Nitrogen-source regulation of yeast γ-glutamyl transpeptidase synthesis involves the regulatory network including the GATA zinc-finger factors Gln3, Nil1/Gat1 and Gzf3

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In *Saccharomyces cerevisiae*, the *CIS2* gene encodes γ-glutamyl transpeptidase (γ-GT; EC 2.3.2.2), the main GSH-degrading enzyme. The promoter region of *CIS2* contains one stress-response element (CCCCT) and eight GAT(T/A)A core sequences, probably involved in nitrogen-regulated transcription. We show in the present study that expression of *CIS2* is indeed regulated according to the nature of the nitrogen source. Expression is highest in cells growing on a poor nitrogen source such as urea. Under these conditions, the GATA zinc-finger transcription factors Nil1 and Gln3 are both required for *CIS2* expression, Nil1 appearing as the more important factor. We further show that Gzf3, another GATA zinc-finger protein, acts as a negative regulator in nitrogen-source control of *CIS2* expression. During growth on a preferred nitrogen source like NH_4^+, *CIS2* expression is repressed through a mechanism involving (at least) the Gln3-binding protein Ure2/GdhCR. Induction of *CIS2* expression during nitrogen starvation is dependent on Gln3 and Nil1. Furthermore, rapamycin causes similar *CIS2* activation, indicating that the target of rapamycin signalling pathway controls *CIS2* expression via Gln3 and Nil1 in nitrogen-starved cells. Finally, our results show that *CIS2* expression is induced mainly by nitrogen starvation but apparently not by other types of stress.

Key words: GATA zinc finger, γ-glutamyl transpeptidase, glutathione, nitrogen regulation, *Saccharomyces cerevisiae*, stress.

INTRODUCTION

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a thiol tripeptide present in most living cells, from micro-organisms to human beings. It plays numerous metabolic roles, such as bioreduction, protection against oxidative stress, detoxification of endogenous toxic metabolites and xenobiotics, transport and enzymic catalysis [1,2]. Yeast can use GSH as an endogenous sulphur source. In the presence of sulphate, excess sulphur can be incorporated into GSH and later mobilized by the cells [3]. Previous studies have shown that the GSH stored in the yeast vacuole can also serve as an alternative nitrogen source during nitrogen starvation [4].

GSH is synthesized by two sequential ATP-requiring reactions, catalysed respectively by γ-glutamylcysteine synthetase (Gsh1p, EC 6.3.2.2, P32477) and glutathione synthetase (Gsh2p, EC 6.3.2.3, Q08220). The encoding genes are respectively *GSH1/YJL101c* and *GSH2/YOL049w* [5]. GSH catabolism in the yeast *Saccharomyces cerevisiae* is mediated by γ-glutamyl transpeptidase (γ-GT; Cis2p, EC 2.3.2.2, Q05902) encoded by *CIS2/ECM38/YLR299w* and by an as yet unidentified cysteinylglycine dipeptidase [6]. γ-GT catalyses the transfer of the γ-glutamyl moiety of GSH and γ-glutamyl compounds to amino acids and also the hydrolytic release of L-glutamate from GSH, γ-glutamyl compounds and S-substituted derivatives (see [2] for a review). Despite the crucial importance of GSH metabolism in cell physiology, it is only in recent years that results on the mechanisms that regulate it have emerged. One finding is that expression of *GSH1* and *GSH2* requires the transcription factor Yap1 [7,8]. Transcription of both genes increases in a Yap1-dependent way under oxidative stress or heat shock [8]. Moreover, transcription factors such as Met4, Met31 and Met32, involved in controlling the metabolism of sulphur-containing amino acids, are required for the regulation of *GSH1* expression in response to cadmium [9].

In the present study, we report that *CIS2* expression is controlled by the nature of the nitrogen source through a regulatory network of transcription factors. We propose that *CIS2* activation during nitrogen starvation or rapamycin treatment involves probably the target of rapamycin (TOR) signalling pathway.

EXPERIMENTAL

Materials

Unless otherwise stated all of the reagents were purchased from Sigma-Aldrich or Fluka (Bornem, Belgium). Products for yeast growth media were obtained from Difco (Becton Dickinson Europe, Meylan Cedex, France) or Gibco (Life Technologies Ltd, Paisley, Scotland, U.K.).

Strains, growth conditions and methods

The *S. cerevisiae* strains used in the present study are all isogenic with the wild-type strain Σ1278b [10], except for the mutations mentioned in Table 1. Cells were grown in a minimal buffered medium (pH 6.1) with 3% (w/v) glucose as the carbon source [11]. To this medium, one or more of the following nitrogen sources were added: L-proline (0.1%), L-glutamate (0.1%), urea (0.1%); L-glutamine (0.1%) or ammonium sulphate (10 mM). When needed, supplements were added to complement auxotrophy. Yeast cells treated with lithium acetate were transformed as described previously [12]. The *Escherichia coli* strain

Abbreviations used: γ-GT, γ-glutamyl transpeptidase; STRE, stress-response element; TOR, target of rapamycin.

