of a number of calcofluor-white-hypersensitive mutants

Klis and coworkers (Ram *et al.*, 1994) isolated 63 calcofluor-white-hypersensitive mutants that were clas-sified into 53 complementation groups. Two of them, *cwh43-1* and *cwh43-2*, were given to us in order to clone wild-type genes by complementation. The *cwh43-2* mutation was mapped on chromosome III between the *LEU2* and *MAT* loci (unpublished data). The trans-formation of this mutant with a genomic library in YCp50, (Rose *et al.*, 1987) followed by selection on SD
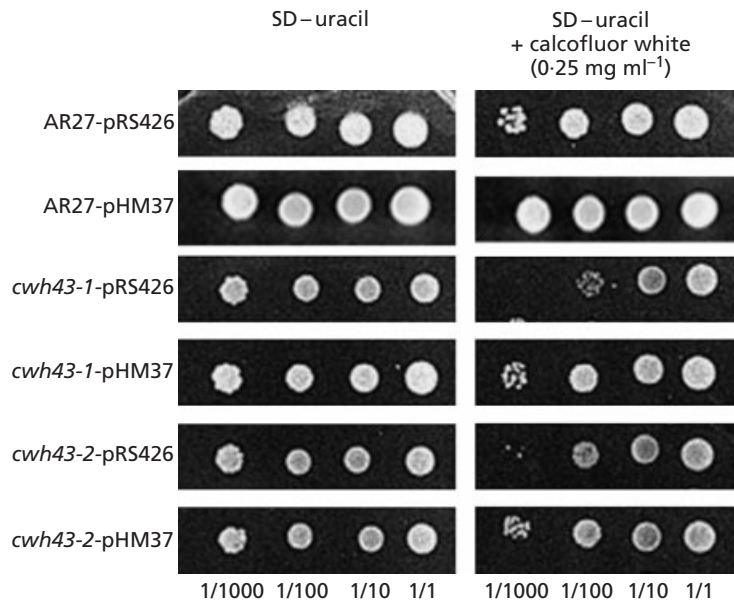
Fig. 1. Suppression of *cwh* mutants by *KNR4*. Transformed wild-type, *cwh43-1* and *cwh43-2* mutant strains with either pRS426 (control plasmid) or pHM37 (pRS426 containing *KNR4*) were grown overnight on SD – uracil and concentrated to OD_{530} 8·0. Serial dilutions of yeast cultures were spotted on SD – uracil plates in the absence or presence of 0·25 mg calcofluor white ml^{-1}. Growth was scored after 2 d at 30 °C.

– uracil containing 0·10 mg calcofluor white ml^{-1} gave rise to six complementing plasmids, among which two contained overlapping 10–14 kb fragments from chromosome VII with several ORFs, including *KNR4*. This gene, subcloned in either a centromeric or a multicopy plasmid, was able to restore wild-type sensitivity of the *cwh43-2* mutant strain to calcofluor white (Fig. 1). We also found that *KNR4* could suppress the *cwh43-1* mutation, which was initially claimed to be allelic to *cwh43-2* (Ram *et al.*, 1994). However, we could not confirm this allelism as the diploid mutant obtained by crossing the two haploid mutants was as sensitive to the drug as the wild-type, and the *cwh43-1* mutation did not show any linkage with markers from chromosome III. Fig. 1 also shows that the control strain AR27 bearing additional copies of *KNR4* was more resistant to calcofluor white. These results suggested that the complementation by *KNR4* was not restricted to a specific interaction with the *cwh43-2* mutation.

To confirm this hypothesis, the centromeric and episomic plasmids bearing *KNR4* were used to transform *cwh* mutants originally classified as having a cell wall mannose/glucose ratio lower than that of the wild-type, and a few others with a mannose/glucose ratio higher than that of the wild-type (Ram *et al.*, 1994), in order to determine whether complementation occurred independently of changes in mannose/glucose ratios. From a series of *cwh* mutants, three mutants (*cwh5*, *cwh9*, *cwh43-2*) with lower levels of mannan and three others (*cwh4*, *cwh39*, *cwh43-1*) having slightly lower levels of *β*-glucan, as compared to those in the control strain AR27 were suppressed by *KNR4* borne on a multicopy plasmid (Table 2).

