Interplay between the transcription factors acting on the GATA- and GABA-responsive elements of *Saccharomyces cerevisiae* UGA promoters

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\[\gamma\text{-Aminobutyric acid (GABA) transport and catabolism in *Saccharomyces cerevisiae* are subject to a complex transcriptional control that depends on the nutritional status of the cells. The expression of the genes that form the UGA regulon is inducible by GABA and sensitive to nitrogen catabolite repression (NCR). GABA induction of these genes is mediated by Uga3 and Dal81 transcription factors, whereas GATA factors are responsible for NCR. Here, we show how members of the UGA regulon share the activation mechanism. Our results show that both Uga3 and Dal81 interact with UGA genes in a GABA-dependent manner, and that they depend on each other for the interaction with their target promoters and the transcriptional activation. The typical DNA-binding domain Zn(II)$_2$-Cys$_6$ of Dal81 is unnecessary for its activity and Uga3 acts as a bridge between Dal81 and DNA. Both the trans-activation activity of the GATA factor Gln3 and the repressive activity of the GATA factor Dal80 are exerted by their interaction with UGA promoters in response to GABA, indicating that Uga3, Dal81, Gln3 and Dal80 all act in concert to induce the expression of UGA genes. So, an interplay between the factors responsible for GABA induction and those responsible for NCR in the regulation of the UGA genes is proposed here.}\]

**INTRODUCTION**

\[\gamma\text{-Aminobutyric acid (GABA) transport and catabolism in *Saccharomyces cerevisiae* cells can produce GABA in the cytosol through the decarboxylation of L-glutamate by glutamate decarboxylase (GAD) (Coleman et al., 2001) and can transport it from the extracellular environment through the specific GABA permease Uga4, the general amino acid permease Gap1 and the proline-specific permease Put4 (Grenson, 1987). GABA is an abundant amino acid that can be found in many natural environments of yeasts; in particular, *S. cerevisiae* is able to use this amino acid as a nitrogen source (Ramos et al., 1985). In a first step, GABA is irreversibly transaminated to \(\alpha\)-ketoglutarate by GABA transaminase (GABA-T) producing glutamate and succinate semialdehyde (SSA). Then, SSA is converted to succinate, a metabolite of the tricarboxylic acid (TCA) cycle, by succinate semialdehyde dehydrogenase (SSADH).\]

In *S. cerevisiae*, the GABA-specific permease and the enzymes GABA-T and SSADH, are encoded by UGA4, UGA1 and UGA2 genes, respectively (André & Jauniaux, 1990a; André et al., 1993; Coleman et al., 2001; Ramos et al., 1985; Vissers et al., 1989). UGA1, UGA2 and UGA4 are subject to a complex regulation that depends on the nutritional status of the cells. The three genes contain 5'--GAT(A/T)A--3' sequences in their promoters and are subject to nitrogen catabolite repression (NCR) (André et al., 1995; Cunningham et al., 1994). Expression of NCR-sensitive genes is coordinated by the prion-like Ure2 protein and four DNA-binding proteins possessing homologous GATA-type zinc fingers: two activators (Gln3 and Gat1/Nil1) and two repressors (Dal80/Uga43 and Gzf3/Deh1/Nil2). In the presence of preferred nitrogen sources, the GATA activators are sequestered in the cytoplasm by Ure2, whereas after depletion of the repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes is activated by Gln3, Gat1 or both. Once in the nucleus, positive factors compete with the negative factors for the GATA binding sites present in the promoters of their target genes and the balance of this competition leads to expression levels that depend on the quality of the nitrogen sources available (Georis et al., 2009). Gzf3 seems to inhibit the expression of genes under nitrogen repressive conditions (Soussi-Boudekou et al.,)

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**Abbreviations:** GABA, \(\gamma\text{-aminobutyric acid; NCR, nitrogen catabolite repression; RT-qPCR, quantitative RT-PCR.**}}

