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RESEARCH ARTICLE

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Genome-wide expression analysis of genes affected by amino acid sensor Ssy1p in *Saccharomyces cerevisiae*

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Abstract *Saccharomyces cerevisiae* Ssy1p is a membrane protein which senses extracellular amino acids and controls the expression of certain amino acid permease genes. Analysis by DNA micro-array newly identified *DIP5* and *MUP1* as the positive targets and *CAN1*, *PUT4* and *GAP1* as the negative targets under Ssy1p control. Interestingly, the effect of *ssy1* deletion was not restricted to amino acid permease genes: the expression of nitrogen catabolite repression (NCR)-sensitive genes and methionine-biosynthesizing genes (*MET* genes) was derepressed by the deletion of *SSY1*. Constitutive overexpression of the genes for glutamine permease (*GNP1*) or methionine permease (*MUP1*) enhanced the assimilation of glutamine or methionine in the *ssy1*Δ strain but could not fully suppress the derepression of the NCR-sensitive genes or *MET* genes. This result suggests that Ssy1p regulates not only the transcription of amino acid permease genes, but also transcription of many other nitrogen-metabolizing genes.

Keywords *SSY1* · Amino acid sensor · Genomic analysis · *Saccharomyces cerevisiae*

Introduction

Amino acid uptake in *Saccharomyces cerevisiae* is mediated by several amino acid permeases, which are transcriptionally and post-translationally regulated in response to the variety and concentration of intracellular amino acids. The expression of the branched-chain amino acid permease genes *BAP2*, *BAP3* and *TAT1* is induced when certain amino acids, such as leucine and phenylalanine, are present in the growth medium. This induction requires the presence of Ssy1p, together with other two components, Ptr3p and Ssy5p, as amino acid sensor (Didion et al. 1998; Iraqui et al. 1999; Klasson et al. 1999; Forsberg and Ljungdahl 2001a; for review, see Forsberg and Ljungdahl 2001b). Ssy1p is a member of a family of amino acid permease-like proteins with 12 transmembrane-spanning domains, but differs from the other members in the presence of a long N-terminal domain. Ssy1p is unable to mediate amino acid transport by itself, but generates a signal that induces certain amino acid permease genes in response to the extracellular presence of specific amino acids. Ssy1p bears a similarity to the two glucose sensors, Snf3p and Rgt2p (Özcan et al. 1996) in *S. cerevisiae*, which in a comparable way are members of the hexose transporter (*HXT*) gene family, contain long C-terminal domains and are unable to mediate glucose transport in themselves but generate signals that induce *HXT* gene transcription in response to extracellular glucose. The C-terminal domains of Snf3p and Rgt2p are required for generating the signals for induction of *HXT* gene expression (Coons et al. 1997; Özcan et al. 1998; Vagnoli et al. 1998). The unusually long N-terminal domain of Ssy1p is likely to play a similar key role in signal transduction. Ssy1p is also required for the induction of some other permease genes, i.e. a broad-specificity amino acid permease gene (*AGP1*), the tyrosine permease gene (*TAT2*), the glutamine permease gene (*GNP1*) and the peptide transporter gene (*PTR2*; Didion et al. 1998; Iraqui et al. 1999).

In contrast, an *ssy1* deletion mutant shows aberrant induction of the general amino acid permease gene (*GAP1*) in amino acid-rich medium (Klasson et al. 1999). Gap1p can transport all naturally occurring amino acids, including citrulline and D-amino acids (Grenson et al. 1970; Jauniaux and Grenson 1990), but Gap1p is strictly regulated at both the transcriptional

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and post-translational levels (Stanbrough and Magasanik 1995). Many nitrogen catabolic genes, in addition to *GAP1*, are transcriptionally repressed in the presence of "preferred" nitrogen sources, such as glutamine, asparagine and ammonia, which phenomenon is referred to as nitrogen catabolite repression (NCR; Wiame et al. 1985; Magasanik 1992; Hofman-Bang 1999). At least four proteins (Gln3p, Nil1p, Dal80p, Nil2p) control the NCR-sensitive genes in coordination, by binding to their upstream regulatory region containing the consensus sequence 5′-GATA-3′ (Daugherty et al. 1993; Stanbrough et al. 1995; Coffman et al. 1997). The increased *GAP1* expression level in an *ssy1*-deletion mutant suggests that Ssy1p may also negatively control other NCR-sensitive genes. In the course of preparation of this study, it was reported that the levels of several NCR-sensitive genes and amino acid biosynthetic genes are affected by leucine addition in an Ssy1p-dependent fashion (Forsberg et al. 2001). Since it has been reported that Ssy1p is able to sense multiple amino acids in addition to leucine (Iraqui et al. 1999; Klasson et al. 1999), there could be other unknown Ssy1p-regulated genes in the presence of multiple amino acids. The aim of this study is to clarify the multiple effects of amino acid signal generated by Ssy1p via the DNA micro-array technique and to investigate how the target genes are regulated.

Materials and methods

Strain and media

Escherichia coli strain JM109 was used as the host for plasmid construction. Growth and handling of *E. coli* and plasmids followed standard procedures (Sambrook et al. 1989). The *S. cerevisiae* strains used in this study are listed in Table 1. Yeast cells were grown at 30 °C in standard media (YPD, SD or SC; Rose et al. 1990). The concentrations of the nitrogen sources in YPD medium used for this study are listed in Table 2. Yeast transformation was performed using the lithium acetate method (Ito et al. 1983). Selections for positive clones were carried out on YPD plates supplemented with 300 mg G418/l or YPD plates supplemented with 10 mM formaldehyde.

Construction of the *ssy1*Δ strain

The wild-type strain X2180-1A was transformed with linearized plasmid pΔSSY1 (Kodama et al. 2001) and the resultant *ssy1* disruptant was named YK001 (*ssy1*Δ).

Constitutive overexpression of the amino acid permease genes in the *ssy1*Δ strain

Plasmid pYCGPY (Kodama et al. 2001) is a centromeric vector that allows expression of genes placed downstream of the yeast pyruvate kinase (*PYK1*) promoter. To create a *Not*I site in pYCGPY, the plasmid was digested with *Sac*I and *Bam*HI and ligated with the phosphorylated oligonucleotides 021 and 022 (Table 3). The resultant plasmid was designated pYCGPY2. The open reading frames of *GNP1*, *DIP5* and *MUP1* were amplified by PCR from genomic DNA of X2180-1A, using primers listed in Table 3. The *GNP1*-coding region was prepared as a *Not*I-*Bam*HI fragment with the oligonucleotide primers 023 and 024, the *DIP5*-coding region as a *Sac*I-*Not*I fragment with the oligonucleotide primers 025 and 026 and the *MUP1*-coding region as a *Sac*I-*Not*I fragment with the oligonucleotide primers 027 and 028. These three fragments were inserted into the *Not*I-*Bam*HI or *Sac*I-*Not*I gap of pYCGPY2 and were denoted pPKGNP1, pPKDIP5 and pPKMUP1, respectively. The *BAP2*-coding region was excised as the *Sac*I-*Bam*HI fragment from pBAP2ORF (Omura et al. 2001) and inserted into the *Sac*I-*Bam*HI gap of pYCGPY2 to create pPKBAP2. All of these plasmids (pPKGNP1, pPKDIP5, pPKMUP1, pPKBAP2) were introduced into strain YK001 (*ssy1*Δ) and the resultant transformants were denoted YK002, YK003, YK004 and YK005, respectively.