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Table 1 Strains used in the present study

Strain	Genotype	Reference
Σ1278b		[40]
KM097	cis2Δ::kanMX2	[6]
24344c	*ura3*	[47]
30505b	*ura3,gln3*Δ	[41]
32164b	*ura3,nil1*Δ	[21]
32164c	*ura3,gln3*Δ, *nil1*Δ	[21]
26854a	*ura3,ure2* (*gdhCR*)	[42]
SBS10	*ura3,gzf3*Δ	[21]

used for plasmid-DNA amplification and purification was JM109. All procedures for manipulating DNA are described in [13].

Plasmids

The centromeric YCp*CIS2-lacZ* plasmid was constructed by inserting a DNA fragment into *Hin*dIII–*Bam*HI sites of YCpAJ152 [14], flanked by *Bam*HI and *Hin*dIII sites, spanning nucleotides −1000 to +8 with respect to the ATG initiation codon of the *CIS2* gene. This DNA fragment was obtained by PCR amplification with primers Z-GGT3 (5′-CGCGGATCC-CACAACAGCATAAC-3′) and Z-GGT5 (5′-CCCAAGCTTG-GAATATCCTGGTG-3′), using total Σ1278b DNA as the template. The same plasmid was further used to transform yeast strains auxotrophic for uracil. Clones expressing *CIS2-LacZ* were selected for their ability to grow in minimal uracil-free medium.

Enzyme assays and GSH content

The intracellular GSH concentration was determined according to the method described in [6]. The γ-GT assays were performed as described in [4]; enzyme activities are expressed in nmol of *p*-nitroaniline produced $h^{-1} \cdot (\text{mg of protein})^{-1}$ at 37 °C. β-Galactosidase assays were performed as described in [14]; enzyme activities are expressed in nmol of *o*-nitrophenol formed $min^{-1} \cdot (\text{mg of protein})^{-1}$ at 30 °C. Protein concentrations were measured by the method of Lowry et al. [15] with BSA as the standard. All assays were performed on cells that had reached a balanced growth phase [16].

RESULTS

CIS2 expression is regulated by the nitrogen source

We have reported previously that γ-GT activity is high in yeast cells using L-glutamate or urea as the sole nitrogen source [17]. In contrast, this activity is minimal when the nitrogen source is ammonium (NH_4^+). This regulation by NH_4^+ is proposed to occur at transcription level, but its exact mechanism remains largely unknown [17]. To investigate this mechanism, we constructed a *LACZ* fusion where the β-galactosidase gene is under the control of the promoter region of *CIS2*. Wild-type cells transformed with the *CIS2-LacZ* plasmid were grown on minimal medium supplemented with different nitrogen sources, and both the β-galactosidase and γ-GT activities were measured (Figure 1). Expression of *CIS2* was maximal in cells using urea, L-glutamate or L-proline as the nitrogen source. As expected, this high level of expression was accompanied by high γ-GT activity. In contrast, the presence of NH_4^+ or L-glutamine or both resulted in strong repression of *CIS2* and low γ-GT activity. Taken together, these results show that *CIS2* expression is regulated by the nature of the nitrogen source.

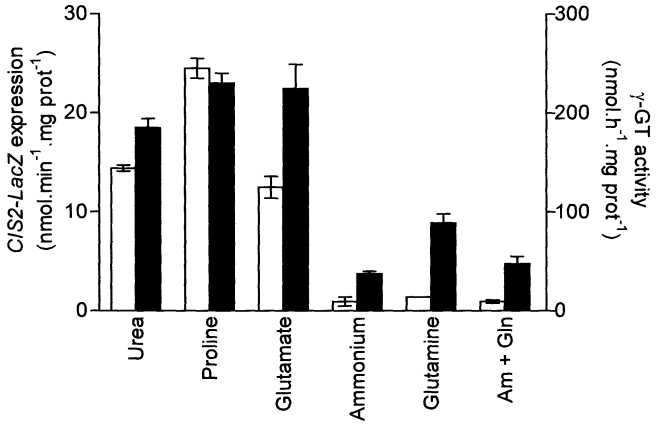

Figure 1 Expression of *CIS2* gene is regulated by nitrogen sources

Wild-type cells transformed with *CIS2-LacZ* gene were grown on minimal medium containing a nitrogen source at the following concentration: 0.1 % urea, 0.1 % L-proline, 0.1 % L-glutamate, 10 mM $(NH_4)_2SO_4$, 0.1 % L-glutamine or 10 mM $(NH_4)_2SO_4$ + 0.1 % L-glutamine (Am + Gln) and both *CIS2-LacZ* expression (□) and γ-GT activity (γ-GT) (■) were measured. Results are means ± S.D. for at least two independent experiments.