Two supplementary figures are available with the online version of this paper.
Expression of UGA genes also depends on GABA induction. This induction requires at least two positive regulatory proteins: the specific Uga3 factor and the pleiotropic Dal81 factor (also called Uga35) that act through a 19 bp GC-rich upstream activating sequence named UASGABA present in the promoters of UGA4 and UGA1 genes (André, 1990; André et al., 1993, 1995; Godard et al., 2007; Ramos et al., 1985; Talibi et al., 1995). It has been proposed that the Uga3 DNA-binding site is an asymmetrical site of 5'-SGCGGNWTT-3' (S=G or C, W=A or T and N=no nucleotide or G). The UASGABA present in UGA4 and UGA1 promoters contains two independent Uga3 binding sites, whereas the UGA2 promoter only contains one consensus binding site for this factor (Idicula, 2002; Idicula et al., 2002). Both factors Uga3 and Dal81 interact in vivo with the UGA4 promoter in a GABA-dependent manner (Cardillo et al., 2010).

Dal81 is a general positive regulator of genes involved in nitrogen utilization related to metabolisms of GABA, urea, arginine and allatoin (Coornaert et al., 1991; Vissers et al., 1990); moreover, Dal81 is involved in the amino acid SPS sensor pathway (Abdel-Sater et al., 2004; Boban & Ljungdahl, 2007; Iraqui et al., 1999). In all these induction processes, Dal81 acts together with an inducer-specific protein; this specific factor is Uga3 in GABA-induction of UGA genes (André, 1990), Dal82/DurM in allolophanate-induction of DUR and DAL genes (André & Jauniaux, 1990b; Jacobs et al., 1980; Olive et al., 1991) and Stp1 in amino acid induction of amino acid permease genes (Abdel-Sater et al., 2004; Boban & Ljungdahl, 2007; Iraqui et al., 1999).

The transcription factors Uga3 and Dal81 belong to the zinc binuclear cluster family. Proteins of this family contain a putative DNA-binding domain that consists of six cysteine residues bound to two zinc atoms [Zn(II)2-Cys6]. This domain is essential for Uga3 activity (Talibi et al., 1995). However, Dal81, like Aspergillus nidulans TamA, does not require the Zn(II)2-Cys6 domain to fully activate the DUR1/2 gene (Bricmont et al., 1991; Davis et al., 1996).

The main purpose of this work was to elucidate the molecular mechanisms and the interplay of transcription factors that lead to the induction of UGA genes. We demonstrated that there is a mutual dependency of Uga3 and Dal81 factors for the interaction with the promoters of UGA genes correlating with the almost undetectable induction levels observed in the absence of either of these two factors. Moreover, we demonstrated that the Zn(II)2-Cys6 domain present in Dal81 is not essential for its activity in GABA induction, and that Uga3 functions as a bridge in the interaction between Dal81 and DNA. We also found that Uga3, Dal81, Gln3 and Dal80 transcription factors are all recruited to UGA1, UGA2 and UGA4 promoters in a GABA-dependent manner. Uga3 and Dal81 affect Dal80 recruitment to these promoters; this result is the first evidence, to our knowledge, of a cross-talk between the transcription factors acting on the GABA-responsive elements and those acting on the GATA-responsive elements.

## METHODS

### Strains and media.

The *S. cerevisiae* strains used in this study, isogenic to the wild-type Σ1278b, are listed in Table 1. Cells were grown in minimal buffered (pH 6.1) medium (Jacobs et al., 1980) with 3% glucose and 10 mM proline as the carbon and nitrogen sources, respectively.

### Strain construction.

The strains generated in this study were constructed using variations of the PCR-based gene deletion strategy (Longtine et al., 1998) and of the *in vivo* site-directed mutagenesis or ‘Delitto perfetto’ strategy (Storici et al., 2001; Storici & Resnick, 2003). All the parental strains are listed in Table 1, and all primers used for PCR are listed in Table 2.

