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Unravelling the transcriptional regulation of *Saccharomyces cerevisiae UGA* genes: the dual role of transcription factor Leu3

Marcos Palavecino-Ruiz, Mariana Bermudez-Moretti and Susana Correa-Garcia*

Abstract

Yeast cells can use γ-aminobutyric acid (GABA), a non-protein amino acid, as a nitrogen source that is mainly imported by the permease Uga4 and catabolized by the enzymes GABA transaminase and succinate-semialdehyde dehydrogenase, encoded by the *UGA1* and *UGA2* genes, respectively. The three *UGA* genes are inducible by GABA and subject to nitrogen catabolite repression. Hence, their regulation occurs through two mechanisms, one dependent on the inducer and the other on nitrogen source quality. The aim of this work was to better understand the molecular mechanisms of transcription factors acting on different regulatory elements present in *UGA* promoters, such as Uga3, Dal81, Leu3 and the GATA factors, and to establish the mechanism of the concerted action between them. We found that Gat1 plays an important role in the induction of *UGA4* transcription by GABA and that Gzf3 has an effect in cells grown in a poor nitrogen source such as proline and that this effect is positive on *UGA4* expression. We also found that Gln3 and Dal80 affect the interaction of Uga3 and Dal81 on *UGA* promoters. Moreover, our results indicated that the repressing activity of Leu3 on *UGA4* and *UGA1* occurs through Dal80 since we demonstrated that Leu3 facilitates Dal80 interaction with DNA. However, when the expression of GATA factors is null or negligible, Leu3 functions as an activator.

INTRODUCTION

Many permeases in plasma membranes and in other intracellular organelles allow *Saccharomyces cerevisiae* cells to adapt to the wide variety of nutrients present in natural environments. In particular, amino acids can be metabolised and used as nitrogen and carbon sources, or as building blocks for protein biosynthesis. The uptake and utilization of these nutrients must be highly coordinated to enable cells to reap their major benefits. For this purpose, not only general transcriptional and translational mechanisms are triggered, but also specialized transcription factors are activated, generating activation or repression of specific sets of genes [1].

Nitrogen catabolite repression (NCR) is a general regulatory mechanism that ensures yeast cells use preferred nitrogen sources in the first place, and only in the absence of such sources can cells use non-preferred nitrogen sources. This occurs by the repression and the de-repression of NCR-sensitive genes. Four GATA factors, Gln3, Gat1/Nil1, Dal80/Uga43 and Gzf3/Deh1, are essential in such a mechanism. Gln3 and Gat1 act as activators that are translocated into the nucleus in the absence of a preferred nitrogen source and in the presence of a non-preferred one such as proline. In contrast, Dal80 and Gzf3 are nuclear factors that usually have a negative effect on the expression of NCR-sensitive genes. The expression of all GATA factors, except Gln3, is regulated by the GATA factors themselves [2, 3].

γ-aminobutyric acid (GABA) can be used by *S. cerevisiae* cells as a nitrogen source. GABA is mainly taken up from the culture medium through the permease Uga4 and is then catabolized to glutamate, which enters the nitrogen central metabolism, and to succinate, an intermediate of the tricarboxylic acid cycle (TCA), by the enzymes GABA transaminase and succinate-semialdehyde dehydrogenase, encoded by the *UGA1* and *UGA2* genes, respectively [4]. *UGA4*, *UGA1* and *UGA2* genes, which form the *UGA* regulon, are inducible by GABA and subject to NCR [5–9]. So, the transcriptional regulation of these genes occurs through two mechanisms, one dependent on the inducer and the other on the quality of the nitrogen source.

The transcription factors, Uga3 and Dal81, are involved in the induction of *UGA* genes by GABA [8, 10]. Both Uga3 and Dal81 interact *in vivo* with *UGA* promoters only when GABA is present in the growth medium, and they act

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Author affiliation: Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Biológica; Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto de Química Biológica de la Facultad de Ciencias Exactas y Naturales (IQUIBICEN). Buenos Aires, Argentina.
***Correspondence:** Susana Correa-Garcia, correa@qb.fcen.uba.ar
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Abbreviations: alpha-IPM, alpha-isopropylmalate; GABA, gamma-aminobutyric acid; NCR, Nitrogen Catabolite Repression.

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together to bind to their target genes [11, 12]. Leu3, a well-known transcription factor of genes involved in the metabolism of branched amino acids [13], also has a negative effect on *UGA4* and *UGA1* expression but does not affect *UGA2* expression [10, 11, 14]. Even though Leu3 could not be detected bound to the *UGA4* promoter, it interferes in the interaction of Dal81 but not of Uga3 with the *UGA4* promoter [11]. The transcription factors Uga3, Dal81 and Leu3 belong to the class III family ($Zn(II)_2Cys_6$ proteins), and they recognize highly related sequences rich in GGC triplets [15].

All *UGA* genes are sensitive to NCR and have 5′-GATA(A/T)-3' sequences in their regulatory regions that are targets of the GATA factors [6, 7, 16]. However, each GATA factor appears to act differently on each gene [17, 18], and their mechanism of action still remains elusive. We found that Gln3, like Uga3 and Dal81, interacts with *UGA* promoters in a GABA-dependent manner [12]. In contrast, Dal80 interacts with *UGA4* and *UGA1* promoters in the absence of the inducer, and is released when GABA is added. Interestingly, in cells deficient in *UGA3* or *DAL81*, Dal80 is recruited to these promoters in both non-induced and GABA-induced conditions [12, 19]. As far as we know, these results are the first *in vivo* evidence that the interaction of transcription factors acting through the UAS_{GATA} element of the *UGA4* gene is modulated by the activity of factors acting through the UAS_{GABA} element [12]. It has also been proposed that Gln3 could facilitate the action of Stp1 and Dal81 on *AGP1* transcription [20]. Moreover, studies on *Aspergillus nidulans* showed that TamA and LeuB, homologues of *S. cerevisiae* Dal81 and Leu3, modulate the recruitment of AreA, a GATA factor functionally related to Gln3 [21–24]. All these results showed that there is an interconnection between the induction process by a specific substrate (such as GABA for *UGA* genes or leucine for the *AGP1* gene) and the NCR mechanism.

