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Flocculation of *Saccharomyces cerevisiae* is induced by transformation with the *GAP1* gene from *Kluyveromyces marxianus*

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A non-flocculent strain of *Saccharomyces cerevisiae* was transformed with the *GAP1* gene which encodes p37, a GAPDH-like protein present in the cell wall of *Kluyveromyces marxianus* flocculent cells. The transformed cells were characterized with respect to flocculation behaviour, morphology, growth, cell wall integrity and GAPDH activity. A flocculent phenotype was acquired by the transformed cells, showing a behaviour in respect to flocculation/deflocculation very similar to that of *K. marxianus*. The presence of p37 in the cell wall was assessed by immunoprecipitation of biotinylated cell wall proteins and an accumulation of p37 was evident in the cell wall of transformed cells. This result was confirmed by studies using a chimeric protein resulting from fusing the p37 with a yeast-enhanced green fluorescent protein, yEGFP. The recombinant protein was localized mainly in the cell wall of the transformed strain, although the presence of p37 in the cytosol was indicated by an increase in GAPDH activity. Calcofluor white sensitivity tests indicated that the cell wall structure is affected by the accumulation of p37. These results provided further evidence of p37 function regarding flocculation and that although lacking a N-terminal signal peptide p37 is targeted to the cell wall. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS — flocculation; *Saccharomyces cerevisiae*; *Kluyveromyces marxianus*; cell wall proteins; GFP

INTRODUCTION

Flocculation of *Saccharomyces cerevisiae* is generally reported as a reversible process of cell aggregation that involves protein–carbohydrate interactions between specific lectins, like surface proteins and the cell wall mannan (Teunissen and Steensma, 1995); this model is supported by the fact that protein denaturation and the presence of sugars can influence flocculation ability (Stratford and Assinder, 1991; Sieiro *et al.*, 1995, 1997).

The complexity of cell wall architecture and composition, as well as its adaptation to culture and environmental conditions (reviewed in Kapteyn *et al.*, 1999), accounts for the fact that the underlying molecular mechanisms of flocculation are still unclear. So far, 33 genes have already been reported to be involved in flocculation or cell aggregation (Teunissen and Steensma, 1995), and different putative flocculins have been identified (Stewart *et al.*, 1976; Holmberg, 1978).

Three dominant flocculation genes have been found by classical genetics: *FLO1*, *FLO5* and *FLO8* (Johnston and Reader, 1982, 1983; Yamashita and Fukui, 1983; Stratford and Assinder, 1991) and recently, two more possible flocculation dominant genes (*FLO9* and *FLO10*) were identified in *S. cerevisiae* (Teunissen and Steensma, 1995). These genes, together with *FLO1*, *FLO5* and *FLO8*, are now considered the 'flocculin family' and encode for proteins with a high degree of homology (Bossier *et al.*, 1997; Teunissen and Steensma, 1995). In 1996, Lo and Draginis reported a new flocculin, encoded by *FLO11*, a yeast gene related to *STA* genes, and later Sieiro *et al.* (1997) cloned a flocculation-conferring gene (*FLO2*) that was able to complement the *flo1* mutation.

Although the flocculation data reported above

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has been obtained using *S. cerevisiae*, there are several reports of similar studies on other yeast strains, such as *Hansenula anomala* (Saito *et al.*, 1990) and *K. marxianus* (Teixeira *et al.*, 1989; Fernandes *et al.*, 1992, 1993), leading to the identification of putative flocculins.

When *K. marxianus* is flocculent, a protein – p37 – accumulates in the cell wall, corresponding to 11% of the total wall proteins released by SDS, suggesting that this protein is involved in the interaction between adjacent cells leading to aggregation (Fernandes *et al.*, 1992, 1993). p37 purification and characterization revealed a high identity to cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fernandes *et al.*, 1992, 1995). Although it seems surprising to find a GAPDH-like protein in the cell wall, a similar situation was described in *Candida albicans* by Alloush *et al.* (1997) for 3-phosphoglycerate kinase and by Gil-Navarro *et al.* (1998) for GAPDH itself.