Strains that express N-terminal-tagged proteins under the control of its natural promoter were generated using the pOM10 plasmid as a template for PCR (Gauss et al., 2005). Strains with a C-terminal tag were generated using pFA6a-3HA-KanMX6 plasmid (Longtine et al., 1998). Strains that contain site-directed mutations in their genome were generated in two steps. First, a strain containing an insertion of the KanMX-KIURA3 module was generated using the pCORE plasmid as template for PCR (Storici et al., 2001; Storici & Resnick, 2003). Second, fragments containing the desired mutations in the promoter of UGA4 gene were generated by PCR using the plasmids Yep357-UASGABA mut and Yep357-UASGABAdel (Cardillo et al., 2010) as templates. Counter-selection with 5-fluoroorotic acid (5-FOA) was carried out to isolate those mutants that had lost the pCORE module and had incorporated the desired mutations. The correct generation of the mutations was verified by DNA sequence analysis.

All yeast transformations were carried out using the lithium method (Gietz & Woods, 2002). Transformants were selected on rich medium containing 200 μg G418 ml⁻¹ or on minimal medium containing 2 mg uracil ml⁻¹ and 24 μg 5-FOA ml⁻¹.

### Plasmids.

The pSBC-UGA3 plasmid was constructed by cloning a fragment containing the promoter, the coding region, and the 3’ non-coding region (positions 800–1930) of the UGA3 gene into the pRS316 plasmid (Sikorski & Hieter, 1989). The UGA3 gene was amplified from genomic DNA of the Σ1278 strain. Functionality of the Uga3 protein encoded by pSBC-UGA3 plasmid was determined by its capacity to restore both UGA gene induction and growth in the presence of GABA as a sole nitrogen source in a uga3Δ strain.

pPB plasmids carrying different mutated versions of the DAL81 gene under its natural promoter were kindly provided by Dr T. Cooper (University of Tennessee, Memphis, USA) (Bricmont et al., 1991). pPB71 encodes the complete protein; pPB67 encodes the protein lacking the polyglutamine stretch between residues 73 and 94 (PolyGln 73-94Δ); pPB68, the polyglutamine stretch between residues 227 and 237 (PolyGln 227-237Δ); pPB70, the zinc binuclear cluster domain (Zn(II)2-Cys6 150-179Δ); and pPB72, both polyglutamine stretches (PolyGln 73-94A 227-237Δ).

### Chromatin immunoprecipitation assays (ChIP).

ChIP experiments were performed according to the method of Cardillo et al. (2010). Normal mouse IgG (Santa Cruz) or monoclonal anti-HA antibody [HA probe (F-7), Santa Cruz) were used. Real-time quantitative PCR was carried out in an Opticon Monitor 3 (Bio-Rad) with primers that amplified promoter regions of the UGA4 (F/R-UGA4PCR; Cardillo et al., 2010), UGA1 (F/R-UGA1-qChIP) and UGA2 (F/R-UGA2-qChIP) genes. A pair of primers that amplified a region located 2.5 kb downstream of UGA4 promoter was used as an anchor.
unbound control (F/R-UGA4 UCqPCR; Cardillo et al., 2010). 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 fold change of binding to the promoter of interest (Livak & Schmittgen, 2001). Results are expressed as the mean ± SEM of three independent experiments.

**Quantitative RT-PCR (RT-qPCR).** Total RNA was extracted from 4 ml cultures as described by Schmitt et al. (1990). Genomic DNA was eliminated after incubating RNA with DNase RQ1 (Promega) for 60 min at 37 °C. cDNA was generated from 1–4 μg total RNA using the RevertAid Reverse Transcriptase (Fermentas) with hexa-random primers following the manufacturer’s recommended protocol. cDNAs were subsequently quantified by real-time PCR using an Opticon Monitor 3 (Bio-Rad) with the primers F/R-qRT-UGA4, F/R-UGA1 RT qPCR, F/R-UGA2 RT qPCR and F/R-TBP qPCR. Expression values correspond to the ratio of concentrations of UGA1, UGA2 or UGA4 over TBP1 specific mRNAs determined in each sample. Results are expressed as the mean ± SEM of three independent experiments.