The main purpose of this work was to obtain further insights into the regulation of *UGA* genes and to establish the mechanism of the concerted action of the transcription factors that target the different regulatory elements present on *UGA* promoters. Our findings contributed to clarifying the role of each GATA factor on *UGA4* regulation and demonstrated that the repressing activity of Leu3 on *UGA4* and *UGA1* genes occurs through Dal80, since Leu3 facilitates Dal80 interaction with DNA. However, when the expression of GATA factors is null or negligible, Leu3 functions as an activator.

METHODS

Strains and media

The *S. cerevisiae* strains used in this study are isogenic to the wild-type Σ1278b and are listed in Table 1.

Cells were grown in a minimal medium containing a 0.17 % Difco yeast nitrogen base (YNB without amino acids and ammonium sulphate), 2 % glucose as the carbon source and 10 mM proline or 10 mM ammonium sulphate as the nitrogen source. The final concentration of the inducer GABA was 0.1 mM.

MPY13, MPY14, MPY15, MPY16, MPY17, MPY18 and MPY19 mutant strains were generated using the PCR-based gene-deletion strategy [25, 26] or modified versions of it [27].

The MPY13 strain (*ura3 leu3Δ::loxP DAL80-3HA-kanMX6)* was generated using the pFA6a-3HA-kanMX6 plasmid [27] as the template and the primers F/R-*DAL80*-Tag previously described [12]. The PCR product was used to transform the MPY09 strain (*ura3 leu3Δ*). The correct insertion of the tag was verified by PCR using the primers F-*DAL80* int, R-*DAL80* down and F-kan int (Table 2).

MPY14, MPY15, MPY16, MPY17, MPY18 and MPY19 strains were generated using the pAG25 plasmid [28] as the template and the primers F/R-leu3 described by Cardillo *et al.* [11]. Correct deletion of *LEU3* was verified by PCR using the primers F-*LEU3* prom, R-*LEU3* int and R-pYM-N (Table 2).

Plasmids

The construction of Yep357-*UGA4-lacZ* and pSBC-*HA-DAL81* plasmids was already described [10, 11]. *UGA3* tagged with six HA epitopes was amplified by PCR from genomic DNA of the strain SBCY13 using the primers F-Eco-*UGA3* and R-Kpn-*UGA3* (Table 2) and cloned into the pRS426 plasmid [29].

β-galactosidase activity assay

Cells grown in a minimal medium up to an absorbance of 0.5–0.9 at 570 nm were harvested and transferred to a fresh medium with or without 0.1 mM GABA. After a 60 min incubation, an aliquot (10 ml) of each culture was collected by centrifugation and resuspended in 2 ml buffer Z [30]. β-galactosidase activity was expressed as Miller units [30]. Results are shown as mean±SD of duplicates within an assay. At least duplicate assays for each of the two independent transformants were performed. The deviation of these values from the mean was less than 15 %.

Quantitative RT-PCR

RT-qPCR experiments were performed according to Cardillo *et al.* [10]. cDNAs were quantified by RT-PCR using an Opticon Monitor 3 (Bio-Rad) with the primers F-*DAL80* RT-qPCR and R-*DAL80* RT-qPCR (Table 2) and F-TBPqPCR/R-TBPqPCR [10]. Expression values correspond to the ratio of concentrations of *DAL80* over *TBP1*-specific mRNAs determined in each sample and represent the mean ±SEM of at least three independent experiments.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) experiments were performed according to Cardillo *et al.* [12]. Normal mouse IgG (Santa Cruz) or monoclonal anti HA antibody (HA probe (F-7), Santa Cruz) were used. RT- qPCR was carried out in an Opticon Monitor 3 (Bio-Rad) with primers that

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Table 1. Strains used in this work

Strain	Genotype	Parent	Primer	Source or reference
Σ1278b	*MATα*	–	–	[46]
23344c	*MATα ura3*	–	–	[47]
SBCY01	*MATα ura3 leu3Δ::kanMX4*	23344c	–	[11]
30505b	*MATα ura3 gln3Δ*	23344c	–	[5]
32164b	*MATα ura3 gat1Δ*	23344c	–	[39]
34411c	*MATα ura3 gln3Δ gat1Δ*	23344c	–	B. André's laboratory
30078c	*MATα ura3 dal80Δ*	23344c	–	[48]
SBS10	*MATα ura3 gzf3Δ*	23344c	–	[43]
50000b	*MATα ura3 dal80Δ gzf3Δ*	23344c	–	[43]
SBCY41	*MATα ura3 DAL80-3HA-kanMX6*	23344c	–	[12]
MPY09	*MATα ura3 leu3Δ::loxP*	23344c	–	[38]
MPY13	*MATα ura3 leu3Δ::loxP DAL80HA-kanMX6*	MPY09	F/R DAL80-Tag	This work
MPY14	*MATα ura3 leu3Δ::natMX4 gln3Δ*	30505b	F/R leu3	This work
MPY15	*MATα ura3 leu3Δ::natMX4 gln3Δ gat1Δ*	34411c	F/R leu3	This work
MPY16	*MATα ura3 leu3Δ::natMX4 dal80Δ*	30078c	F/R leu3	This work
MPY17	*MATα ura3 leu3Δ::natMX4 gzf3Δ*	SBS10	F/R leu3	This work
MPY18	*MATα ura3 leu3Δ::natMX4 gat1Δ*	32164b	F/R leu3	This work
MPY19	*MATα ura3 leu3Δ::natMX4 dal80Δ gzf3Δ*	50000b	F/R leu3	This work