The involvement of p37 in flocculation was supported by the fact that a *K. marxianus* mutant, deficient in the synthesis of p37, is no longer able to flocculate (Falcão Moreira *et al.*, 1998).

Although p37 has been localized in the cell wall of *K. marxianus* (Fernandes *et al.*, 1992, 1993), the sequencing data of the *GAP1* gene (Fernandes *et al.*, 1995) revealed that the protein has no *N*-terminal leader sequence targeting it to the endoplasmic reticulum.

The present paper reports on the transformation of a non-flocculent strain of *S. cerevisiae* with the *GAP1* gene from *K. marxianus*. The results provide further evidence that p37 function is correlated with flocculation phenotype, since the transformant cells acquired the ability to flocculate. Furthermore, we show that p37 is incorporated in the cell wall of *S. cerevisiae*, although lacking a known targeting sequence. The role of the protein as a flocculin in *K. marxianus* and its capacity of inducing cell aggregation is discussed in the present work, providing evidence that mechanisms leading to flocculation of non-conventional yeast can be used to induce flocculation of *S. cerevisiae*.

MATERIALS AND METHODS

Strains and plasmids

The yeast strain of *S. cerevisiae* W303 1A (*leu*2–3, 112 *ura* 3–1, *trp*1–1, *his*3–11,15, *ade*2–1, *can*1–100) was used in this study. The *E. coli* strain employed was XL1 blue. The vector pYES2 is an autonomous shuttle vector containing the ampicilin resistance gene, the *URA3* gene and the *GAL1* promoter induced by galactose (Figure 1). The vector pGAPKm1 is a pBS^{TD} KS^{+} (Stratagene)-derived vector containing the coding region of *K. marxianus GAP1* gene, which encodes the p37 protein. pYPD–ADH–yEGFP3 is a pYPB–ADH–derived vector obtained by insertion of the

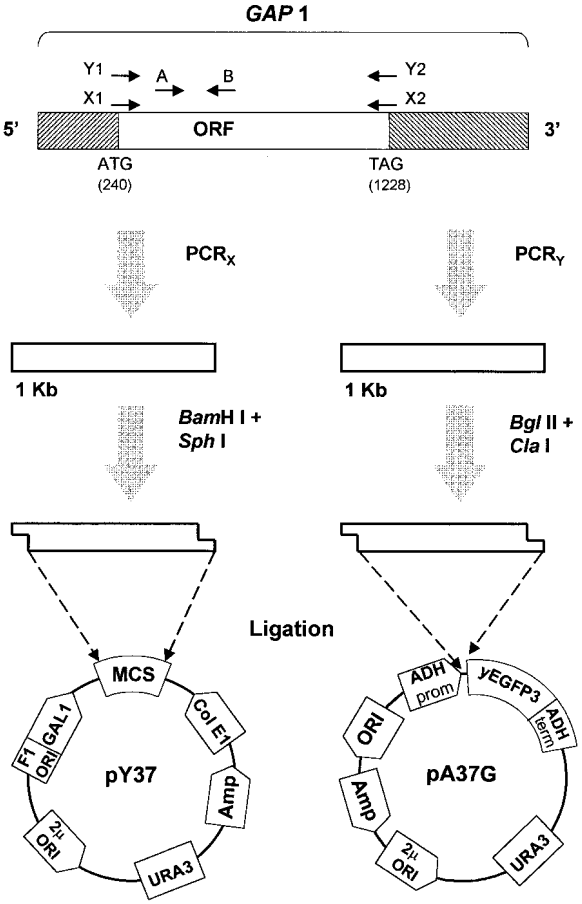

Figure 1. PCR$_X$: construction of p37 expression vector by insertion of *GAP1* gene encoding p37 in the multicloning site of pYES2. The amplification of *GAP1* gene with primers X1 and X2 resulted in a PCR product which, after digestion with *Bam*HI and *Sph*I, was ligated with the *Bam*HI/*Sph*I-digested pYES2 vector. PCR$_Y$: Construction of a p37–yegfp expression vector by insertion of *GAP1* gene encoding p37 in front of the *ADH* promoter and in frame with *yEGFP*, present in pYPB1–ADH–YEGFP. The amplification of *GAP1* gene with primers Y1 and Y2 resulted in a PCR product which, after digestion with *Bgl*II and *Cla*I, was ligated with the *Bgl*II/*Cla*I-digested plasmid.

