Identification of direct and indirect targets of the Gln3 and Gat1 activators by transcriptional profiling in response to nitrogen availability in the short and long term

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Abstract

Nitrogen catabolite repression (NCR) consists in the specific inhibition of transcriptional activation of genes encoding the permeases and catabolic enzymes needed to degrade poor nitrogen sources. Under nitrogen limitation or rapamycin treatment, NCR genes are activated by Gln3 or Gat1, or by both factors. To compare the sets of genes responding to rapamycin or to nitrogen limitation, we used DNA microarrays to establishing the expression profiles of a wild type strain, and of a double *gln3*Δ–*gat1*Δ strain, grown on glutamine, after addition of rapamycin, on proline, or after a shift from glutamine to proline. Analysis of microarray data revealed 392 genes whose expression was dependent on the nitrogen source quality. 91 genes were activated in a GATA factor-dependent manner in all growth conditions, suggesting a direct role of Gln3 and Gat1 in their expression. Other genes were only transiently up-regulated (stress-responsive genes) or down-regulated (genes encoding ribosomal proteins and translational factors) upon nitrogen limitation, and this regulation was delayed in a *gln3*Δ–*gat1*Δ strain. Repression of amino acid and nucleotide biosynthetic genes after a nitrogen shift did not depend on Gcn4. Several transporter genes were repressed as a consequence of enhanced levels of NCR-responsive permeases present at the plasma membrane.

Introduction

In *Saccharomyces cerevisiae*, nitrogen regulation (nitrogen catabolite repression, NCR) refers to the difference in gene expression during steady-state growth on optimal nitrogen sources (glutamine, asparagine, ammonia) compared to the growth on poorer nitrogen sources (proline, urea, γ-amino-butyrate). Yeast, like most microorganisms, transports, accumulates and utilizes good nitrogen sources in preference to poor ones, and NCR is the mechanism for achieving this selectivity. NCR consists in the specific inhibition of transcriptional activation systems of genes encoding permeases and catabolic enzymes needed to degrade poor nitrogen sources. When readily used nitrogen sources are available, NCR-sensitive genes are expressed at low basal levels. Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes is activated by Gln3 or Gat1, or more frequently by both GATA factors (Mitchell & Magasanik, 1984; Courchesne & Magasanik, 1988; Stanbrough *et al*., 1995). In the presence of good nitrogen sources, these two GATA transcriptional activators are restricted to the cytoplasm by interaction with Ure2, whereas in the presence of poor nitrogen sources these GATA factors accumulate in the nucleus, and activate NCR-sensitive transcription (Beck & Hall, 1999). Addition of the immunosuppressant drug rapamycin to a good nitrogen source transiently mimics a low-quality nitrogen source, which results in activation of NCR gene expression by Gln3 and Gat1 (Beck & Hall, 1999; Cardenas *et al*., 1999; Hardwick *et al*., 1999; Bertram *et al*., 2000). Rapamycin inhibits a conserved signaling cascade required for cell proliferation. This cascade involves the two protein kinases Tor1 and Tor2, protein phosphatases such as Sit4, and the phosphatase regulatory subunit Tap42 (Como & Arndt, 1996; Thomas & Hall, 1997; Jiang & Broach, 1999). It has been proposed that Gln3 and Gat1 are phosphorylated in a Tor-dependent manner and thereby tethered to the cytoplasmic Ure2 protein. Upon rapamycin treatment, Gln3 and Gat1 are dephosphorylated by Sit4, released from Ure2 inhibition and translocated to the nucleus, where they activate NCR

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genes (Beck & Hall, 1999; Bertram *et al.*, 2000). This signaling mechanism has been extrapolated to the cell's response to nitrogen starvation and nitrogen availability (reviewed in Cooper, 2002; Crespo & Hall, 2002; Magasanik & Kaiser, 2002; Rohde & Cardenas, 2004). However, it was shown recently that on a poor nitrogen source Gln3 is localized in the nucleus, despite its hyperphosphorylation (Cox *et al.*, 2004). These data suggest that rapamycin treatment, a short-term response, and growth on poor nitrogen sources, and with a long-term response, generates similar cellular responses, but likely do so by different mechanistic pathways. These observations led us to investigate whether the set of genes expressed after rapamycin treatment is identical to that expressed under nitrogen limitation, either by steady-state growth on a poor nitrogen source or after a shift from an optimal nitrogen source to a poor one. Moreover, we determined whether all these genes were controlled in a Gln3- and Gat1-dependent manner.

To address these questions, we used DNA microarrays comparing the expression profiles under steady-state growth conditions on an optimal nitrogen source (M.Gln) and on a poor nitrogen source (M.Pro), and under more transient conditions such as addition of rapamycin to M.Gln, or shifting cells from a good nitrogen source (M.Gln) to a poorer one (M.Pro). These growth conditions allow discrimination between rapid adaptation to a cellular nitrogen stress (short-term response), and the long-term adaptation during growth on nitrogen sources of different qualities. In contrast to other whole-genome analyses (Cox *et al.*, 1999; Shamji *et al.*, 2000), we used a double *gln3*Δ–*gat1*Δ strain to identify the entire set of genes regulated by nitrogen, as expression of many NCR genes is abolished only in the absence of both regulators.

Materials and methods

Strains and media

All the strains used in this work are derived from the BY strain family, isogenic to S288c (Brachmann *et al.*, 1998). The long flanking homology strategy was used to perform deletion of *GLN3*, *GAT1* and *URE2* (Wach, 1996). The different coding sequences were replaced by the kanMX4 cassette, which confers resistance to geneticin, yielding strains 4709ΔGLN3 (*MAT*α, *ura3*, *gln3::kanMX4*), 4709ΔGAT1 (*MAT*α, *ura3*, *gat1::kanMX4*), 4700ΔGAT1 (*MATa*, *ura3*, *gat1::kanMX4*), and 4709ΔURE2 (*MAT*α, *ura3*, *ure2::kanMX4*). To construct strain 03167b (*MATa*, *ura3*, *gln3::kanMX4*, *gat1::kanMX4*), we crossed strains 4709ΔGLN3 and 4700ΔGAT1. To construct strain 1C22182b (*ura3*, *leu2*, *his3*, *lys2*, *met15*, *msn2::kanMX4*, *msn4::kanMX4*), we crossed strains 4742ΔMSN2 (*MAT*α, *ura3*, *leu2*, *his3*, *lys2*, *msn2::kanMX4*) and 4741ΔMSN4 (*MATa*, *ura3*, *leu2*, *his3*, *met15*, *msn4::kanMX4*). Strains 4742ΔMSN2, 4741ΔMSN4, 4742ΔNPR1 (*MAT*α, *ura3*, *leu2*, *his3*, *lys2*, *npr1::kanMX4*) and 4742ΔGAP1 (*MAT*α, *ura3*, *leu2*, *his3*, *lys2*, *gap1::kanMX4*) were obtained from the *Saccharomyces* Genome deletion collection (Winzeler *et al.*, 1999).

Strains F113 (*MATa*, *ura3-52*, *ino1*, *can1*), F212 (*MATa*, *ura3-52*, *ino1*, *can1*, *gcn4*Δ) and H396 (*MATa*, *ura3-52*, *ino1*, *can1*, *GCN4*^{c} inserted at the *URA3* locus) were gifts from Alan Hinnebusch.

Yeast strains were grown on synthetic medium (YNB) without ammonia, without amino acids and with 3% glucose, supplemented with 1 mg mL^{-1} glutamine or 1 mg mL^{-1} proline as a nitrogen source. The shift from glutamine (M.Gln) to proline (M.Pro) was achieved by filtering the cells grown on M.Gln and cultivating them on fresh M.Pro for 2 h. Rapamycin was used at a final concentration of 200 ng mL^{-1}. Stock solution (1 mg mL^{-1}) of rapamycin (Sigma, St Louis, MO) was prepared in a 90% ethanol, 10% Tween-20 solution. When required, the medium was supplemented with 25 μg mL^{-1} of uracil, leucine, histidine, lysine and methionine.

Yeast cells were harvested during the exponential stage of growth (OD_{660} 0.40, measured with a Genesys 20 spectrophotometer from Thermo Spectronic, Rochester, NY). Addition of rapamycin or the shift from glutamine to proline were also performed at the same OD.

RNA preparation

Total RNAs were extracted following the method of Schmitt *et al.* (1990), and purified using the RNeasy kit (Qiagen, Chatsworth, CA).

Northern blot analysis

Northern blot analysis was performed as described by Foury & Talibi (2001). DIG-DNA probes of about 500 bp were generated by PCR using appropriate oligonucleotides, and labeled using a PCR DIG probe synthesis kit (Roche, Rotkreuz, Switzerland). Hybridizations were carried out according to standard procedures. Detection of digoxigenin-labeled nucleic acids was performed by enzyme immunoassay with luminescence following the supplier's procedure (Roche). The Hybond-N+nylon membranes were analyzed with a chemiluminescence camera (Chemi-Smart from Vilbert-Lourmat, Torcy, France). The exposure time varied between 30 and 120 min.The RNA signals were quantified using the Bio-1D analysis program from Vilbert-Lourmat.

