Down-regulation of the expression of *PKC1* and *SRB1/PSA1/VIG9*, two genes involved in cell wall integrity in *Saccharomyces cerevisiae*, causes flocculation

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The cell wall integrity determinants *PKC1* and *SRB1/PSA1/VIG9* of *Saccharomyces cerevisiae* were expressed under the control of the tightly regulated promoter *pMET3*. Substitution of the cell-cycle-regulated *SRB1/PSA1* native promoter with *pMET3* led to faster cell growth, larger cell volumes, and a twofold reduction of the steady-state *SRB1/PSA1* mRNA level. In addition, the new pattern of expression of *SRB1/PSA1* resulted in a dominant flocculation phenotype at all phases of batch growth. By contrast, expression of *PKC1* from *pMET3* increased the flocculation capacity of cells only at stationary phase. Methionine-mediated repression of either *PSA1/SRB1* or *PKC1* resulted in enhanced cell clumping. Cells in which both these genes had been replaced with their respective *pMET3*-regulated cassettes were highly flocculent under both expression and repression conditions. These results suggest that greater exposure of flocculin on the cell surface, caused by either cell wall distortion (through depletion of Pkc1p) or aberrant regulation of mannosylation (through constitutive production of Srb1p), results in an increased flocculation ability.

Keywords: *MET3* promoter, *SRB1/PSA1*, *PKC1*, cell wall, yeast flocculation

INTRODUCTION

The yeast cell wall is a dynamic organelle responsible for a number of cellular functions, the most important being physical and osmotic protection, selective permeability, and cell–cell recognition and adhesion during mating and flocculation (Stratford, 1994). Unlike adhesion in mating, which is induced by highly specific pheromones, flocculation is an asexual aggregation of cells which is a very useful characteristic in industrial yeast strains. Flocculation is exploited in fermentations such as beer-brewing, wine-making, and fuel ethanol production because it leads to efficient separation of cells from the fermentation liquor (Esser & Kues, 1983).

Two types of flocculation phenotypes have been described (Stratford & Assinder, 1991). The Flo1 type, caused by *FLO1/FLO5/FLO8*, is Ca^{2+} -dependent and

inhibited by mannopyranoses. The NewFlo phenotype, on the other hand, is prevented by both manno- and glucopyranoses. The *FLO1* gene, which is located on chromosome I, has been reported to encode a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein with its amino terminus exposed to the medium (Bony *et al.*, 1997; Straver *et al.*, 1994). *FLO5* is highly homologous to *FLO1* (Bidard *et al.*, 1994) and is also found on chromosome I. *FLO8*, previously mapped to chromosome I and said to be allelic to *FLO1* (Teunissen *et al.*, 1995b), has recently been reassigned to chromosome V and demonstrated to mediate flocculation via transcriptional activation of *FLO1* (Kobayashi *et al.*, 1996). More recently, a new flocculation gene, named *FLO2*, has been cloned and localized to chromosome XII; its function remains unclear, although it can complement *flo1* mutations (Sieiro *et al.*, 1997). Other genes, such as *TUP1* and *SNN6*, also act on yeast cell flocculation via transcriptional regulation (Teunissen *et al.*, 1995a).

Flocculation in *Saccharomyces cerevisiae* is thought to

Abbreviation: GPI, glycosylphosphatidylinositol.

be a result of interactions between lectin-like cell-surface proteins (termed flocculins), encoded by the *FLO* genes, and the cell wall mannan (Miki *et al.*, 1982; Stratford, 1992). This hypothesis is supported by the following findings: loss of flocculation capacity following protease treatment (Eddy & Rudin, 1958; Nishihara *et al.*, 1977, 1982), efficient dispersion of flocs by mannose and its derivatives (Mill, 1964; Taylor & Orton, 1978), and the failure of certain *mn* mutant cells to co-flocculate with flocculent cells (Stratford, 1992). So far, studies of flocculation have centred on the cloning and characterization of dominant flocculation genes and the elucidation of their transcriptional regulation. Less attention has been paid to the effect of changes of cell wall structure on flocculation, although it has been found that mutations in genes that encode proteins involved in cell wall synthesis, such as *KRE6* (Roemer & Bussey, 1991) and *CHS2* (Sudoh *et al.*, 1991), may also produce a flocculent phenotype in non-flocculating strains.