yEGFP3 gene after the *C. albicans ADH1* promoter. The ADH promoter is a strong and constitutive yeast promoter and yEGFP is an EGFP gene codon-optimized for yeasts (Cormack *et al.*, 1997).

Cell growth conditions

E. coli cells were grown in LB (1% sodium chloride, 1% bactotryptone and 0.5% yeast extract) and transformed *E. coli* cells were grown in LB supplemented with ampicilin (50 μg/ml) for selection.

S. cerevisiae were grown in minimal medium (2% galactose, 0.67% Yeast Nitrogen Base) supplemented with leucine (0.008%), histidine (0.004%), tryptophan (0.004%), adenine (0.004%) and, for wild-type cells, uracil (0.004%). Plates used in the calcofluor white sensitivity tests were 1.5% agar, 2% galactose, 2% bactopeptone, 1% yeast extract, supplemented with calcofluor white (50 μg/ml). For the confocal microscopy experiments, cells were grown in 2% glucose, 0.67% Yeast Nitrogen Base.

PCR

The PCR reactions were performed using 40 ng of plasmids and PCR primers: X1 (5′ AAAGGATCCAGATGGTTTCTATTGCTAT 3′); X2 (5′ GGAGCATGCCTAAGCAACGTGTTCGACCA 3′); Y1 (5′ TTTTAAAAGAGATCTATGGTTTCTATTGCTA 3′); Y2 (5′ AGGAAATAACATCGATAGCAACGTGTTC 3′); the positions of the primers are shown in Figure 1). A final volume of 100 μl was used and the conditions were as follows: 1 mM each primer, 100 mM Tris–HCL (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 200 mM dNTPs. One unit of AmpliTaq DNA polymerase (Perkin-Elmer) was added after an initial denaturation step of 10 min at 94°C, followed, in PCR_X, by 30 cycles of 94°C for 40 s, 60°C for 1 min and 72°C for 1 min and, in PCR_Y, by 30 cycles of 94°C for 40 s, 62°C for 1 min and 72°C for 2 min. The PCR products were analysed on a 0.8% agarose gel.

Construction of p37 expression vectors

For the overexpression of p37 in *S. cerevisiae*, a pYES2-derived expression vector was constructed (Figure 1). *GAP1* gene, encoding p37, was amplified by PCR reaction of the plasmid pGAPKm1 with primers X1 and X2. Since these primers included *Bam*HI (X1 primer) and *Sph*I (X2 primer) restriction sites, the PCR product was digested with these enzymes and subsequently inserted in the *Bam*HI/*Sph*I digested pYES2 vector. The new vector was designated pY37.

For the expression of the chimeric protein p37–yegfp, the *GAP1* gene was amplified by PCR reaction of the plasmid pGAPKm1 with primers Y1 and Y2. Since these primers included a *Bgl*II (Y1 primer) and *Cla*I (X2 primer) restriction sites, the PCR product was digested with these enzymes and subsequently inserted into the pYPD–ADH–yEGFP3 vector. The *GAP1* gene was, in this way, inserted in front of the ADH promoter, before the yEGFP3 gene and in frame with the last. The new vector was designated pA37G (Figure 1).

Plasmids were prepared using the PROMEGA Minipreps DNA Purification System.

Bacteria transformation

E. coli transformation was performed using the $CaCl_2$ method (Ausubel *et al.*, 1987). Transformed cells were plated in LB medium with ampicillin (for selection), solidified with 1.5% Bacto-agar.