Because *KNR4* borne on a centromeric plasmid was also known to be able to suppress the calcofluor white sensitivity of some of the *cwh* mutants tested, it was suggested that a small change in the amount of

Knr4p affects cell wall structure. In favour of this interpretation, we found that the deletion of one copy of *KNR4* in a diploid rendered this strain sensitive to calcofluor white, and that the sensitivity to this drug was more pronounced in a haploid strain deleted for this gene (Fig. 2) and in a diploid strain homozygous for Δ*knr4* (not shown). In addition, and in contrast to a previous report of Fishel *et al.* (1993) who cloned the same gene (named *SMI1* for *s*uppressor of *MAR inhibition 1*), deletion of this gene did not result in a thermosensitive growth arrest phenotype in two different genetic backgrounds.

Changes in cell wall composition induced by additional copies of *KNR4*

Since calcofluor sensitivity is basically dependent on the chitin content of the cells, we analysed the cell wall composition in *cwh* mutants transformed with control and *KNR4*-containing plasmids. Results of these measurements are shown in Table 2. It can be seen that, except for the *cwh39* and *cwh43-1* mutant strains, other *cwh* mutants transformed with the control plasmid (pRS426) contained two- to fivefold more chitin than the control strain AR27. This result agrees with previous data showing that modifications leading to changes in cell wall content of either mannan or glucan are accompanied by an increase in chitin (Daran *et al.*, 1997; Popolo *et al.*, 1997; Kapteyn *et al.*, 1997; Dallies *et al.*, 1998). Upon transformation with the multicopy plasmid pHM37, one group of four *cwh* mutants which were complemented by *KNR4* showed a significant decrease in chitin levels. In a second group of two mutants also complemented by this gene, changes in chitin levels were barely detectable. In a third group of two *cwh* mutants which were not complemented by *KNR4*, one of them (*cwh3*) showed reduced levels of chitin in the presence of additional copies of *KNR4*, whereas in the other (*cwh53-*

Table 2. Effect of overexpression of *KNR4* on cell wall carbohydrate composition of control and *cwh* mutant strains

Cell walls were obtained from exponentially growing cells cultivated on SD – uracil. Results are expressed as percentages of cell wall dry mass and values are the means from two independent experiments. The standard deviation was 5 %.   , Not determined.

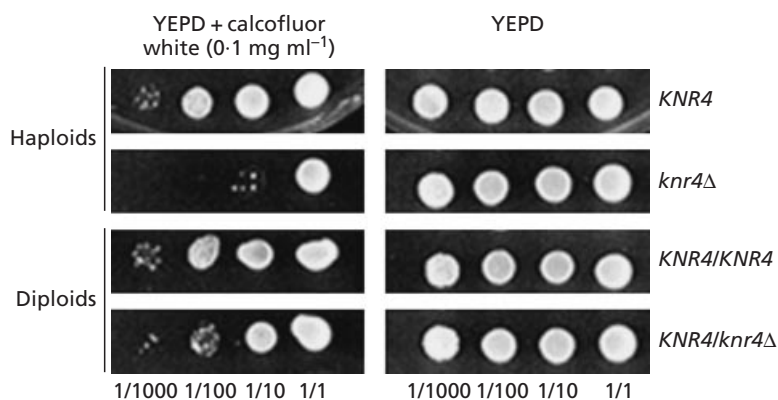| Strain	Complementation by *KNR4*	Glucan		Mannan		Chitin	
		pRS426	pHM37	pRS426	pHM37	pRS426	pHM37
cwh5	+	68·1	70·3	26·2	26·6	5·6	3·0
cwh9	+	73·1	70·2	22·0	25·8	4·9	4·0
cwh43-2	+	65·3	65·0	28·2	31·4	6·5	3·6
cwh4	+	53·4	62·0	41·0	34·2	4·7	3·8
cwh39	+	55·7	60·0	42·2	37·7	2·1	2·3
cwh43-1	+	58·5	49·4	39·2	49·1	2·5	2·5
cwh3	−	62·8	72·1	32·4	24·0	4·8	4·0
cwh53-1	−	37·7	35·0	47·9	50·7	14·4	14·3
AR27		59·3		38·0		2·7	