Table 2 Number of upstream regulatory sequences found in the promoting region of genes related to GSH and sulphur metabolism

Enzyme	Gene name	GAT(T/A)A sequence	CCCCT sequence
γ-Glutamyl-cysteine synthetase	*GSH1/YJL101c*	7	1
Glutathione synthetase	*GSH2/YOL049w*	3	0
γ-Glutamyl transpeptidase	*CIS2/YLR299w*	8	1
Glutathione reductase	*GLR1/YPL091w*	2	0
Glutaredoxin	*GRX1/YCL035c*	9	1
Glutaredoxin	*GRX2/YDR513w*	7	3
Glutathione S-transferase	*GTT1/YIR038c*	4	3
Glutathione S-transferase	*GTT2/YLL060c*	4	0
Glutathione peroxidase	*GPX1/YKL026c*	5	4
Glutathione peroxidase	*GPX2/YBR244w*	3	1
Glutathione peroxidase	*GPX3/YIR037w*	6	1
Cell-surface glutathione transporter	*HGT1/YJL212c*	6	2
Vacuolar glutathione transporter	*YCF1/YDR135c*	5	1
γ-Cystathionine synthase	*CYS4/YOR155w*	9	1
β-Cystathionase	*YFR055w*	8	0
β-Cystathionine synthase	*MET7/YOR241w*	6	0

The GATA zinc-finger proteins Gln3 and Nil1/Gat1 both contribute to *CIS2* expression

In general, the promoter regions of yeast genes responding to nitrogen control contain several GAT(T/A)A sequences acting as upstream regulatory elements. These sequences constitute DNA-binding domains for transcription factors of the GATA zinc-finger family (see [18] for a review). Analysis of the *CIS2* promoter region showed that it contains eight GAT(T/A)A sequences, probably involved in nitrogen-source-regulated transcription (Table 2). In *S. cerevisiae*, GATA zinc-fingers Gln3 and Nil1/Gat1 positively regulate the expression of nitrogen-source-responsive genes [19,20]. To investigate the role of these transcription factors in *CIS2* expression, we introduced the *CIS2*-

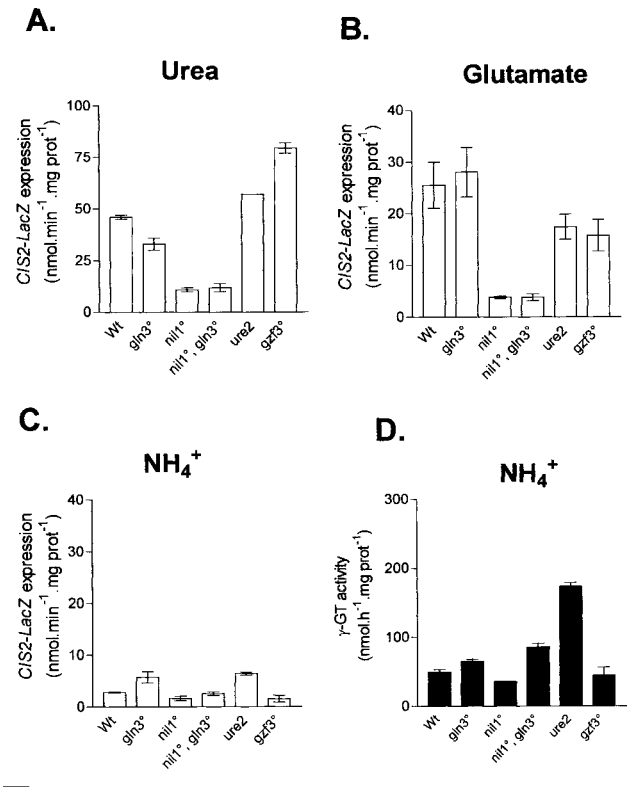

Figure 2 GATA zinc-finger transcription factors control expression of the *CIS2* gene

Cells transformed with the *CIS2-LacZ* gene were grown on minimal medium containing either 0.1% urea, 0.1% glutamate or 20 mM NH_4^+ as the sole nitrogen source and *CIS2-LacZ* expression or γ-GT activity was measured in wild-type (Wt) and the indicated mutant cells. Results are means ± S.D. for at least two independent experiments.