## RESULTS

### Uga3 and Dal81 transcription factors depend on GABA to interact with UGA genes

It has been demonstrated by genetic analysis that both of the transcription factors Uga3 and Dal81 are essential for the induction of UGA genes in response to GABA (André et al., 1993; Godard et al., 2007; Talibi et al., 1995; Vissers et al., 1989). Using RT-qPCR assays, we corroborated those results and showed that the three genes of the UGA regulon, UGA1, UGA2 and UGA4, were inducible by GABA in a strictly Uga3- and Dal81-dependent manner (Fig. 1).

Although in vitro assays pointed to a GABA-independent binding of Uga3 transcription factor to the UAS_{GABA} element on the promoters of UGA1 and UGA4 genes (Ielicula et al., 2002), we recently demonstrated that Uga3 and also Dal81 transcription factors interact in vivo with the UGA4 promoter after the addition of GABA (Cardillo et al., 2010). So, we decided to extend our study by analysing the in vivo binding of these two factors to the promoters of UGA1 and UGA2 genes. Results showed a GABA-dependent binding of both factors, HA-Uga3 and HA-Dal81, to the promoter regions of UGA1 and UGA2 (Fig. 2a, b).

### Dal81 transcription factor is necessary for Uga3 binding to UGA promoters

We demonstrated that the GABA-dependent interaction between Dal81 and the UGA4 promoter was impaired by the presence of extracellular leucine and that this effect was a consequence of a signal triggered by the SPS amino acid sensor (Cardillo et al., 2010). Interestingly, Uga3 binding to UGA4 promoter responded to the presence of extracellular leucine in the same way as Dal81 (Cardillo et al., 2010).

<table>
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<th>Strain</th>
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<th>Primer</th>
<th>Source or reference</th>
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Table 1. Strains used in this work
Since Uga3 is an inducer-specific transcription factor of UGA genes and there were no previous reports linking Uga3 with the amino-acid-responsive pathway, this result was unexpected. One possible explanation was that Uga3 would need Dal81 to some extent to properly bind to the UGA4 promoter. To test this hypothesis, we decided to study the in vivo binding of HA-Uga3 to the UGA promoters in a dal81Δ strain. In the absence of Dal81, no interaction between HA-Uga3 and the UGA promoters was detected (Fig. 2c). Moreover, when DAL81 expression was

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**Table 2. Primers used in this work**

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<td>R-UGA3</td>
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</tr>
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**Fig. 1.** The induction of UGA genes depends on GABA and on Uga3 and Dal81 transcription factors. mRNA levels of UGA4 (a), UGA1 (b) and UGA2 (c) were determined in wild-type (23344c strain), uga3Δ (26790a strain) and dal81Δ (SBCY17 strain) cells treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. mRNA levels were quantified by RT-qPCR. UGA1, UGA2 and UGA4 values were normalized with TBP1 and represent the mean ± SEM of three independent experiments.
restored by transforming the \( \text{dal}81\Delta \) strain with the plasmid \( \text{pPB71} \) that encodes the full-length \( \text{DAL81} \) gene, HA-Uga3 recovered its ability to interact with \( \text{UGA} \) promoters in response to GABA (Fig. 2d). So, in \( \text{dal}81\Delta \) cells, neither of the two transcription factors responsible for GABA induction, Uga3 and Dal81, bound to the \( \text{UGA} \) promoters, which correlated with the extremely low levels of induction of \( \text{UGA} \) genes in these cells (Fig. 1).

The Dal81 polyglutamine domain spanning residues 73–94, but not the \( \text{Zn(II)}_2\)-Cys\(_6\) domain, is necessary for its activity