amplified promoter regions of *UGA4, UGA1* and *UGA2* [11, 12]. A pair of primers that amplified a region located 2.5 Kb downstream of the *UGA4* promoter (F-UC/R-UC) was used as an unbound control [11]. ChIP DNA was normalized to input DNA and calculated as a signal-to-noise ratio over an IgG control ChIP. The ΔΔCt method was used to calculate the fold change of binding to the promoter of interest [31]. Results are expressed as the mean±SEM of three independent experiments.

RESULTS

UGA promoter sequences were analysed *in silico* using the website *Regulatory Sequence Analysis Tools* (RSAT, http://fungi.rsat.eu/) [32] and the databases YEASTRACT (www.yeastract.com/) [33] and SCPD (http://rulai.cshl.edu/SCPD/) [34]. The regulatory regions of the *UGA4* and *UGA1* genes contain a UAS_{GABA} element (5′-AAAAACCGCCGGCGGCAAT-3′) target of Uga3 and Dal81 factors. Within this element there is a consensus site for Leu3 [8, 10, 11, 35]. In contrast, *UGA2* has a consensus site for Uga3 (5′-SGCGGNWTT-3′) but not a defined UAS_{GABA} element or a Leu3 putative site. On *UGA* promoters we also found various and different consensus sites for the GATA factors (5′-GAT(A/T)A-3′). Only the *UGA4* promoter possesses a region with three closely spaced GATA sites (5′-CTTATCGCTAATCGCTTATCGCTTATCG-3′) that form a UAS_{GATA} element [6, 16]. In spite of these differences, all *UGA* genes are subject to NCR [8, 16–18].

Table 2. Primers used in this work

Primer group and name	Sequence (5′ to 3′)
Oligonucleotides for strain construction	
F-Eco-*UGA3*	CGCGGAATTCCCGGAATCACA TTTGCCCAAGA
R-Kpn-*UGA3*	CGCGGTACCCACTGGCACGTCG TATGCAGGA
F-*LEU3* prom	AGGTGCCGCCTAATTTATCG
R-*LEU3* int	ACTTCTGCTGACGACATTCC
F-*DAL80* int	ACTACCTCTATCAGATCAACG
R-*DAL80 down*	GCTCACATCTCATCAAACTGC
R-pYM-N	TGTGATGTGAGAACTGTATCC
F-kan int	CATCCTATGGAACTGCCTCG
Oligonucleotides for RT-qPCR	
F-*DAL80* RT-qPCR	TGCTGCTGGAGTGGATGATTG
R-*DAL80* RT-qPCR	TGGGTCGTTAGAATGGGTATTGG

Previous reports indicated that the GATA factors involved in the NCR mechanism can have different activities depending on the target gene, on the growth conditions and even on the genetic background [8, 12, 17, 36]. Therefore, we decided to analyse the participation of GATA factors on *UGA4* regulation. The induction of *UGA4* by GABA was similar in wild-type and *gln3Δ* cells, whereas diminished in *gat1Δ* cells (Fig. 1a), indicating a significant effect of Gat1 on *UGA4* regulation. In cells deficient in both activators, *GLN3* and *GAT1*, *UGA4* expression was high, even in the absence of the inducer GABA (Fig. 1a). This result was expected since in the absence of both activators, the repressors, Dal80 and Gzf3, are not expressed [2, 37].

The high expression of *UGA4* found in *dal80Δ* cells (Fig. 1b) can be attributed to the activity of the activators Gln3 and Gat1 that could bind freely to the *UGA4* promoter, since they do not compete with Dal80 for their target sequences. Besides, it has been reported that in *dal80Δ* cells the expression of Gat1 is high [3].