Generation and analysis of microarray data

The yeast DNA chips were manufactured by Eurogentec (Seraing, Belgium) and by Plateforme Transcriptome

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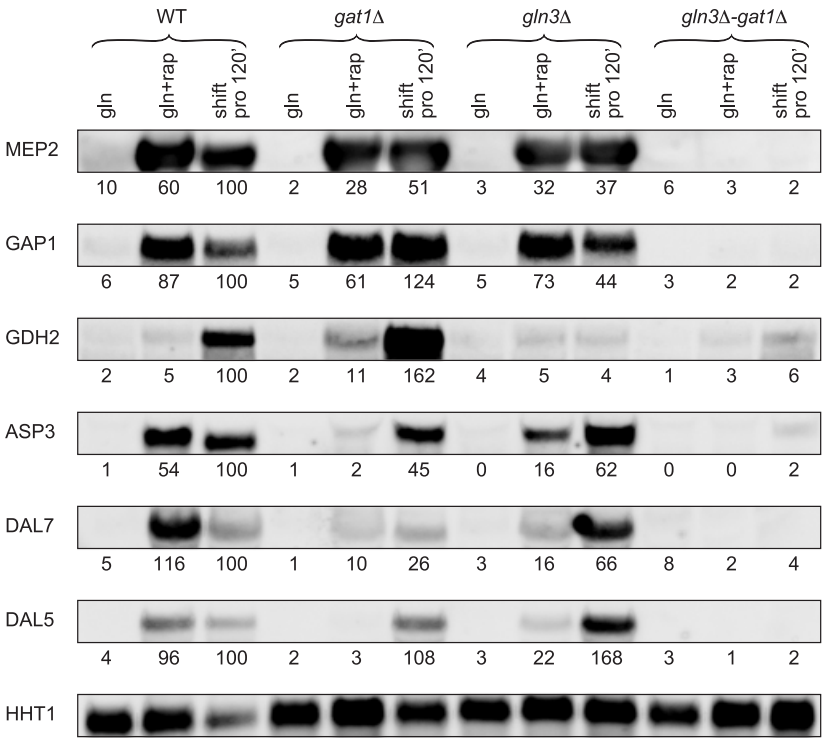

Fig. 1. Effect of single or combined deletions of the GATA factors on the expression of NCR-sensitive genes. Total RNAs were isolated from a wild-type strain (BY4709), a *gat1*Δ strain (4709ΔGAT1), a *gln3*Δ strain (4709ΔGLN3), and a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln+rapamycin for 20 min, after a 2-h shift from M.Gln to M.Pro. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *MEP2*, *GAP1*, *GDH2*, *ASP3*, *DAL7*, *DAL5* and *HHT1* encoding histone H3, as a loading control. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain after a shift from M.Gln to M.Pro.

Biopuces (Centre de Bioingénierie Gilbert Durand, INSA, Toulouse, France). Fluorescent cDNA synthesis for microarray hybridization was performed according to Foury & Talibi (2001), using Cy3-dCTP or Cy5-dCTP (Amersham Biosciences, Sunnyvale, CA). Hybridizations were performed according to Foury & Talibi (2001). The hybridization signal was measured using a GSM418 laser scanner. Image analysis for each array was processed using the GenePix Pro 4.0 (Axon Instruments Inc., Sunnyvale, CA) software package, which measures fluorescence intensity pairs for each gene. Following image acquisition, a visual inspection of the individual spots on each microarray (size, signal-to-noise ratio, background level, and spot uniformity) completed the flagging (present/not present, good/bad) of the data. To maximize sensitivity, two scans were made, one at high laser power and high photomultiplier tube (PMT) gain to detect the faintest spots, and a second one at low laser power and a low PMT gain to avoid saturation. The values for spots presenting $\geq 5\%$ saturation in the first scan were calculated based on an extrapolation after linear regression analysis of the intensities from both scans. These data were then imported in the GeneSpring 7.1 (Silicon Genetics, Palo Alto, CA) software package, applying a per spot per chip intensity-dependent (Lowess) normalization for further analysis.

The *Saccharomyces* Genome Database (SGD, http://www.yeastgenome.org) and the MIPS Comprehensive Yeast Genome Database (CYGD, http://mips.gsf.de/genre/proj/yeast) were used to retrieve information about specific gene function and biological process.

Results

Transcriptional response to nitrogen availability through the two regulators Gln3 and Gat1

To demonstrate the usefulness of strain *gln3*Δ–*gat1*Δ (03167b) instead of either *gln3*Δ(4709ΔGLN3) or *gat1*Δ (4709ΔGAT1) strains, the expression of well-known NCR genes (*MEP2*, *GAP1*, *GDH2*, *ASP3*, *DAL7*, *DAL5*) was examined by northern blot analysis (Fig. 1). These data show that single deletions of *GLN3* or *GAT1* reduce only weakly the induction of *MEP2* and *GAP1*, whereas for *DAL5*, *DAL7* and *ASP3*, deletion of one regulator impairs mainly the response to rapamycin; for all these genes, except for *GDH2*, only the double deletion completely abolishes their induction. Therefore, we chose to perform microarray analysis using only the double-mutated strain, as the sensitivity of our microrrays was not sufficient to reveal such weak variations.

We used cDNA microarray technology to compare the genome-wide expression profiles of a wild-type strain (BY4700) (Fig. 2, experiments 02, 04 and 06) or the isogenic strain with genes *GLN3* and *GAT1* deleted (experiments 01, 03 and 05) grown in YNB medium with glutamine as nitrogen source (M.Gln) against the wild-type strain grown

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in M.Gln after addition of rapamacyn (20 min) (experiments 01 and 02), or M.Pro (experiments 03 and 04) or after a 2-h shift from M.Gln to M.Pro (experiments 05 and 06), all growth conditions known to modify the expression of genes involved in nitrogen utilization. These microarrays allowed identification of the set of genes that were up- or downregulated in response to the quality of the nitrogen source. To evaluate whether the majority of genes responding to the nitrogen source were dependent on Gln3 and Gat1, we compared the expression profiles of the wild-type strain and of the isogenic strain with genes *GLN3* and *GAT1* deleted (03167b: *ura3*, *gln3*Δ, *gat1*Δ), when both strains were grown on M.Gln+rapamycin (Fig. 2, experiment 07), or M.Pro (experiment 08), or after a shift from M.Gln to M.Pro (experiment 09). We also used an independent means of identifying Gln3–Gat1-regulated genes by comparing the expression profiles of wild-type and *ure2*Δ(4709ΔURE2) strains on M.Gln medium (experiment 10).

The microarray experiments were independently repeated several times (at least twice, including dye swapping), and in this paper we present data from 10 representative microarray experiments comparing different strains and different growth conditions (Fig. S1). Microarray data can be obtained from the GEO database (accession number GSE2891 (http://www.ncbi.nlm.nih.gov/geo/)).

Our microarray experiments identified 367 genes showing five-fold or greater changes in one or several experiments, but in a first step we retained the genes presenting at least a two-fold change of expression in one of the 10 experiments. This resulted in a list of 2853 genes responding to variations in nitrogen nutrients, directly or indirectly through Gln3 and Gat1. To further refine this list, we retained only the genes with at least a 1.5-fold expression change in at least four out of six of the experiments using different nitrogen growth conditions (Fig. 2, experiments 01–06), as well as in at least two out of four experiments comparing wild-type and mutant strains (experiments 7–10). The cut-off value of 1.5 was chosen to avoid elimination of several well-known NCR genes. This analysis led to a list of 335 genes belonging to three categories: 100 genes were upregulated, 92 genes were downregulated, and 143 were downregulated but independently of Gln3 and Gat1. We completed these three categories with additional genes, either known to be regulated by nitrogen or known to be involved in the same biological process, ending up with 111 upregulated genes (group I for inducible), 124 downregulated genes (group R-1 for repressible) and 157 genes belonging to the third category (group R-2). A cluster analysis of these 392 genes clearly illustrates the three categories selected from the analysis of the different types of experiments (Fig. S1). Table S1 shows the normalized ratios for the 392 genes in the 10 experiments.

In the I group, 20 out of 111 genes were poorly derepressed on M.pro (Fig. 2b, experiments 04 and 08) but responded to the addition of rapamycin (experiments 01, 02 and 07) and to the shift from glutamine to proline (experiments 05, 06 and 09), leading to subgroup I-2, whereas the remaining 91 genes constitute group I-1. The mean expression profile of each group and the names of the genes annotated following their biological process are shown in Fig. 2.

Group I-1 comprises many of the well-known NCR-regulated genes encoding catabolic enzymes, transporters and proteins involved in proteolysis. Our analysis has increased the number of transport and proteolysis genes, as well as genes of unknown function (Fig. 2a). Group I-2 contains several genes responding to stress or generating precursor metabolites or energy (Fig. 2b). Group R-1 mainly contains genes involved in amino acid and nucleotide metabolism, protein biosynthesis and protein transport (Fig. 2c). Most of the genes in group R-2 are required for protein biosynthesis (Fig. 2d).

