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Involvement of *GFA1*, which encodes glutamine–fructose-6-phosphate amidotransferase, in the activation of the chitin synthesis pathway in response to cell-wall defects in *Saccharomyces cerevisiae*

Arnaud Lagorce, Veronique Le Berre-Anton, Blanca Aguilar-Uscanga, Helene Martin-Yken, Adilia Dagkessamanskaia and Jean François

Centre de Bioingenierie Gilbert Durand, UMR-CNRS 5504, UR-INRA 792, Département de Génie Biochimique et Alimentaire, Complexe Scientifique de Rangeuil, Toulouse, France

Cell-wall damage caused by mutations of cell-wall-related genes triggers a compensatory mechanism which eventually results in hyperaccumulation of chitin reaching 20% of the cell-wall dry mass. We show that activation of chitin synthesis is accompanied by a rise, from 1.3-fold to 3.5-fold according to the gene mutation, in the expression of most of the genes encoding enzymes of the chitin metabolic pathways. Evidence that *GFA1*, which encodes glutamine–fructose-6-*P*hosphate amidotransferase (Gfa1p), the first committed enzyme of this pathway, plays a major role in this process was as follows. Activation of chitin synthesis in the cell-wall mutants correlated with activation of *GFA1* and with a proportional increase in Gfa1p activity. Overexpression of *GFA1* caused an approximately threefold increase in chitin in the transformed cells, whereas chitin content was barely affected by the joint overexpression of *CHS3* and *CHS7*. Introduction of a *gfa1-97* allele mutation in the cell-wall-defective *gas1*Δ mutant or cultivation of this mutant in a hyperosmotic medium resulted in reduction in chitin synthesis that was proportional to the decrease in Gfa1p activity. Finally, the stimulation of chitin production was also accompanied by an increase in pools of fructose 6-*P*hosphate, a substrate of Gfa1p. In quantitative terms, we estimated the flux-coefficient control of Gfa1p to be in the range of 0.90, and found that regulation of the chitin metabolic pathway was mainly hierarchical, i.e. dominated by regulation of the amount of newly synthesized GFA1 protein. In the search for the mechanism by which *GFA1* is activated in response to cell-wall perturbations, we could only show that neither *MCM1* nor *RLM1*, which encode two transcriptional factors of the MADS box family that are required for expression of cell-cycle and cell-wall-related genes, was involved in this process.

Keywords: cell wall; chitin; *GFA1*; metabolic control; *Saccharomyces cerevisiae*.

Yeasts and fungi are surrounded by a cell wall, which is a complex structure essential for maintenance of the cell shape, prevention of lysis and protection against harmful environmental conditions. The cell wall of the yeast *Saccharomyces cerevisiae* is a supramolecular structure composed of three classes of macromolecules, namely mannoproteins, β-glucans and chitin, which are interconnected by covalent bonds [1,2] and which accounts for 20–30% of the cell dry mass [3,4]. Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine residues, which only amounts to 1–3% of the cell wall dry mass, is indispensable for the survival of yeast cells [5]. The major part of chitin is localized as a ring at the base of the bud [6]. A small part of it is deposited in dispersed fashion in the lateral walls [5] and appears to be covalently linked to β-1,3-glucans and β-1,6-glucans [7]. These linkages may account for the presence of alkali-soluble and alkali-insoluble β1,3-glucans and β1,6-glucans [2,3]. Chitin synthesis is performed by three distinct chitin synthases encoded by *CHS1*, *CHS2* and *CHS3*. The three isoenzymes differ in enzymatic properties and are spatially and temporarily regulated (for a review, see [5]). However, Chs3p activity is responsible for the synthesis of ≈90% of chitin during vegetative growth of yeast cells [8,9]. This enzyme requires the products of the *CHS4*, *CHS5*, *CHS6* and *CHS7* genes for optimal activity, with Chs7p being the most important in the enhancement of the '*in vitro*' activity of Chs3p [10–13]. Moreover, it has been shown that Chs3p is temporarily sequestered in chitosomes, which are endosomal structures distinct from vacuoles and mature secretory vesicles [14,15]. Although the biological significance of this structure is still unclear, it appears that the optimal function of Chs3p may depend on proper Chs3p synthesis, transport and localization [5,13].

Yeast cells live in environmental conditions that can weaken their walls, so they have developed mechanisms for cell-wall rearrangement to combat cell lysis. Consistent with this idea, mutations in genes involved in the biosynthesis of β-1,3-glucans, mannans, or cell-wall component assembly are accompanied by dramatic changes in the molecular architecture of the wall [1]. A well-established event resulting from these insults is a large increase in chitin formation, which can reach up to 20% of the cell wall dry mass

Correspondence to J. M. François, Centre de Bioingenierie Gilbert Durand, UMR-CNRS 5504, UR-INRA 792, Département de Génie Biochimique et Alimentaire, Complexe Scientifique de Rangeuil, 31077 Toulouse, France. Fax: + 33 5 61 55 9400, Tel.: + 33 5 61 55 9492, E-mail: fran_jm@insa-tlse.fr

Abbreviations: Gfa1p, glutamine–fructose-6-*P*hosphate amidotransferase; Chs, chitin synthase.

Note: a web page is available at http://www.insa-tlse.fr/gba/

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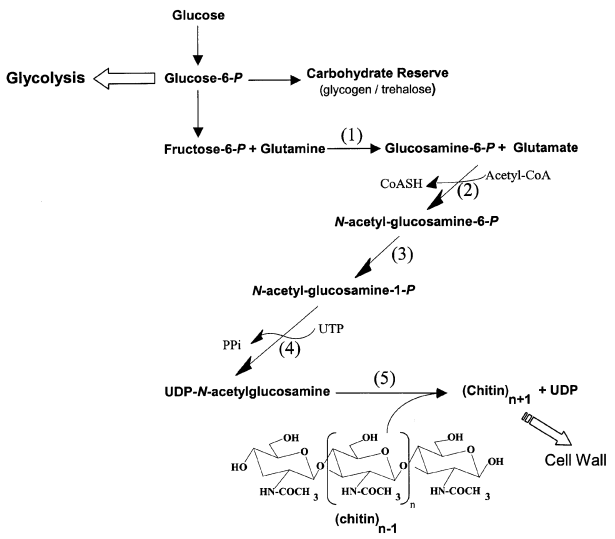

Fig. 1. Interaction of the chitin synthesis pathway with glycolysis and cell-wall synthesis. (1) glutamine–fructose 6-phosphate amidotransferase encoded by *GFA1*; (2) glucosamine phosphate *N*-acetyltransferase encoded by *GNA1*; (3) phospho-*N*-acetylglucosamine mutase encoded by *AGM1*; (4) UDP-*N*-acetylglucosamine pyrophosphorylase encoded by *UAP1*; (5) chitin synthase encoded by *CHS1*, *CHS2*, *CHS3*.