Southern blot and hybridization

The DNA (plasmids or PCR products) was separated in a 0.8% agarose gel and transferred onto Hybond-N nylon membrane (Amersham) under alkaline conditions (Sambrook *et al.*, 1989). The DNA was covalently bound to the membrane by UV irradiation and subsequently prehybridized for 1 h at 65°C with 6× SSC, 5× Denhardt's solution, 0.5% SDS and 20 mg/ml herring sperm DNA. The blot was hybridized in the same solution at 65°C for 16 h using as probe the ^{32}P-labelled PCR fragment obtained from the amplification of the initial part (350 bp) of the coding region of *GAP1* with primers A (5′ ATTCGCTCATTCAACTGGTTTCGG 3′) and B (5′ AGGCAGTCAGATGATGCTTTTATG 3′) and using as template the plasmid pGAPKm1. The positions of primers A and B are shown in Figure 1.

Yeast transformation

S. cerevisiae cells were transformed by the electroporation method (Ausubel *et al.*, 1987). Transformed cells were plated in uracil-deficient minimal medium supplemented with sorbitol 1 M,

leucine, histidine, tryptophan and adenine, and solidified with 1.5% Bacto-agar.

Quantification of biomass and flocculation measurements

Aliquots (50 ml) of cell culture were filtered under vacuum using a preweighed filter (ME 25/41 ST membrane filter, Schleicher and Schuell). The cells were washed twice with sterile water and the filter was dried at 80°C for 24 h. The filter was cooled in a dessicator and weighed to determine the amount of biomass present, expressed in g/l.

A preculture was used to inoculate a culture at a concentration of 2×10^6 cells/ml in an Erlenmeyer flask plugged with cotton wool on a shaker at 100 rpm. Cells were grown for 3 days at 26°C. The cells were harvested by centrifugation ($2500 \times g$, 4°C, 5 min), washed four times with distilled water and finally suspended in order to have an absorbance of 1.5 at 640 nm. The test tubes, containing the cell suspension, were sealed and rotated upside down on a mechanical device for 15 min. After agitation, the tubes were allowed to stand undisturbed for 5 min and 2 ml of the suspension were pipetted and its absorbance measured at 640 nm. After a further 10 min, the procedure was repeated (adapted from Kihn *et al.*, 1988).

Immunoprecipitation of biotinylated cell wall proteins

Yeast cells wall proteins were biotinylated according to the method described in Mrsa *et al.* (1997). Subsequently the SDS-extractable proteins were obtained and immunoprecipitated as described previously (Fernandes *et al.*, 1992). The anti-p37 serum was produced in rabbit using recombinant protein expressed in *E. coli*.

Electrophoresis and blotting

The proteins were then analysed by SDS–polyacrylamide gel electrophoresis (PAGE) (12.5%), using the method described by Laemmli (1970). To visualize immunoprecipitated biotin-labelled proteins, they were blotted to nitrocellulose membranes and then detected with Streptavidin–horseradish peroxidase conjugate, as described in Mrsa *et al.* (1997).

Confocal microscopy

A preculture was used to inoculate a culture at a concentration of 2×10^6 cells/ml in an Erlenmeyer flask plugged with cotton wool on a shaker at 200 rpm. Cells were grown at 26°C until they reached the stationary phase. All preparations were observed with a BioRad MRC 600 confocal laser microscope.

Calcofluor white sensitivity tests

The tests were performed according to the method described by Ram *et al.* (1994), with the following modifications: cells were incubated in 5 ml minimal medium and incubated at 26°C until OD (640 nm) $=8$ was reached. Dilutions of $10\times$, $100\times$, $1000\times$ and $10\,000\times$ were prepared and 5 µl of each were spotted on a galactose (2%); bactopeptone (2%) and yeast extract (1%) agar plate containing 50 µg/ml calcofluor white. The plates were incubated at 26°C and growth was evaluated after 2 days of incubation (F.M. Klis, personal communication).

Enzyme assays

Cells were harvested at an OD (640 nm) of 1 and glyceraldehyde-3-phosphate dehydrogenase activity was estimated using the method described by Holland and Weshead (1973).