The major components of the yeast cell wall are glucans and mannoproteins. Glucan consists of two polymers: a large, linear 1,3- β -glucan and a smaller, highly branched 1,6- β -glucan moiety. *KRE* genes, identified by killer resistance, are involved in 1,6- β -glucan synthesis (Klis, 1994). The synthesis of 1,3- β -glucan is controlled by *RHO1* and the *PKC1* signal transduction MAP kinase pathway (Drzonova *et al.*, 1996; Qadota *et al.*, 1996; Cid *et al.*, 1995). Mannoprotein, the other major cell wall component, is a complex of proteins modified by the attachment, via *N*- and *O*-glycosidic bonds, of mannose-containing carbohydrate chains of different length and structure. It is these carbohydrate chains that act as receptors in flocculation (Stratford, 1992). Recently, *PSA1/SRB1* has been shown to encode GDP-mannose pyrophosphorylase (Hashimoto *et al.*, 1997; G. Tomlin, S. G. Oliver & L. I. Stateva, unpublished). This enzyme produces GDP-mannose, one of the main substrates for the biosynthesis of cell wall mannoproteins and GPI anchors (Abeijon & Hirschberg, 1992). Although *PKC1* and *SRB1/PSA1* may both be classified as morphogenes, they contribute to cell wall biogenesis in completely different ways. Null mutants of *PKC1* can grow in the presence of osmotic stabilizers, while a *SRB1/PSA1* null mutation is lethal. Loss of *PKC1* function by repressing its expression while under the control of the *GAL1* promoter results in a cell-cycle-specific osmotic stability defect (Levin & Bartlett-Heubusch, 1992), whereas loss of *SRB1/PSA1* function by inhibiting the expression of *PSA1* under the control of the *MET3* promoter, which depletes cells of a key substrate for protein modifications, has a very pleiotropic effect, leading to defects in bud growth, bud site selection, and cell separation, in addition to an increase in cell permeability and cell lysis (Zhang, 1997).

Here, we report that regulation of the expression of *PKC1* and *PSA1/SRB1* by replacing their own promoters with that of the *MET3* gene, has profound effects on flocculation.

METHODS

Strains and media. The *S. cerevisiae* strains used in this study, together with their genotypes and sources, are listed in Table 1. They consist of an isogenic set. The strains were grown in minimal medium (SD) prepared by the method of Kaiser *et al.* (1994). Nutritional supplements were added to the medium as necessary.

Plasmids and transformation. The plasmid *SRB1.9e*, containing *pMET3-SRB1/PSA1*, is based on *pRS316* (N. Zhang and others, unpublished). Yeast transformation was performed by the method of Hill *et al.* (1986).

Northern analysis. Yeast total RNA was extracted and separated on a 1.5% (w/v) denaturing agarose gel according to the protocol of Kaiser *et al.* (1994). Following blotting onto a nylon membrane (positively charged; Boehringer Mannheim), it was hybridized with the appropriate DNA probe labelled with [α -³²P]dCTP (10 mCi ml⁻¹, 1 Ci = 3.7 × 10¹⁰ Bq) using a Rediprime kit (Amersham Pharmacia Biotech). Pre-hybridization, hybridization and washing steps were carried out as described by Engler-Blum *et al.* (1993). After washing, the membrane was wrapped in Saran-Wrap and exposed to a Bio-Rad imaging screen (type BI) which was then developed with the Bio-Rad phosphorimager (GS-363) and analysed with Molecular Analyser software (Bio-Rad).

Methionine regulation of *pMET3* expression cassettes. Cultures of strains containing *pMET3*-regulated cassettes were grown in selective SD medium at 250 r.p.m. and 30 °C until OD₆₀₀ 0.05, at which point they were split into two halves. Methionine was added to one half of each culture, to a final concentration of 2 mM; to the other half, an equivalent volume of distilled water was added. The two cultures were then incubated under the same conditions as before.

Construction of isogenic wild-type strain ZO123. Used as the control in this study, the isogenic wild-type strain was constructed by transforming a PCR product containing the *PKC1* gene into strain ZO124. The *PKC1* coding sequence was PCR-amplified using YEplac195-*PKC1* as the template, a forward primer of sequence 5' GAAAGCGCCGCAAAA-GTAAGTATAGTATCAC 3', and a reverse primer of sequence 5' CCTGAGCTCCAAGATAGGTACGAACAAAA 3'. Those transformants which could grow without 10% (w/v) sorbitol, but could not grow without a leucine supplement, were further checked for correct integration. This was done by diagnostic PCR, using one oligonucleotide, 5' TGTCGATTGGTGGGCATTGGG 3', which anneals to *PKC1* but not the deletant sequence and another, 5' CGTC-ATGAAGTCTCGCGGATTTGATA 3', which anneals outside the *PKC1* fragment. This should give a 0.85 kb fragment, as is the case with ZO123 (data not shown).