LacZ fusion into mutant cells lacking the *GLN3* gene, the *NIL1* gene or both, and monitored β-galactosidase activity during growth on various nitrogen sources (Figure 2). As already shown, *CIS2* expression was high in wild-type cells grown on urea. It was slightly reduced in *gln3* cells and almost abolished in *nil1* and *nil1,gln3* double-mutant cells. In cells growing on L-glutamate, *CIS2* expression was almost abolished in *nil1* mutants, but unaffected by the *gln3* mutation, indicating that Nil1 is the main transcription activator. This result was quite unexpected, since it is generally assumed that glutamate inhibits the action of Nil1 [19]. To our knowledge, this is the first example of gene expression requiring Nil1 in cells growing on glutamate. Taken together, these results show that Nil1 and Gln3 both act as positive regulators of *CIS2* expression, Nil1 appearing as the main positive factor.

It has been proposed that during growth on a preferred nitrogen source, Nil1-dependent gene expression is inhibited by the action of a GATA zinc-finger factor called Gzf3 [21,22], whereas Gln3-dependent expression is inhibited by the Gln3-binding protein Ure2/GdhCR [23,24]. Since *CIS2* expression is down-regulated by NH_4^+, we tested the involvement of these two negative factors in this regulation. As already shown (Figure 1), *CIS2* expression was strongly reduced in cells grown on NH_4^+. Deletion of the *GZF3* gene did not relieve this repression, but *CIS2* expression and γ-GT activity increased slightly when the *URE2/GDHCR* gene was mutated, indicating that Ure2/GdhCR can participate in *CIS2* repression. Increased expression of *CIS2* was also observed in *gln3* mutant cells, but this was not accompanied by a significant increase in γ-GT activity. Finally, deletion of *GZF3* resulted in increased *CIS2* expression in urea-grown but not in glutamate-grown cells, indicating that Gzf3 might inhibit Nil1-dependent *CIS2* expression through a nitrogen-source-controlled mechanism. In conclusion, our results show that the well-known regulatory network of GATA zinc-finger proteins controlling the expression of genes involved in nitrogen metabolism also regulates *CIS2* expression.

Nitrogen starvation induces *CIS2* expression

We have shown previously that nitrogen starvation increases γ-GT activity [17]. To test whether this regulation occurs at the transcription level, we monitored *CIS2* expression during nitrogen starvation in both the wild-type and the *gln3,nil1* double-mutant cells (Figure 3A). When ammonium-grown wild-type cells were transferred to a minimal medium lacking a nitrogen source, *CIS2* expression increased gradually. This derepression

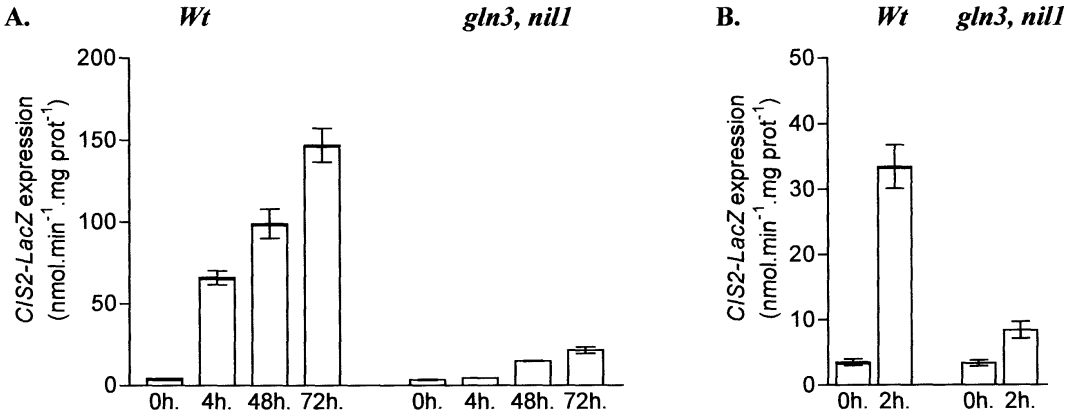

Figure 3 Nitrogen starvation and rapamycin treatment increase *CIS2* gene expression

Wild-type (Wt) and *gln3,nil1* mutant cells transformed with the *CIS2-LacZ* gene were grown on minimal medium containing 20 mM NH_4^+ and *CIS2-LacZ* expression was measured before and at various times after transferring the cells to (**A**) a minimal medium containing no nitrogen source or (**B**) minimal 20 mM NH_4^+ medium supplemented with 1 μM rapamycin. Results are means ± S.D. for at least two independent experiments.