RESULTS

pY37 expression vector

Several lines of evidence indicate that the accumulation of p37 (encoded by *GAP1* gene) in the cell wall of *Kluyveromyces marxianus* is correlated with flocculation, namely from the fact that *gap1* mutant no longer exhibits a flocculent phenotype and the absence of p37 affects the cell wall structure (Falcão Moreira *et al.*, 1998). To determine whether the *K. marxianus* p37 protein in *Saccharomyces cerevisiae* would produce a flocculent phenotype, a vector for overexpressing p37 was constructed. This was performed by insertion of the ORF (open reading frame) of *GAP1* (1 kb) into the multicloning site of pYES2 vector, under the control of *GAL1* promoter (Figure 1). To confirm the subcloning of the *GAP1* gene in the pYES2 vector, the plasmid was prepared from *E. coli* and screened by PCR and Southern blotting. The PCR product (obtained using primers X1 and X2) was separated

on a agarose gel 0.8%, revealing the DNA fragment of the expected size of 1.2 kb. The constructed plasmid and its PCR product were then hybridized with a ^{32}P-labelled probe specific for the coding region of *GAP1*, and positive bands were visualized in both cases, indicating the presence of the ORF encoding p37.

Characterization of the S. cerevisiae *transformant*

S. cerevisiae cells were transformed by electroporation, either with the empty vector pYES2 or with the vector pY37, and the effects of *GAP1* overexpression in *S. cerevisiae* were characterized regarding flocculation behaviour, growth, biomass, cell wall integrity and GAPDH activity. All cells were grown in minimal medium supplemented with galactose.

Flocculation behaviour and effect of ion concentration The transformed *S. cerevisiae* acquired a stable flocculation phenotype, absent in *S. cerevisiae* wild-type cells or transformed with the empty vector. Considering 100% as the percentage of cells in suspension in the beginning of the flocculation measurements, the pY37-transformed *S. cerevisiae* had only 13% of cells in suspension after 15 min of rest, when grown in galactose-rich medium. In contrast, 70% of control cells (wild-type or transformed with the empty vector) are in suspension at that time.

The effect of Ca^{2+} was analysed and the flocculation measurements indicated that in the presence of the ion, pY37 transformants cell aggregation was increased and this effect is dependent to the calcium concentration (0, 5, 50, 150, 200 mM; Table 1). Wild-type cells and cells transformed with the empty vector both presented no alteration in the flocculation phenotype due to calcium presence. Deflocculation of transformed cells was only achieved with high concentration of EDTA (250 mM) and high mechanical agitation (above 200 rpm).

Cell growth and biomass Cell growth was estimated at 26°C and in minimal medium with galactose. The results concerning cell growth and biomass values of the *S. cerevisiae* wild-type cells or transformed with the empty vector were similar. With respect to the pY37-transformed cells, the results indicated that growth (Figure 2a) of these cells presented a longer lag phase, followed by a shorter exponential phase that led to relatively higher final biomass. The results obtained for biomass (Figure 2b) showed that the dry weight values of the pY37-transformed cells were 18% higher than those of control cells, at equivalent OD (640 nm) values.

GAPDH activity The results obtained in *K. marxianus* revealed that p37 contributes to the total GAPDH activity (Falcão Moreira *et al.*, 1998). Therefore, estimation of the total enzyme activity in the *S. cerevisiae* transformant was performed. Cytosolic GAPDH activity in the transformed *S. cerevisiae* was about 30% higher compared to control cells (wild type or transformed with the empty vector), which indicates that the p37 protein is synthesized in an enzymatic active form in these cells (Table 2).

p37 cellular localization

Immunoprecipitation of biotinylated cell wall proteins *S. cerevisiae* cell wall proteins were labelled with Sulfo-NHS-LC-Biotin, immunoprecipitated with anti-p37 serum and visualized via a streptavidin-mediated colour reaction. Using a biotinylation reagent that does not cross the plasma membrane of the cell, we were able to

Table 1. Percentage of cells still in suspension after 5 min of undisturbed rest in the flocculation measurements.