Relationship between Gln3- and Gat1-dependent gene expression and occurrence of GATA factor-binding sites in the promoter

Previous studies have shown that the target of Gln3 and Gat1 contained at least two GATAAG or several GATT/AA core sequences in the NCR-regulated promoters (Cunningham *et al.*, 1996). Computer analysis of the promoters (from

Fig. 2. Mean expression profile of the different categories identified by the microarray analysis and listing of the genes annotated following their biological process. The mean normalized ratios of (a) 91 genes belonging to group I-1, (b) 20 genes belonging to group I-2, (c) 124 genes belonging to group R-1 and (d) 157 genes belonging to group R-2 are plotted on the *y*-axis, and the different experiments (1–10) are listed along the *x*-axis. Number 1–10 correspond to 10 independent experiments where the expression profiles of the following pairs of strains were compared: WT (BY4700) on M.Gln+rapamycin (20 min)/*gln3*Δ–*gat1*Δ (03167b) on M.Gln (01); WT (BY4700) on M.Gln+rapamycin (20 min)/WT (BY4700) on M.Gln (02); WT (BY4700) on M.Pro/*gln3*Δ–*gat1*Δ (03167b) on M.Gln (03); WT (BY4700) on M.Pro/WT (BY4700) on M.Gln (04); WT (BY4700) after a 2-h shift from M.Gln to M.Pro/03167b (*gln3*Δ–*gat1*Δ) on M.Gln (05); WT (BY4700) after a 2-h shift from M.Gln to M.Pro/WT (BY4700) on M.Gln (06); WT (BY4700) on M.Gln+rapamycin (20 min)/*gln3*Δ–*gat1*Δ (03167b) M.Gln+rapamycin (20 min) (07); WT (BY4700) on M.Pro/*gln3*Δ–*gat1*Δ (03167b) on M.Pro (08); WT (BY4700) after a 2-h shift from M.Gln to M.Pro/*gln3*Δ–*gat1*Δ (03167b) after a 2-h shift from M.Gln to M.Pro (09); and *ure2*Δ (4709ΔURE2) on M.Gln/WT (BY4700) on M.Gln (10). The genes are listed in subgroups corresponding to the major biological processes in which they are involved.

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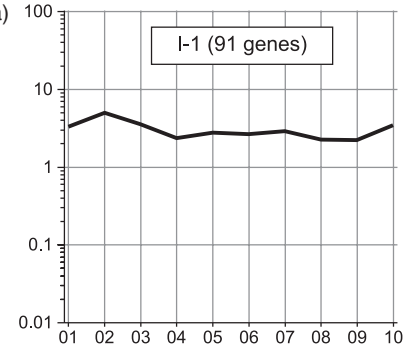
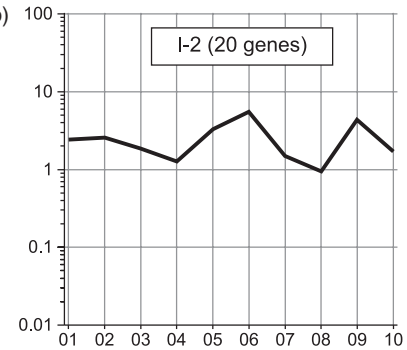
–500 to –10 bp) of the entire genome and of all the genes identified by our microarray experiments revealed that only the I-1 category was enriched in genes containing several GATAAG or GATT/AA sequences in their promoters (Fig. 3). These results suggest that only the genes of the I-1 group would be direct targets of Gln3 and Gat1, whereas

(a)



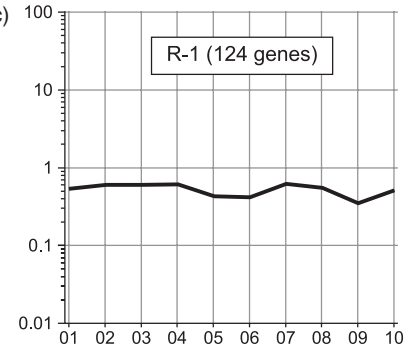
Biological process unknown	28 out of 91 genes, 31%
ALT1, FMP16, ICY1, LEE1, MOH1, NIT1, PHM8, PST2, SNA2, UGX2, YBR085C-A, YBR139W, YDR090C, YER030W, YET1, YIL087C, YIL165C, YKL137W, YKR033C, YLR149C, YLR164W, YLR257W, YMR181C, YMR196W,YOL019W, YOR052C, YPR098C, ZSP1	
Transport	**19 out of 91 genes, 20%**
AVT1, AVT4, AVT7, CAN1, DAL4, DAL5, DIP5, DUR3, GAP1, MEP2, OPT1, OPT2, PMP1, PMP3, PTR2, PUT4, QDR2, UGA4, VBA1	
Amino acid and allantoin metabolism	**15 out of 91 genes, 17%**
ASP3-1, ASP3-2, ASP3-3, ASP3-4, BAT2, DAL1, DAL2, DAL3, DAL7, DUR1,2, GDH2, GLN1, GLT1, PUT1, SDL1	
Proteolysis	**9 out of 91 genes, 10%**
CPS1, LAP3, LAP4, PBI2, PEP4, PRB1, PRC1, RPN6, RPT2	
Transcription	**4 out of 91 genes, 4%**
ACE2, DAL80, GAT1, INO4	
Other	**16 out of 91 genes, 18%**
ALD4, APE2, COX12, ECM15, ECM38, GPG1, GRX1, HOR7, HXK1, NRK1, RAS2, RNY1, YFR055W, YHR138C, YJR096W, YMR226C	

(b)



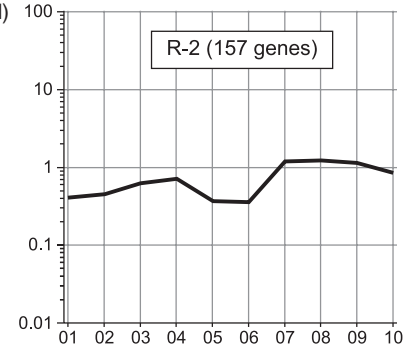
Response to stress	5 out of 20 genes, 25%
DDR2, HSP104, HSP12, HSP26, TTR1	
Generation of precursor metabolites and energy	**5 out of 20 genes, 25%**
ATP18, COX7, QCR7, STF2, TIM11	
Other	**10 out of 20 genes, 50%**
ARA1, ATG19, ATG22, BIG1, GTT1, PAI3, PNC1, PRX1, TFS1, YNL274C	

(c)



Amino acid and nucleotide metabolism	58 out of 124 genes, 47%
AAT2, ADE2, ADE3, ADE4, ADE5,7, ADE6, ADE8, ADO1, ARG5,6, ARO1, ARO2, ARO4, ASN2, ASP1, BAT1, CPA1, CPA2, CYS3, ECM17, GLY1, HIS1, HIS4, HIS7, HOM2, HOM3, ILV1, ILV3, ILV5, ILV6, IMD1, IMD2, IMD3, IMD4, LEU1, LYS1, LYS12, LYS2, LYS20, LYS21, LYS4, MET1, MET10, MET13, MET2, MET6, PRO2, PRS1, SAM1, SAM2, SAM4, SER1, SER3, STR3, THR1, THR4, TRP4, TRP5, URA2	
Protein biosynthesis	**23 out of 124 genes, 19%**
ANB1, CAM1, CDC60, DED81, FRS2, GCD7, GRS1, ILS1, KRS1, MES1, PMT2, PRT1, RLI1, RPL10, RPS14B, SSB1, SSB2, SUI2, SUI3, TEF4, TYS1, YDR341C, YHR020W	
Transport	**18 out of 124 genes, 14%**
ATO3, BAP2, CTP1, FUI1, GNP1, HIP1, MUP1, PSE1, SAM3, SEC61, SUL2, TAT1, TAT2, TNA1, VHT1, ZRC1, ZRT1, ZRT2	
Biological process unknown	**7 out of 124 genes, 6%**
ARB1, NEW1, YAR075W, YBR187W, YJL200C, YLR413W, YOR271C	
Other	**18 out of 124 genes, 14%**
AGA2, CDC19, FAS2, GND1, ISU2, PAC10, PDC1, PDC5, PGI1, PGU1, RHR2, RPA135, RPC40, TKL1, TPI1, UTP5, UTR2, YGR251W	

(d)