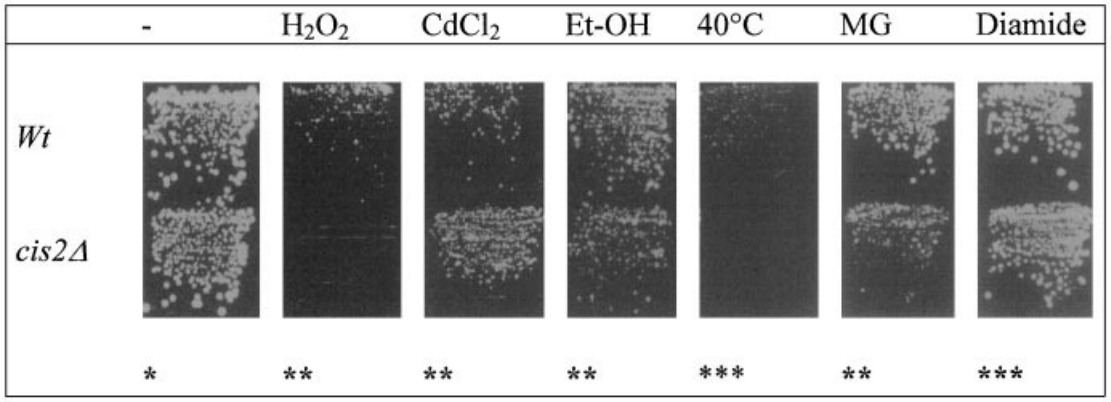

Figure 4 Effect of γ-GT deprivation on growth under different types of stress

Wild-type (Wt) and *cis2*Δ cells were grown on minimal L-glutamate medium containing one of the following toxic compounds: 5 mM H_2O_2, 50 μM $CdCl_2$ 8% ethanol (Et-OH), 2 mM methylglyoxal (MG) and 0.5 mM diamide. Cell growth was estimated after (*) 2 days, (**) 3 days or (***) 4 days of incubation. Results are representative of three independent experiments.

was strongly impaired in the *gln3,nil1* double-mutant cells, indicating that Gln3, Nil1 or both are required for derepression of *CIS2* during nitrogen starvation.

The TOR-dependent signalling pathway plays a critical role in the nutrient-responsive regulation of gene expression [25,26]. In *S. cerevisiae*, TOR proteins bind to and inhibit the activity of some nutrient-responsive transcription factors, including the GATA zinc-finger factors Gln3 and Nil1 [25]. To investigate whether the TOR signalling pathway might be involved in nitrogen-regulated expression of *CIS2*, we tested the effect of rapamycin, an inhibitor of TOR function, on *CIS2* expression (Figure 3B). Addition of rapamycin to NH_4^+-growing wild-type cells caused an increase in *CIS2* expression similar to that detected during nitrogen starvation. As expected, this induction was much reduced in *gln3,nil1* double-mutant cells. Taken together, these results suggest that the TOR signalling pathway is involved in the regulation of *CIS2* expression via Gln3 and Nil1.

CIS2 expression under adverse conditions

The promoter region of the *CIS2* gene contains one copy of the stress-response element (STRE), 5′-CCCCT-3′ (Table 2). This sequence, present in one or multiple copies in the promoter region of a gene, is generally required for increased expression of that gene under various types of stress, such as heat shock, nitrogen starvation or oxidative stress [27,28]. To examine whether γ-GT is required for cell resistance to stress, we compared the growth phenotype of wild-type cells and *cis2*Δ cells lacking γ-GT, under various types of stress (Figure 4). Cells lacking *CIS2* grew just as well as wild-type cells on glutamate medium. The growth of wild-type cells on this medium was reduced when H_2O_2, cadmium chloride, ethanol, methylglyoxal or diamide was added to the medium, and also after heat-shock treatment. Deletion of *CIS2* rendered the cells more sensitive to H_2O_2, methylglyoxal and heat shock (to different degrees), but not to cadmium, ethanol or diamide. Since the intracellular level of GSH is similar in *cis2*Δ and wild-type cells (K. Mehdi and M. J. Penninckx, unpublished work), these results favour a role for γ-GT in the response of yeast to various types of stress. To investigate this possibility, we studied the effect of various types of stress on *CIS2* expression (Figure 5). As shown before, both nitrogen deprivation and rapamycin treatment caused *CIS2* expression to increase markedly (Figure 3), but other types of stress had no effect. Hence, γ-GT biosynthesis in the Σ1278 background appears less sensitive to stress when compared with nitrogen regulation, at least under the conditions tested.