Construction of ZO125. ZO125 is based on ZO123 with *pMET3-PSA1-LEU2* integrated at the chromosomal *PSA1* locus. *SRB1.9e* (N. Zhang and others, unpublished) was digested with *ApaI* and *Bst1107I*. The fragment containing the *PSA1* upstream region, *pMET3-PSA1-LEU2* and *PSA1* downstream region was transformed into ZO123. The correct integration was confirmed both phenotypically (by the inability of the transformants to grow in the presence of 2 mM methionine) and by diagnostic PCR, using primers which anneal to the *MET3* promoter sequence (5' ACGACAGAG-AGAGACCCAAG 3'), and outside the *MET3-PSA1-LEU2* fragment (5' CATGCTGCCTATGTTGCA 3'), respectively.

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

Yeast strains	Genotype	Source or reference
ZO123	<i>MATa his3 leu2 trp1 ura3</i>	This study
ZO124	<i>MATa his3 leu2 trp1 ura3</i> <i>pkc1::LEU2</i>	D. Levin
ZO125	<i>MATa his3 leu2 trp1 ura3</i> <i>psa1Δ::pMET3-PSA1-LEU2</i>	This study
ZO126	<i>MATa his3 leu2 trp1 ura3</i> <i>pkc1Δ::TRP1-pMET3-PKC1</i>	N. Zhang and others, unpublished
ZO127	<i>MATa his3 leu2 trp1 ura3</i> <i>pkc1Δ::TRP1-pMET3-PKC1</i> <i>psa1Δ::pMET3-PSA1-LEU2</i>	N. Zhang and others, unpublished

Table 2. Flocculation tests under different conditions

Strain and conditions	Relevant genotype	Cells flocculated (%)
ZO123, –Met	<i>PSA1, PKC1</i>	13
ZO123, +Met	<i>PSA1, PKC1</i>	15
ZO126, –Met	<i>pMET3-PKC1</i>	24
ZO126, +Met	<i>pMET3-PKC1</i>	54
ZO126, +Met, 1 M glucose	<i>pMET3-PKC1</i>	52
ZO126, +Met, 1 M mannose	<i>pMET3-PKC1</i>	0
ZO123+FY23, –Met		3
FY23, –Met		0
ZO125, –Met	<i>pMET3-PSA1</i>	66
ZO125, –Met, 1 M glucose	<i>pMET3-PSA1</i>	60
ZO125, –Met, 1 M mannose	<i>pMET3-PSA1</i>	0
ZO125, +Met	<i>pMET3-PSA1</i>	65
ZO123/SRB1.9e, –Met	<i>PSA1, pMET3-PSA1</i>	60
ZO127, –Met	<i>pMET3-PKC1</i> , <i>pMET3-PSA1</i>	97
ZO127, +Met	<i>pMET3-PKC1</i> , <i>pMET3-PSA1</i>	94

This diagnostic PCR amplification should result in a 1.1 kb band, which was exactly the case (data not shown).

The other two strains used in this study ZO126 and ZO127 (see Table 1 for genotypes) were constructed by N. Zhang and others (unpublished).

Flocculation measurements. A cell culture (100 ml) was grown in SD medium containing 2% (w/v) glucose for 48 h until stationary phase had been reached. A portion (50 ml) of this culture was placed in a 50 ml Falcon tube. Cells were collected by centrifugation at 5000 r.p.m. for 5 min. The medium was poured away and cells were washed once with 250 mM EDTA and twice with sterile water before resuspension in water to a final concentration of $1-4 \times 10^9$ cells ml⁻¹. An aliquot (1 ml) of this suspension was used for quantification of flocculation as described by Stratford & Assinder (1991). Sugar dispersal of flocs and co-flocculation were carried out according to Stratford (1993).

RESULTS AND DISCUSSION

pMET3 regulation of *PKC1* causes flocculation under conditions of methionine repression

Strain ZO126 (Table 1), carrying the *pMET3-PKC1* cassette, was grown until early exponential phase, when methionine (2 mM) was added to half the culture in order to repress the expression of *PKC1*. Incubation was then continued at 30 °C for a further 48 h. The other half of the culture was grown for the same time in the absence of methionine. Flocs formed in the stationary culture grown under repression conditions. Cells were harvested and flocculation was quantified as described in Methods. The wild-type strain ZO123, grown under similar conditions, with and without methionine, was included as a control. The results (Table 2) show that the wild-type cells had a very weak flocculation ability, irrespective of whether methionine was present or not.