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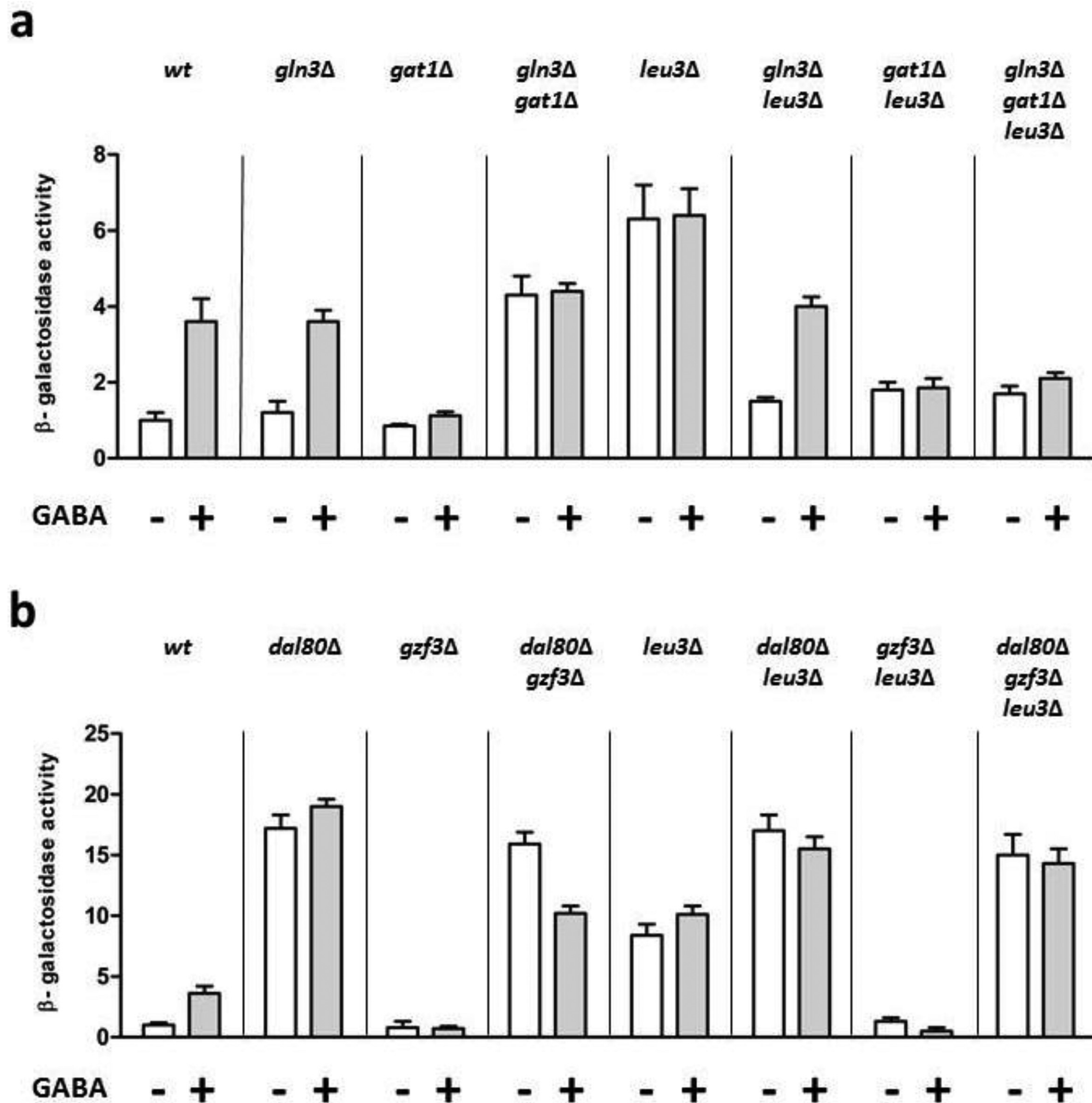

Fig. 1. (a) Effect of GATA activators and Leu3 on *UGA4* expression. Wild-type (23344c), *leu3*Δ (SBCY01), *gln3*Δ (30505b), *gat1*Δ (32164b), *gln3*Δ *gat1*Δ (34411c), *gln3*Δ *leu3*Δ (MPY14), *gat1*Δ *leu3*Δ (MPY18), *gln3*Δ *gat1*Δ *leu3*Δ (MPY16) cells carrying the Yep-357-*UGA4*-lacZ plasmid were grown on 10 mM proline and incubated (grey bars) or not (white bars) with 0.1 mM GABA. After 60 min, cells were harvested and β-galactosidase activity was measured. (b) Effect of GATA repressors and Leu3 on *UGA4* expression. Wild-type (23344c), *leu3*Δ (SBCY01), *dal80*Δ (30078c), *gzf3*Δ (SBS10), *dal80*Δ *gzf3*Δ (50000b), *dal80*Δ *leu3*Δ (MPY15), *gzf3*Δ *leu3*Δ (MPY17), *dal80*Δ *gzf3*Δ *leu3*Δ (MPY19) cells carrying the Yep-357-*UGA4*-lacZ plasmid were grown on 10 mM proline and incubated (grey bars) or not (white bars) with 0.1 mM GABA. After 60 min, cells were harvested and β-galactosidase activity was measured. Results expressed as Miller units were normalized to uninduced wild-type cells and represent the mean±SD of duplicates within an assay.

UGA4 expression significantly decreased in *gzf3*Δ cells (Fig. 1b), indicating that Gzf3 does not have the same function as Dal80; in contrast, Gzf3 appears to have an activating role. This striking result might be due to an increase in *DAL80* levels in a *gzf3*Δ strain [2]. We also propose that in the absence of Gzf3, Gat1 could not interact with its target genes since these factors need each other, as Georis and collaborators have demonstrated by co-immunoprecipitation experiments [3].

Although there is a putative consensus site for Leu3 within the UAS_{GABA} element of *UGA4* and *UGA1* genes, we were not able to detect any interaction between Leu3 and the *UGA4* promoter [11]. However, whether directly or indirectly, Leu3 has a negative effect on basal expression levels of *UGA4* and *UGA1* [10, 11]. Considering that Leu3 seemed to participate in the regulation of *UGA4* and *UGA1* genes in the absence of the inducer GABA, and Dal80 is the transcription factor responsible for *UGA4* repression in cells