It has been demonstrated that the \( \text{Zn(II)}_2\)-Cys\(_6\) domain of the Dal81 protein (residues 150–179) is not essential for the induction of \( \text{DUR1}/2 \) gene, measured indirectly through the enzymic activity of urea amidolyase (Bricmont \textit{et al.}, 1991). Similar observations were made for the \( A. \text{nidulans} \) protein TamA, a protein closely related to Dal81 that is involved in nitrogen utilization (Davis \textit{et al.}, 1996). So, we wondered whether the \( \text{Zn(II)}_2\)-Cys\(_6\) domain and also the polyglutamine stretches (residues 73–94 and 227–237) present in the Dal81 protein were required for GABA induction of \( \text{UGA} \) regulon genes. For this purpose, cells deficient in \( \text{DAL81} \) were transformed with plasmids carrying different mutated versions of \( \text{DAL81} \) (Bricmont \textit{et al.}, 1991) and mRNA levels of \( \text{UGA4} \) were measured (Fig. 3). In \( \text{dal}81\Delta \) cells, GABA induction of the \( \text{UGA4} \) gene was almost undetectable, but this deficiency was complemented by the complete Dal81 protein (\( \text{DAL81} \)). Dal81 proteins lacking the \( \text{Zn(II)}_2\)-Cys\(_6\) domain [\( \text{Zn(II)}_2\)-Cys\(_6\) 150–179\)] or the polyglutamine stretch between residues 227 and 237 (PolyGln 227–237\)) also complemented the \( \text{dal}81\Delta \) deletion. However, the protein without the polyglutamine stretch between residues 73 and 94 (PolyGln 73–94\) and PolyGln 73–94\) 227–237\)) was not able to restore GABA induction of \( \text{UGA4} \). When mRNA levels of the other members of the \( \text{UGA} \) regulon, \( \text{UGA1} \) and \( \text{UGA2} \), were measured, similar results were obtained (Supplementary Fig. S1, available with the online version of this paper). These data clearly indicate that the \( \text{Zn(II)}_2\)-Cys\(_6\) domain and the polyglutamine stretch between residues 227 and 237 of Dal81 are not essential for GABA induction of \( \text{UGA} \) genes, whereas the polyglutamine stretch between residues 73 and 94 is essential in this process.

Uga3 factor acts as a bridge between Dal81 and DNA

Dal81 was able to bind to \( \text{UGA4} \) (Cardillo \textit{et al.}, 2010), \( \text{UGA1} \) and \( \text{UGA2} \) promoters (Fig. 2b). However, the \( \text{Zn(II)}_2\)-Cys\(_6\) domain of the Dal81 protein, usually essential for the interaction of many fungal transcription factors with the DNA, was not essential for its role in GABA induction of \( \text{UGA} \) genes (Fig. 3 and Supplementary Fig. S1). These results
suggested that Dal81 might interact with UGA promoters through another protein. The fact that Uga3 and Dal81 act together through the UAS\textsubscript{GABA} element present in the promoters of UGA1 and UGA4 genes (Idicula et al., 2002; Talibi et al., 1995), made Uga3 a good candidate to be acting as a bridge between DNA and Dal81. To assess this hypothesis, we assayed the binding of Dal81 to the UGA promoters in a uga3Δ strain. No interaction between HA-Dal81 and the UGA promoters was detected in the absence of Uga3 (Fig. 2c). However, when UGA3 expression was restored by transforming the uga3Δ HA-DAL81 strain with the plasmid pSBC-UGA3, encoding the full-length UGA3 gene, the binding of Dal81 to UGA promoters was detected again (Fig. 2f). These results indicated that Dal81 needed Uga3 to bind the UGA promoters, suggesting that this interaction might occur through this protein. Again, the fact that in uga3Δ cells, none of the two transcription factors responsible for GABA induction could interact with their target promoters, correlated with the extremely low levels of UGA gene induction measured in these conditions (Fig. 1).