Fig. 2. Effect of a *KNR4* deletion in haploid and isogenic diploid strains on their sensitivity to calcofluor white. Strains were grown overnight on YEPD to stationary phase and concentrated to OD_{530} 8·0. Serial dilutions were spotted on YEPD plates in the absence or presence of 0·1 mg calcofluor white ml^{-1}.

1) mutant bearing a mutation in *FKS1*, (encoding the catalytic subunit of 1,3-β-glucan synthase; Ram *et al.*, 1995), no effect on chitin levels could be detected. It can also be seen in Table 2 that the effects of overexpressing *KNR4* on mannan and glucan levels were moderate, specific to each mutant strain, and that no link between changes in these two cell wall polymers and the suppressor effect of this gene could be found.

Since the *KNR4* suppressor effect was observed on media containing calcofluor white, the presence of which is known to increase chitin levels (Roncero & Duran, 1985), we thought that additional copies of *KNR4* would antagonize this increase, and therefore could account for the complementation of *cwh* mutants by this gene. If this was the case, the effect of additional copies of the gene on chitin levels would be more pronounced in the presence of calcofluor white. Unfortunately, attempts to conduct cell wall analysis with yeast grown in the presence of 0·1 mg calcofluor white ml^{-1} failed because of the formation of a large amount of yellow precipitate resistant to hot acidic hydrolysis. Different methods to dissolve this precipitate were unsuccessful.

Effect of *KNR4* on the transcription of genes encoding chitin synthases

The fact that on one hand, a deletion of *KNR4* resulted in a fourfold increase in chitin content and a 50 % decrease in β-glucan content, and that on the other hand, additional copies of this gene suppressed the calcofluor white sensitivity of yeast mutants associated, in some cases, with reduction in cell wall chitin prompted us to investigate the effect of *KNR4* on the transcription of key genes involved in cell wall polymer biosynthesis by Northern analysis. As shown in Fig. 3, transcripts for *CHS1*, *CHS2* and *CHS3*, encoding chitin synthases, were strongly reduced both in the control strain AR27 and in *cwh* mutants transformed with either a centromeric vector or an episomic vector bearing *KNR4*. There was, however, no effect on expression of *FKS1*
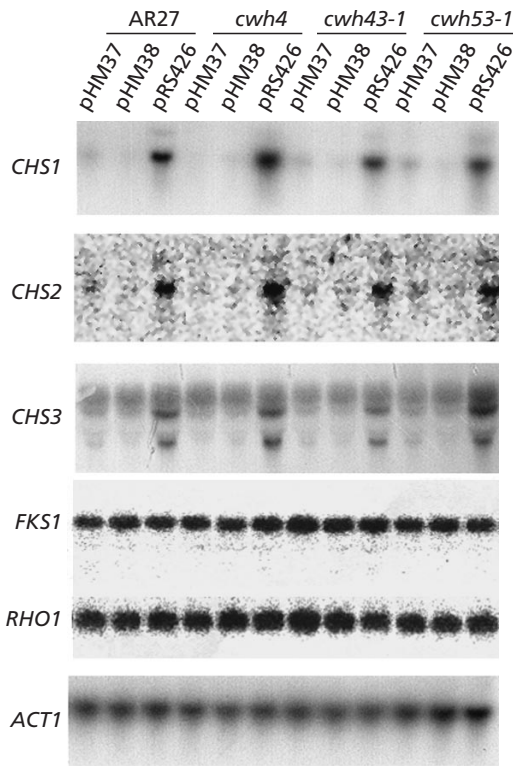
Fig. 3. Effect of overexpression of *KNR4* on expression of *CHS1*, *CHS2*, *CHS3*, *FKS1* and *RHO1*. Total RNA (15 μg per lane) was analysed by Northern blot experiments as outlined in Methods. *ACT1* transcripts were used as a loading control.