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grown on non-induced conditions, we decided to obtain a deeper insight into the mechanism by which Leu3 regulates the *UGA4* gene. For this purpose, *UGA4* expression was measured in cells deficient in *LEU3* and in the GATA factors (Fig. 1). The high expression of *UGA4* observed in *leu3*Δ cells in the absence of GABA decreased in *gln3*Δ *leu3*Δ cells and in *gat1*Δ *leu3*Δ cells. This indicates that the high *UGA4* expression in cells deficient in the *LEU3* gene depends on Gln3 and Gat1; moreover, in this mechanism the role of Gat1 could not be accomplished by Gln3 and vice versa. Hence, these results demonstrated that, although deficiency in *GLN3* has no apparent effect on *UGA4* expression, Gln3 is involved in *UGA4* transcription. In the double *gln3*Δ *gat1*Δ mutant, *UGA4* expression was high in the presence or absence of GABA, probably due to the low levels of Dal80 and Gzf3 in this strain [2, 37]. However, *UGA4* expression in the triple *gln3*Δ *gat1*Δ *leu3*Δ mutant was lower than in the double *gln3*Δ *gat1*Δ mutant, suggesting that in conditions where the expression of the four GATA factors is null or negligible, Leu3 could be acting as an activator (Fig. 1a).

UGA4 expression was high and independent of GABA when Leu3 or Dal80 were absent (Fig. 1b), indicating that both Leu3 and Dal80 have a negative effect on *UGA4*. However, it is interesting to note that expression levels of *UGA4* observed in a *leu3*Δ mutant were lower than those obtained in a *dal80*Δ mutant. This could be due to an increase in *GAT1* expression in cells devoid of *DAL80* [3] and/or to Leu3 acting as an activator in the absence of Dal80. Taking into account that *UGA4* expression was similar in *dal80*Δ *gzf3*Δ cells and *dal80*Δ *gzf3*Δ *leu3*Δ cells (Fig. 1b), we cannot attribute such *UGA4* levels to a positive activity of Leu3. On the other hand, the positive effect of Gzf3 on *UGA4* expression did not depend on Leu3 (Fig. 1b).

In order to better understand the mechanism of action of GATA factors, we studied the effect of Gln3 and Dal80, the two GATA factors that we assume could interact with *UGA* promoters [12], on the interaction of Uga3 and Dal81. In the absence of the inducer GABA, Uga3 and Dal81 did not bind to *UGA* promoters in any of the strains assayed (Fig. 2). The addition of GABA promoted Uga3 interaction on *UGA4* and *UGA1* promoters regardless of the strain, even though in cells deficient in *GLN3* or *DAL80* this interaction weakened (Figs 2a, c). The effect of Gln3 and Dal80 on the binding of Uga3 was stronger on *UGA1* than on the *UGA4* promoter. So, these results suggested that Uga3 binding might be facilitated by Gln3 and Dal80 mainly on the *UGA1* promoter. In addition, Uga3 interaction with the *UGA2* promoter was independent of Gln3 and Dal80 (Fig. 2e).

On the other hand, neither Gln3 nor Dal80 affected Dal81 recruitment on the *UGA4* promoter (Fig. 2b), whereas Dal80 appeared to interfere with the binding of Dal81 on the *UGA1* promoter (Fig. 2d) and Gln3 with the binding of Dal81 on *UGA2* (Fig. 2f). Further studies are needed to clarify if the effect of Gln3 and Dal80 on the strength of the binding of Uga3 and Dal81 on *UGA* promoters has a physiological function.

Due to the fact that Dal80 and Leu3 had a similar effect on *UGA4* expression, we decided to explore the possibility of Leu3 affecting the interaction of Dal80 on *UGA* promoters.

In the absence of GABA, Dal80 interacted with *UGA4* and *UGA1* promoters but not with *UGA2*; in the presence of GABA the interaction diminished (Fig. 3a–c) [12]. Interestingly, the binding of Dal80 on *UGA4* and *UGA1* promoters in cells deficient in *LEU3* was negligible (Fig. 3a, b). These results contribute to explaining the high basal expression levels of *UGA1* and *UGA4* in *leu3*Δ cells. *UGA2* is not regulated by Leu3, and Dal80 is not the transcriptional factor responsible for maintaining its low basal levels. The fact that Dal80 did not affect Uga3 or Dal81 binding on the *UGA2* promoter (Fig. 2e, f) is in agreement with the finding that Dal80 does not interact with the *UGA2* promoter (Fig. 3c).

Georis and collaborators proposed that the interaction of Dal80 with its target promoters *in vivo* is solely controlled by its expression level [3]. Hence, we measured Dal80 expression in cells deficient in *LEU3,* and we found that Leu3 did not regulate *DAL80* (Fig. 3d). So, the very low recruitment of Dal80 observed in these cells is probably due to a direct effect of Leu3 on the interaction of Dal80 with its target promoters rather than a consequence of a decrease in *DAL80* levels.

Altogether, our results demonstrate that Leu3 facilitates the interaction of Dal80 with its recognition sites and also support our idea that there is a coordinated action of transcription factors acting on different UAS elements.

DISCUSSION

The aim of this work was to increase our understanding of the molecular mechanisms involved in the transcriptional regulation of *UGA* genes, in particular to reveal details of the cross-talk between transcription factors acting on different regulatory elements.

Previously, we demonstrated that Uga3, Dal81, Gln3 and Dal80 interact with *UGA* promoters in a GABA-dependent manner. However, it should be noted that the recruitment of Uga3, Dal81 and Gln3 on *UGA* promoters occurs in the presence of GABA, whereas Dal80 binding occurs only in uninduced conditions [11, 12, 38]. Moreover, we demonstrated that Dal81 and Uga3 affect the binding of Dal80 to *UGA4* and *UGA1* promoters. These results suggested to us that there is a concerted mechanism of action of the GATA transcription factors acting on the UAS_{GATA} element in response to the quality of the nitrogen source and the transcription factors acting on the UAS_{GABA} element in response to the inducer GABA.