[4,16–18]. Hence, the activation of chitin synthesis probably contributes to the strength of the cell wall, and is a general compensation mechanism activated by cell wall damage.

As illustrated in Fig. 1, the chitin metabolic pathway consists of five steps, initiated at the level of fructose 6-*P*hosphate and glutamine by the glutamine–fructose-6-*P*hosphate amidotransferase (Gfa1p) encoded by *GFA1* [19]. From studies carried out with mutants defective in the major β1,3-glucan synthase (*fks1*) or a major glycosylphosphatidylinositol-anchor cell-wall protein (*gas1*), it was proposed that the increase in chitin in response to cell-wall defects succeeded activation of chitin synthase 3 encoded by *CHS3* [20,21]. However, this conclusion was hampered by inconsistencies in data on Chs3p activity and by the fact that it was not clear whether this putative activation of Chs3p resulted from a transcriptional or a post-translational effect. Moreover, one cannot draw the conclusion that there is major regulation of the chitin-biosynthetic pathway by Chs3p from the fact that loss of *CHS3* function prevents activation of chitin synthesis in these mutants. According to the metabolic control analysis theory, it is unlikely that a single step in a pathway is rate-limiting, i.e. the enzyme catalysing this step has a flux-coefficient control close to 1 [22]. However, it was previously reported that exposure of haploid yeast cells to mating pheromones caused an increase in chitin that was almost proportional to the rise in transcriptional activation of *GFA1* [23]. This finding suggests Gfa1p, which catalyses the first committed step of the hexosamine/chitin metabolic pathway, as an important controlling pacemaker of this pathway. We therefore investigated the importance of the amidotransferase in the activation of chitin synthesis in response to cell-wall defects in yeast.

MATERIALS AND METHODS

Construction of strains and plasmids

Yeast strains used in this work are listed in Table 1. Strain JF292 was used as the recipient for transformation with episomic plasmids YEpGW42 (YEp352::*GFA1* [19]), pRS424::*CHS3* (gift from C. Roncero, Salamanca, Spain) or pAL2 (pRS425::*CHS7*, this study). To construct pAL2, the 4.3-kb *Pvu*I–*Pvu*I fragment containing *CHS7* was cut from pTM14 (pRS426::CHS7 [13]) and cloned into a *Pvu*I site of pRS425 plasmid. The *rlm1*::*LEU2* mutant (strain JF1409) was obtained by transformation with the 4.0-kb

Table 1. List of yeast strains used in this work.

Strain	Genotype	Source or reference
X2180-1A	*MAT α MNN9 SUC2 mal mel gal2 CUP1*	ATCC
MNN9	*S288c mnn9*	ATCC
W303-1A	*MAT a ade2-1 ura3-1 leu2,3-112 trp1-1 his3-11,15 can1-100*	This study
JF1409	W303-1 a *rlm1*Δ::LEU2	This study
AR27	*MATα ura3-52*	[62]
HM10	*AR27 knr4:: KanMX4*	[26]
CWH48	*AR27 cwh48 (kre6)*	[62]
FY834	*MATα his3*-Δ200 ura3-52 leu2Δ1 lys2Δ202 trp1Δ63	[18]
CWH53	*FY834 cwh53 (fks1)*	[62]
W303-1B	*MATα ade2-1 ura3-1 leu2,3-112 trp1-1 his3-11,15 can1-100 GAS1*	[17]
WB2d	*MATα ade2-1 ura3-1 leu2,3-112 trp1-1 his3-11,15 can1-100, gas1*Δ::LEU2	[17]
8534-8A	*MAT a leu2-3112 ura3-52 his3-11,15 MCM1*	[61]
JF1382	*MAT a leu2-3112 ura3-52 his3-11,15 MCM1 gas1*Δ::LEU2	This study
RM1-6C	*MAT a leu2-3112 ura3-52 his3-11,15 mcm1-1*	[61]
JF1383	*MAT a leu2-3112 ura3-52 his3-11,15 mcm1-1 gas1*Δ::LEU2	This study
CH1305	*MAT α leu2-3112 ura3-52 his3 trp1 ade2 ade3 GFA1 GAS1*	[47]
JF1007-97	*MAT α leu2-3112 ura3-52 his3 trp1 ade2 ade3 gfa1-97 GAS1*	[47]
JF1408	*MAT α leu2-3112 ura3-52 his3 trp1 ade2 ade3 GFA1 gas1*Δ::LEU2	This study
JF1411	*MAT α leu2-3112 ura3-52 his3 trp1 ade2 ade3 gfa1-97 gas1*Δ::LEU2	This study

*Hin*dIII–*Sac*I fragment of the plasmid pRLM1::LEU2 (gift from D. Levin, Baltimore, MD, USA) in the W303-1A strain. The *mcm1-1 gas1*Δ::LEU2 (JF1383) and *gfa1-97 gas1*Δ::LEU2 mutant (strain JF1411) were obtained by transformation with a 5.4-kb *Nde*I–*Nco*I fragment of the plasmid pBSG-7L13 (gift from L. Popolo, University degli Studi, Milan, Italy) in strain RM1-6C (gift from Bik-Tye, Cornell University, Ithaca, NY, USA) and JF1007-97 (gift from J. Cannon, University of Missouri, Minneapolis, MN, USA), respectively. Transformation of yeast was carried out by the lithium acetate method of Schietsl & Gietz [24]. Genetic manipulation of yeast cells for sporulation and tetrad dissection was carried out as described in [25].

Growth conditions and phenotypic tests

Yeast cells were grown in YEPD (10 g yeast extract, 20 g bactopeptone, and 20 g glucose per litre) or on SD medium (0.17 g yeast nitrogen base without amino acids and ammonium, 0.5 $g{\cdot}L^{-1}$ ammonium sulfate, 20 g glucose per litre and supplemented with auxotrophic requirements). Unless otherwise stated, the growth was performed at 30 °C. For cell-wall extraction, metabolite and enzymatic assays, yeast cells were collected at the mid-exponential phase of growth (D_{600} between 0.6 and 1.0, which corresponded to 1–1.5 10^7 $cells{\cdot}mL^{-1}$). For phenotypic tests on different drugs, i.e. Calcofluor White, Congo red, aminoglucoside G418 and killer toxin K1 or HM1 from *Hansenula mrakii*, yeast cells grown in liquid SD supplemented with auxotrophic requirements or YEPD medium in late exponential phase ($D_{660} \approx 2.0$). Cells were harvested, washed once with sterile water, and resuspended in sterile water at D_{660} of 10. Serial dilutions of 1 : 1, 1 : 10, 1 : 100 and 1 : 1000 were spotted on solid medium containing the drugs at different concentrations, and prepared as described previously [26]. Growth on plates was scored after 2 days of incubation at 30 °C.