(a)

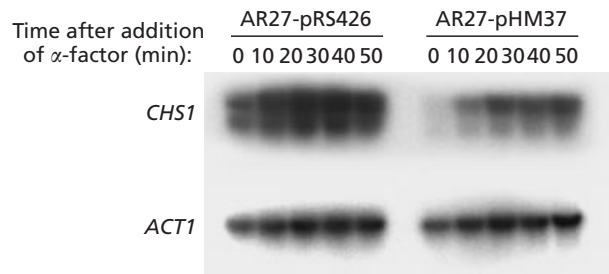| Time after addition of α-factor (h)	Percentage of shmoos formed	
	AR27-pHM37	AR27-pRS426
0	0	0
1	0	9
2	39	79
3	52	82
4	69	83
5	74	85

(b)

Fig. 4. Effect of overexpression of *KNR4* on shmoo formation and induction of *CHS1* upon exposure of yeast to α-factor. Exponentially growing cells of strain AR27 transformed with pRS426 (control plasmid) or pHM37 (pRS426 containing *KNR4*) on SD – uracil were exposed to 2×10^{-6} M α-factor. (a) The percentage of shmoos was determined by counting about 300 cells under the optical microscope. (b) Induction of *CHS1* in response to mating pheromone was analysed by Northern blots of *CHS1* mRNA. The blot was also hybridized with *ACT1* probe used as a loading control.

and *RHO1*. Interestingly, the repressing effect of additional copies of *KNR4* was observed in all mutant strains, even in those that were not complemented by this gene. The case of the *cwh53-1* mutant is particularly striking. Although this mutant strain contained a huge amount of chitin, mRNA corresponding to the three *CHS* genes was not higher than in the wild-type control. In contrast, the overexpression of *KNR4* in the *cwh53-1* mutant was without effect on chitin, though it caused a strong reduction in *CHS* transcripts (Fig. 3). We also found that deletion of *KNR4* in the control strain AR27 caused a fourfold increase in chitin levels without changing the expression pattern of *CHS* genes (results not shown).

Additional copies of *KNR4* affect pheromonal response in a wild-type strain but not the growth of a *chs1 chs2* double mutant

To illustrate a potential physiological role of *KNR4* in regulating expression of *CHS* genes, we investigated the effect of overexpressing this gene on the induction of *CHS1* (Appeltauer & Achstetter, 1989) and shmoo formation of haploid cells in response to pheromone. Fig. 4 shows that activation of *CHS1* was strongly inhibited, and shmoo formation was delayed in these

*MAT**a** cells bearing multicopies of *KNR4* incubated with 2×10^{-6} M α-factor. This delay was most likely due to the effect of additional copies of *KNR4* in reducing levels of *CHS2* and *CHS3* transcripts in transformed cells since recent data have shown no relationship between *CHS1* expression and mating efficiency (Santos *et al.*, 1997). To further strengthen a potential role of *KNR4* in chitin formation, we transformed a *chs1 chs2* double mutant which can survive due to the presence of *CHS3* with pHM37 bearing *KNR4* on a multicopy vector. As expected, the levels of *CHS3* transcript were dramatically reduced, in a similar way to that illustrated in Fig. 3, with no detectable effect on bud formation or on cell growth (results not shown). The same results were obtained for a *chs2 chs3* double mutant strain transformed with a plasmid bearing *KNR4* under the control of the *GAL10/CYC1* hybrid promoter upon transfer from a glucose- to a galactose-containing medium.