Amino acid and ammonia assimilation analysis

Cells were cultured in YPD broth for 5 h and the cultures were centrifuged to remove the yeast cells. The supernatants and original YPD broth were diluted 10-fold with 0.02 N HCl and were analyzed for amino acid and ammonia concentration with an amino acid analyzer (model L-8800, Hitachi). The amount of assimilated amino acids was estimated as the difference between the amount of amino acids present in the medium at the start and the end of cultivation. The whole-cell amino acid pool concentrations were determined according to the method of Ohsumi et al. (1988).

GeneChip expression analysis

Cells were grown to mid-exponential phase in YPD broth at 30 °C. Total RNA was extracted according to the standard method (Rose et al. 1990) and poly(A)$^+$ RNA was purified from total RNA, using the Oligotex-dt30 mRNA purification kit (Takara). Poly(A)$^+$ RNA was amplified, biotin-labeled and hybridized to oligonucleotide arrays (GeneChip S98 arrays, Affymetrix, Santa Clara, Calif.); and hybridization intensities were analyzed and normalized as described by Jelinsky and Samson (1999). Genes whose expression increased or decreased were listed, based on the following criteria: the average change of two experiments was more than 3-fold, if the change in each experiment was greater than 1.5-fold and the change in values was above the background values in both comparisons (Wyrick et al. 1999). The raw data can be retrieved from our ftp server: gscarray.gsc.riken.go.jp (username: anonymous, password: your e-mail address). The files are under the directory: /pub/Kodama_et al.

Table 1. *Saccharomyces cerevisiae* strains used in this study. *YGSC* Yeast Genetics Stock Center, University of California, Berkeley

Strain	Genotype	Source or reference
X2180-1A	*MAT*a *SUC2 mal mel gal2 CUP1*	YGSC
YK001	*MAT*a *SUC2 mal mel gal2 CUP1 ssy1::SFA1*	This study
YK002	*MAT*a *SUC2 mal mel gal2 CUP1 ssy1::SFA1* with pPKGNP1 [*PYK1p-GNP1*]	This study
YK003	*MAT*a *SUC2 mal mel gal2 CUP1 ssy1::SFA1* with pPKDIP5 [*PYK1p-DIP5*]	This study
YK004	*MAT*a *SUC2 mal mel gal2 CUP1 ssy1::SFA1* with pPKMUP1 [*PYK1p-MUP1*]	This study
YK005	*MAT*a *SUC2 mal mel gal2 CUP1 ssy1::SFA1* with pPKBAP2 [*PYK1p-BAP2*]	This study
YK006	*MAT*a *SUC2 mal mel gal2 CUP1 gap1::AUR1-C*	Kodama et al. 2001
YK007	*MAT*a *SUC2 mal mel gal2 CUP1 gap1::AUR1-C ssy1::SFA1*	Kodama et al. 2001

Table 2. Concentrations of nitrogen sources in YPD medium (see text for details)

Nitrogen source	Concentration (mM)
Aspartate	2.05
Threonine + glutamine	3.01
Serine + asparagine	3.20
Glutamate	7.03
Glycine	4.45
Alanine	8.21
Cysteine	0.29
Valine	4.02
Methinone	0.92
Isoleucine	2.77
Leucine	5.75
Tyrosine	1.92
Phenylalanine	3.50
Ornithine	0.37
Lysine	3.47
Histidine	0.66
Tryptophan	0.47
Arginine	5.55
Ammonia	2.50

Table 3. Oligonucleotides used in this study. The nucleotides *in italics* were added to generate restriction sites

Number	Sequence
021	5′-CATAGCGGCCGCATAG-3′
022	5′-GATCCTATGCGGCCGCTATGAGCT-3′
023	5′-*GCGGCCGC*ATGACGCTTGGTAATAGACGC-3′
024	5′-*GGATCC*TGTAGGAAGGCT GGGATTTGA-3′
025	5′-*GAGCTC*ATGAAGATGCCTCTAAAGAAGATG-3′
026	5′-*GCGGCCGC*GGCATCTAGAAGATATTACCCA-3′
027	5′-*GAGCTC*ATGTCGGAAGGAAGAACGTT-3′
028	5′-*GCGGCCGC*TTACAGCGATTTTTCTTGTTCA-3′

Northern analysis

Total RNA (20 μg) was fractionationed in a 1% denaturing agarose gel and transferred to a Nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). PCR products approximately 700 bp long were synthesized using gene-specific oligonucleotide primers and were labeled using the Alkphos-direct system (Amersham Pharmacia Biotech). Hybridization with *MET3* and *MET14* probes was carried out using a ^{32}P-labeled probe. Transcription of the pyruvate dehydrogenase E1α subunit-encoding *PDA1* (Wenzel et al. 1995) was used as an internal standard. Hybridization and signal detection were carried out according to the supplier's instructions.

Results

The assimilation of each amino acid shows a variable response to SSY1 disruption

We investigated the assimilation of amino acids in YK001 (*ssy1*Δ) and X2180-1A (wild type) during growth in YPD broth at 30 °C for 5 h. The two strains exhibited almost the same growth rate (data not shown). As shown in Fig. 1, the assimilation of aspartate, threonine plus glutamine, serine plus asparagine, glutamate, methionine, leucine and phenylalanine decreased in strain YK001. This result suggests that Ssy1p is required for the uptake of these amino acids. The reduced assimilation of leucine, phenylalanine and glutamate in the *ssy1*Δ strain is consistent with an earlier report on reduced transport of these amino acids in an *ssy1*Δ strain (Klasson et al. 1999). In contrast, the assimilation of arginine and ammonia increased in strain YK001. These data suggest that the amino acid signal generated by Ssy1p positively and negatively regulates the assimilation of various amino acids and ammonia.