Protein biosynthesis	119 out of 157 genes, 76%
EFT1, EFT2, GCD6, MRPL7, NIP1, PAB1, PMT6, RPL11A, RPL12A, RPL12B, RPL13A, RPL13B, RPL14A, RPL15B, RPL16A, RPL16B, RPL17A, RPL17B, RPL18A, RPL18B, RPL19A, RPL1A, RPL1B, RPL20A, RPL20B, RPL21A, RPL21B, RPL22A, RPL22B, RPL23B, RPL24A, RPL26A, RPL26B, RPL27A, RPL27B, RPL29, RPL3, RPL30, RPL31A, RPL31B, RPL32, RPL33A, RPL33B, RPL34A, RPL35A, RPL35B, RPL36B, RPL37A, RPL37B, RPL39, RPL40A, RPL42A, RPL42B, RPL43A, RPL43B, RPL4A, RPL5, RPL6A, RPL6B, RPL7A, RPL7B, RPL8A, RPL8B, RPL9B, RPP0, RPP1B, RPP2A, RPS0A, RPS0B, RPS10A, RPS10B, RPS11A, RPS11B, RPS12, RPS13, RPS14A, RPS15, RPS16A, RPS16B, RPS17A, RPS17B, RPS18A, RPS18B, RPS19A, RPS1A, RPS1B, RPS2, RPS21A, RPS21B, RPS22A, RPS22B, RPS23A, RPS23B, RPS24A, RPS24B, RPS25B, RPS26A, RPS26B, RPS27A, RPS28A, RPS29A, RPS29B, RPS3, RPS30A, RPS31, RPS4A, RPS4B, RPS5, RPS6A, RPS6B, RPS7A, RPS7B, RPS8A, RPS8B, RPS9A, RPS9B, SRO9, TIF3, TIF5	
Other	**38 out of 157 genes, 24%**
ADE13, ADE17, AGP1, CGR1, CYS4, ELP2, FOL1, GPM1, GUK1, HEK2, HEM15, HOR2, KAP123, MAP1, MCA1, MEP3, MET3, PHO11, RBF18, RIB4, RPA49, RPB8, RPE1, RRP12, SCP160, SEC26, SIK1, SNU13, SRP40, SVL3, URA1, URA5, URA6, UTP9, YBR191W-A, YJL010C, YJR141W, YPL207W	

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genes of groups I-2 and R-1 would be indirectly controlled by the GATA factors.

All genes upregulated by nitrogen limitation do not respond similarly to the three regulators Gln3, Gat1 and Ure2

In order to validate the results obtained by our DNA microarray analysis, we performed northern blotting experiments with the same RNAs used in the microarray experiments, using a set of DNA probes from I-1 group genes. All the northern blotting experiments were repeated at least twice with RNA extracted from independently grown cells.

ASP3 was chosen as a typical NCR-regulated gene; other genes were selected either because they were not described as nitrogen-responsive genes, or because they appeared in previous global analyses but without confirmation (Cox *et al.*, 1999; Komeili *et al.*, 2000; Shamji *et al.*, 2000; Tai *et al.*, 2005). We compared the expression of the different genes in a wild-type strain and in a *gln3*Δ–*gat1*Δ strain grown on M.Gln, M.Gln+rapamycin, M.Pro, and after a 2-h shift from M.Gln to M.Pro, and in a *ure2*Δ strain grown on M.Gln (Fig. 4).

These genes can be classified into different groups. *ASP3*, *OPT2*, *AVT7* and *NRK1* are induced after addition of rapamycin, after growth on M.Pro and after a 2-h shift from M.Gln to M.Pro. Expression of these genes is constitutive in a *ure2*Δ strain, and their activation is abolished in the *gln3*Δ–*gat1*Δ strain under the different tested growth conditions. These are 'classic' NCR-responsive genes. Expression of *YIL165c*, *BAT2* and *YFR055w* is upregulated in response to the different nitrogen signals, is constitutive in a *ure2*Δ strain, and is abolished in a *gln3*Δ–*gat1*Δ strain after addition of rapamycin and growth on M.Pro, but is only weakly affected after a shift from M.Gln to M.Pro. Such genes would belong to the family of NCR genes, but during the shift their expression would be transiently activated in a Gln3- and Gat1-independent manner. Expression of *SDL1* and *YLR164w* is also induced in response to nitrogen availability, dependent on Gln3 and Gat1 but not on Ure2. In the last group, several genes (*PBI2*, *PRB1*, *HOR7*, *YBR139w* and *YOR052c*) are also nitrogen-sensitive genes under the control of Ure2, but the absence of Gln3 and Gat1 has only a minor effect. For these genes, the basal level of expression on M.Gln is rather high, resulting possibly from activation by an unknown factor that could operate more efficiently in the absence of Gln3 and Gat1, compensating for the lack of Gln3–Gat1 activation.

To establish if these genes are the direct targets of Gln3 and Gat1, it is essential that their promoters contain a UAS_{GATA}, consisting of at least two GATAAG or several GATT/AA sequences. For this study, we chose the region between –10 and –500 from the ATG as containing potential UAS_{GATA} elements, since a genome-wide GATA sequence

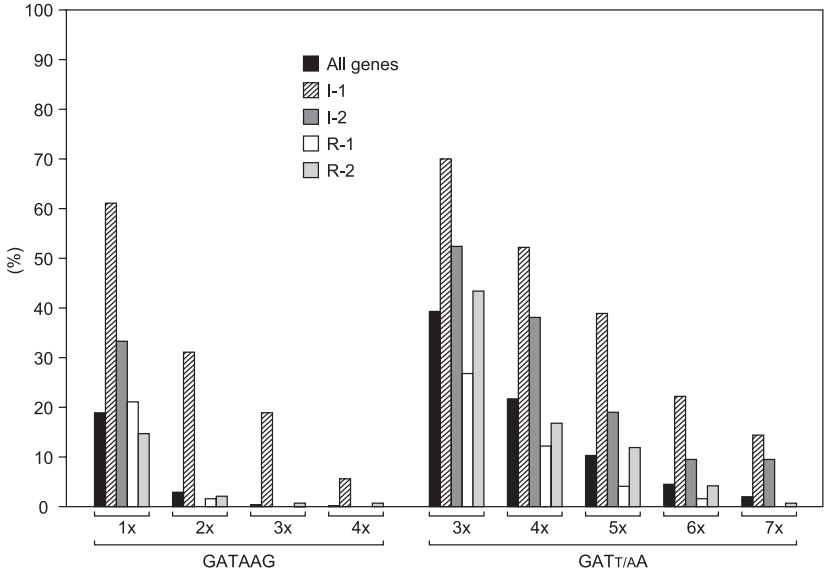

Fig. 3. Relationship between Gln3- and Gat1-dependent gene expression and occurrence of GATA factor-binding sites in the promoter. The frequency of occurrence of GATAAG or GATT/AA sequences in the promoters (from –500 to –10 bp) of the genes in the different categories identified by the microarray analysis was compared to that of the promoters of all genes in the genome. On the *y*-axis is plotted the percentage of genes containing one or several GATAAG or GATT/AA elements in their promoter in each category (all genes, I-1, I-2, R-1 and R-2). On the *x*-axis is plotted the number of GATAAG (one to four) or GATT/AA (three to seven) elements in the promoters. We used the genome-scale DNA-pattern search tool on the 'Regulatory Sequence Analysis Tools' website (http://rsat.scmbb.ulb.ac.be/rsat/) to retrieve a list of occurrences and positions of GATAAG or GATT/AA elements in the promoters of all genes (from –500 to –10 bp) (van Helden *et al.*, 1998). This list was queried to determine the number of GATA elements in the promoters of all the genes in each category.