Localization of *KNR4* using the GFP fusion protein

In previous work aimed at identifying genes involved in chromatin reorganization during mitosis, a gene named *SMI1* 100 % identical to *KNR4* was isolated (Fishel *et al.*, 1993). These authors generated anti-SMI1 antibodies
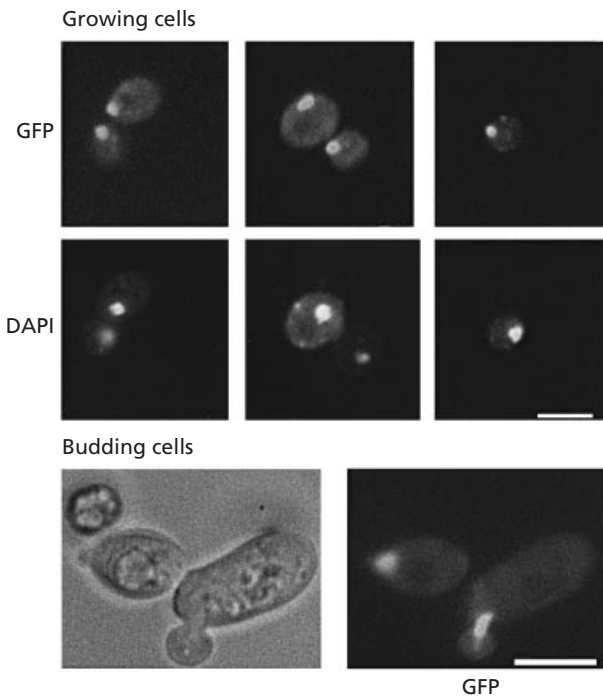
Growing cells

GFP

DAPI

Budding cells

GFP

Fig. 5. Localization of GFP–Knr4p fusion protein in a Δ*knr4* strain transformed with pGFP-KNR4. Cells of strain HM10 were grown, fixed and visualized as described in Methods. Time exposure for GFP–fusion protein was 15 s, and for DAPI 2 s. Bar, 5 μm.

raised against 18 amino acids of the C terminus region of the protein to use for immunolocalization in animal cells. As these antipeptide antibodies reacted with intranuclear materials, these authors concluded that Smi1p homologues exist in the nucleus of animal cells (Fishel *et al.*, 1993). Very surprisingly, they did not use these anti-SMI1 antibodies for indirect immuno-fluorescent experiments in yeast. Therefore, we re-investigated the cellular localization of the *SMI1/KNR4* gene product in yeast cells, using the GFP fusion methodology (Chalfie *et al.*, 1994). To this end, we constructed two centromeric plasmids, one with GFP at the C terminus, and the other at the N terminus of Knr4p. Only the first construction turned out to be functional since it fully complemented a Δ*knr4* strain for the ability to restore wild-type sensitivity to calcofluor white and to K9 killer toxin. Transformed cells growing exponentially on SD medium without methionine were examined by fluorescence microscopy. This experiment revealed that the fusion protein localized as cytoplasmic patches near the presumptive bud site in unbudded cells, distinct from the nucleus (top left of Fig. 5), and that it localized at the incipient bud site in cells with small buds (bottom right of Fig. 5). During growth of the daughter cells, the GFP–Knr4p remained in the neck of the bud. Before nuclear division, the fusion protein separated into two parts, with one migrating to the cytoplasm of the daughter cell and the other remaining in the mother

cell (not shown). The same results were obtained with cells growing in the presence of methionine, except that the fluorescence intensity was weaker.