	Cells still in suspension (%) after 5 min				
Strain	Ca 0 mM	Ca 50 mM	Ca 100 mM	Ca 150 mM	Ca 200 mM
W	90±0.1	88±0.3	88±0.2	87±0.1	88±0.3
W+empty vector	89±0.2	86±0.5	88±0.2	85±0.3	86±0.1
G	40±0.2	32±0.1	30±0.3	24±0.2	21±0.1

Results are expressed as percentage of cells still in suspension±standard deviation and represent the average of eight independent experiments. Effect of calcium concentration on flocculation degree. W, wild-type *S. cerevisiae* cells; W+empty vector, empty vector-transformed *S. cerevisiae*; G, pY37-transformed *S. cerevisiae* (cells grown in minimal medium with galactose).

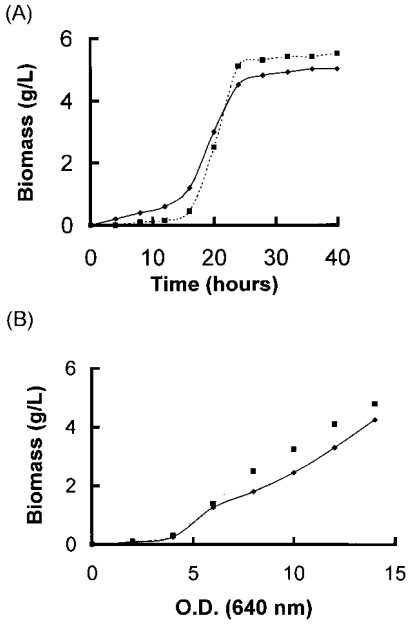

Figure 2. (A) Growth of *S. cerevisiae* wild-type (—) and transformed *S. cerevisiae* (- - -) in minimal medium with galactose at 26°C. Growth is expressed in biomass in relation to the time of growth of cells. (B) Wild-type *S. cerevisiae* cells' biomass (—) and transformed *S. cerevisiae* cells' biomass (- - -) in relation to cell $OD_{640\ nm}$. Biomass was measured in dry weight. The results are the average of four independent experiments. Results for cells transformed with the empty vector are identical to the ones obtained with the wild-type.

eliminate cytosolic contaminations in the protein extract. The results obtained showed the presence of p37 in the cell wall of transformed cells, whereas no protein could be detected in the wild-type cell walls (Figure 3).

Localization of p37 using a fusion with green fluorescent protein To confirm p37 presence in the cell wall, this protein was fused with yegfp. For that purpose, the GAP1 ORF (coding for p37) was inserted into the shuttle vector pYPB–ADH–yEGFP3 between the ADH promoter and the yEGFP ORF (Figure 1). After transforming *S. cerevisiae* cells with this vector, pA37G, we were able to locate the fusion protein p37–yegfp *in vivo* by confocal microscopy. As can be observed in Figure 4, fluorescence is mainly localized in the cell wall of the transformant cells expressing p37–yegfp, in contrast to control cells transformed with pYPB–ADH–yEGFP3. This accumulation of p37–yegfp seems not to be random, as dots of fluorescence appear throughout the cell wall. The cytoplasm of pA37G-transformed cells also stains, indicating the presence of the protein p37–yegfp.

Table 2. Assay of GAPDH activity in wild-type and transformed *S. cerevisiae*.

Strain	GAPDH activity/min/$mg_{total\ protein}$
W	0.62 ± 0.05
W + empty vector	0.59 ± 0.08
G	0.80 ± 0.06

Values are expressed as GAPDH activity/min/$mg_{total\ protein} \pm$ SD and represent the average of seven independent experiments. W, wild-type *S. cerevisiae* cells; W + empty vector, empty vector-transformed *S. cerevisiae*; G, pY37-transformed *S. cerevisiae* (cells grown in minimal medium with galactose).