There are two independent binding sites for Uga3 placed in the central core of the UAS\textsubscript{GABA} element at the UGA4 promoter (Idicula et al., 2002). If the interaction of Dal81 with the UGA4 promoter effectively occurs through Uga3, no interaction between Dal81 and UGA4 promoter lacking the consensus binding site for Uga3 would be detected. To test this hypothesis we generated two strains containing a mutation or a deletion in the central core of the UAS\textsubscript{GABA} element of the UGA4 gene (Fig. 4a). First, we found that UGA4 mRNA levels in these strains were approximately four times higher than in the wild-type strain and no GABA-induction was observed (Fig. 4b), confirming our previous observations obtained using the lacZ reporter gene where we proposed that Leu3 is acting as a repressor on the UAS\textsubscript{GABA} element (Cardillo et al., 2010). Next, we studied the in vivo interaction of HA-Uga3 and HA-Dal81 with the mutated UGA4 promoters. As expected, no interaction of HA-Uga3 with the UGA4 promoters that lack the central core of the UAS\textsubscript{GABA} element was detected, while binding to the internal positive control UGA1 promoter was detected (Fig. 5a, c). Similarly, no binding of the HA-Dal81 protein to the mutated versions of UGA4 promoter was detected (Fig. 5b, d). These results supported the idea that both factors, Uga3 and Dal81, interacted with the UGA promoters through the Uga3 binding sites. Therefore, the absence of not only Uga3 but also an intact UAS\textsubscript{GABA} element impaired the binding of Dal81 to the UGA4 promoter, suggesting that this factor interacted with the DNA through Uga3 and that the GABA-induced transcription of all the members of the UGA regulon requires the concerted action of both transcription factors.

There is an interplay between Gln3 and Dal80 GATA factors and Uga3 and Dal81

The genes of the UGA regulon are subject to NCR and are positively regulated by Gln3 and negatively regulated by Dal80 (André et al., 1995; Cunningham et al., 1994). In the presence of a poor nitrogen source, these factors compete for the GATA binding sites to activate transcription (Coffman et al., 1997; Soussi-Boudekou et al., 1997), and it has been proposed that Gln3 trans-activation cannot occur until GABA is added (André et al., 1995; Talibi et al., 1995). To test the dynamics of Gln3 and Dal80 recruitment to UGA promoters we analysed the in vivo binding of these factors to their target DNA. Gln3-HA bound to the three UGA promoters only in the presence of GABA (Fig. 6a). In contrast, Dal80-HA was detected bound to UGA1 and UGA4 promoters in the absence of the inducer and its binding decreased after the addition of GABA (Fig. 6b). These observations are in agreement with the proposal that both GATA factors, Gln3 and Dal80, compete with each other for their DNA binding sites. To study the influence of Uga3 and Dal81 on the binding dynamics of these GATA factors, we decided to analyse the interaction of Gln3 and Dal80 to UGA promoters in the absence of Uga3 or Dal81. The strains deficient in UGA3 or DAL81 expressing the tagged Gln3 grew slower than the wild-type in a poor nitrogen source but not in a rich one. Besides this, we had technical problems during the ChIP assays with these strains, since we were not able to detect binding of Gln3 even in the internal positive control GAP1. These technical difficulties may be linked to the growth problems these strains have (data not shown). On the other hand, in both uga3Δ and dal81Δ, Dal80-HA remained bound to UGA1 and UGA4 promoters, showing
that this binding was enhanced in the presence of GABA (Fig. 6c, d). These results suggested that the transcription factors acting on the UAS_{GABA} element influence the binding of GATA factors to these promoters. Interestingly, in the wild-type strain, Dal80-HA binding to the UGA2 promoter was barely detected (Fig. 6b). However, Dal80-HA interaction with this promoter slightly increased in the absence of Uga3 and Dal81 factors (Fig. 6c, d). Previously, we demonstrated that Leu3 transcription factor negatively regulates UGA4 and UGA1 genes, but not UGA2 (Cardillo et al., 2010, 2011), so it would be interesting to study whether Leu3 affects Dal80 interaction with promoters.

These data represent the first in vivo evidence, to our knowledge, of cross-talk between the GABA-dependent transcription factors Uga3 and Dal81 acting through the Uga3 binding sites, and the GATA factors acting through the GATA sequences.