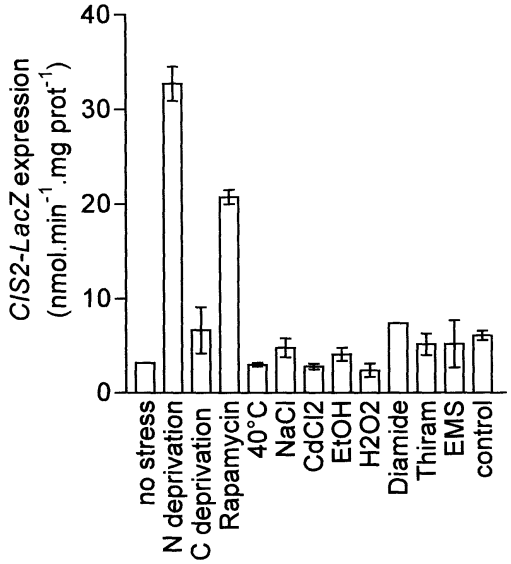

Figure 5 Effect of various types of stress on *CIS2* expression

Wild-type cells transformed with the *CIS2-LacZ* gene were grown on minimal medium containing 20 mM NH_4^+ and *CIS2-LacZ* expression was measured before and 2 h after transferring the cells to the minimal medium lacking a nitrogen or carbon source or after treating the cells with 1 μM rapamycin, 300 mM NaCl, 1 mM $CdCl_2$, 8% ethanol, 5 mM diamide, 1 mM H_2O_2, 1 mM thiram or 0.05% ethylmethane sulphonate (EMS). Control means *CIS2-LacZ* expression in cells allowed to remain in the initial minimal NH_4^+ medium without any stress throughout the 2 h of the experiment. Results are means ± S.D. for atleast two independent experiments.

DISCUSSION

In the present study, we show that *CIS2* expression is regulated according to the nature of the nitrogen source. Expression is high when yeast is grown on a poor nitrogen source, such as urea or L-proline, and low when a good nitrogen source, such as NH_4^+ or L-glutamine, is provided. This regulation is a common feature

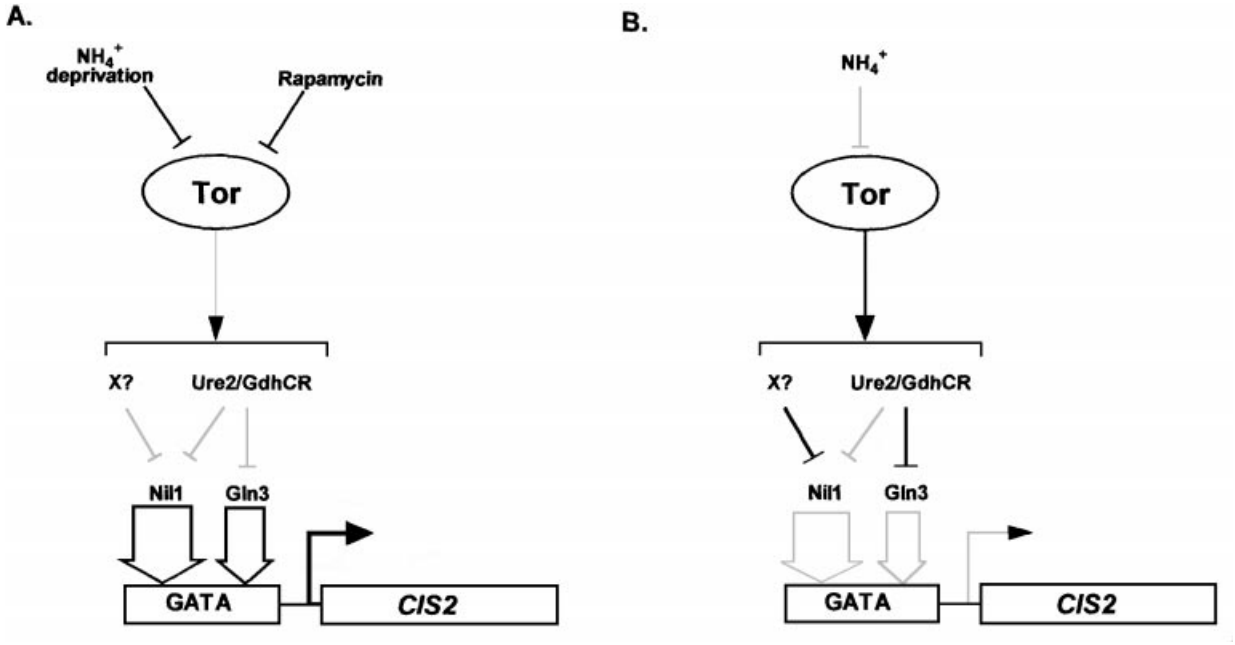

Figure 6 Regulatory network involved in nitrogen-regulated *CIS2* expression

(**A**) When cells are grown on a medium containing a poor nitrogen source or they are nitrogen-starved or treated with rapamycin, the TOR signalling pathway is in an inactive state allowing the positive GATA zinc-fingers Nil1 and Gln3 to activate *CIS2* expression. (**B**) When NH_4^+ is present in the medium, the TOR pathway is active and transcription factors Gln3 and Nil1 are prevented from activating *CIS2* by the action of the Gln3-binding protein Ure2/GdhCR and of an unknown protein labelled X.