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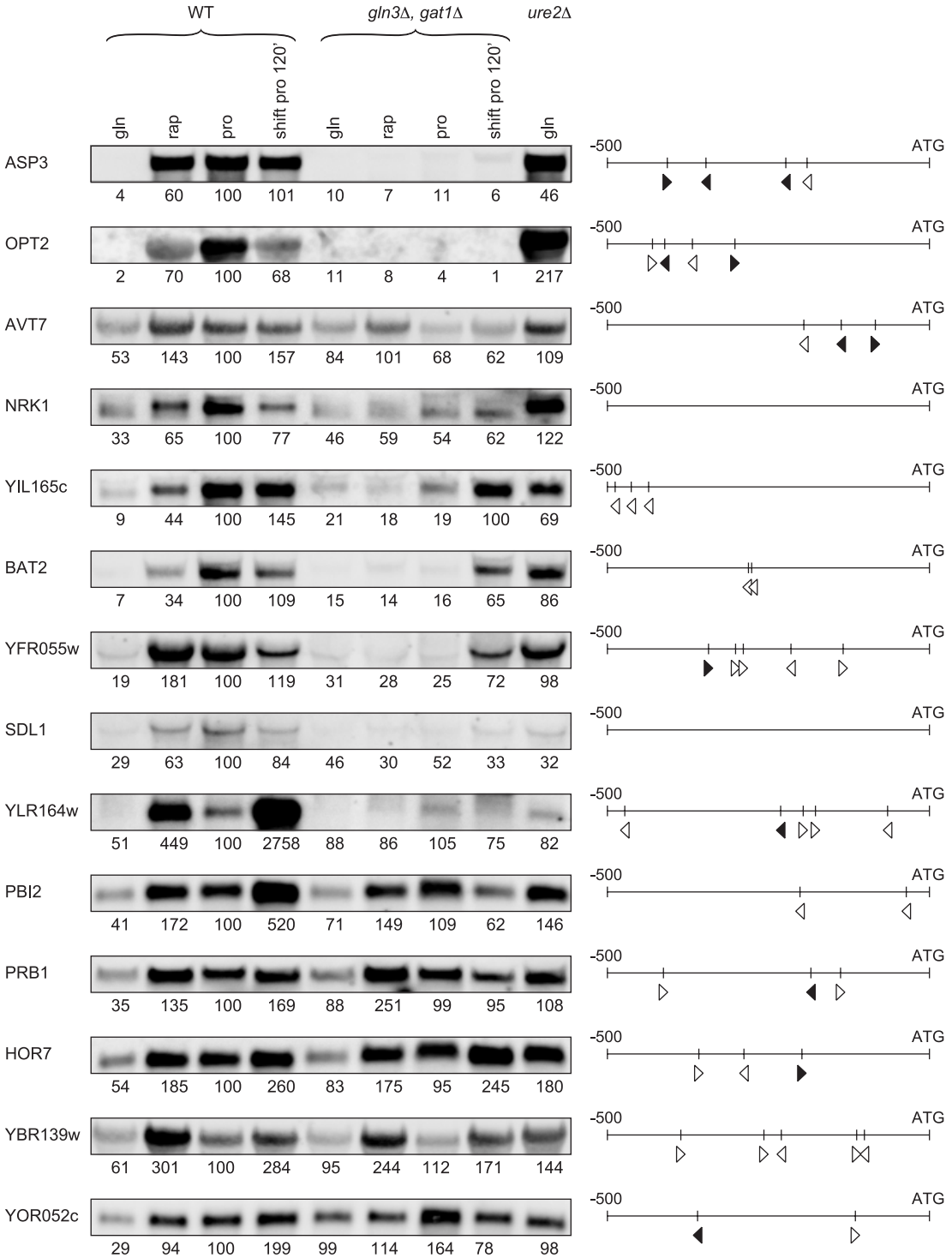

Fig. 4. Transcriptional activation of I-1 class genes in response to nitrogen availability and to Gln3 and Gat1 activators. Total RNAs were isolated from a wild-type strain (BY4700), a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln + rapamycin for 20 min, on M.Pro and after a 2-h shift from M.Gln to M.Pro, and a *ure2*Δ strain (4709ΔURE2) grown on M.Gln. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *ASP3*, *OPT2*, *AVT7*, *NRK1*, *YIL165c*, *BAT2*, *YFR055w*, *SDL1*, *YLR164w*, *PBI2*, *PRB1*, *HOR7*, *YBR139w* and *YOR052c*. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount (transcript not shown), taking as 100% the RNA value obtained for the wild-type strain grown on M.Pro. The right side of the figure shows a schematic drawing of the promoters of each gene (from –10 to –500 bp), in which are positioned GATAAG sequences (black triangles) and GATT/AA sequences (open triangles). The orientation of the triangles indicates if the sequence is 5′–3′ towards the ATG or on the complementary DNA strand.

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analysis had been performed in a region within 500 bp upstream of the ORF site (van Helden *et al.*, 1998). We have analyzed the promoters of the genes for which induction of expression was confirmed by northern blot analysis. The occurrence of GATAAG and GATT/AA sequences is presented on the right side of Fig. 4. Among the upregulated genes analyzed, eight promoters contain at least one GATAAG and several GATT/AA sequences, four promoters contain at least two GATT/AA sequences, but two genes, *SDL1* and *NRK1*, do not contain such sequences, suggesting for these two last genes an indirect control by the GATA factors. It is nevertheless difficult to ascertain the direct interaction of the GATA factors with a gene promoter without further analysis, since some promoters require an auxiliary binding site TTGT/GT, or a binding site for Abf1 or Rap1, and since some significantly upregulated genes such as *GDH2*, *PUT4* and *GLN1* contain few canonical GATT/AA sequences (reviewed in Magasanik & Kaiser, 2002). Moreover, the presence of the sequences GATAA does not imply function, since only two of eight such sequences situated upstream of the well-characterized *DAL5* gene, can be demonstrated to function *in vivo* (Rai *et al.*, 1989).

Induction of stress-responsive genes after a shift from glutamine to proline is delayed in a *gln3–gat1* strain

Subgroup I-2 contains 20 genes that are poorly derepressed on *gln3*Δ–*gat1*Δ but respond to the addition of rapamycin and to the shift from glutamine to proline. Among these genes, we find five stress-responsive genes and five genes involved in generation of metabolites and energy. Northern blot experiments for two stress-responsive genes (*HSP12* and *DDR2*) confirm that these genes strongly respond to addition of rapamycin and to the shift from M.Gln to M.Pro, and this effect is reduced in a *gln3*Δ–*gat1*Δ strain (Fig. 5a). It is noteworthy that the expression levels of *HSP12* and *DDR2* are very high on M.Pro in the *gln3*Δ–*gat1*Δ strain, which could be the consequence of a stress imposed on this strain while it is growing very slowly on M.Pro. This activation could occur through Msn2 and Msn4 regulators (Martinez-Pastor *et al.*, 1996), and thus this high gene expression should be abolished in a strain with simultaneous deletion of *GLN3*, *GAT1*, *MSN2* and *MSN4*. Indeed, *HSP12* and *DDR2* belong to a family of multistress-responsive genes (STRE family) and they are known to be also induced after rapamycin treatment through activation by Msn2 and Msn4 transcription factors. Rapamycin treatment or different stresses result in the rapid translocation of Msn2 and Msn4 to the nucleus (Beck & Hall, 1999).

We established the time course of *DDR2* expression after a shift from M.Gln to M.Pro and after addition of rapamycin (Fig. 5b, c). Induction of *DDR2* expression in a wild-type strain is very rapid under both growth conditions. In contrast, in the *gln3*Δ–*gat1*Δ strain, induction is delayed and reduced under both growth conditions. This induction is almost abolished in an *msn2*Δ–*msn4*Δ strain. These results suggest that shifting the cells from an optimal nitrogen source (M.Gln) to a poorer nitrogen source (M.Pro) induces a transient stress leading to activation of STRE genes, which is in agreement with the weaker induction of *HSP12* and *DDR2* genes when the cells grow on M.Pro under steady-state conditions. This response is delayed in a *gln3*Δ–*gat1*Δ mutant strain, which could result from its very slow growth on M.Pro. However, since the basal levels of *HSP12* and *DDR2* are reduced in the *gln3*Δ–*gat1*Δ strain on M.Gln compared to the wild-type strain, and as the expression of these genes is significantly increased in a *ure2*Δ strain, it is not excluded that the absence of Gln3, Gat1 and Ure2 proteins could lead to inappropriate activation of Msn2 and Msn4.

Shifting cells from glutamine to proline represses expression of amino acid and nucleotide biosynthetic genes

The expression of 124 genes (R-1 group) is downregulated in response to variations in the nitrogen source and is dependent on Gln3, Gat1 and Ure2. Of these genes, 47% are involved in amino acid and nucleotide metabolism (Fig. 2c). Among others, 19% of the genes, many of which encode aminoacyl tRNA synthetases, are involved in protein biosynthesis, and 14% of the genes encode transporters. This last category will be addressed in the next paragraph. Northern blot analysis confirms the moderate repression of *ILV3*, *IMD2* and *CDC60* by addition of rapamycin or growth on M.Pro, and after a shift from M.Gln to M.Pro (Fig. 6a). The repression induced by rapamycin still occurs in the absence of Gln3 and Gat1 (Fig. 6b), whereas the repression induced by the shift is significantly reduced in a *gln3*Δ–*gat1*Δ strain as well as in an *msn2*Δ–*msn4*Δ strain (Fig. 6c). This last result suggests an indirect role of these four regulators.

As Gcn4 controls many of these genes, we tested whether this repression was dependent on this protein. To address this point, we compared the *ILV3* and *IMD2* mRNA levels in a wild-type strain, a strain in which Gcn4 is constitutively expressed, and in a *gcn4*Δ strain. Repression of these genes during a shift from M.Gln to M.Pro still occurs, although to a lesser extent, in the *gcn4*Δ and *GCN4*^{c} mutant strains, suggesting that Gcn4 is not involved in this repression mechanism (Fig. 6d). Moreover, the amount of Gcn4 determined by western blot using a Gcn4–Myc strain or by β-galactosidase assays using *GCN4–LacZ* fusion did not vary under these different growth conditions or in a *gln3*Δ–*gat1*Δ strain (data not shown).