Fig. 1. Flocs of ZO125 (pMET-PSA1) and ZO123 (PSA1) transformed by SRB1.9e (pMET3-PSA1) grown under expression conditions (–Met, no methionine). Samples (5 µl) of exponential-phase cultures grown under expression conditions (–Met) were pipetted onto a glass slide, covered by a cover slip and examined under a light microscope. The wild-type strain ZO123 was included as the control. (a) ZO123, (b) ZO125, (c) ZO123/SRB1.9e. Bar, 0.1 mm.

The flocculation ability of the strain in which *PKC1* is under the control of pMET3 was about 50% higher than that of the wild-type under expression (no methionine) conditions. However, this strain showed at least a three to four times greater ability to flocculate when methionine was added to repress the expression of *PKC1*. The flocs obtained under the repression condition could be dispersed by 250 mM EDTA and reversed by adding CaCl_2 , confirming that the aggregates were not induced by chain formation.

There are two types of flocculation phenotypes, Flo1 type, which can be inhibited by mannopyranoses, and

NewFlo type, which is prevented by both mannopyranoses and glucopyranoses (Stratford & Assinder, 1991). The flocs caused by *PKC1* repression could be dispersed by 1 M mannose but not by 1 M glucose (see Table 2), suggesting that *PKC1* repression-induced flocculation belongs to the Flo1 phenotype.

PKC1 has been shown to be involved in glucan biosynthesis (Cid *et al.*, 1995). Electron micrographs of the cell wall of a temperature-sensitive *pkc1* mutant show that the wall loses its normal organization at the restrictive temperature. At the permissive temperature, the wall consists of an inner transparent layer and an outer, dark-stained, mannoprotein layer. By contrast, at the restrictive temperature, the transparent layer almost completely disappears and the entire wall stains dark, suggesting that the inner (skeletal) layer of the wall has been affected (Paravicini *et al.*, 1992; Klis, 1994). Upon addition of methionine to a ZO126 culture, cells would be gradually depleted of Pkc1p. This is expected to result in much thinner inner cell walls, which should render the mannose side-chains in the incorporated mannoprotein and/or flocculin more accessible to each other, leading to a higher ability for flocculation.

If this flocculation was induced by more exposed mannose side-chains after *PKC1* was repressed, this would mean that the parent strain, ZO123, with normal *PKC1* expression, would have exposed flocculin on the surface and would co-flocculate with a wild-type non-flocculent strain. To test this hypothesis, the wild-type ZO123 cells were mixed with the standard laboratory strain, FY23, which has been shown to be non-flocculent due to absence of transcription of the main flocculation gene, *FLO1* (Liu *et al.*, 1996). ZO123 and FY23 cells were washed with 250 mM EDTA once, and with sterile water twice, and were then dispersed in 20 mM EDTA. An approximately equal number of ZO123 and FY23 cells were suspended in flocculation buffer and flocculation was induced by adding 50 mM CaCl_2 . These two strains did not co-flocculate (Table 2), suggesting that *PKC1*-repressed cells are characterized by a distorted cell wall which may allow greater exposure of the flocculins on the cell surface, leading to a greater ability for flocculation. Stratford & Carter (1993) showed that flocculins were synthesized early in cell growth, stored in the wall and activated many hours later. Flocculation could be activated artificially by a brief burst of heat, which may act by disturbing cell wall structure. Moreover, the fact that other cell wall mutants, such as *kre6* (Roemer & Bussey 1991) and *chs2* (Sudoh *et al.*, 1991), also display a flocculation phenotype supports the hypothesis that distorted cell wall exposes more flocculins on the cell surface and, as a result, enhances flocculation ability.