DISCUSSION

We report in this paper that additional copies of the *KNR4* gene can suppress the calcofluor white hyper-sensitivity of several *cwh* mutants. Although the loss of function of this gene caused a 50 % decrease in β-glucan (Hong *et al.*, 1994a), the suppressor effect of over-expression of *KNR4* could not be explained by partial recovery of this cell wall polymer because it was obtained with mutants containing β-glucan levels at both lower and higher levels than those of the control strain. As it was recently shown that changes in either β-glucan or mannan in yeast cell walls were accompanied by an increase in chitin levels (Popolo *et al.*, 1997; Dallies *et al.*, 1998), and as the perturbating effect of calcofluor white on cell wall assembly is caused by its binding to chitin (Elorza *et al.*, 1983), we suggested that additional copies of *KNR4* might cause a decrease in chitin levels in *cwh* mutants. Hence, this would make these mutant strains less sensitive to calcofluor white, simply because they would have fewer binding sites for the drug. In addition, this suggestion was supported by the fact that deletion of *KNR4* led to a fourfold increase in chitin (Hong *et al.*, 1994b). Quantitative cell wall analysis showed that additional copies of *KNR4* actually lowered the chitin content in the wall of some, but not all, *cwh* mutants. We believe that this effect was not observed in all cases because the experiments were performed with cells grown in the absence of calcofluor white, whose presence is known to activate chitin polymerization (Roncero & Duran, 1985). Therefore, the decreased sensitivity of *cwh* mutants overexpressing *KNR4* could be due to its effect of counteracting the increase in chitin in mutants cultivated in the presence of calcofluor white. Unfortunately, this hypothesis could not be verified because of an inability to extract the sugar components of cell walls from yeast cultivated under this condition.

To further understand how *KNR4* could affect cell wall assembly, we studied the expression of key genes involved in biosynthesis of chitin and 1,3-β-glucan in yeast cells deleted for *KNR4* and in those containing additional copies of this gene. The results of these experiments showed that as the transcripts correspond-ing to genes encoding the three membrane-bound chitin synthases were severely reduced in cells containing at least one additional copy of *KNR4*, chitin levels were either reduced or unchanged. This was particularly the case with the *cwh53-1* mutant, as its very high content of chitin was not affected by overexpression of *KNR4*, despite a strong decrease in *CHS* transcripts. In contrast, the message levels of these three genes were not modified in a Δ*knr4* mutant, even though the chitin content in this mutant was increased fourfold. Moreover, deletion or overexpression of *KNR4* did not affect expression of *FKS1* and *RHO1*, two genes involved in 1,3-β-glucan

synthesis. Taken together, these results indicate that there is no proportional correlation between *CHS* mRNA and chitin levels, and that cell wall modifications induced by changes in the amount of Knr4p cannot be solely mediated by transcriptional effects on the genes encoding key enzymes in the biosynthesis of cell wall polymers. Furthermore, the transcriptional down regulation of *CHS* genes by *KNR4* does not seem to have important physiological relevance for chitin biosynthesis because the potent repression of *CHS3* expression in a *chs1 chs2* double mutant (ECY36-3C) by *KNR4* over-expression was not accompanied by any detectable effect on bud formation, cell morphology and vegetative growth. These data suggest that chitin biosynthesis is not merely regulated at the transcriptional level. A similar conclusion was given by Choi *et al*. (1994) who showed an absence of correlation between the activity of the three chitin synthases and the transcript levels of the three corresponding genes. However, if very low levels of *CHS* mRNAs, as we observed in the presence of excess of Knr4p, are sufficient for normal growth, why then would the expression of chitin-synthase-encoding genes need to be so high in wild-type cells? We propose that in normal growth conditions, an excess of transcription of *CHS* genes leads to deposition of enzymes in cytoplasmic microvesicles named chitosomes (Leal-Morales *et al*., 1988, 1994), most likely in an inactive (zymogenic) form. Just prior to bud formation, or in response to environmental signals such as pheromones, these vesicles deliver their chitin synthases to the plasma membrane where they become activated. This process could be part of the cell wall repair mechanism which is activated in response to cell wall weakening, and which is characterized by an increase in chitin and in the cross-linking between *β*-glucan and chitin (Kapteyn *et al*., 1997; Popolo *et al*., 1997; Daran *et al*., 1997).