Genome-wide expression analysis newly identified several genes as targets under Ssy1p control

In order to clarify the multiple effects of Ssy1p-dependent amino acid signal, we carried out genome-wide expression analyses using DNA micro-arrays. The GeneChip methodology developed by Affimetrix was employed to monitor global gene expression in YK001 (*ssy1*Δ) and X2180-1A (wild type). Approximately 7,000 open reading frames (ORFs) containing recognized and non-annotated ORFs, as suggested by SAGE analysis (Velculescu et al. 1997), were analyzed. The ORFs whose transcripts decreased more than 3-fold

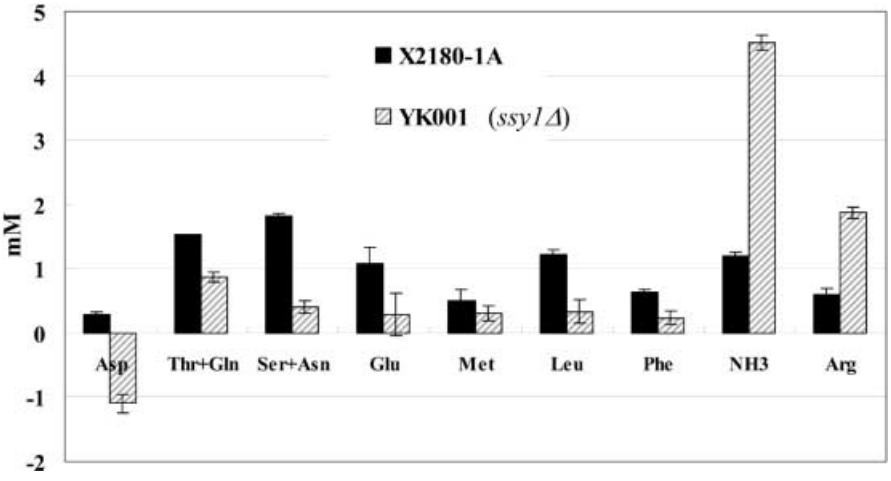

Fig. 1. Amino acid and ammonia assimilation in strain X2180-1A (wild type) and strain YK001 (*ssy1*Δ). The amount of assimilation was estimated as the difference between the amounts in the medium at the beginning and at the end of cultivation. The amount of aspartate in the medium increased in YK001

(average of two experiments) in the *ssy1*Δ strain, compared with the wild-type strain, are listed in Table 4. Thirty ORFs are included in this group, eight of them assigned as genes for amino acid permeases. The expression level of amino acid permease genes reported to be under the control of Ssy1p (Didion et al. 1998; Iraqui et al. 1999; Klasson et al. 1999; Forsberg et al. 2001) was found to decrease 6.7- to 52.9-fold. These results indicate that the lack of Ssy1p leads to an inability to properly induce these amino acid permease genes.

There are 22 other genes whose transcripts decreased more than 3-fold. Amino acid-metabolizing genes, such as the serine–threonine dehydratase gene (*CHA1*) and the arginosaccinate synthetase gene (*ARG1*), are included in this group, suggesting that these genes are positively regulated by Ssy1p. It is possible that the other genes with unknown functions in Table 4 are related to amino acid-metabolizing genes that could be under the positive control of Ssy1p.

The ORFs whose transcripts increased more than 3-fold in the *ssy1*Δ strain, compared with the wild-type strain, are listed in Table 5. The group includes 35 ORFs. YPD broth contains so-called ''preferred'' or ''rich'' nitrogen sources, such as glutamine, asparagine, glutamate and ammonia. In this type of medium, many nitrogen catabolite genes are transcriptionally repressed. Surprisingly, many NCR-sensitive genes, such as allantoin-catabolizing genes (*DAL* genes), urea-catabolizing genes (*DUL* genes) and a proline-catabolizing gene (*PUT1*), showed increased expression levels in strain YK001. The increased expression level of the arginase gene (*CAR1*) was not consistent with the previous report that the *CAR1* mRNA in the *ssy1*-deleted strain decreased in the presence of leucine (Klasson et al. 1999). It is possible that the increased expression level of *CAR1* in YK001 was due to increased arginine assimilation, as shown in Fig. 1. The expression levels of NCR-sensitive amino acid permease genes (*GAP1*, *CAN1*, *PUT4*) and the ammonia permease gene (*MEP2*) also increased in YK001 (*CAN1* is not listed in Table 5, as the expression level of *CAN1* increased by 2.4-fold in YK001, compared with the wild-type strain).

The NCR of susceptible genes is established through transcriptional regulation at the upstream 5′-GATA-3′ sequences of each gene. Four distinct GATA factors, the two activators (Gln3p, Nil1p/Gat1p) and two repressors (Uga43p/Dal80p, Gzf3p/Nil2p/Deh1p; Coffman et al. 1997; Rowen et al. 1997; Soussi-Boudekou et al. 1997),

Table 4. Open reading frames (ORFs) whose transcripts are decreased by > 3-fold in YK001 (*ssy1*Δ)

ORF	Gene	*X*-fold change	Function
Amino acid permease genes			
YDR046C	*BAP3*	52.9	Branched-chain amino acid permease
YPL265W	*DIP5*	25.2	Dicarboxylic amino acid permease
YBR068C	*BAP2*	23.5	Branched-chain amino acid permease
YBR069C	*TAT1*	14.8	Amino acid permease for valine, leucine, isoleucine and tyrosine
YDR508C	*GNP1*	14.0	High-affinity glutamine permease
YOL020W	*TAT2*	8.0	High-affinity tryptophan permease
YCL025C	*AGP1*	6.7	Amino acid permease
YGR055W	*MUP1*	4.8	High-affinity methionine permease
Other genes			
YNL333W	*SNZ2*	∼42.0[b]	Snooze: stationary phase-induced gene family
NBR015C[a]		38.6	
YGR243W		∼16.1	Strong similarity to hypothetical protein YHR162w
YPL230W	*USV1*	∼10.6	Putative finger transcription factor
YOR049C		∼8.5	
YDL222C		7.1	
YCL064C	*CHA1*	6.1	Catabolic serine (threonine) dehydratase
YGR052W		5.2	Similarity to ser/thr protein kinases
YHR033W		5.2	Strong similarity to glutamate 5-kinase
YNL194C		∼5.2	Strong similarity to YDL222c and similarity to Sur7p
YHR209W		∼4.7	Putative methyltransferase
YGR043C		4.4	(Strong similarity to)transaldolase <
YGL117W		3.9	
YBR092C	*PHO3*	3.9	Acid phosphatase
YOL058W	*ARG1*	3.8	Arginosuccinate synthetase
YEL073C		∼3.8	
YNL140C		∼3.8	
YIL122W		∼3.6	
NGR122W[a]		3.5	
NLR044W[a]		3.3	
YPR193C	*HPA2*	∼3.2	Histone and other protein acetyltransferase
YGR146C		3.1	

[a] Non-annotated SAGE ORF (Velculescu et al. 1997)
[b] Hybridization intensity in the wild-type strain was below a certain threshold and was therefore increased to an arbitrary, low value for the purpose of this calculation. Values marked ''∼'' are therefore approximate