Expression of *SRB1/PSA1* under the control of the *MET3* promoter causes flocculation at all stages of the batch growth cycle

The growth of strain ZO125, harbouring the pMET3-*PSA1* cassette, in SD medium under expression con-

Strain and medium	<i>PSA1</i> transcript	<i>ACT1</i> transcript	Ratio (<i>PSA1/ACT1</i>)	Wild-type expression (%)
ZO123, -Met (1)	64068	7320	8.76	100
ZO125, -Met (2)	26924	7180	3.75	43
ZO125, +Met (3)	415	6756	0.06	0.7

Fig. 2. Northern analysis of *PSA1* transcription in ZO125 grown under expression (–Met) or repression (+Met) conditions; 15 µg RNA from each sample was used. *ACT1* was employed as the internal control. The *SRB1/PSA1* transcript signal was analysed by Molecular Analyser software (signals quantified in arbitrary units) and normalized against the *ACT1* signal to obtain the relative transcription level.

ditions (without methionine), was characterized by the following phenotypes. The culture was ‘flaky’ throughout batch growth. The flocculated cells at the exponential phase are shown in Fig. 1. This suggests that the expression of *SRB1/PSA1* from *pMET3*, unlike that of *PKC1* (above), leads to flocculation at any growth phase. ZO125 cells grew faster, with a doubling time of 1.9 h (whereas the doubling time of the wild-type strain ZO123 was 2.1 h) and appeared to have bigger cell volumes (Fig. 1), in comparison to the wild-type isogenic control, ZO123. When the cells from both strains were grown to stationary phase and subjected to the flocculation test, the cells carrying the *pMET3-PSA1* expression cassette had a five times higher ability for flocculation than the control strain (Table 2).

It has recently been shown that *SRB1/PSA1/VIG9* encodes the GDP-mannose pyrophosphorylase of *S. cerevisiae* (Hashimoto *et al.*, 1997) and so is responsible for providing one of the main substrates for the glycosylation of proteins, including cell wall mannoproteins. Benton *et al.* (1996) also reported that the transcription of this essential gene is cell-cycle regulated, with a peak of expression occurring at the G1 phase. The flocculation phenotype accompanying the expression of *SRB1/PSA1* under the control of *pMET3* could be due either to the loss of the normal cell-cycle regulation of expression, or to overexpression or underexpression. Therefore, we decided to study the level of transcription of *SRB1/PSA1* from *pMET3* in the absence of the repressor (methionine) and compare it with that from its native promoter. For this purpose, ZO125 and ZO123 were grown to mid-exponential phase and used for total RNA isolation. The RNA was subjected to standard Northern analysis, as described in Methods. As shown in Fig. 2, the transcript level of *SRB1/PSA1* from the *MET3* promoter is about half that from its normal promoter. These results permit us to rule out *SRB1/PSA1* overexpression as the reason for the flocculation phenotype.

The flocculation phenotype caused by *pMET3-PSA1* expression is dominant

We further addressed the problem of how *SRB1/PSA1* expression from the *MET3* promoter caused flocculation by investigating whether *pMET3-PSA1* led to flocculation in a wild-type strain. The plasmid *SRB1.9e*, which carries the *pMET3-PSA1* cassette in a centromeric vector, was transformed into the wild-type strain ZO123, which has a wild-type copy of *SRB1/PSA1* under the control of its own promoter. The transformants were grown in the same medium as that for ZO125, the strain containing an integrated copy of *pMET3-PSA1* at the *SRB1/PSA1* chromosomal locus. A flocculation phenotype the same as that of ZO125 was observed in all growth phases and the cell volume appeared larger compared with that of the wild-type (Fig. 1). The flocculation test, which was performed after the cells had been grown to stationary phase, showed that this transformant flocculates to the same degree as ZO125 (Table 2). This result confirmed that the flocculation phenotype caused when *SRB1/PSA1* is transcribed from *pMET3* is not due to the gene's underexpression but, rather, is the result of its constitutive expression. When *SRB1/PSA1* is expressed from its own promoter, its transcription level increases some four- to sixfold at START (Benton *et al.*, 1996). Thus the constitutive expression of *SRB1/PSA1* from *pMET3* would hyperactivate glycosylation at all other cell-cycle phases; it may well increase the expression of many genes, including flocculation genes, which in turn leads to enhanced cell growth and flocculation.

The flocs formed by constitutive *SRB1/PSA1* expression could be easily dispersed by 250 mM EDTA and reversed by adding CaCl_2 . Using sugars, we demonstrated (Table 2) that these flocs can only be inhibited by mannose but not by glucose, showing that this flocculation phenotype also belongs to Flo1 type. It would be interesting to determine whether the transcript levels of known *FLO*

genes are up-regulated. However, it has been found that expression of *pMET3-PSA1* in the non-flocculent strain FY23 did not lead to flocculation (Zhang, 1997). FY23 is a direct derivative of S288C (Winston *et al.*, 1995), a strain that has been reported to have a nonsense mutation in *FLO8* (Liu *et al.*, 1996) that, in turn, controls the transcription of the main flocculation gene, *FLO1*. Taken together, our results indicate that the interaction between *SRB1/PSA1* expression and the dominant flocculation genes is important in determining a flocculation phenotype.