The *UGA4*, *UGA1* and *UGA2* genes, which form the *UGA* regulon, share many regulatory features; however they present many important differences. Leu3 negatively regulates *UGA4* and *UGA1* genes, but it does not have any effect on

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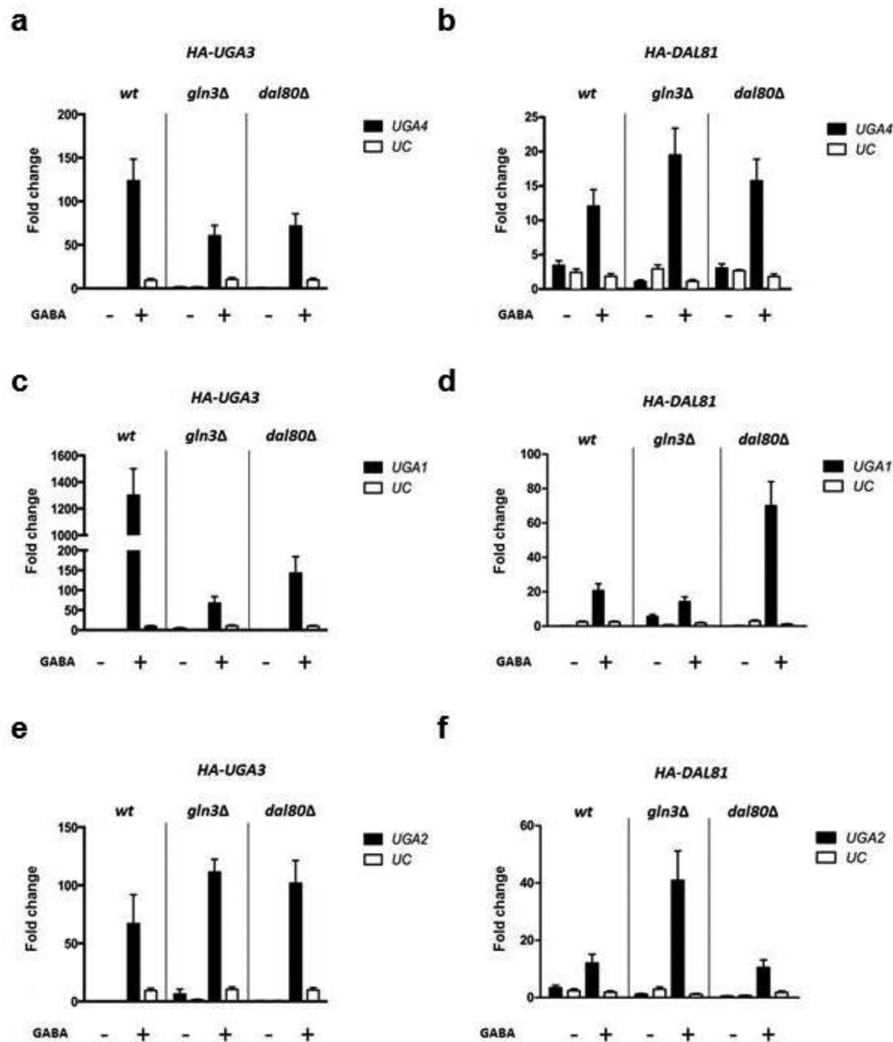

Fig. 2. Role of Gln3 and Dal80 in Uga3 and Dal81 interaction on *UGA* promoters. Wild-type (23344c), *gln3*Δ (30505b) and *dal80*Δ (30078 c) cells expressing HA-Uga3 (pMP-*HA-UGA3* plasmid), or expressing HA-Dal81 (pSBC-*HA-DAL81* plasmid) were grown on proline and incubated or not with 0.1 mM GABA for 30 min. ChIPs assays were carried out using antibodies against the HA epitope. qPCR was performed with primers that amplify a region of the *UGA4*, *UGA1* and *UGA2* promoters (black bars) and a region 2.5 kb downstream of the *UGA4* promoter (white bars) used as a negative control (Unbound Control, UC). Results are expressed as the fold change of binding to each promoter and are the mean±SEM of three independent experiments.

the *UGA2* expression [10], correlating with the fact that there is not a consensus site for Leu3 in the regulatory region of *UGA2*. Dal80 was not detected bound to the *UGA2* promoter in any of the conditions assayed [12].

Several authors suggested a central role for Gat1 in the regulation of genes involved in the utilization of poor nitrogen sources [3, 16, 17, 39]. Here we demonstrated that Gat1 plays an important role in *UGA4* expression (Fig. 1). Moreover, our results showed that Gln3 is not indispensable for Uga3 and Dal81 binding on *UGA* promoters (Fig. 2). It was also previously reported that the absence of Gln3 does not impede cell growth in GABA as the sole nitrogen source [40]. The regulatory role of Gln3 on *UGA4* transcription becomes evident since *UGA4* expression levels in cells devoid of *LEU3* are significantly higher than in cells devoid of both *LEU3* and *GLN3* (Fig. 1).

We confirmed that Dal80 represses *UGA4* expression in the absence of the inducer GABA (Fig. 1) [3, 8, 9, 37, 41]. The high expression of *UGA4* in cells deficient in *DAL80* could be due to the binding of Gat1 and Gln3 to the promoter. This is supported by previous results showing that in the absence of Dal80, Gat1 expression levels and its recruitment to the *UGA4* promoter increased in cells grown in proline [3].