RNA analysis

Total RNA was extracted from exponentially growing cells (D_{600} = 0.6) as described by Hauser *et al.* [27] using RNeasy Midi Kit (Qiagen), separated on formaldehyde/agarose gels (30 μg per lane) as described by Siderius *et al.* [28] and transferred to Nylon-N^+ filters (Amersham Pharmacia Biotech). Each ORF of interest was amplified by PCR using commercial gene-specific oligonucleotides (purchased from Research Genetics, Huntsville, AL, USA) and genomic DNA isolated from yeast strain CENPK113-7D [29]. The PCR products were gel-purified (Qiaquick from Qiagen) and labelled with [α-^{32}P]dATP using a random-priming method (Megaprime™ DNA labelling system; Amersham Pharmacia Biotech). Unincorporated radioactivity was separated from the probe on a MicroSpin™ S-200 HR column (Amersham Pharmacia Biotech). Blots were prehybridized in 7% SDS/0.5 M Na_2HPO_4/NaH_2PO_4 (pH 7.2)/1 mM EDTA at 65 °C. Labelled probes were heat-denatured, quickly cooled on ice, and added to prehybridized filters. Hybridization was carried out overnight at 65 °C. The filters were first washed once briefly in 0.1% SDS/1% NaCl/Cit at room temperature and washed again (2 × 10 min) in 0.1% SDS/1% NaCl/Cit at room temperature. *PDA1* transcript was used as a loading control. Northern blots were quantified with a phosphofluoroimager STORM 860 (Molecular Dynamics) using the ImageQuant software from Molecular Dynamics (Amersham Biotech).

Assay of Gfa1p

Exponentially growing cells were collected by centrifugation, washed once with cold 20 mM Hepes buffer, pH 7.1, containing 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethanesulfonyl fluoride, and resuspended in 1 mL of the same buffer. Cell extract was obtained by vigorously shaking the yeast cell suspension on a vortex mixer in the presence of 1 g glass beads (0.45–0.55 mm diameter) for six periods of 30 s with intervals of 30 s on ice. The extract was centrifuged for 10 min at 4 °C, and, unless otherwise stated, the supernatant (0.2 mL) was loaded on to a Sephadex G-25 column (1 × 25 cm) equilibrated with the same buffer to remove small metabolites before the enzymatic assay.

Gfa1p was assayed in a total volume of 3.6 mL in a buffered solution containing 20 mM Hepes, pH 7.1, 75 mM KCl, 1 mM EDTA, 12 mM glucose 6-*P*, 4 mM fructose 6-*P* and 4 mM glutamine. The reaction was initiated by the addition of 100–500 μL gel-filtered cell extract. The reaction was stopped by mixing 0.5 mL of the reaction with 0.5 mL water in a bath maintained at 90 °C for 3 min. After the mixture had cooled down, glucosamine 6-*P* was determined by the colorimetric procedure modified from Ride & Drystale [30] as follows. To 250 μL of the assay reaction were added 250 μL $NaNO_2$ solution (5%, w/v) and 250 μL $KHSO_4$ solution at 5% (w/v). The mixture was shaken for 15 min. Then, 250 μL $NH_4SO_3NH_2$ solution at 12.5% (w/v) + 250 μL 3-methyl-2-benzothiazolone hydrazone hydrochloride solution at 0.5% (w/v) were added, and this new mixture was incubated in a boiling water bath for 3 min After the mixture had cooled down, 250 μL of a $FeCl_3$ solution at 0.5% (w/v) was added, and the suspension allowed to stand at room temperature for 30 min before the A_{650} was read.

Glucosamine 6-*P* was quantified using a calibration curve constructed with standard solutions of glucosamine 6-*P*. Both the calibration curve and reagents were prepared daily. The enzymatic activity is expressed as the amount of the enzyme that catalyses the production of 1 μmol glucosamine 6-*P* per min under these assay conditions.

Protein concentration in cell extracts was measured by the Bradford Coomassie-dye method using BSA as a standard.

Analytical procedures

Extraction, acid hydrolysis of yeast cell wall, and determination of β-glucans, mannans and chitin by high-performance anionic exchange chromatography (HPAEC) were carried out as described by Dallies *et al.* [4]. Chitin was also determined using chitinase from *Serratia marcescens* (purchased from Sigma) following the procedure of Bulawa *et al.* [31], and the *N*-acetylglucosamine residues released by the enzymatic hydrolysis were quantified by a colorimetric method [32]. Collection of yeast cells and metabolite extraction were as described previously [33]. Glycolytic intermediates (glucose 6-*P*, fructose 6-*P*, ATP) were measured by conventional spectrofluorimetric techniques [34].

Table 2. β-Glucans, mannans and chitin content in cell walls of wild-type and cell-wall-defective mutants as determined by glucose, mannose and glucosamine liberated after acid hydrolysis, and determination of the chitin content by measurement of glucosamine after enzymatic treatment on whole cells with commercial chitinase. The values reported are the mean ± SD from three independent experiments.

				Cell wall components [μg·(mg cell dry mass)$^{-1}$]			
				Chitin		Mannan and β-glucans as	
Strains	Genotype	Growth rate (h^{-1})	Cell wall mass (% of dry mass)	Enzymatic digestion	Acid hydrolysis	Mannose	Glucose
AR27	Wild-type	0.37	24	3.3 ± 0.30	4.8 ± 1.0	88 ± 3.0	144 ± 6.0
CWH48	*kre6*	0.28	22	15.8 ± 1.5	17.40 ± 1.8	100 ± 5.0	97 ± 6.0
HM10	*knr4*Δ	0.30	22	12.5 ± 1.5	14.6 ± 3.0	129 ± 6.0	75.6 ± 5.0
FY834	Wild-type	0.36	23	5.80 ± 0.5	6.3 ± 1.20	82 ± 3.5	148 ± 3.0
CWH53	*fks1*	0.24	17	17.7 ± 1.5	16.60 ± 1.5	96.5 ± 5.0	58.7 ± 3.5
S288C	Wild-type	0.39	24	4.40 ± 0.30	ND	82 ± 3.0	150 ± 3.0
MNN9	*mnn9*	0.23	18	20.4 ± 2.5	24.5 ± 2.017	11.4 ± 2.0	134 ± 4.0
W303-1B	Wild-type	0.36	24	3.80 ± 0.5	4.9 ± 1.5	93.3 ± 5.0	137.4 ± 5.5
WB2d	*gas1*Δ	0.22	17	ND	25.8 ± 2.0	ND	ND

RESULTS

Determination of chitin levels in yeast cell-wall mutants by two different methods

In a previous study, we reported that the chitin levels in *fks1* and *mnn9* mutants reached between 10–20% of the cell wall dry mass [4]. As this was estimated from the amount of glucosamine released after total cell-wall hydrolysis in hot H_2SO_4, it could not be excluded that part of the glucosamine came from the *N*-acetylglucosamine units present in the highly abundant N-mannosylated proteins of the cell wall [35]. To resolve this, we compared these data with those obtained with the specific enzymatic assay developed by Bulawa *et al.* [31] using the commercial chitinase from *S. marcescens*. As can be seen in Table 2, levels of chitin obtained with the enzymatic assay were actually 7–12% higher than those determined by the H_2SO_4 method. Data from Table 2 also confirmed other previous findings that defects in β-glucan led to increased levels of mannan, clearly illustrating the readjustment of the cell-wall carbohydrate composition in response to cell wall mutations.