Whilst additional copies of *KNR4* clearly reduced the expression of genes encoding the three chitin synthases, we did not find the product of this gene in the nucleus. Rather, using the powerful GFP technique allowing '*in vivo*' visualization (Chalfie *et al*., 1994; Niedenthal *et al*., 1996), we found the GFP–Knr4p at the neck between mother and daugther cells in the early stages of budding, and in patches near the presumptive bud site in unbudded cells. Further work will be necessary to investigate in more detail the localization of Knr4p during the life cycle and to compare it with that of other proteins involved in bud formation and chitin deposition (De Marini *et al*., 1997; Santos & Snyder, 1997). The non-nuclear localization of Knr4p in our experiments is in contrast with the data of Fishel *et al*. (1993) who isolated the same gene, designated *SMI1*, in a screen for proteins that bind MAR (matrix-associated regions) on DNA, and found an intranuclear localization of Smi1p homologues in animal cells by indirect immunofluorescence using SMI1-antipeptide antibodies. The reason for nuclear localization of this protein is not clear, unless the antipeptide antibodies raised against an 18 amino acid basic-rich region located at the C terminus of the yeast Smi1p could react artefactually with nuclear

material in animal cells. It is also not clear why these authors did not use these antibodies for immuno-fluorescence experiments in *S. cerevisiae*. We however can provide a convincing explanation of why *SMI1/ KNR4* was isolated in a search for MAR-binding proteins. The screening of mutants affected in this binding was based on a temperature-sensitive inducible colour enhancement on X-Gal plates. The major drawback of this genetic approach is to isolate mutant cells highly permeable to the chromophore. Yeast cells mutated at the *KNR4* locus actually displayed such a phenotype, even though quantitative assays of *β*-galactosidase performed on crude extract from *knr4* mutants and wild-type cells were identical (Hong *et al*., 1994a; our unpublished result). Another intriguing difference between our results and those of Fishel *et al*. (1993) was our inability to observe a thermosensitive growth arrest phenotype with different strains.

The localization of Knr4p at the incipient bud site and bud neck is in good agreement with the fact that *KNR4* was isolated by complementation of a mutant resistant to K9 killer toxin from *H. mrakii* (Hong *et al*., 1994a) whose cytocidal action has been shown to take place at the site of new bud appearance (Komiyama *et al*., 1996). In addition, this localization is very similar to that of proteins involved in chitin biosynthesis, including Chs3p, Chs4p and Bni4p (Santos & Snyder, 1997; De Marini *et al*., 1997), suggesting that Knr4p may be a component of this enzymic machinery. A main difference with these latter proteins is that Knr4p has an apparent symetrical distribution accross the neck. Such a localization makes this protein unlikely to act as a direct transcriptional repressor of *CHS* genes, but the fact that *KNR4* affected all three *CHS* genes reveals for the first time the occurrence of a common regulatory mechanism. One possible explanation is that an excess of Knr4p can titrate a factor required for transcriptional regulation of genes encoding chitin synthases. Alternatively, Knr4p could be a component of a complex regulator involved in global regulation of cell wall assembly. Lack of this protein would disorganize this complex protein, leading to a decrease in 1,3-*β*-glucan synthase activity and stimulation of chitin synthase, whereas an excess of this protein could have perturbating effects on as yet unknown components involved in the feedback regulation of genes encoding chitin synthases. Work is under way to investigate potential interactions of Knr4p with components of the chitin biosynthetic machinery and to unravel other potential partners.

ACKNOWLEDGEMENTS

from INRA (Institut National de la Recherche Agronomique), France. A.D. is a post-doctoral fellow from CNRS (Centre National de la Recherche Scientifique), France.

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