Table 5. ORFs whose transcripts are increased by > 3-fold in YK001(*ssy1*Δ). *NCR* Nitrogen catabolite repression

ORF	Gene	*X*-fold change	Function
NCR-sensitive genes			
YIR028W	*DAL4*	~92.5[b]	Allantoin permease
YIR027C	*DAL1*	~66.3	Allantoinase
YIR031C	*DAL7*	63.2	Malate synthase 2
YKR034W	*DAL80*	~50	Negative regulator of multiple nitrogen-catabolizing genes
YLR142W	*PUT1*	31.4	Proline oxidase
YBR208C	*DUR1,2*	26.3	Urea amidolyase
YIR032C	*DAL3*	26.0	Ureidoglycolate hydrolase
YHL016C	*DUR3*	24.7	Urea transport protein
YNL142W	*MEP2*	20.0	Ammonia transport protein
YIR029W	*DAL2*	18.2	Allantoicase
YKR039W	*GAP1*	13.9	General amino acid permease
YOR348C	*PUT4*	12.4	Putative proline-specific permease
YJR152W	*DAL5*	5.9	Allantoate permease
YFL021W	*GAT1*	5.8	Transcriptional activator with GATA-1-type Zn finger DNA-binding motif
YIR030C	*DCG1*	~4.1	Involved in cell wall biogenesis and architecture
YPL111W	*CAR1*	3.1	Arginase
Methionine-biosynthesizing genes			
YJR010W	*MET3*	15.2	ATP sulfurylase
YNL277W	*MET2*	12.0	Homoserine *O*-trans-acetylase
YBR294W	*SUL1*	11.2	Putative sulfate permease
YKL001C	*MET14*	10.3	Adenylylsulfate kinase
YFR030W	*MET10*	7.8	Subunit of assimilatory sulfite reductase
YIR017C	*MET28*	6.7	Transcriptional activator of sulfur amino acid metabolism
YLR092W	*SUL2*	5.8	High-affinity sulfate permease
YPR167C	*MET16*	5.6	3′ Phosphoadenylylsulfate reductase
YKR069W	*MET1*	5.0	Siroheme synthase
YJR137C	*MET5*	4.9	Sulfite reductase
YER091C	*MET6*	3.3	Homocysteine methyltransferase
Other genes			
NDL032W[a]		8.6	
YLL061W	*MMP1*	7.8	High affinity *S*-methylmethionine permease
NIR009W[a]		~7.4	
YDR281C	*PHM6*	~5.4	Phosphate metabolism; transcription is regulated by PHO system
YLL062C	*MHT1*	4.6	*S*-Methylmethionine:homocysteine methyltransferase
YGL184C	*STR3*	4.4	Cystathionine beta-lyase
YAR071W	*PHO11*	4.0	Acid phosphatase, secreted
YKL120W	*OAC1*	3.2	Mitochondrial transporter for oxaloacetate and sulfate

[a] non-annotated SAGE ORF (Velculescu et al. 1997)
[b] Hybridization intensity in the wild-type strain was below a certain threshold and was therefore increased to an arbitrary, low value for the purpose of this calculation. Values marked "~" are therefore approximate

can bind to these 5′-GATA-3′ sequences and thus affect the transcription level. The present analysis shows that the expression level of each of the GATA factors in YK001 is as follows: no change in *GLN3*, a 5.8-fold increase in *NIL1*/*GAT1*, a 50-fold increase in *UGA43*/*DAL80* and no change in *GZF3*/*NIL2*/*DEH1*. The expression of *NIL1*/*GAT1* and *UGA43*/*DAL80* was earlier reported to be NCR-sensitive (Cunningham and Cooper 1991; Coffman et al. 1996). These results suggest that the deregulation of NCR-sensitive genes may be a secondary consequence of deregulation of GATA factors in *ssy1*Δ cells.

The expression of methionine biosynthetic genes (*MET* genes) was also induced in the *ssy1*Δ strain (Table 5). The expression of most methionine biosynthetic genes is known to be repressed in the presence of methionine in the medium (Hinnebusch 1992; Thomas and Surdin-Kerjan 1997) and the amount of methionine in YPD broth is sufficient for this mechanism to work. In YK001 (*ssy1*Δ), the expression level of *MET3*, *MET2*, *MET14*, *MET10*, *MET28*, *MET16*, *MET1*, *MET5* and *MET6* increased by 3.3- to 15.2-fold, compared with the wild-type strain. Furthermore, the expression of the sulfate permease genes (*SUL1*, *SUL2*), which were also known to be repressed in the presence of methionine (Cherest et al. 1997), increased by 11.2-fold and 5.8-fold, respectively. These results indicate that the lack of Ssy1p also causes the deregulation of the expression of methionine biosynthetic genes.

There are eight more genes whose expression increases in the *ssy1*Δ strain, four genes out of the eight being sulfur-related metabolic genes (*MMP1*, *MHT1*, *STR3*, *OAC1*). However, transcriptional regulation of these genes (e.g. the effect of amino acid concentration in the medium) has not been well established.

The expression of genes potentially regulated by Ssy1p was confirmed by Northern analysis

In order to confirm the expression pattern of genes that are potentially regulated by Ssy1p, we carried out Northern analysis of some of the genes listed in Tables 4 and 5 in yeast cells growing in synthetic media with or without amino acids (in SC or SD medium). As shown in Fig. 2, the expression of *BAP2*, *MUP1*, *PHO3*, *CHA1* and *YDL222c* was induced by amino acids in the wild-type strain (lanes 1, 3), while this induction was almost lost in the *ssy1*Δ strain (lanes 2, 4). These results indicate that Ssy1p is a requisite for the transcriptional induction of these genes, in response to extracellular amino acids. We also investigated the expression of a selection of NCR-sensitive genes and methionine-biosynthesizing genes, using Northern analysis. The expression of *DAL3* and *DAL7* was repressed in the wild-type strain growing in SC medium, while this repression was lost in the *ssy1*Δ strain (Fig. 2). The expression of *GAP1*, *MEP2*, *MET3* and *MET14* in the *ssy1*Δ strain was derepressed in YPD medium whereas, in the wild-type strain, these genes were repressed (Fig. 3, lanes 1, 2). These results indicate that most of the results obtained from DNA micro-array analysis are reproducible and reliable.