Induction of genes related, or not, to cell-wall metabolism in response to cell-wall mutations

To understand the mechanism that promotes chitin activation in response to cell-wall damage, we focused our analysis on hexosamine and chitin metabolic-related genes. Genes with significantly increased expression (*i.e* more than twofold over the wild-type) in the different mutants included *GFA1* and *AGM1*, which encode, respectively, Gfa1p and *N*-acetylglucosamine-6-*P* mutase [19,36], and *CHS1* and *CHS3*, which encode two of the three chitin synthases (Fig. 2). Other genes, including *CHS4* and *CHS7* which encode regulatory proteins of Chs3p, or *GNA1* and *UAP1,* which encode, respectively, the glucosamine 6-*P* acetyltransferase [37] and UDP-*N*-acetylglucosamine pyrophosphorylase [38], were either weakly modified (Fig. 2) or not modified at all (not shown). A fairly good correlation between levels of *GFA1* and *CHS3* transcripts with those of chitin in the different cell-wall mutants could be drawn (Fig. 3), whereas such a correlation was not as good as for *AGM1* and *CHS1* (not shown). In quantitative terms, it could be seen that the fold rise in the transcripts of *GFA1* coincided much better than that of *CHS3* with the fold increase in chitin in the cell-wall mutants tested.

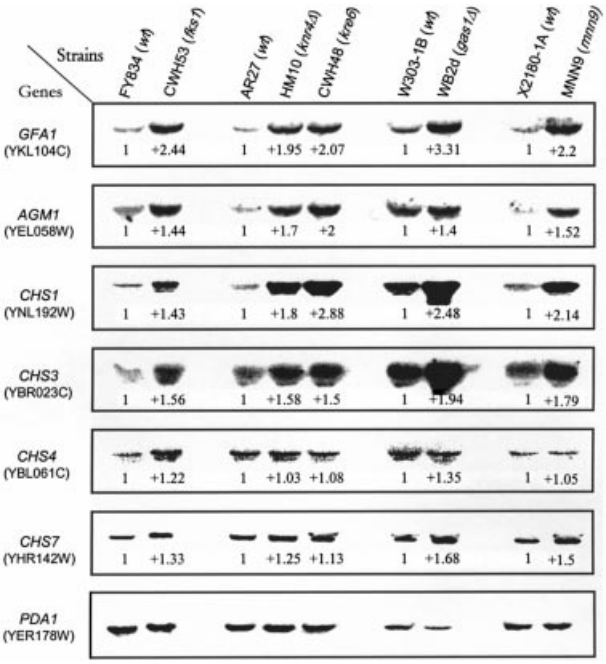

Fig. 2. Effect of mutations in cell-wall-related genes on the expression of genes involved in the hexosamine and chitin metabolic pathways. Yeast strains were cultivated in YPD to early exponential phase ($D_{660} = 0.6$). Cultures were harvested for RNA extraction and Northern-blot analysis as described in Materials and Methods. *PDA1* mRNA was used as the internal reference. The values gave the fold changes in the transcript compared with *PDA1* mRNA taken as the internal standard as determined by phosphoimager analysis.

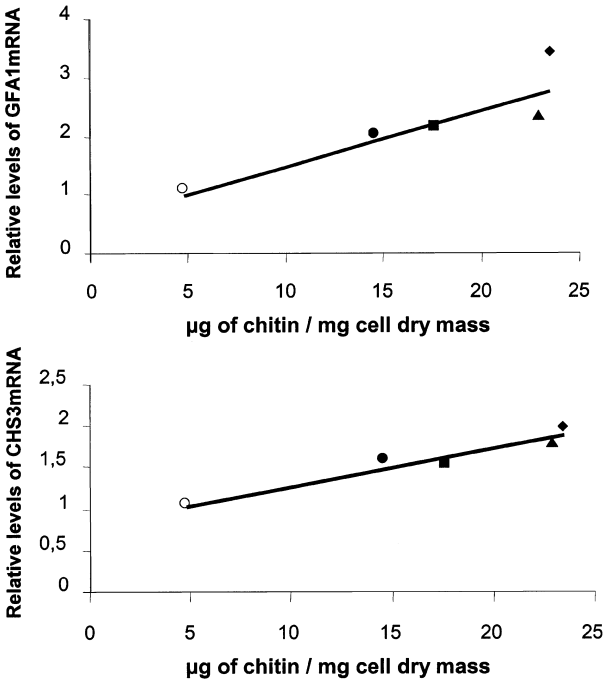

Fig. 3. Correlation between transcripts levels of *GFA1*, *CHS3* and chitin content in cell-wall-defective mutants. (○) Control strains (AR27, FY834, X2180-1a, W303-1B); (●) HM10 (*knr4*Δ); (■) CWH48 (*kre6*); (▲) MNN9 (*mnn9*); and (◆) WB2d (*gas1*Δ) mutant strains. The relative mRNA levels of *GFA1 and CHS3* were calculated from the ratio between signal intensity of *GFA1 and CHS3* mRNA compared with that of *PDA1* mRNA taken as the reference.

As observed in response to various stress situations [39,40], expression of genes involved in the cell-wall integrity pathway and not directly related to cell-wall function were also modified in these mutants. Although a genomic-scale analysis in response to cell-wall defects will be presented elsewhere (A. Lagorce & J. François, unpublished), we report in Fig. 4 a typical example of some upregulated

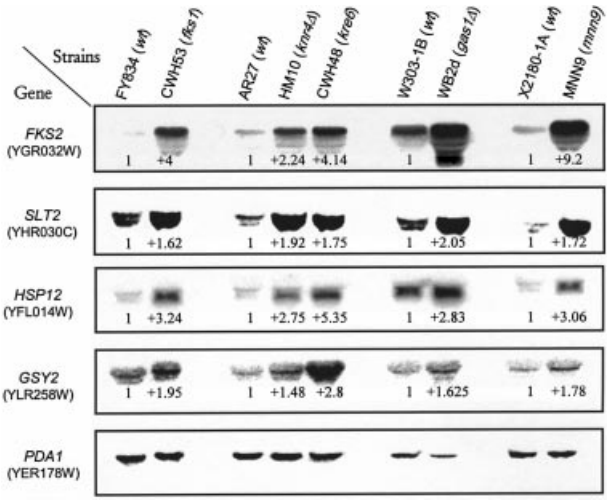

Fig. 4. Effect of mutation of cell-wall-related genes on expression levels of genes related to cell-wall integrity pathway (*SLT2*, *FKS2*) and in genes not related to cell-wall function (*HSP12* and *GSY2*). The experiment was as in Fig. 2. *PDA1* mRNA was used as the internal reference.