Cell wall integrity

Calcofluor white sensitivity Calcofluor white is a negatively charged fluorescent dye that is used to detect cell wall mutants, due to its interference with cell wall assembly (Ram *et al.*, 1994). As the protein p37 accumulates in the cell wall, we addressed the question whether there was an alteration of the wall structure and indeed the results (Figure 5) show the transformed *S. cerevi-*

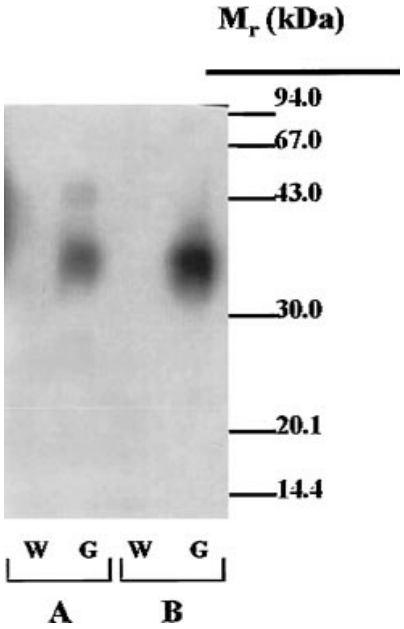

Figure 3. Immunoprecipitation of biotinylated cell wall proteins (A, 50 μg of total protein; B, 150 μg of total protein) of wild-type *S. cerevisiae* (W) and transformed (G) cells.

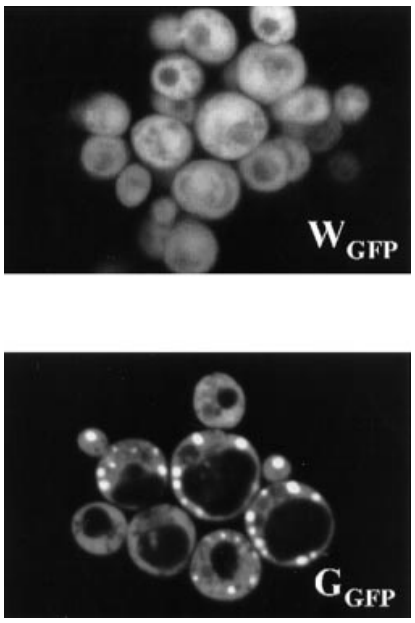

Figure 4. Detection of p37–yEGFP using confocal microscopy: W_{GFP}, W303 1A transformed with pYPB–ADH–yEGFP3; G_{GFP}, W303 1A transformed with pA37G.

siae cells exhibiting higher resistance to calcofluor white, compared with the effect on wild-type cells. *S. cerevisiae* cells transformed with the empty vector present a similar sensitivity to the drug as wild-type cells.

DISCUSSION

In *K. marxianus*, the protein, p37, has been associated with flocculation, as it is accumulated in the cell wall of cells grown in culture conditions that induce flocculation. Aiming at elucidating the role of p37 as a flocculin, the gene *GAP1* was introduced and over-expressed in a non-flocculent *Saccharomyces cerevisiae*. The p37 expression vector was constructed, inserting the *GAP1* open reading frame into the multicloning site of pYES2 vector, under the control of the *GAL1* promoter (Figure 1). The transformed cells acquired a flocculent phenotype, showing a behaviour in respect to flocculation/deflocculation very similar to that of *K. marxianus*, and the degree of flocculation is dependent on the presence of calcium in the culture medium. In *K. marxianus*, the calcium is essential to promote flocculation; however, in the transformed *S. cerevisiae* cells there is only a partial correlation between the amount of calcium present and the degree of flocculation (Table 1). The role of calcium in the aggregation of cells mediated by p37 is most likely to promote the contact and to establish interactions between cells, by neutralizing negative charges at the surface of the cell. The differences of composition/structure between cell walls of the two strains may explain the unequal effect of calcium in their flocculation behaviour.