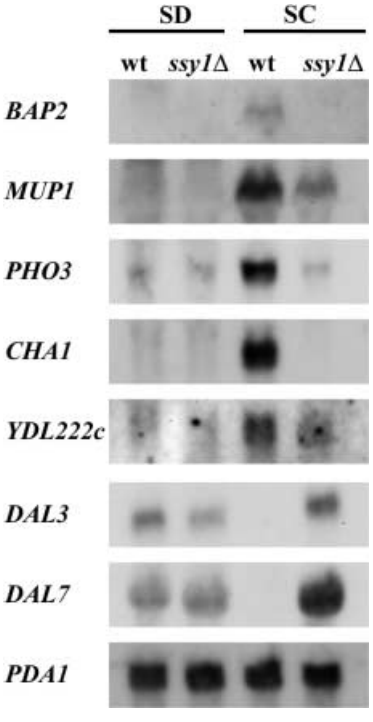

Fig. 2. The mRNA expression level of genes potentially regulated by Ssy1p in X2180-1A (wild type; *wt*) and YK001 (*ssy1*Δ), growing in SD medium or SC medium (Rose et al. 1990). *PDA1* was used as an internal standard. Cells were pre-grown overnight in SD or SC medium at 30 °C. From these pre-cultures, main cultures were inoculated at an optical density at 600 nm (OD_{600}) = 0.5 in fresh SD or SC medium, and subsequently grown to an OD_{600} = 1.0 (for 3 h) at 30 °C

The constitutive overexpression of amino acid permeases in a *ssy1*Δ strain partially suppressed the derepression of NCR-sensitive genes and methionine-biosynthesizing genes

In order to clarify the role of Ssy1p in the repression of nitrogen-catabolizing genes or methionine-biosynthesizing genes, we attempted to examine the effects of constitutive overexpression of the glutamine permease gene (*GNP1*), the methionine permease gene (*MUP1*), the dicarboxylic amino acid permease gene (*DIP5*) and the branched-amino acid permease gene (*BAP2*) in strain YK001 (*ssy1*Δ). The ORFs of *GNP1*, *DIP5*, *MUP1* and *BAP2* were inserted into the centromeric vector pYCGPY2 and put under the control of the constitutive *PYK1* promoter. These plasmids were introduced into YK001 (*ssy1*Δ) and the resultant transformants were denoted as YK002, YK003, YK004 and YK005, respectively. Constitutive overexpression of these amino acid permease genes in each transformant was confirmed by Northern analysis (Fig. 3). To verify that these constructs encoded functional amino acid permeases, amino

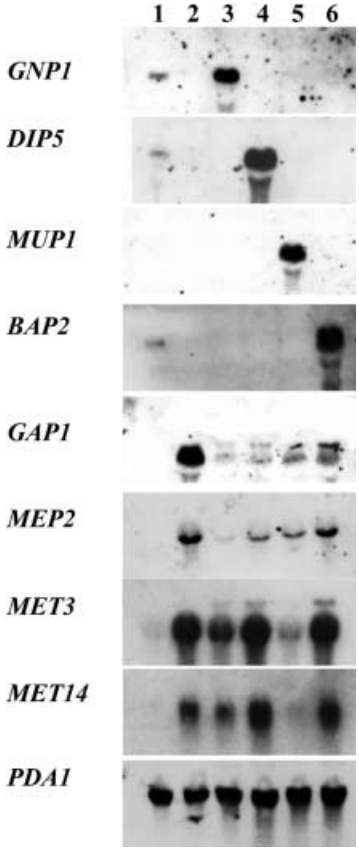

Fig. 3. Northern analysis of the transcription of *GNP1*, *DIP5*, *MUP1*, *BAP2*, *GAP1*, *MEP2*, *MET3*, *MET14* and *PDA1* in six strains: *lane 1*X2180-1A (wild type), *lane 2* YK001 (*ssy1*Δ), *lane 3* YK002 (*PYK1p-GNP1 ssy1*Δ), *lane 4* YK003 (*PYK1p-DIP5 ssy1*Δ), *lane 5* YK004 (*PYK1p-MUP1 ssy1*Δ) and *lane 6* YK005 (*PYK1p-BAP2 ssy1*Δ). *PDA1* was used as internal standard. Total RNA was isolated after cultivation for 5 h in YPD medium at 30 °C

acid assimilation during growth in YPD broth and the intracellular amino acid pools in each transformant were analyzed. Each transformant with overexpression of a specific amino acid permease showed increased assimilation and amino acid pools of corresponding amino acids, compared with YK001, and even, in some cases, with the wild-type strain X2180-1A (Fig. 4). Using Northern analysis, we investigated the expression of NCR-sensitive genes (*MEP2*, *GAP1*) and methionine-biosynthesizing genes (*MET3*, *MET14*) in these transformants (Fig. 3). The expression of *MEP2* and *GAP1* in YK002, YK003, YK004 and YK005 was repressed, compared with YK001 (*ssy1*Δ), but was not as low as in the wild-type strain X2180-1A. Similarly, the expression level of *MET3* and *MET14* in the *MUP1*-constitutive strain YK004 decreased, compared with YK001 (*ssy1*Δ), but was not as low as in X2180-1A. Furthermore, we investigated genome-wide gene expression in the strains YK002 and YK004, compared with that of X2180-1A, using GeneChip expression analysis. The expression levels of amino acid permease genes and several genes with unknown functions (YNL333w, NBR015C, YGR052W, YHR033W, YEL073C, YIL122W) listed in Table 4 were still decreased more than 3-fold in the *GNP1*-constitutive strain YK002 and the *MUP1*-constitutive strain YK004, compared with X2180-1A (data not shown). The expression level of several NCR-sensitive genes (*DAL1–7*, *DUR1–3*, *PUT1*, *PUT4*, *MEP2*, *GAP1*) in the *GNP1*-constitutive strain YK002 and that of several methionine synthetic genes (*MET2*, *3*, *10*, *14*) in the *MUP1*-constitutive strain YK004 decreased, compared with YK001 (*ssy1*Δ), but, once again, not to the very low levels found in X2180-1A (data not shown). These results indicate not only that the constitutive overexpression of *GNP1* or *MUP1* suppresses the derepression of NCR-sensitive genes or *MET* genes in an *ssy1*Δ strain, but also that this suppression is incomplete. The latter observation suggests the existence of additional unknown Ssy1p-dependent pathway(s) independent of amino acid uptake for the repression of NCR-sensitive genes and methionine-biosynthesizing genes.