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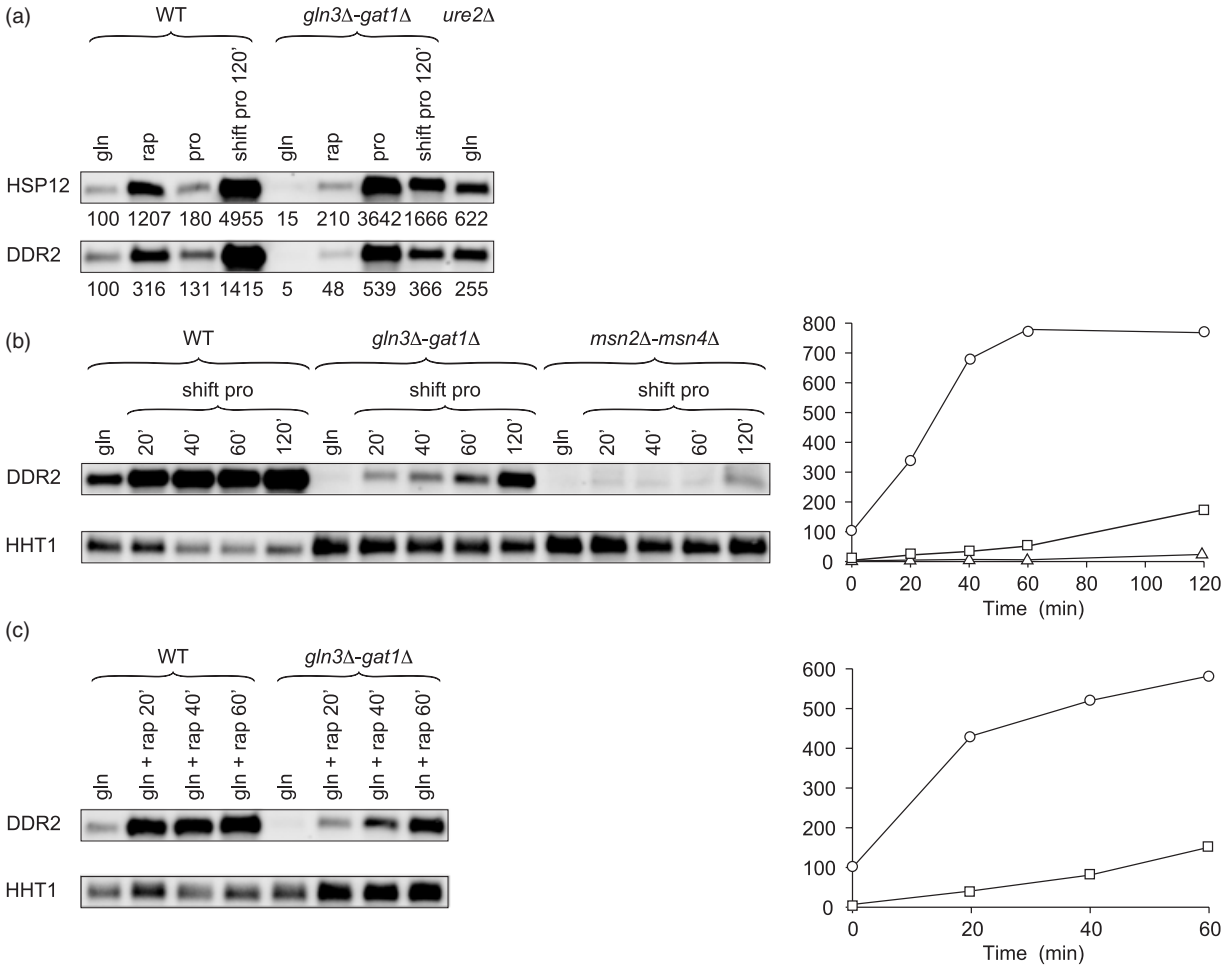

Fig. 5. Transient transcriptional activation of stress-responsive genes in response to nitrogen availability. (a) Total RNAs were isolated from a wild-type strain (BY4700), a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln+rapamycin for 20 min, on M.Pro and after a 2-h shift from M.Gln to M.Pro, and a *ure2*Δ strain (4709ΔURE2) grown on M.Gln. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *HSP12* and *DDR2*. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln. (b) Total RNAs were isolated from a wild-type strain (BY4700) (circles), a *gln3*Δ–*gat1*Δ strain (03167b) (squares) and an *msn2*Δ–*msn4*Δ strain (1C22182b) (triangles) grown on M.Gln and shifted to M.Pro. Aliquots were taken at different time points. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probe *DDR2*. The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes. (c) Total RNAs were isolated from a wild-type strain (BY4700) (circles), and a *gln3*Δ–*gat1*Δ strain (03167b) (squares) grown on M.Gln and on M.Gln+rapamycin for 20, 40 and 60 min. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probe *DDR2*. The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes.

Repression of transporter genes in response to nitrogen availability depends on induction of nitrogen catabolite repression-sensitive transporters

Among the genes whose expression is repressed in response to nitrogen limitation in a Gln3-, Gat1- and Ure2-dependent manner, we find several genes encoding amino acid transporters (*BAP2*, *GNP1*, *HIP1*, *MUP1*, *SAM3*, *TAT1* and *TAT2*) and three zinc transporters *(ZRC1*, *ZRT1* and *ZRT2*). These results are confirmed by northern blot analysis (Fig. 7a), and suggest that Gln3 and Gat1 negatively control the expression of these genes. However, none of the promoters of these genes contains canonical UAS_{GATA} sequences. It has been reported that Gap1 could be a major regulator of the yeast plasma membrane regulatory system responding to external amino acids (Bakkoury *et al.*, 2001). These authors proposed that Gap1 exerts a negative effect on the transcriptional induction of these genes and that this repression is relieved once Gap1 is downregulated by ubiquitination,

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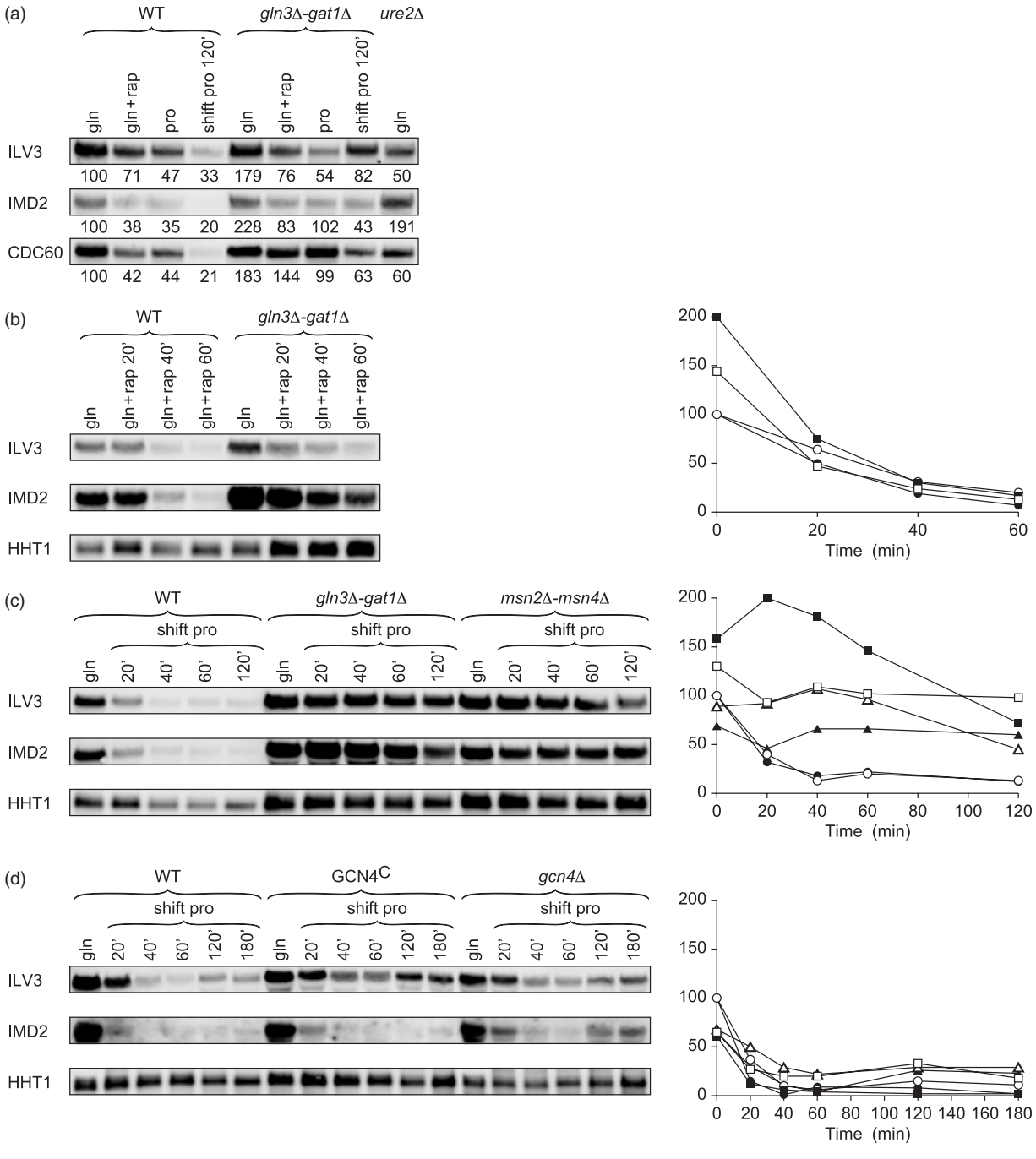
endocytosis and degradation. As *GAP1* is regulated in an opposite way from other transporter genes (Fig. 7a), the presence of Gap1 at the plasma membrane might be responsible for the repression of several transporter genes by an unknown mechanism. The results shown in Fig. 7b entirely fit with this view. Indeed, deletion of *GAP1* prevents partially or completely the repression of *GNP1*, *TAT2* and *ZRT1* in response to nitrogen availability. In addition, the loss of repression is also observed in an *npr1*Δ strain, in which Gap1 is not present at the plasma membrane (Grenson, 1983; De Craene *et al.*, 2001).