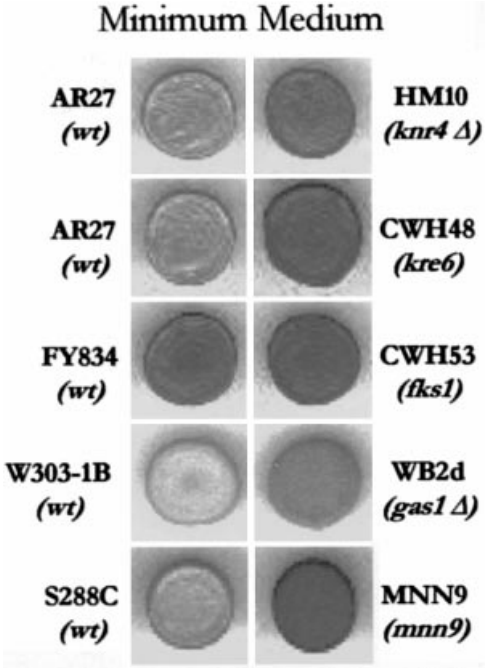

Fig. 5. Levels of glycogen in wild-type and cell-wall-defective mutants. Exposure to iodine vapour was made after 2 days of growth of the different yeast strains on an SD agar plate containing 2% glucose and supplemented with auxotrophic requirements.

genes. With regard to cell-wall function, we found a potent upregulation of *FKS2*, a gene that encodes the minor subunit of β-1, 3-glucan synthase [41], and an approximately twofold increase in the expression of the *SLT2* gene, which encodes the ultimate mitogen-activated protein (MAP) kinase of the PKC1-MAP kinase pathway [42]. These results are consistent with previous studies showing that the *PKC1-SLT2* cell-wall integrity pathway is activated in response to cell-wall perturbations, such as a temperature shock [39], or treatment of the cells with Calcofluor White or β-glucanase [43]. Expression levels of genes probably related to general stress responses, such as *HSP12*, which encodes a heat shock protein, and *GSY2*, which encodes the major isoform of glycogen synthase, were also dramatically increased in response to cell-wall defects (Fig. 4). Interestingly, the activation of *GSY2* was confirmed by an increase in glycogen levels, as judged by coloration of the colonies under iodine vapour (Fig. 5).

Activation of chitin synthesis by overexpression of *GFA1*

It is well accepted that Chs3p encoded by *CHS3* is responsible for ≈90% of chitin formation during vegetative growth [8]. Recently, it was shown that the deletion of *CHS3* in *gas1* or *fks1* null mutants that otherwise hyper-accumulate chitin led to a severe reduction in this polymer [20,21]. However, it cannot be inferred from these data that the activation of chitin synthesis in these cell-wall-related mutants was due only to an increase in Chs3p activity. This consideration is actually consistent with the finding that a very marginal increase in chitin was measured in cells that jointly overexpressed *CHS3* and *CHS7* (Table 3 and [13]), and that the additional presence of *CHS4* did not improve

Table 3. Levels of chitin and activity of Gfa1p in yeast strain JF292 transformed by multicopy plasmids bearing *CHS3*, *CHS7* or *GFA1*. The values reported are the mean ± SD from three independent experiments.

JF292 cells transformed with	Corresponding genes	Chitin za[μg·(mg dry mass)$^{-1}$]	Chitin Fold change	Gfa1p activity [mU·(mg protein)$^{-1}$]
pRS424	None	9.5 ± 1.20	1	28 ± 3.0
pRS424::CHS3	*CHS3*	13.2 ± 2.45	1.38	25 ± 3.0
pRS425	None	8.8 ± 0.95	1	23 ± 4.0
pAL2	*CHS7*	8.25 ± 1.20	1	29 ± 3.0
pRS424 + pRS425	None	10.9 ± 0.85	1	25 ± 3.0
pRS424::CHS3 + pAL2	*CHS3* + *CHS7*	13.70 ± 1.45	1.25	28 ± 4.0
YEp352	None	9.5 ± 0.80	1	25 ± 3.0
YepGW42	*GFA1*	25.2 ± 2.40	**2.65**	**160 ± 6.0**
pRS424 + YEp352	None	10.10 ± 1.12	1	23 ± 3.0
pRS424::CHS3 + pYEpGW42	*CHS3* + *GFA1*	24.9 ± 2.45	**2.46**	**165 ± 6.5**
YEp352 + pRS425	None	10.40 ± 1.0	1	26 ± 3.0
pGFA1 + pCHS7	*GFA1* + *CHS7*	22.0 ± 1.50	**2.20**	**175 ± 8.9**
pRS424 + pRS425 + YEp352	None	10.40 ± 0.85	1	24 ± 3.0
pRS424::CHS3 + pAL2 + YEpGW42	*GFA1* + *CHS3* + *CHS7*	29.90 ± 2.5	**2.90**	**153 ± 7.52.90**

this effect very much ([13] and data not shown). Thus, because of the correlation between *GFA1* mRNA and chitin levels (Fig. 3), and on the fact that this gene encodes the enzyme that catalyses the first committed step of the chitin synthesis [19], we investigated the effects of overproduction of *GFA1* alone or together with *CHS* genes on chitin levels in yeast. As shown in Table 3 and Fig. 6, transformed cells with high-copy-number plasmids bearing *GFA1* exhibited fivefold higher Gfa1p activity, and this overproduction was accompanied by ≈ threefold increase in the chitin content and a hypersensitivity of the transformed cells to Calcofluor White. The overexpression of *GFA1* in combination with *CHS3* and *CHS7* only slightly improved these effects (Fig. 6). These results conclusively showed that the synthesis of chitin was dependent on changes in *GFA1* expression rather than changes in *CHS3* expression.