The repression of NCR-sensitive genes and methionine-biosynthesizing genes by D-amino acids in a gap1Δ strain was partially Ssy1p-dependent

It has been reported that D-leucine is able to induce the transcription of *BAP2*, *PTR2* and *CAR1* in an Ssy1p-dependent manner (Didion et al. 1998; Klasson et al. 1999). We carried out Northern analysis and found that the expression of NCR-sensitive genes and methionine-biosynthesizing genes were repressed by D-glutamine,

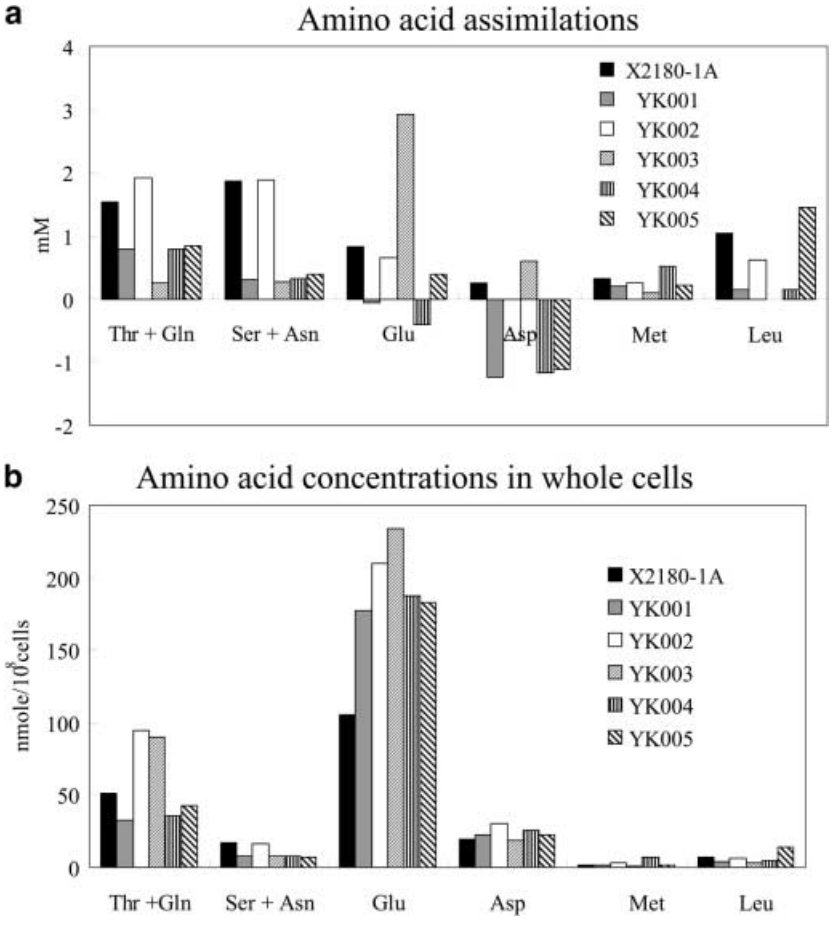

Fig. 4. Amino acid assimilations (**a**) and whole-cell amino acid concentrations (**b**) in the yeast strains: X2180-1A (wild type), YK001 (*ssy1*Δ), YK002 (*PYK1p-GNP1 ssy1*Δ), YK003 (*PYK1p-DIP5 ssy1*Δ), YK004 (*PYK1p-MUP1 ssy1*Δ) and YK005 (*PYK1p-BAP2 ssy1*Δ). The assimilation was estimated as the difference between the amounts of amino acids present in the medium at the start and at the end of cultivation

D-methionine and D-leucine in a *gap1*Δ strain (data not shown). We also compared the expression levels of *BAP2*, *MET3*, *MET25* and *MEP2* between the *gap1*Δ strain (YK006) and the *gap1*Δ *ssy1*Δ strain (YK007) in the presence of D-glutamine, D-methionine and D-leucine. As shown in Fig. 5, the expression level of *BAP2* was reduced in the *gap1*Δ *ssy1*Δ strain (lane 2), compared with the *gap1*Δ strain (lane 1) in all cases. This result shows that these D-amino acids induce the expression of *BAP2* in an Ssy1p-dependent manner. In contrast, the expression levels of *MET3*, *MET25* and *MEP2* were slightly increased in the *gap1*Δ *ssy1*Δ strain (lane 2), compared with the *gap1*Δ strain (lane 1). Similar results were obtained in the expression of *MET16* and *DUR1* (data not shown). These results suggest that Ssy1p could sense the existence of these D-amino acids and generate signals for the repression of NCR-sensitive genes and methionine-biosynthesizing genes.

Discussion

The significant impact of an *ssy1* deletion on the assimilation of amino acids suggested that the amino acid signal from Ssy1p could affect positively and negatively the expression of a wide variety of genes; and this prompted us to analyze genome-wide expression in the *ssy1*Δ strain. Out of the approximately 7,000 ORFs in the yeast genome, the number of ORFs whose transcripts decreased or increased more than 3-fold in strain YK001 (*ssy1*Δ) were 30 and 35, respectively. Most of these were amino acid permease genes, NCR-sensitive genes or methionine-biosynthesizing genes. Employing Northern analysis, we investigated further the expression level of some of these genes, which are putatively under the control of Ssy1p; and we confirmed that they were indeed under the control of Ssy1p.

The results of the genome-wide expression analysis revealed that amino acid permease genes could be grouped into three classes: genes that are under positive regulation of Ssy1p (*BAP2*, *BAP3*, *TAT1*, *TAT2*, *GNP1*, *DIP5*, *MUP1*, *AGP1*), genes that are under negative regulation of Ssy1p (*CAN1*, *PUT4*, *GAP1*) and genes that are not regulated by Ssy1p (e.g. *HIP1*, encoding histidine permease, *LYP1*, encoding lysine permease; data not shown). These various changes in the expression of amino acid permease genes in the *ssy1*Δ strain correlated well with the change in assimilation of amino acids. The decreased expression of *BAP2*, *BAP3*, *TAT1*, *TAT2*, *GNP1*, *DIP5*, *MUP1* was consistent with the decreased assimilation of their substrate amino acids, leucine, phenylalanine, serine plus asparagine, threonine plus glutamine, aspartate, glutamate and methionine, respectively; and the increased assimilation of arginine seemed to be the result of the increased expression of *CAN1* and *GAP1*. However, it was not obvious from the results of the amino acid assimilation that Ssy1p also regulates NCR-sensitive genes and methionine-biosynthesizing genes. Although it was reported that the non-transporter gene (*CAR1*) was under the control of the Ssy1p signal (Klasson et al. 1999), the genome-wide expression analysis revealed for the first time that Ssy1p regulates not only amino acid permease genes but also, directly or indirectly, many nitrogen-metabolizing genes. While it is known that *BAP2*, *BAP3* and *TAT1* contain regulatory elements for transcriptional induction by amino acids in their promoter region (De Boer et al. 1998), it is unlikely that all of the Ssy1p-dependent genes identified in this study contain the same regulatory promoter elements. However, NCR-sensitive genes and *MET* genes are known to contain elements in their promoter regions that are responsible for this kind of regulation, which these genes are subject to (Hinnebusch 1992; Thomas and Surdin-Kerjan 1997; Hofman-Bang 1999). The external amino acid signal generated by Ssy1p might thus be eventually transduced to several transcriptional factors, resulting in the regulation of a wide range of genes.