Repression of ribosomal protein genes after a shift from glutamine to proline is delayed in a *gln3*Δ*–gat1*Δ strain

Several groups have already shown that Tor signaling is a prerequisite for the induction of r-protein gene



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transcription that occurs in response to improved nutrient conditions (Cardenas *et al.*, 1999; Hardwick *et al.*, 1999; Powers & Walter, 1999). In the R-2 group, 76% of the genes encode proteins involved in protein biosynthesis. According to the average expression profile, these genes are mainly repressed by addition of rapamycin and after a shift from M.Gln to M.Pro, independently of the regulators Gln3, Gat1 and Ure2 (Fig. 2d). These results have been confirmed for two genes, *RPL3* and *RPL30* (Fig. 8a). In agreement with previous published data, a robust repression of ribosomal genes (*RPL30*) requires a long exposure to rapamycin (at least 40 min), and this repression is not impaired in a *gln3*Δ–*gat1*Δ strain (Fig. 8b). However, in this mutant strain there is a delay in the repression of *RPL30* when cells are shifted from M.Gln to M.Pro (Fig. 8c). The same delay is observed in an *msn2*Δ–*msn4*Δ strain. Thus, for r-protein genes, shifting cells from a good nitrogen source to a poorer one leads to the same response as addition of rapamycin. This repression is independent of the regulators activated by the Tor cascade (Gln3, Gat1, Msn2 and Msn4), even if the response to the shift condition appears to be delayed in the absence of these regulatory factors.

Discussion

We used whole-genome expression profiling to investigate the cellular adaptation to different nitrogen conditions, focusing on the role of the transcriptional activators Gln3 and Gat1 and their inhibitor Ure2. The microarray analysis of the yeast genome-wide response to variations in the quality of the nitrogen source allowed the identification of several hundreds of genes up- or downregulated under conditions of steady-state growth or with transient variations in growth conditions. However, only a more thorough analysis by northern blotting experiments allowed discrimination between direct activation or repression by the GATA factors and secondary metabolic consequences. Time course experiments, as well as the use of different mutated strains, were essential to establish the network of genes responding through Gln3 and Gat1 to the quality of the nitrogen source.

Our work has enlarged the set of genes whose expression responds through Gln3 and Gat1 to the quality of the nitrogen source at steady state. We have identified expected nitrogen-regulated genes, such as *DAL2*, *DAL3*, *DAL5*, *DAL7*, *ASP3*, *GAP1*, *MEP2*, *CPS1* and *PEP4*, and others. Moreover, the number of genes involved in transport and in proteolysis is increased and many genes of unknown function seem to be regulated by nitrogen. Among the genes identified by our microarray experiments appeared genes that were known to be regulated through the Tor cascade in response to rapamycin or to severe nutrient starvation as reported previously (Cardenas *et al.*, 1999; Powers & Walter, 1999), but we showed here that these genes also respond transiently to a sudden change in the quality of the nitrogen source (shift from M.Gln to M.Pro). Indeed, in a wild-type strain, a sudden change in the quality of the nitrogen source triggers activation or repression of a large set of genes similar to the response induced by rapamycin or by severe nutrient starvation. The shift from a good nitrogen source to a poorer one seems to provoke a transitory stress response, leading to induction of some heat shock genes and repression of genes involved in protein synthesis, such as ribosomal and amino acid biosynthetic genes. In contrast, in a strain deprived of the nitrogen regulators Gln3 and Gat1, the responses to rapamycin and to a nitrogen shift are not equivalent and may vary with the gene category. In a *gln3*Δ–*gat1*Δ strain, induction of heat shock genes is strongly reduced in response to both rapamycin and to the nitrogen shift, whereas repression of ribosomal and biosynthetic genes by rapamycin occurs independently of Gln3 and Gat1. However, in the *gln3*Δ–*gat1*Δ strain as well as in the *msn2*Δ–*msn4*Δ strain, repression of these genes is delayed or abolished after a nitrogen shift, suggesting that the response to rapamycin or to a nitrogen shift could involve distinct

Fig. 6. Transcriptional repression of genes involved in amino acid and nucleotide biosynthesis in response to nitrogen availability. (a) Total RNAs were isolated from a wild-type strain (BY4700), a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln+rapamycin for 20 min, on M.Pro and after a 2-h shift from M.Gln to M.Pro, and a *ure2*Δ strain (4709ΔURE2) grown on M.Gln. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *ILV3*, *IMD2* and *CDC60*. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln. (b) Total RNAs were isolated from a wild-type strain (BY4709) (circles), and a *gln3*Δ–*gat1*Δ strain (03167b) (squares) grown on M.Gln and on M.Gln+rapamycin 200 ng mL^{-1} for 20, 40 and 60 min.Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *ILV3* (open symbols) and *IMD2* (filled symbols). The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes. (c) Total RNAs were isolated from a wild-type strain (BY4709) (circles), a *gln3*Δ–*gat1*Δ strain (03167b) (squares) and an *msn2*Δ–*msn4*Δ strain (1C22182b) (triangles) grown on M.Gln and shifted to M.Pro. Aliquots were taken at different time points. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *ILV3* (open symbols) and *IMD2* (filled symbols). The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes. (d) Total RNAs were isolated from a wild-type strain (F113) (circles), a *GCN4*^{c} strain (H396) (squares) and a *gcn4*Δ strain (F212) (triangles) grown on M.Gln and shifted to M.Pro. Aliquots were taken at different time points. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *ILV3* (open symbols) and *IMD2* (filled symbols). The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes.

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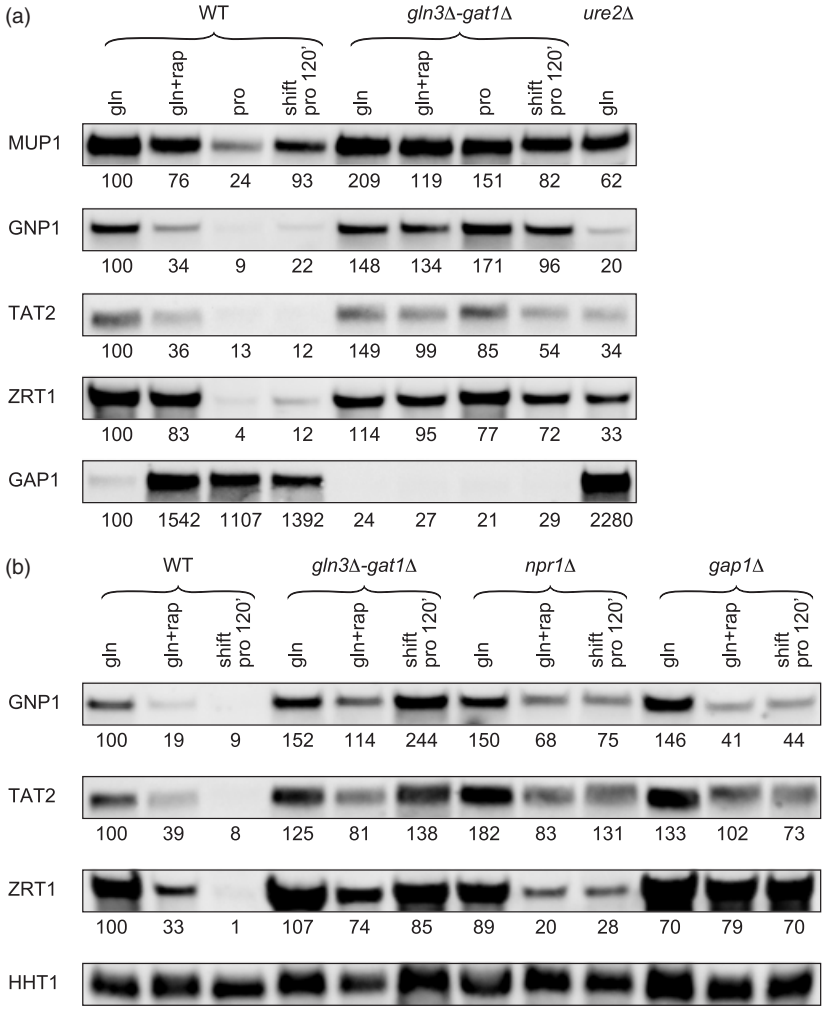