It has been proposed that Dal80 and Gzf3 play different roles in the regulation of nitrogen-sensitive genes: Dal80

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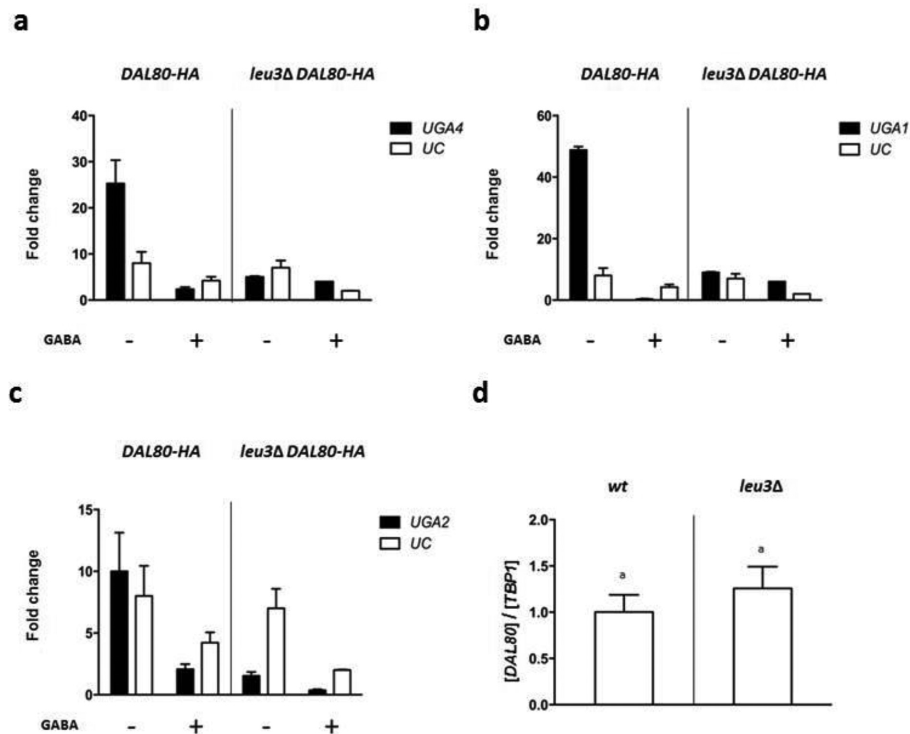

Fig. 3. Effect of Leu3 on Dal80 interaction on *UGA* promoters. Wild-type (SBCY41) and *leu3*Δ (MPY13) cells expressing Dal80-HA were grown on proline and incubated or not with 0.1 mM GABA for 30 min. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with primers that amplify a region of *UGA4* (a), *UGA1* (b) and *UGA2* (c) promoters (black bars) and a region 2.5 kb downstream of the *UGA4* promoter (white bars) used as a negative control (UC). Results are expressed as the fold change of binding to the promoter of interest and are the mean±SEM of three independent experiments. (d) Effect of Leu3 on *DAL80* expression. Wild-type (23344c) and *leu3*Δ (MPY09) cells were grown on proline, and mRNA levels were measured by RT-qPCR. *DAL80* values were normalized with *TBP1* and to wild-type cells.

represses the transcription of those genes in a poor nitrogen source and Gzf3 does so in a rich one [42, 43]. We found that Gzf3 has an effect on *UGA4* expression in cells grown on proline, a poor nitrogen source, and that this effect is positive since *UGA4* does not express in the absence of Gzf3 (Fig. 1). Similar results were previously reported for *GAP1* and *DAL5*, two genes highly sensitive to NCR, like *UGA4* [16, 36, 44, 45]. Georis and collaborators demonstrated by co-immunoprecipitation assays that there is an interaction between Gzf3 and Gat1 in cells grown in proline [3]. Thus, the low expression levels of *UGA4* in a *gzf3*Δ strain could be attributed to the high levels of *DAL80* in this strain and also to the fact that Gat1 could not interact with the *UGA4* promoter in this strain and consequently, an increase in the recruitment of Dal80 occurred. In the absence of Dal80 and Gzf3, *UGA4* expression could be due to the free interaction of Gln3 on the *UGA4* promoter since it is assumed that Dal80 and Gzf3 act on NCR genes by competing with Gat1 and Gln3 [2, 3, 6, 7, 16, 17, 43]. The Dal80 deficiency is epistatic with Gzf3.

Taking into account our previous and present works and the results obtained by Georis and collaborators [3], here we propose a concerted regulatory mechanism of action between the transcription factors acting on the UAS_{GATA} element and those acting on UAS_{GABA} (Fig. 4). In the absence of the inducer GABA, Dal80 interacts with *UGA4* and *UGA1* promoters [12]. When the inducer is added, Uga3, Dal81 and Gln3 bind to *UGA* promoters [11, 12], and Gln3 and Gat1 compete with Dal80 for their target sequences and as a result *UGA* genes are induced. In contrast, in the absence of Leu3, Dal80 cannot be recruited to *UGA4* and *UGA1* promoters, and Gln3 and Gat1 do interact with the DNA, producing high levels of expression even in the absence of inducer. In the absence of Dal80, Leu3 does not have a negative effect on *UGA4* and *UGA1* transcription, indicating that even though both Dal80 and Leu3 act as repressors on *UGA4* and *UGA1* genes, Dal80 is actually the factor that represses, whereas Leu3 acts only by stabilizing Dal80 interaction. We found that Dal80 does not interact with the *UGA2* promoter and that Leu3 does not regulate *UGA2* expression (the *UGA2* promoter does not present any Leu3 consensus site). These findings reinforce our conclusion that Dal80 acts coordinately with Leu3 in the interaction with *UGA4* and *UGA1* promoters. Further studies are needed to determine the mechanism by which *UGA2* is