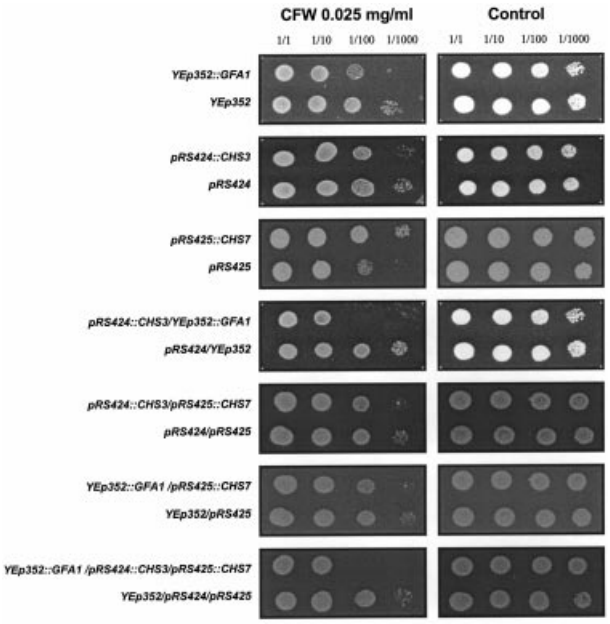

Fig. 6. Calcofluor White sensitivity of strains transformed with multicopy plasmids bearing *GFA1*, *CHS3* or *CHS7* alone or in combination. The strain JF292 was transformed with YEp352::*GFA1*, pRS423::*CHS3* and pRS425::*CHS7*. Transformed cells were grown overnight in liquid SD medium supplemented with auxotrophic requirements until late exponential phase ($D_{660} = 2$). The cells were collected by centrifugation and resuspended in sterile water at $D_{530} = 8.0$. Serial dilution from 1 : 1 to 1 : 1000 of yeast cultures was spotted on SD agar plates in the absence or presence of 0.25 mg·mL^{-1} Calcofluor White. Growth was scored after 2 days at 30 °C.

Flux through the chitin metabolic pathway is mainly dependent on Gfa1p activity

Although a good correlation between transcript levels of *GFA1* and chitin content in the cell-wall mutants was obtained, the magnitude of changes in chitin was apparently superior to that of *GFA1* expression, suggesting additional effects at the post-translational level. We therefore measured Gfa1p activity in cell-wall mutants. As can be seen in Fig. 7, a good correlation ($r = 0.95$) between Gfa1p activity and the content of chitin in the different mutants could be drawn. As this correlation was very similar to that obtained between *GFA1* mRNA and chitin levels, it is very unlikely that any post-translational changes had occurred to this enzyme. This assumption was confirmed by the fact that Gfa1p in gel-filtered extract from wild-type and mutant cells exhibited similar kinetic properties, i.e. similar K_m values for glutamine (0.3–0.4 mM) and fructose 6-*P* (0.7–0.8 mM), and identical sensitivity to UDP and UDP-*N*-acetylglucosamine 6-*P* (data not shown). Moreover, various attempts to verify whether the activity of Gfa1p in a gel-filtered extract from wild-type or mutants could be modified by protein phosphorylation after incubation in the presence of ATPMg and cAMP, as has been shown for other microbial species [44,45], were unsuccessful (data not shown).

Finally, the '*in vivo*' rate of Gfa1p could also be affected by changes in substrate concentration, provided that the enzyme was not yet saturated by its substrates. Therefore, we determined levels of hexose phosphates in wild-type and

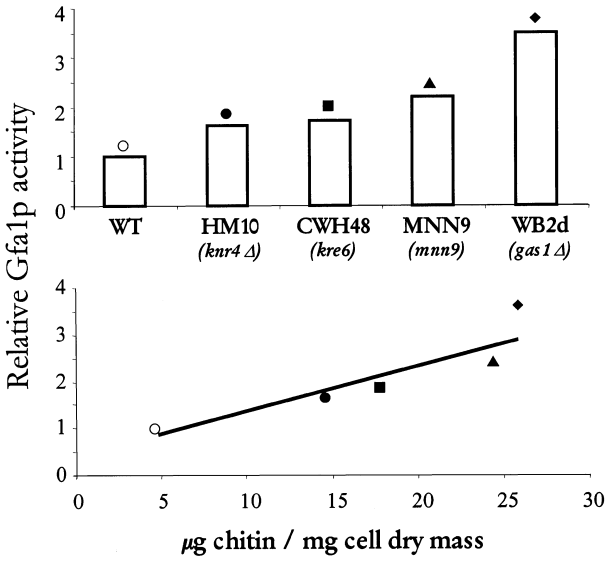

Fig. 7. Correlation between chitin levels and activity of Gfa1p in control and cell-wall-defective mutant strains. Yeast cells cultured on YEPD were harvested at the mid-exponential phase of growth ($D_{600} \approx 1.0$). (○) control strains (AR27, FY834, X2180-1A, W303-1B); (●) HM10 (*knr4*Δ); (■) CWH48 (*kre6*); (▲) MNN9 (*mnn9*); (◆) WB2d (*gas1*Δ) mutant strains. Gfa1p activity measured in the different control strains and from three independent experiments was 9 ± 2.0 mU·(mg protein)$^{-1}$. This value was set at 1.0, and the ratio between the activity of Gfa1p from cell-wall-defective mutant and that from the control gave an estimation of the fold increase in Gfa1p in the mutant strains.

in *gas1* and *mnn9* mutants. Interestingly, we found that the intracellular pools of fructose 6-*P* were about twofold to threefold higher in these two mutants than in the control strain (i.e. 0.7 mM in *gas1* and *mnn9* cells compared with 0.3 mM in the wild-type). As this concentration was in the K_m value of the glutamine–fructose 6-*P* amidotransferase (0.6–0.8 mM [19]), this rise in fructose 6-*P* may cause a further twofold increase in the rate of Gfa1p activity.

To further reinforce the notion that levels of Gfa1p affect chitin synthesis, we took another approach by investigating whether conditions known to reduce chitin levels were accompanied by a comparable reduction in Gfa1p activity, and reciprocally, whether a reduction in the latter enzyme caused a reduction in chitin content in the cells. With respect to the first situation, Turchini *et al.* [46] reported that the addition of 0.5 M KCl to the medium improved the growth of a *gas1* null mutant and that levels of chitin in this mutant cultivated in this hyperosmotic medium were reduced by 50%. We confirmed their findings and showed that the twofold reduction in chitin was associated with a proportional reduction in Gfa1p activity (Table 4). The second condition was to investigate the effect of introducing the *gfa1-97* allele mutation in a *gas1*Δ mutant on chitin levels. The justification of this experiment came from the fact that the *gfa1-97* mutant contains only 30% of the wild-type Gfa1p activity [47] (see Table 4). Accordingly, a *gfa1-97 gas1*Δ mutant contained about two times less chitin than a *gas1* mutant, and this reduction was accompanied by a twofold reduction in Gfa1p activity (Table 4).