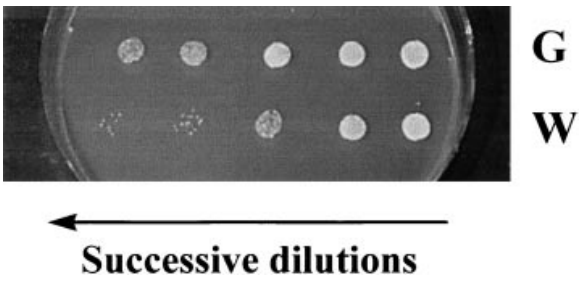

Figure 5. Wild-type (W) and transformed (G) *S. cerevisiae* cells grown on agar plates with 2% galactose, 2% Bactopeptone and 1% Yeast Extract, supplemented with calcofluor white (50 μg/ml). Cell growth of empty vector transformants is identical to wild-type cells.

Immunoprecipitation of biotinylated cell wall proteins showed an accumulation of p37 in the cell wall of transformed cells, whereas no protein could be detected in the wild-type cell walls (Figure 3). Confirmation of the presence of p37 in the cell wall was provided when p37 was fused with yegfp. Fluorescence is mainly detected in the cell wall of the transformant cells expressing the chimeric protein, in contrast to control cells transformed with pYPB–ADH–yEGFP3 (Figure 4). These results show that p37 incorporation in the cell wall occurs independently of the absence of a classical targeting signal. The mechanisms involved in its secretion have not yet been elucidated, as it also occurs for various glycolytic enzymes, found in the cell wall of *C. albicans* (Chaffin *et al.*, 1998) or secreted by *S. cerevisiae* (Pardo *et al.*, 1999).

The presence of p37 in the cell wall affects somehow the cell wall structure, as it is suggested by the calcofluor white (CFW) sensitivity tests: the transformed cells, when over-expressing p37, are more resistant to CFW (Figure 5). In contrast, *K. marxianus gap1* mutant cells are more sensitive to CFW than the wild-type (Falcão Moreira *et al.*, 1998). The association of a GAPDH-like protein with a yeast cell wall has also been reported by Gil-Navarro *et al.* (1998) and it was shown that this protein could interact with laminin and

fibronectin. Beside cell wall stability, p37 overexpression influences the behaviour of *S. cerevisiae* cells in respect to other parameters. The transformed cells present higher values of biomass (Figure 2), probably due to changes of the cell size, as transformed *S. cerevisiae* cells are larger than the wild-type. An altered morphology can be expected when a cell is genetically manipulated (Veenhuis *et al.*, 1978; Wright *et al.*, 1988; Binder *et al.*, 1991; Simon *et al.*, 1992; Vergéres *et al.*, 1993). The differences concerning growth and biomass between wild-type cells and pY37 transformants indicate that p37 presence affects the cell metabolism. The results are in agreement with the ones obtained previously with the *gap1* mutant, deficient in p37.

The enzymatic nature of p37 was confirmed, as GAPDH activity in the transformed cells was about 30% higher compared to that registered for the wild-type cells (Table 2), which is consistent with the results obtained with the *gap1* mutant (Falcão Moreira *et al.*, 1998) and with the fact that some fluorescence can be detected in the cytoplasm of cells expressing p37-yegfp.

In summary, there is a net correlation between the presence of p37 in the cell wall and the ability of cells to flocculate (either of *K. marxianus* or *S. cerevisiae*). The ubiquity of GAPDH has been the subject of several reports in the literature (Huitorel and Pantolini, 1985; Kawamoto and Caswell, 1986; Allen *et al.*, 1987; Meyer-Siegler *et al.*, 1991; Pancholi and Fischetti, 1992; Singh and Green, 1993; Robbins *et al.*, 1995; Gil-Navarro *et al.*, 1997) and the results presented in this paper clearly show that this GAPDH protein (p37) has other functions than its glycolytic enzymatic activity, as its oversynthesis influences growth, biomass yield, cell wall stability, cell morphology and aggregation. There is now evidence that p37 can induce cell aggregation in *Saccharomyces*, although it is a *Kluyveromyces* protein. The fact that there is no description of a *Saccharomyces* flocculin similar to p37 shows that this protein, which is involved in a mechanism that is specific for a non-conventional yeast such as *K. marxianus*, can be used as a 'tool' to induce flocculation in *S. cerevisiae*.

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