of genes involved in nitrogen uptake and utilization, similar to amino acid permeases, NAD-glutamate dehydrogenase or arginase (see [16] for a review). Our results show a good correlation between *CIS2* expression (as measured in reporter-gene expression assays) and γ-GT activity, yet some differences were noted. For instance, reporter-gene expression was only half as high in glutamate-grown cells when compared with proline-grown cells, whereas γ-GT activity was similar. Perhaps, the expression must reach a certain threshold for γ-GT activity to develop or there exist additional post-transcriptional regulations. Such regulations, according to the nature of the nitrogen source, have been extensively studied in the case of the amino acid permease Gap1. Targeting of Gap1 to the vacuole and its ubiquitination and degradation are tightly controlled by the nitrogen source [45,46]. Similar regulation might exist for γ-GT and contribute in addition to transcriptional regulation, to the regulation of enzyme activity.

Yeast cells can use GSH as an alternative nitrogen source during nitrogen limitation. This suggests that nitrogen and GSH metabolism might share a common regulation [2,4]. In agreement with this hypothesis, we show here that high *CIS2* expression requires the well-known GATA zinc-finger transcription activators Gln3 and Nil1/Gat1, both acting, most probably, through GAT(T/A)A core sequences in the promoter region of *CIS2* (Figure 6). The Gln3 and Nil1/Gat1 factors are required to various degrees for expression of genes involved in nitrogen metabolism (see [18] for a review). Both of these factors contribute to increasing *CIS2* expression, but Nil1/Gat1 seems to play a more important role. In cells growing on glutamate as the sole nitrogen source, only Nil1/Gat1 appears to activate *CIS2* transcription. This result was unexpected, since it was proposed previously that a high intracellular L-glutamate concentration inhibits the action of Nil1 [9]. To our knowledge, this is the first report indicating that Nil1/Gat1 might be active even in the presence of glutamate, but the exact reason for this remains unknown. *CIS2* expression is also slightly reduced in NH_4^+-grown *gln3* deletion mutants. A similar effect has been described in reports on expression of the amino acid permease *GAP1* [17,22]. The explanation proposed in these reports was that *gln3* cells might be unable to produce Dal80/Uga43, a GATA zinc-finger protein capable of competing with Nil1 [43]. Whether Dal80/Uga43 can inhibit Nil1-dependent expression of *CIS2* remains to be investigated.

Another GATA zinc-finger protein, called Gzf3, is known to inhibit the action of Nil1/Gat1 during nitrogen repression by preventing its binding to GATAA sequences [21,44]. Yet deletion of *GZF3* did not restore high *CIS2* expression in NH_4^+-growing cells, indicating that Gzf3 is not required for nitrogen repression of *CIS2*, and that the main factor inhibiting Nil1 under these conditions remains to be identified. The *gzf3* mutation is associated, however, with increased *CIS2* expression in urea-grown cells, suggesting that Gzf3 might somehow inhibit Nil1 activity under certain conditions related to the nitrogen source. We further show that the *ure2/gdhCR* mutation restores moderate expression of *CIS2* in NH_4^+-grown cells. This limited effect might be due to the weak contribution of Gln3 to *CIS2* expression or to the fact that Ure2 has some ability to bind to Nil1 [43]. This result indicates participation of Ure2/GdhCR as a negative factor controlling *CIS2* expression. Interestingly, it has been shown that Ure2, which shares sequence homology with glutathione S-transferase, binds to GSH with high affinity, a process that might affect Ure2 oligomerization [29]. It is therefore tempting to speculate that GSH might also act as a regulator of Ure2 activity. In keeping with this view, previous work indicates that intracellular GSH may repress to some extent γ-GT biosynthesis [3].

To date, little has been published on the molecular mechanisms involved in regulating the enzymes involved in GSH metabolism.