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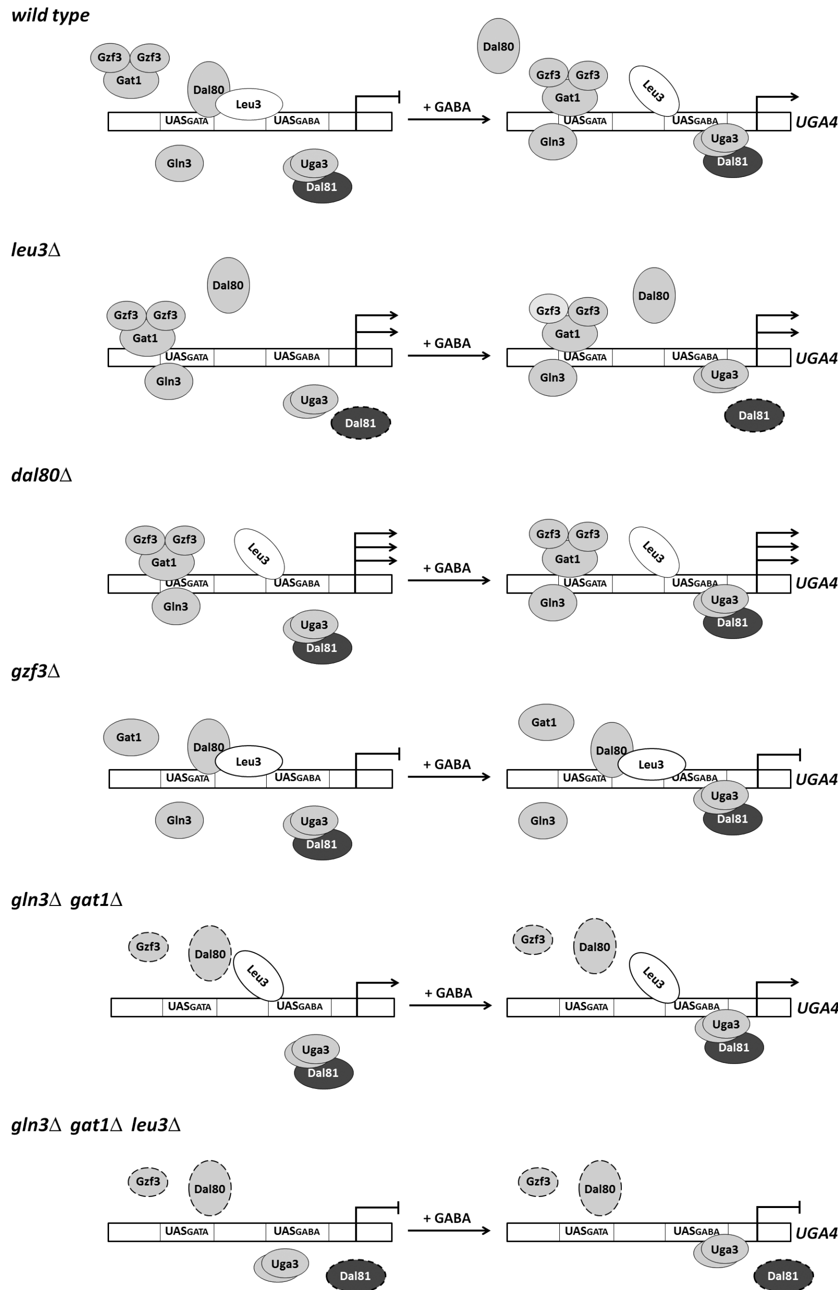

Fig. 4. Model depicting the concerted action of the transcription factors acting on *UGA4* regulation. We showed that Leu3 and Dal80 repress *UGA4* expression and that the binding of Dal80 requires the presence of Leu3. Moreover, in agreement with a previous report [3], we propose that Gat1 activity depends on the presence of Gzf3. This model was elaborated taking into account interactions like those of Gln3, Dal80, Uga3 and Dal81, we have demonstrated in the present and past works [11, 12, 19], and also considering the interaction of Gat1 demonstrated by Georis and collaborators [3]. On the other hand, we propose other interactions like those of Leu3 or of Gat1 in the presence of GABA, that until now we have not been able to detect. Dash contours indicate low levels of the transcription factors in question.

repressed in the absence of an inducer. We also propose that Gzf3 indirectly and positively regulates the expression of *UGA4*. The low expression of *UGA4* in *gzf3*Δ cells could be due to the increased levels of Dal80 and/or to the inability of Gat1 and Gln3 to bind to the *UGA4* promoter in the absence of Gzf3.

The genetic background and growth conditions we used in the present and past works lead to a low concentration of leucine and a high concentration of α-isopropylmalate (α-IPM) [13], as we previously showed using cells with different α-IPM-synthesizing capacities [11]. Under these conditions, it is expected that Leu3 acts as an activator on its

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target genes [13]. *UGA4* expression in the *gln3*Δ *gat1*Δ background revealed that Leu3 has a positive effect, in agreement with the previous statement. Therefore, the repressing activity of Leu3 on *UGA4* (Fig. 1) and *UGA1* genes [10] that we observed in the wild-type background even in the presence of high concentration of α-IPM, occurs because Leu3 functions in conjunction with the GATA repressor, Dal80.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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