Down-regulation of *SRB1/PSA1* strongly enhances flocculation

To quantify the effect of *SRB1/PSA1* repression on flocculation, ZO125 was grown under expression conditions (–Met) until early exponential phase; 2 mM methionine was then added and the culture grown for another 48 h before carrying out a flocculation test. As shown in Table 2, repression of *SRB1/PSA1* in ZO125 led to a fivefold increase in flocculation compared to wild-type cells. Repression or loss of *SRB1/PSA1* expression has also been found to result in flocculation in a number of other circumstances. For instance, loss of a plasmid-borne copy of the wild-type *SRB1/PSA1* gene from a *srb1-1* host strain has been found to cause clumping (G. Hamilton & L. Stateva, unpublished). Moreover, repression of *SRB1/PSA1* expression in the standard laboratory strain FY23 causes not only cell lysis, but also flocculation (Zhang, 1997).

Srb1p/Psa1p activity results in the production of GDP-mannose, an activated sugar that is involved, either directly or via the dolichol pathway, in all kinds of protein glycosylation, including GPI-anchor synthesis. The product of the dominant flocculation determinant, *FLO1*, has been shown to be a GPI-anchored cell-surface protein (Bony *et al.*, 1997). Thus, it is conceivable that repression of *SRB1/PSA1* results in a failure to incorporate Flo1p into the cell wall, or in its failure to function properly if incorporated. If this is the case, then the flocculation that occurs in the absence of *SRB1/PSA1* expression might be due to a different mechanism to that which operates when the gene is constitutively expressed. Ballou *et al.* (1980) have reported that mutants, such as *mn9*, that lack the outer polymannose chain on their mannoproteins grow slowly and display a clumpy morphology. Thus, the flocculation resulting from *SRB1/PSA1* repression could be due to a profound change in cell-surface characteristics, including hydrophobicity.

Flocculation ability of a double (*pMET3-PSA1*, *pMET3-PKC1*) mutant

Strain ZO127 (see Table 1), carrying both the *pMET3-PSA1* and *pMET3-PKC1* cassettes, was used to test flocculation under non-repressing (–Met) and repressing (+Met) conditions. Under both conditions, cells from this strain formed very large flocs (Fig. 3) at all

Fig. 3. Flocs of ZO127 grown under expression or repression conditions. Samples (5 µl) of exponential-phase cultures grown under (a) expression (–Met, no methionine) or (b) repression (+Met, 2 mM methionine) conditions were pipetted onto a glass slide, covered by a cover slip and examined under a light microscope. Bar, 0.1 mm.

growth phases. After growth for 48 h, cells were harvested and subjected to a flocculation test (Table 2). Around 95 % of the cells, from either the –Met or the +Met culture, formed large aggregates, which could be dispersed by 250 mM EDTA and reversed by adding CaCl_2 . This indicates that ZO127 cells have a higher ability for flocculation than those bearing the single cassettes (compare results in Table 2), suggesting that the flocculation effects resulting from *pMET3*-regulated *PKC1* and *SRB1/PSA1* expression or repression are additive.

The expression of morphogenes from their native promoters is subject to a range of controls that can regulate the synthesis of the cell wall in response to the demands of the cell cycle and the influences of stress and nutritional conditions. The replacement of the upstream regions of individual cell wall genes provides a means of investigating their dosage-dependent effects on cell wall functions, including flocculation. In this paper, we have demonstrated that regulation of *PKC1* and *SRB1/PSA1*

is directly linked to flocculation, one of the functions of the cell wall. These results suggest that morphogenes in yeast must obey cell-cycle controls whilst responding to environmental changes.

Flocculation provides a means of efficient separation of cells from the medium. However, most cell wall mutants with enhanced flocculation capacity are affected in cell growth or cell viability, like *kre6* (Roemer & Bussey, 1991) and *mn9* (Ballou *et al.*, 1980). The flocculation resulting from *SRB1/PSA1* expression from the *MET3* promoter is particularly interesting because it has no overt effect on cell growth and viability. This could be exploited to improve the flocculation ability of industrial strains. Concomitant repression of *SRB1/PSA1* and *PKC1* leads to extensive cell lysis and flocculation (N. Zhang and others, unpublished, and the present report), making the system useful in heterologous protein expression and downstream operations in which controlled cell lysis and efficient separation of cell ghosts from the medium can be realized at the same time.

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