Fig. 7. Transcriptional repression of transporter genes as a consequence of induction of nitrogen catabolite repression-sensitive transporters in response to nitrogen availability. (a) Total RNAs were isolated from a wild-type strain (BY4700), a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln+rapamycin for 20 min, on M.Pro and after a 2-h shift from M.Gln to M.Pro, and a *ure2*Δ strain (4709ΔURE2) grown on M.Gln. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *MUP1*, *GNP1*, *TAT2*, *ZRT1* and *GAP1*. (b) Total RNAs were isolated from a wild type (BY4742), *gln3*Δ–*gat1*Δ strain (03167b), and *npr1*Δ (4742ΔNPR1) and *gap1*Δ (4742ΔGAP1) strains grown on M.Gln, on M.Gln+rapamycin for 20 min and after a 2-h shift from M.Gln to M.Pro. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *GNP1*, *TAT2*, *ZRT1* and *GAP1*. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln.

mechanistic pathways. This distinction would be more striking in the mutated strains, since they might have a fast adaptation deficiency. Moreover, it is also possible that the absence of the three nitrogen regulators tested (Gln3, Gat1 and Ure2) leads to artificial cross-talk between the stress and nitrogen signaling pathways, as some components of these signaling pathways are shared.

To date, as for the repression of r-protein genes, the factors involved in the repression of amino acid and nucleotide genes in response to nitrogen availability remain uncharacterized. Among the amino acid- and nucleotide-repressed genes, we have mainly identified genes involved in lysine, branched-chain amino acid, methionine, adenine and uracil biosynthesis, which is in agreement with recent observations from T. Powers' group (personal communication). In contrast to data previously reported by others (Hardwick *et al.*, 1999; Shamji *et al.*, 2000; Rohde *et al.*, 2004), we did not observe induction of arginine, histidine and tryptophan biosynthetic genes in response to rapamycin treatment.

It is also noteworthy that in our experiments we did not retrieve the retrograde RTG target genes, such as *CIT2*, because these genes were only induced after rapamycin treatment, and not under the other growth conditions, since

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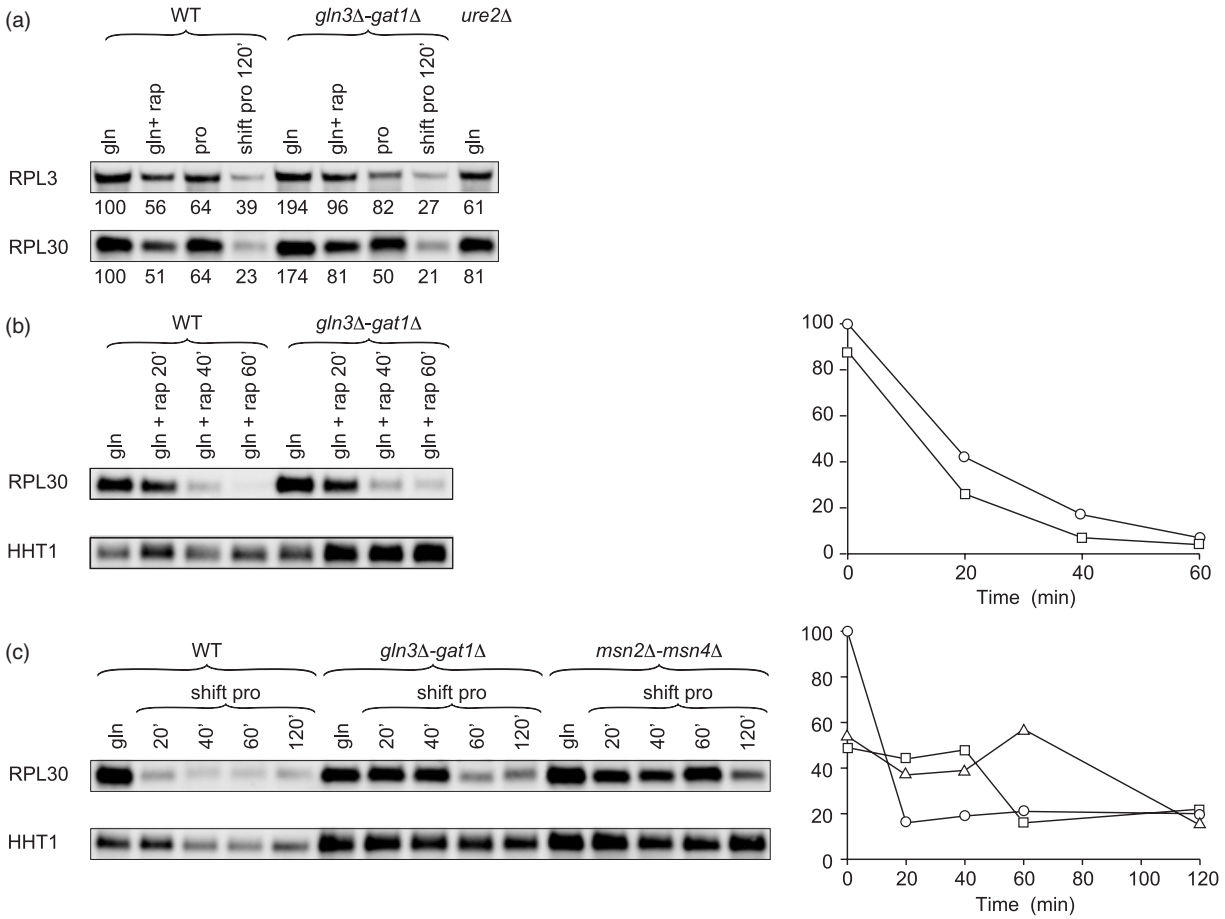

Fig. 8. Transcriptional repression of r-protein genes in response to nitrogen availability. (a) Total RNAs were isolated from a wild-type strain (BY4700), a *gln3*Δ–*gat1*Δ strain (03167b) grown on M.Gln, on M.Gln+rapamycin for 20 min, on M.Pro and after a 2-h shift from M.Gln to M.Pro, and a *ure2*Δ strain (4709ΔURE2) grown on M.Gln. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probes *RPL3* and *RPL30*. The numbers under each signal represent the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln. (b) Total RNAs were isolated from a wild-type strain (BY4709) (circles), and a *gln3*Δ–*gat1*Δ strain (03167b) (squares) grown on M.Gln and on M.Gln+rapamycin for 20, 40 and 60 min.Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probe *RPL30*. The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes. (c) Total RNAs were isolated from a wild-type strain (BY4709) (circles), a *gln3*Δ–*gat1*Δ strain (03167b) (squares) and an *msn2*Δ–*msn4*Δ strain (1C22182b) (triangles) grown on M.Gln and shifted to M.Pro. Aliquots were taken at different time points as indicated in the figure. Thirty micrograms of RNA were analyzed by northern blotting with DIG-labeled probe *RPL30*. The *y*-axis represents the amount of RNA normalized to the *HHT1* mRNA amount, taking as 100% the RNA value obtained for the wild-type strain grown on M.Gln; the *x*-axis represents time points in minutes.

we used proline as a poor nitrogen source and not urea. Indeed, it is known that glutamate provided by proline degradation represses RTG target genes (Komeili *et al*., 2000; Tate *et al*., 2002; Dilova *et al*., 2004).

Repression of some transporter genes by addition of rapamycin, growth on a poor nitrogen source or shifting cells from a good nitrogen source to a poorer one has already been reported (Komeili *et al*., 2000). However, we have shown that this repression, although depending on the presence of Gln3 and Gat1, is indirect, since this repression is relieved in a *gap1*Δ strain. Occupancy of the plasma membrane by transporters such as Gap1, whose expression is induced by Gln3 and Gat1, could trigger transcriptional repression of other transporter genes, which is in agreement with data showing that Gap1 prevents Agp1 from being induced by low concentrations of external amino acids (El Bakkouri *et al*., 2001). Our microarray analysis allowed us to extend this regulation to a series of transporters, not only amino acid transporters such as Gnp1, Bap2, Tat1, Tat2 and Mup1, but also to zinc transporters (Zrc1, Zrt1 and Zrt2).

The role of the Tor cascade in the response to the presence of preferred nitrogen or carbon sources could be to maintain the adequate expression level of different gene networks to

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ensure optimal growth. Upon transient depletion of nutrients, the cell would activate or repress at once all the genes under Tor signaling (nitrogen, ribosomal, biosynthetic and stress-responsive genes) to further select and maintain the precise adaptation response.

Supplementary material

The following supplementary material is available for this article online.

Fig. S1. Hierarchical gene tree clustering analysis of the results from 10 microarray experiments.

Table S1. The normalized ratios for the 392 genes selected from the analysis of 10 experiments.

The material is available as part of the online article from http://www.blackwell-synergy.com

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