Collectively, our data show that Gfa1p may be 'rate-limiting' in the process of chitin activation in response to cell-wall defects, or at least, using the modern terminology of Metabolic Control Analysis, that this enzyme has a high flux-coefficient control [22]. To satisfy this consideration, we calculated a control coefficient for Gfa1p using data from Table 2, assuming that the amount of chitin determined in the cell-wall mutants is a direct measure of the synthesis of the polymer during growth. Thus, the rate of chitin of production can be calculated by multiplying the content of chitin by the growth rate (μ). Taking into account this simplification, we plotted the logarithm of the rate of chitin production (*y* axis) against the logarithm of Gfa1p activity (*x* axis), and as described in [22], the tangent of the slope gives the coefficient control of the enzyme (Fig. 8A). We estimated a flux-coefficient control in the range of 0.90, indicating that this enzyme is indeed 'rate-limiting' in the process of chitin activation in response to cell-wall defects. Other information that we could get from data in Fig. 7 and Table 2 was to estimate whether regulation of the chitin metabolic pathway is hierarchical, i.e. dominated by regulation at the gene expression level, or metabolic, i.e. dependent on a change in effectors, substrates, or covalent modification of Gfa1p. To obtain this information, we followed the methods proposed by Ter Kuile & Westerhoff

Table 4. Correlation between activity of Gfa1p and levels of chitin. The values are from three independent experiments (with a standard deviation of less than 10%).

Strain	Genotype	Growth conditions	Chitin levels [μg glucosamine·(mg dry mass)$^{-1}$]	Gfa1p activity [mU·(mg protein)$^{-1}$]
WB2d	*gas1*Δ	YPD	24.6	28.35
WB2d	*gas1*Δ	YPD + KCl 0.5 M	15	16.0
[a]CH1305	*GFA1GAS1*	YPD	4.20	4.30
[a]JF1007-97	*gfa1-97GAS1*	YPD	3.80	1.38
[a]JF1408	*GFA1gas1*Δ	YPD	9.8	9.50
W303-1A	*RLM1*	YPD	4.20	9.0
JF1409	*rlm1*Δ	YPD	4.80	9.0
88534-8A	*MCM1*	YPD	4.8	10
JF1382	*MCM1gas1*Δ	YPD	22.8	25.5
JF1384	*mcm1-1 gas1*Δ	YPD	22.9	25.4

[a]Cultures were performed at 25 °C.

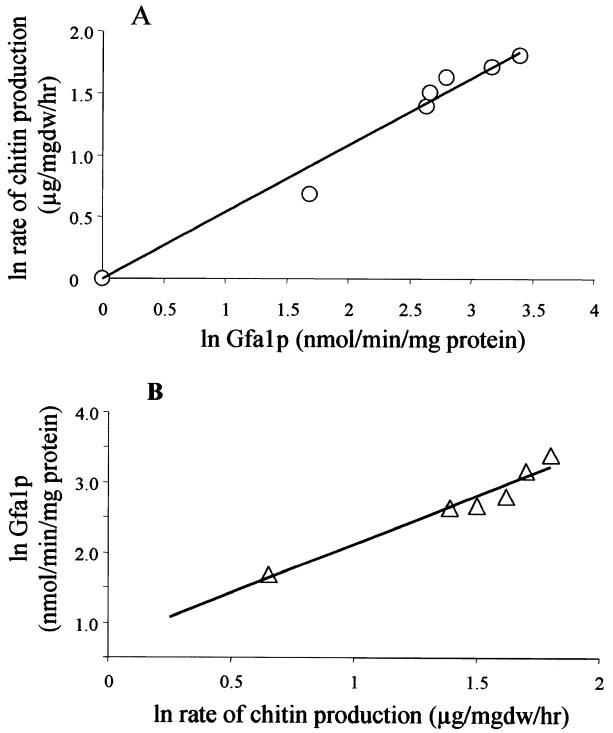

Fig. 8. Double-logarithmic plots of (A) rate of chitin production against activity of Gfa1p, used for the calculation of flux-coefficient control of this enzyme, and (B) Gfa1p activity against rate of chitin production in the different cell-wall mutants, used to quantify hierarchical and metabolic control coefficient. The line drawn in (A) represents the best fitted tangent to the hypothetical curve joining all the points. See refs [22,48] for further mathematical explanations.

[48] by plotting the logarithm of Gfa1p activity against the logarithm of the rate of chitin production in the mutants (Fig. 8B). The slope of this plot provided what the latter authors have called 'the hierarchical regulation coefficient ρ_h'. We estimated a ρ_h of ≈ 0.94, which indicates that the activation of chitin synthesis in response to cell-wall mutations was mainly due to an increase in *GFA1* expression. The latter conclusion could be actually inferred from data in Fig. 3.

Transcriptional activation of *GFA1* in response to cell-wall damage does not depend on Mcm1 and Rlm1 transcription factors

Our data show that activation of chitin synthesis in response to cell-wall damage causes transcriptional activation of *GFA1*. It is well established that conditions that affect cell-wall integrity result in transcriptional activation of many cell-wall-related genes, as exemplified by genome wide analysis of a *fks1*Δ mutant defective in the major β-glucan synthase [49]. Most of these genes are activated by the Rlm1p transcription factor, one of the targets of the PKC1 cell-wall integrity pathway [50] and which is activated on cell-wall perturbation [1,42,43]. We therefore investigated whether the loss of *RLM1* function led to a reduction in chitin and *GFA1* expression. Because deletion of this gene was synthetically lethal with *knr4*, *gas1*, *fks1* or *mnn9* mutation ([49], and our unpublished data), we could only measure effects of *RLM1* deletion in wild-type cells, and we found that neither Gfa1p activity nor *GFA1* transcripts were modified by this mutation (Table 4). Alternatively, the activation of *GFA1* may be dependent on *MCM1*, encoding a MADS-box transcriptional factor involved in numerous cell functions including mating and cell cycle [51], as well as in the expression of *GFA1* at the transition of M to G1 phase of the cell cycle [52]. As the loss of *MCM1* function is also lethal in yeast [53], we had to rely on a *mcm1-1* allelic mutation to investigate this hypothesis. As seen in Table 4, neither a *mcm1* mutation alone (not shown) nor the introduction of this mutation in a *gas1*Δ mutant had any effect on chitin levels or Gfa1p activity.

DISCUSSION

This study was conducted in order to understand the mechanism by which chitin synthesis is dramatically increased in response to cell-wall damage. In a previous report [4], we showed that chitin levels could reach up to 20% of the cell wall dry mass in a *mnn9* mutant lacking an α-1,6-mannosyltransferase [35]. As the original procedure to quantify chitin was based on the measurement of total glucosamine released after H_2SO_4 treatment, we suspected that part of this product might arise from the *N*-glucosamine residues from the N-linked glycosylated proteins, which amount to $\approx 40\%$ of the cell wall dry mass in wild-type cells and the levels of which are also affected in cell-wall mutants. In comparing the data obtained with the acid method with those of the specific enzymatic method, we found that chitin was 7–12% higher with the latter method. This result was actually consistent with our previous data suggesting that the recovery of chitin after acid hydrolysis was ≈ 85–90% [4]. Therefore, one can conclude that the enzymatic procedure is the preferred method for a quantitative and reliable measurement of the chitin content in the cells.