**DISCUSSION**

The aim of this work was to elucidate the molecular mechanisms and interplay of transcription factors that lead to the induction of UGA genes. Here, we demonstrated...
that in spite of the differences between the promoters of the three genes that comprise the UGA regulon, UGA1, UGA2 and UGA4 (Supplementary Fig. S2), they respond to GABA stimuli in a similar way. First, UGA genes are inducible by GABA, and Uga3 and Dal81 transcription factors are both essential for this induction (Fig. 1) (André et al., 1993; Godard et al., 2007; Talibi et al., 1995). These transcription factors interact with UGA1 and UGA2 promoters in a GABA-dependent manner (Fig. 2a, b), as we previously reported for the UGA4 gene (Cardillo et al., 2010). Idicula et al. (2002) used in vitro assays to show that Uga3 appears to bind to each binding site in the UASGABA element of UGA1 and UGA4 genes independently of the other one and that an interaction between two molecules of Uga3 increases its affinity for DNA. However, although the UGA2 promoter contains a unique binding site for the Uga3 factor, we detected in vivo interaction of this factor with the UGA2 promoter (Fig. 2a), indicating that, at least for this promoter, the presence of one binding site is sufficient for transcriptional activation.

Dal81, but not Uga3, has been previously related to the SPS amino acid signalling pathway (Abdel-Sater et al., 2004; Boban & Ljungdahl, 2007; Iraqui et al., 1999). However, we demonstrated that, as with Dal81, the GABA-dependent recruitment of Uga3 to the UGA4 promoter is impaired by a signal triggered by the amino acid SPS sensor in response to extracellular leucine (Cardillo et al., 2010). Results presented in Fig. 2(c, d) showed that Uga3 needs Dal81 to interact with UGA promoters and consequently to activate transcription (Fig. 1). Our results resemble the observations made for the AGP1 gene, where it was shown that Dal81 is necessary for the proper induction of this permease and that it enhances Stp1 binding to the AGP1 promoter in response to amino acids (Boban & Ljungdahl, 2007).

In the absence of Dal81, induction of UGA genes was almost undetectable (Fig. 1), correlating with the fact that in the dal81Δ strain neither of the two transcription factors responsible for GABA induction was recruited to the UGA promoters after the addition of GABA (Fig. 2b, c). Taken together, these results suggest that Dal81 would act as an amplifier of the specific signal triggered by GABA on the expression of UGA genes, as was already proposed for amino acid induction of AGP1 gene (Boban & Ljungdahl, 2007).

As observed for the induction of the DUR1/2 gene mediated by Dal81 transcription factor (Bricmont et al., 1991), the Zn(II)2-Cy6 domain of Dal81 is not essential for its activity in GABA-induction of UGA genes (Fig. 3 and Supplementary Fig. S1), indicating that the interaction of Dal81 with DNA could be occurring through another protein. In fact, experiments performed in the absence of Uga3 showed no interaction between Dal81 and UGA promoters, suggesting that Uga3 could be acting as a bridge between DNA and Dal81 (Fig. 2e, f). There are previous data showing that Dal81 acts on DNA through another protein. Scott et al. (2000) proposed the action of Dal81 on DUR1/2 gene through Dal82/DurM. Since it has been reported that Stp1 and Dal81 exert their function via the same regulatory sequence of AGP1 (Boban & Ljungdahl, 2007) and global analysis reported interaction of Dal81 with AGP1 promoter (Harbison et al., 2004), it is possible that this interaction may be occurring through the Stp1 transcription factor. Moreover, our proposal was reinforced by the evidence that showed no interaction between Dal81 and the UGA4 promoter when the consensus binding site for Uga3 was altered (Fig. 5b, d).