We show here for the first time that the GATA zinc-finger proteins Gln3, Nil1/Gat1 and Gzf3 and the Gln3-binding protein Ure2/GdhCR are involved in the regulation of *CIS2* expression. This establishes a clear link between the regulation of GSH metabolism and nitrogen metabolism [4]. Our previous study showing that cells can use GSH as a sulphur-storage compound and that the intracellular level of γ-GT is regulated by sulphate, additionally suggests a possible link between sulphur metabolism and GSH metabolism [3]. In keeping with this, Dormer et al. [9] have recently shown that the transcription factors Met4, Met31 and Met32, regulating the sulphur pathway, are also involved in *GSH1* gene expression. GSH thus appears as a key element interconnecting nitrogen and sulphur metabolism. Database searches show that several genes involved in GSH metabolism, such as the genes encoding γ-glutamylcysteine synthetase, glutathione peroxidase 3, glutaredoxin 1, the GSH cell-surface and vacuolar transporters, and also some genes involved in sulphur metabolism, such as those encoding γ-cystathionine synthase, β-cystathionase and β-cystathionine synthase, contain a number of GATA sequences in their promoter region (Table 2). Genes involved in GSH and sulphur metabolism might thus be regulated by nitrogen via common transcription factors belonging to the GATA zinc-finger family. Furthermore, microarray data reveal that expression of γ-GT, glutathione S-transferases, glutaredoxins, glutathione peroxidases and enzymes involved in sulphate assimilation are induced by nitrogen or amino acid depletion [30]. Control of the expression of S-metabolism genes by the nitrogen source reinforces the idea that nitrogen, sulphur and GSH metabolisms share common regulatory elements.

We have also shown here that the TOR signalling pathway is apparently involved in regulating CIS2 expression. TOR proteins play a critical role in nutrient-regulated transcription [25,31]. The mechanism by which the TOR pathway controls expression of nitrogen-regulated genes probably involves inhibition of the Gln3 and Nil/Gat1 transcription factors [25]. The TOR pathway is most likely involved in Nil1- and Gln3-dependent regulation of *CIS2*, since rapamycin treatment mimics the effect of nitrogen depletion on *CIS2* expression (Figure 6). When cells are deprived of a nitrogen source, *CIS2* expression increases and the amount of GSH stored in the vacuole decreases [4,6]. This mechanism enables yeast to use GSH as an alternative nitrogen supply. In agreement with this, we have observed that *cis2* cells lacking γ-GT grow more slowly and reach a lower stationary-phase plateau than wild-type cells after nitrogen depletion (K. Mehdi and M. J. Penninckx, unpublished work). Additional work further suggests that the TOR pathway controls GSH metabolism. Microarray results, for instance, show that rapamycin induces high-level expression of glutathione transferase *GTT1* in diploid yeast cells [32]. More recently, TOR was found also to play an essential role in the response to salt stress by regulating Gln3- and Nil1/Gat1-mediated transcription of the *ENA1* gene encoding a P-type ATPase [33]. Taken together, these results highlight the major role of the TOR signalling pathway in GSH metabolism and, in general, cell response to environmental stresses.

There is increasing evidence suggesting that in yeast, GSH-associated enzymes play an essential role in the stress response (see [34,35] for reviews). Our *CIS2* expression results clearly show that γ-GT biosynthesis in *S. cerevisiae* is induced by nitrogen starvation, but apparently not by oxidants such as H_2O_2, diamide or thiram. This contrasts with the observed responses of other genes of GSH metabolism such as those encoding the GSH-biosynthetic enzyme Gsh1p [36], glutathione reductase [37] or glutathione peroxidases [38]. With one exception (the glutathione reductase gene), the promoter regions of each of these various genes contain at least one copy of the STRE sequence (Table 2). Exactly how and to what extent this STRE is involved in GSH-associated systems remains to be studied. We have also shown that cadmium, ethanol, heat stress and osmotic stress have no effect on *CIS2* expression. This absence of regulation by stress might be due to an intrinsic property of the Σ1278b background, but this seems unlikely because cells devoid of γ-GT activity are more sensitive to some adverse conditions such as H_2O_2, methylglyoxal or heat shock. The precise involvement of γ-GT in the response of cells to stress remains to be elucidated. The sensitivity of cells lacking γ-GT is not due to a general GSH deficiency, since the level of reduced GSH is similar in *cis2* and wild-type cells. Perhaps, there is a change in the GSH content of some subcellular compartment important for the cellular stress response, such as the vacuole. In keeping with this hypothesis, we have shown recently that the vacuolar GSH transport capacity is decreased 3-fold upon *CIS2* deletion [6]. Deficient transport of GSH and GSH conjugates into the vacuole might be involved in cell sensitivity to stress. It has also been suggested that in animal tissues, γ-GT might counteract oxidative stress by breaking down extracellular GSH and making its component amino acids available to the cells (reviewed in [39]). Yet a similar extracellular role in yeast seems very unlikely, since γ-GT is a vacuolar enzyme [6]. Additional experiments are certainly needed to improve our understanding of the interaction between GSH, γ-GT and the stress response.

This work was supported in part by the government of the Walloon Region and by a grant from the Belgian National Fund for Scientific Research (FNRS). We thank Bruno André for the gift of strains and for a critical reading of the manuscript.

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Received 6 December 2002/10 January 2003; accepted 15 January 2003
Published as BJ Immediate Publication 15 January 2003, DOI 10.1042/BJ20021893