While this study was being prepared, Forsberg et al. (2001) reported the effects of leucine addition to wild-type and *ssy1* null-mutant cells, using genome-wide transcription profile analysis. The expression of several amino acid biosynthetic genes and NCR-sensitive genes is modulated by Ssy1p (Forsberg et al. 2001). However, they did not distinguish between the direct and indirect effects of Ssy1p on these genes. In our experiments, a simple explanation for the derepression of NCR-sensitive genes and methionine-biosynthesizing genes in the *ssy1*Δ strain is that derepression is caused by a lack of intracellular glutamine or methionine, due to inactivation of the corresponding amino acid permeases. The glutamine permease (*GNP1*)-constitutive strain or the methionine permease (*MUP1*)-constitutive strain suppressed, albeit not completely, the derepression of NCR-sensitive genes and methionine-biosynthesizing genes, respectively, as compared with the wild-type strain. These results indicate that the repression of nitrogen catabolite genes or methionine-biosynthesizing genes is

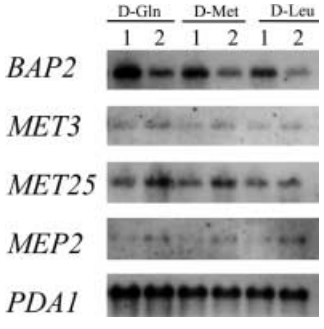

Fig. 5. Northern analysis of the transcription of *BAP2*, *MET3*, *MET25*, *MEP2* and *PDA1* in YK006 (*gap1*Δ, *lane 1*) andYK007 (*gap1*Δ *ssy1*Δ, *lane 2*). *PDA1* was used as internal standard. Cells were pre-grown overnight in SD medium at 30 °C. From these pre-cultures, main cultures were inoculated at $OD_{600} = 0.5$ in fresh SD medium containing either 0.1% D-glutamine (*D-Gln*), 0.1% D-methionine (*D-Met*) or 0.1% D-leucine (*D-Leu*) and subsequently grown for 30 min at 30 °C

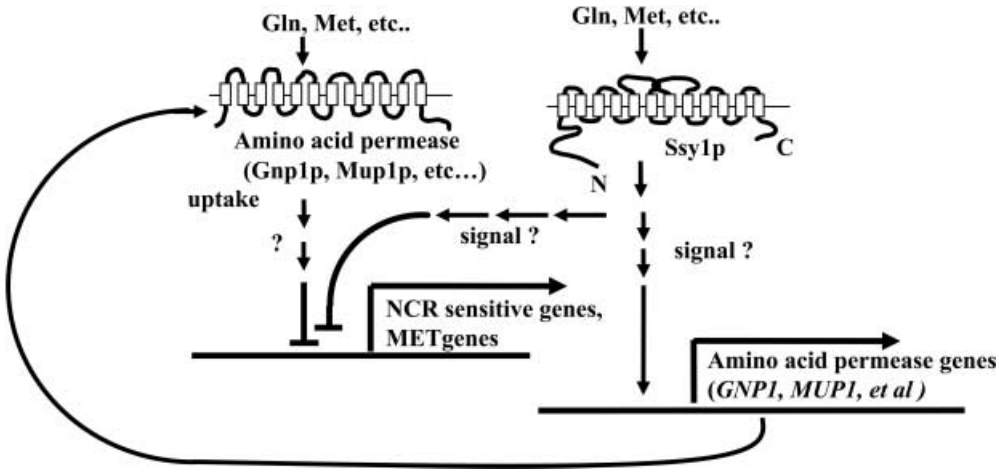

Fig. 6. Model for the Ssy1p-dependent transcriptional regulation of nitrogen-catabolizing genes and methionine-biosynthesizing genes

primarily an indirect effect caused by the activity of glutamine or methionine permease. However, the incomplete suppression by constitutive overexpression of the permease genes suggests that additional, unknown Ssy1p-dependent regulation may be operating in the repression of these genes independently of amino acid uptake, as shown in Fig. 6.

How Ssy1p generates the signal for the expression of the nitrogen-metabolizing genes in response to extracellular amino acids remains elusive. However, it has been reported that the long cytoplasmic N-terminal domain of Ssy1p plays an important role in signal transduction (Klasson et al. 1999; Bernard and Andre 2001a; Forsberg and Ljungdahl 2001a). It may be possible that the binding of amino acids to the transmembrane-spanning domain of Ssy1p induces a conformational change, which is in turn transmitted to the cytoplasmic N-terminal domain. The signal of extracellular amino acids may be transmitted to the other components of the amino acid sensor, Ptr3p and Ssy5p (Klasson et al. 1999; Forsberg and Ljungdahl 2001a), via the cytoplasmic N-terminal domain of Ssy1p.

Iraqui et al. (1999) has reported that the F-box protein Grr1p plays a central role in the Ssy1p-mediated induction of *AGP1*, as well as in transducing signals generated by the Snf3p and Rgt2p glucose sensors (Li et al. 1997). In the pathway of glucose-induced transcription of *HXT* genes by Snf3p and Rgt2p, Grr1p is involved in the modification of Rgt1p, a transcriptional regulator of *HXT* genes, in response to a glucose signal (Johnston 1999; Özcan and Johnston 1999). Furthermore, It has reported that ubiquitin and the SCF^{Grr1} ubiquitin ligase complex are involved in the signaling pathway for the induction of *AGP1* and *PTR2* (Bernard and Andre 2001b). It is conceivable that SCF^{Grr1} is involved in the modification of regulators of Ssy1p-regulated genes, including the nitrogen-catabolizing genes and the methionine-biosynthesizing genes, in response to an increased concentration in external amino acids. On the other hand, it has been reported that Tor1p and Tor2p (TOR kinases) regulate the transcription of NCR-sensitive genes by promoting the association of the GATA transcription factor Gln3p with the cytoplasmic protein Ure2p (Beck and Hall 1999). TOR kinases promote the formation of a cytoplasmic Gln3p-Ure2p complex and thereby prevent nuclear accumulation of Gln3p, in response to the presence of "preferred" nitrogen sources. The "external amino acid signal" generated by Ssy1p may activate this TOR-signaling pathway, dependent or independent of the induction of the amino acid permease genes. Elucidation of the function of Ssy1p in relation to other signal transduction systems could be the key to understanding the overall nitrogen metabolic network.