Gln3 transcription factor is important for the transcriptional regulation of the UGA4 gene in response to GABA (André et al., 1995; Luzzani et al., 2007), whereas its importance for
UGA1 induction is controversial. While Talibi et al. (1995) reported a 60% reduction of UGA1 induction in the absence of Gln3, Daugherty et al. (1993) reported that this GATA factor does not participate in UGA1 regulation. In the presence of a poor nitrogen source, the positive GATA transcription factors translocate to the nucleus and compete with the negative GATA factors for the binding sites present in the promoters of their target genes to activate transcription (Coffman et al., 1997; Soussi-Boudekou et al., 1997). However, it has been suggested that under these conditions, Gln3-mediated trans-activation cannot occur until the addition of the inducer GABA (André et al., 1995). Similarly, it has been proposed that Gln3 trans-activation on AGPI, another NCR-sensitive gene, does not occur without the prior action of Stp1 and Dal81 transcription factors (Abdel-Sater et al., 2004). We found that Dal80 strongly bound to UGA1 and UGA4 promoters in the absence of GABA and this binding weakened after the addition of the inducer (Fig. 6b). In an opposite way, Gln3 bound to UGA promoters in the presence of the inducer (Fig. 6a). In addition, we demonstrated that both Uga3 and Dal81 are responsible for the observed modulation of the binding of Dal80 (Fig. 6c, d). Although we were not able to perform similar experiments for the binding of Gln3, our results suggest that the outcome of the competition between Gln3 and Dal80 for the binding sites present in UGA1 and UGA4 promoters depends on the action of Uga3 and Dal81 factors in response to GABA (Fig. 6). Previous studies demonstrated that, in cells growing in a poor nitrogen source, the absence of the negative factor Dal80 greatly increased the interaction of Gat1, the other positive GATA factor, with the UGA4 promoter (Georis et al., 2009). After the addition of GABA, Uga3 and Dal81 could favour the binding of Gln3 and Gat1 to the GATA binding sites or could displace the negative factor Dal80.

The fact that GABA increases the binding of Gln3 while decreasing the binding of Dal80 to UGA promoters suggests that an interaction between GATA factors and the factors responsible for induction could be occurring. Using LexA-Gln3 and LexA-Dal82 constructions, the interaction of Gln3 and Dal82/DurM with different components of the SAGA complex was demonstrated and it has also been proposed that this complex could be mediating the functional relationship existing between Gln3 and Dal82/DurM (Scott et al., 2000). Uga3 for UGA genes would be the equivalent to Dal82/DurM for the DAL or DUR genes. On the other hand, studies on TamA of A. nidulans, a Dal81-related protein, demonstrated an interaction with AreA protein, a GATA-family transcription factor functionally related to Gln3 (Small et al., 2001), suggesting that a direct interaction of Gln3 and Dal81 factor could be occurring. Similarly, it has been reported that TamA also interacts with LeuB, a protein that shares homology with the S. cerevisiae Leu3 (Polotnianka et al., 2004) and it has been proposed that TamA could have a function in stabilizing AreA and LeuB interactions with DNA. Since we previously reported a negative regulation of UGA4 and UGA1 genes mediated by Leu3 (Cardillo et al., 2010; 2011), it would be interesting to study the interplay occurring between Gln3, Dal80, Uga3, Dal81 and Leu3, among other factors.

Recently, Sylvain et al. (2011) reported that Uga3 and Dal81 transcription factors are able to interact with different components of the SAGA complex and that Uga3 is necessary for the interaction of Dal81 with UGA1 promoter. They also showed data that suggest that Uga3 is necessary for the transcriptional activation via SAGA and Gal11, a component of the mediator. These results, along with similar observations made for DUR/DAL regulation by Dal82/DurM (Scott et al., 2000), support our results and extend the observations to the interaction of Uga3 and Dal81 with the transcriptional machinery for induction of expression.

We also showed that, as was observed for the induction of DUR1/2 gene (Bricmont et al., 1991), the polyglutamine stretch located between residues 73 and 94 of the Dal81 protein is essential for its function in GABA induction or for its stability (Fig. 3 and Supplementary Fig. S1). Further studies should be carried out to study the role of this polyglutamine domain.

In summary, in this work we used in vivo techniques to demonstrate that both factors Uga3 and Dal81 interact with UGA promoters in a GABA-dependent manner and that they depend on each other for both the interaction with the promoters and the transcriptional activation. We also demonstrated that Dal81 interacts with UGA promoters through Uga3 factor. Finally, Gln3 interacts with all three UGA promoters in response to GABA while Dal80 responds in the opposite way, with Uga3 and Dal81 being responsible for this behaviour. Taken together, our observations along with those available in the literature and discussed above, suggest that Uga3, Dal81, Gln3 and Dal80 act in concert to interact with the transcriptional machinery promoting the expression of UGA genes in response to the inducer GABA.

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