The genes and their products involved in the metabolic pathways leading to chitin formation have been characterized. It is now well established that Chs3p encoded by *CHS3* is the major enzyme participating in the formation of chitin during vegetative growth [5,8]. Activity of this enzyme required the presence of at least four other gene products. Chs4p is needed for anchoring of Chs3p to the septin ring [10], Chs5p is involved in the polarized transport of Chs3p in specialized vesicles [12], Chs6p is required for Chs3p transport at a later stage than Chs5p [54], and, finally, Chs7p regulates Chs3p activity by controlling its export from the endoplasmic reticulum [13]. Of these four regulatory proteins, the joint overproduction of Chs3p together with Chs7p led to a fourfold increase in the activity of Chs3p as measured *in vitro*. However, this activated form of Chs3p was accompanied by a very marginal increase in chitin ([13]; this work), suggesting that the provision of UDP-*N*-acetylglucosamine, the substrate of the chitin synthases, could be limiting in activation of chitin synthesis. In favour of this hypothesis, we showed that cells transformed with a multicopy plasmid bearing *GFA1*, which encodes the first committed enzyme of the hexosamine pathway, led to a threefold increase in chitin and to hypersensitivity of transformed cells to Calcofluor White. Furthermore, we found that the increase in chitin in the different cell-wall-

related mutants was associated with an almost proportional increase in *GFA1* mRNA and Gfa1p activity. This finding suggests that Gfa1p is the major controlling step in the chitin metabolic pathway. Indeed, we could estimate that the flux-coefficient control of Gfa1p was in the range of 0.90. Such a high value for a single enzyme in a metabolic pathway has been rarely obtained [22,55], although this estimate should be treated with caution because we assumed steady-state production of chitin during growth. However, several experimental data argue in favour of Gfa1p being the 'rate-limiting' step in chitin synthesis, such as the reduction in chitin levels by modification of the growth medium being accompanied by a proportional decrease in Gfa1p activity, and reciprocally, a lowering of Gfa1p causing a proportional reduction in chitin content.

The fact that the correlation between transcriptional activation of *GFA1* and the rise in chitin were not strictly quantitative suggests that there may be an additional control either at this stage through post-translational effects or at a step further downstream in the chitin pathway. We therefore investigated possible modifications of Gfa1p activity by means of protein phosphorylation, on the basis that enzymes from the yeast pathogen *Candida albicans* [44] and the aquatic fungus *Blastocladiella emersonii* [56] are subjected to this metabolic control, and by measuring levels of fructose 6-*P*, a substrate of Gfa1p. With respect to the first issue, we were unable to find any post-translational effect on Gfa1p by protein phosphorylation. In contrast, the fact that fructose 6-*P* levels in *gas1* and *mnn9* mutants were about twice as high as in wild-type, and considering the K_m value of Gfa1p for this substrate, one could estimate an actual '*in vivo*' rate of Gfa1p activity in *gas1* and *mnn9* mutants that was about 4–5 times higher than in the control strain. This rate was roughly comparable to the 4–5-fold stimulation of chitin synthesis measured in these cell-wall mutants. Another way to quantitatively distinguish between the amount of regulation through synthesis of new Gfa1p (the so-called 'hierarchical regulation' characterized by the coefficient ρ_h') and regulation at the metabolic level by changes in effector concentration and catalytic activity of the enzyme (the so-called 'metabolic regulation' characterized by the coefficient ρ_m') is to use the recent methods and terminology proposed by Ter Kuile & Westerhoff [48]. Following their proposal, we found that ρ_h was close to 1.0, indicating that regulation of the chitin metabolic pathway is largely hierarchical.

These explanations, however, do not exclude the possibility that steps downstream of the Gfa1p reaction may be involved in chitin activation, and, in particular, an increase in Chs3p could be welcome in promoting this process. Consistent with this idea, we observed that the increase in *CHS3* mRNA was in the same trend as that of *GFA1* in the different cell-wall mutants investigated. Transcriptional activation of *CHS3* has been reported by Terashima *et al.* [49] in their genomic-wide screening of genes upregulated in a *fks1*Δ mutant, and also by Hughes *et al.* [57], who employed DNA chips to investigate expression profiling in several stress conditions and cell-wall-related mutants. Interestingly, these authors found that *GFA1 and CHS3* transcripts were coregulated with a correlation coefficient close to 0.83. Other authors [20,21] did not find any change in *CHS3* transcripts in a *fks1*Δ and in *gas1*Δ mutants and emphasized the fact that the increased synthesis of chitin may result from greater transfer of Chs3p activity resulting from delocalization of Chs3p from septa towards the cell surface [21]. Whatever the exact mechanism leading to an increase in Chs3p activity, our data indicate that activation of chitin synthesis in response to cell-wall damage may be largely explained by a push–pull mechanism resulting from a concomitant increase in the rate of the first enzyme, Gfa1p, and the last enzyme, Chs3p, of the chitin metabolic pathway.

One major objective of this work is to understand the molecular mechanism that underlies the transcriptional activation of *GFA1* and *CHS3* as well as of the large spectrum of genes in response to cell-wall damage. The cellular responses have been regarded as a compensatory mechanism to maintain cell-wall integrity [1]. Work from Levin's group [58] provided evidence that the PKC1-MPK1/SLT2 signalling pathway is essential in the preservation of cell integrity. Using an allelic mutation of *MPK1/SLT2* that causes hyperactivation of the SLT2 kinase activity, they claimed that almost all of the upregulated genes were dependent on the Rlm1p transcription factor. A genome-wide analysis of gene expression in a *fks1*Δ mutant also showed upregulation of certain genes, including *GFA1*, that were apparently dependent on Rlm1p, and a DNA-binding consensus for this factor was inferred from consensus alignment in the promoter of *GFA1* [49]. Our data do not contradict this assumption, although deletion of *RLM1* in a wild-type strain did not affect Gfa1p activity or chitin levels. Therefore, our results only show that the basal expression of *GFA1* is not controlled by *RLM1*, but they do not exclude the possibility that the latter factor may be necessary for *GFA1* activation in response to cell-wall damage. Although the PKC1-MPK1/SLT2 pathway plays an important role in cell integrity, we also found upregulation and downregulation of many genes not known to be regulated by this signalling pathway. Many of them are known to be targets of nutrient-signalling pathways (e.g. protein kinase A, high osmolarity glycerol pathways [59]), the expression of some of them have been shown to be controlled by Msn2/Msn4p and Hsf1p regulons [60], and some are activated by as yet unknown transcriptional factors (A. Lagorce & J. François, unpublished). These results indicate the existence of cross-talking between different signalling pathways to optimize the response, and thus to preserve the integrity of the cell in response to external aggression.

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