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References

Beck T, Hall MN (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402:689–692

Bernard F, Andre B (2001a) Genetic analysis of the signalling pathway activated by external amino acids in *Saccharomyces cerevisiae*. Mol Microbiol 41:489–502

Bernard F, Andre B (2001b) Ubiquitin and the SCF^{Grr1} ubiquitin ligase complex are involved in the signalling pathway activated by external amino acids in *Saccharomyces cerevisiae*. FEBS Lett 496:81–85

Cherest H, Davidian JC, Thomas D, Benes V, Ansorge W, Surdin-Kerjan Y (1997) Molecular characterization of two high affinity sulfate transporters in *Saccharomyces cerevisiae*. Genetics 145:627–635

Coffman JA, Rai R, Cunningham T, Svetlov V, Cooper TG (1996) Gat1p, a GATA family protein whose production is sensitive to nitrogen-catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol Cell Biol 16:847–858

Coffman JA, Rai R, Loprete DM, Cunningham T, Svetlov V, Cooper TG (1997) Cross regulation of four GATA factors that control nitrogen catabolic gene expression in *Saccharomyces cerevisiae*. J Bacteriol 179:3416–3429

Coons DM, Vagnoli P, Bisson LF (1997) The C-terminal domain of Snf3p is sufficient to complement the growth defect of *snf3* null mutations in *Saccharomyces cerevisiae*: *SNF3* functions in glucose recognition. Yeast 13:9–20

Cunningham TS, Cooper TG (1991) Expression of the *DAL80* gene, whose product is homologous to the GATA factors and is a negative regulator of multiple nitrogen catabolic genes in *Saccharomyces cerevisiae*, is sensitive to nitrogen catabolite repression. Mol Cell Biol 11:6205–6215

Daugherty JR, Rai R, Berry HM el, Cooper TG (1993) Regulatory circuit for responses of nitrogen catabolic gene expression to the GLN3 and DAL80 proteins and nitrogen catabolite repression in *Saccharomyces cerevisiae*. J Bacteriol 175:64–73

De Boer M, Bebelman JP, Goncalves P.M, Maat J, Van Heerikhuizen H, Planta RJ (1998) Regulation of expression of the amino acid transporter gene *BAP3* in *Saccharomyces cerevisiae*. Mol Microbiol 30:603–613

Didion T, Regenberg B, Jørgensen MU, Killand-Brandt MC, Andersen HA (1998) The permease homolog Ssy1p controls the expression of amino-acid and peptide transporter genes in *Saccharomyces cerevisiae*. Mol Microbiol 27:643–650

Forsberg H, Ljungdahl PO (2001a) Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol Cell Biol 21:814–826

Forsberg H, Ljungdahl PO (2001b) Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. Curr Genet 40:91–109

Forsberg H, Gilstring CF, Zargari A, Martinez P, Ljungdahl PO (2001) The role of the yeast plasma membrane SPS nutrient sensor in the metabolic response to extracellular amino acids. Mol Microbiol 42:215–228

Grenson M, Hou C, Crabeel M (1970) Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. J Bacteriol 103:770–777

Hinnebusch AG (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In: Jones EW, Pringle JR, Broach JR (eds) Molecular and cellular biology of the yeast *Saccharomyces*. Gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 319–414

Hofman-Bang J (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol Biotechnol 12:35–73

Iraqui I, Vissers S, Bernard F, Craene JO de, Boles E, Urrestarazu A, Andre B (1999) Amino acid signaling in *Saccharomyces cerevisiae*: a permease-like sensor of external amino acids and F-box protein Grr1p are required for transcriptional induction of the *AGP1* gene, which encodes a broad-specificity amino acid permease. Mol Cell Biol 19:989–1001

Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168

Jauniaux JC, Grenson M (1990) *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur J Biochem 190:39–44

Jelinsky SA, Samson LD (1999) Global response of *Saccharomyces cerevisiae* to an alkylating agent. Proc Natl Acad Sci USA 96:1486–1491

Johnston M (1999) Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. Trends Genet 15:29–33

Klasson H, Fink GR, Ljungdahl PO (1999) Ssy1p and Ptr3p are plasma membrane components of a yeast system that senses extracellular amino acids. Mol Cell Biol 19:5405–5416

Kodama Y, Omura F, Ashikari T (2001) Isolation and characterization of a gene specific to lager brewing yeast that encodes a branched-chain amino acid permease. Appl Environ Microbiol 67:3455–3462

Li FN, Johnston M (1997) Grr1 of *Saccharomyces cerevisiae* is connected to the ubiquitin proteolysis machinery through Skp1: coupling glucose sensing to gene expression and cell cycle. EMBO J 16:5629–5638

Magasanik B (1992) Regulation of nitrogen utilization, In: Jones EW, Pringle JR, Broach JR (eds) Molecular and cellular biology of the yeast *Saccharomyces*. Gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 283–317

Ohsumi Y, Kitamoto K, Anraku Y (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion. J Bacteriol 170:2676–2682

Omura F, Kodama Y, Ashikari T (2001) The basal turnover of yeast branched-chain amino acid permease Bap2p requires its C-terminal tail. FEMS Microbiol Lett 194:207–214

Özcan S, Johnston M (1999) Function and regulation of yeast hexose transporters. Microbiol Mol Biol Rev 63:554–569

Özcan S, Dover J, Rosenwald AG, Wolfl S, Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc Natl Acad Sci USA 93:12428–12432

Özcan S, Dover J, Johnston M (1998) Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J 17:2566–2573

Rose MD, Winston F, Hieter P (1990) Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Rowen DW, Esiobu N, Magasanik B (1997) Role of GATA factor Nil2p in nitrogen regulation of gene expression in *Saccharomyces cerevisiae*. J Bacteriol 179:3761–3766

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Soussi-Boudekou S, Vissers S, Urrestarazu A, Jauniaux JC, Andre B (1997) Gzf3p, a fourth GATA factor involved in nitrogen-regulated transcription in *Saccharomyces cerevisiae*. Mol Microbiol 23:1157–1168

Stanbrough M, Magasanik B (1995) Transcriptional and post-translational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. J Bacteriol 177:94–102

Stanbrough M, Rowen DW, Magasanik B (1995) Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. Proc Natl Acad Sci USA 92:9450–9454

Thomas D, Surdin-Kerjan Y (1997) Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 61:503–532

Vagnoli P, Coons DM, Bisson LF (1998) The C-terminal domain of Snf3p mediates glucose-responsive signal transduction in *Saccharomyces cerevisiae*. FEMS Microbiol Lett 160:31–36

Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett DE Jr, Hieter P, Vogelstein B, Kinzler KW (1997) Characterization of the yeast transcriptome. Cell 88:243–251

Wenzel TJ, Teunissen AW, Steensma HY de (1995) *PDA1* mRNA: a standard for quantitation of mRNA in *Saccharomyces cerevisiae* superior to *ACT1* mRNA. Nucleic Acids Res 23:883–884

Wiame JM, Grenson M, Arst HNJ (1985) Nitrogen catabolite repression in yeasts and filamentous fungi. Adv Microbiol Physiol 26:1–87

Wyrick JJ, Holstege FC, Jennings EG, Causton HC, Shore D, Grunstein M, Lander ES, Young RA (1999) Chromosomal landscape of nucleosome-dependent gene expression and silencing in yeast. Nature 402:418–421