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

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γ-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.

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INTRODUCTION

γ-Aminobutyric acid (GABA) is a ubiquitous non-protein amino acid which is widely found in prokaryotic and eukaryotic organisms. *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 α-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).

Abbreviations: GABA, γ-aminobutyric acid; NCR, nitrogen catabolite repression; RT-qPCR, quantitative RT-PCR.

Two supplementary figures are available with the online version of this paper.

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.*,

1997), while Dal80 represses NCR-sensitive genes in the presence of poor nitrogen sources (Cunningham *et al.*, 2000).

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 UAS_{GABA} 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 UAS_{GABA} 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 allophanate-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 [$Zn(II)_2$-Cys_6]. This domain is essential for Uga3 activity (Talibi *et al.*, 1995). However, Dal81, like *Aspergillus nidulans* TamA, does not require the $Zn(II)_2$-Cys_6 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 $Zn(II)_2$-Cys_6 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-KlURA3* 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-UAS_{GABA}mut and Yep357-UAS_{GABA}del (Cardillo *et al.*, 2010) as templates. Counter-selection with 5-fluorootic 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 *uga3*Δ 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 ($Zn(II)_2$-Cys_6 150-179Δ); and pPB72, both polyglutamine stretches (PolyGln 73-94Δ 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-*UGA4*qPCR; 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

Table 1. Strains used in this work

Strain	Genotype	Parent	Primer	Source or reference
Σ1278b	*MATα*	–	–	Bechet *et al.* (1970)
23344c	*MATα ura3*	–	–	M. Grenson
26790a	*MATα ura3 uga3*Δ	–	–	Talibi *et al.* (1995)
SBCY10	*MATα ura3 6HA-DAL81*	–	–	Cardillo *et al.* (2010)
SBCY13	*MATα ura3 6HA-UGA3*	–	–	Cardillo *et al.* (2010)
SBCY17	*MATα ura3 dal81*Δ::*natMX4*	–	–	Cardillo *et al.* (2010)
SBCY25	*MATα ura3 dal81*Δ::*natMX4 6HA-UGA3*	SBCY17	F/R-Tag-*UGA3*	This study
SBCY27	*MATα ura3 uga3*Δ *6HA-DAL81*	26790[a]	F/R-Tag-*UGA35*	This study
SBCY28	*MATα ura3 uga4*::*URA3-kanMX*	23344c	F/R-Delitto P_UAS_{GABA}	This study
SBCY29	*MATα ura3 UGA4*::UAS_{GABA}*mut*	SBCY28	F/R-*UGA4* DP	This study
SBCY30	*MATα ura3 UGA4*::UAS_{GABA}*del*	SBCY28	F/R-*UGA4* DP	This study
SBCY33	*MATα ura3 UGA4*::UAS_{GABA}*mut 6HA-UGA3*	SBCY29	F/R-Tag-*UGA3*	This study
SBCY34	*MATα ura3 UGA4*::UAS_{GABA}*del 6HA-UGA3*	SBCY30	F/R-Tag-*UGA3*	This study
SBCY35	*MATα ura3 UGA4*::UAS_{GABA}*mut 6HA-DAL81*	SBCY29	F/R-Tag-*UGA35*	This study
SBCY36	*MATα ura3 UGA4*::UAS_{GABA}*del 6HA-DAL81*	SBCY30	F/R-Tag-*UGA35*	This study
SBCY41	*MATα ura3 DAL80-6HA-KanMX6*	23344c	F/R-*UGA43*-Tag	This study
SBCY43	*MATα ura3 uga3*Δ *DAL80-6HA-KanMX6*	26790[a]	F/R-*UGA43*-Tag	This study
SBCY44	*MATα ura3 dal81*Δ::*natMX4 DAL80-6HA-KanMX6*	SBCY17	F/R-*UGA43*-Tag	This study

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 $\Delta\Delta C_t$ 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 (Idicula *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 2. Primers used in this work

Primer group and name	Sequence (5′–3′)
Oligonucleotides for plasmid construction	
F-*UGA3*	CGCGGAATTCCCGGAATCACATTTGCCCAAGA
R-*UGA3*	CGCGGATCCCACTGGCACGTCGTATGCAGGA
Oligonucleotides for strain construction	
F-Tag-*UGA3*	CATGTATGGATGCCAAGAAAACAAAGTTTTTTAAAGTGAGGTATGTGCAGGTCGACAACCCTTAAT
R-Tag-*UGA3*	CATGCTTCGAATATTTCAATTTCAGCTTCTCCACGCCATAATTGCGGCCGCATAGGCCACT
F-Tag-*UGA35*	TGTTTAGACGAGCGGCAGAACGACAGGCAGCCATACTATCAAATGTGCAGGTCGACAACCCTTAAT
R-Tag-*UGA35*	CTTCGTAGGCGATGCGGCATTATCAGCTGGTGATTGGTGAGGGTCGCGGCCGCATAGGCCACT
F-Delitto P_UAS_{GABA}	TAAGGTACTCTTATCGCTAATCGCTTATCGCTTATCGTGCGCCGAGCTCGTTTTCGACACTGG
R-Delitto P_UAS_{GABA}	GGTAATAGATTGCAAAATCCAACATGACATAAAACATCTCGAATCCTTACCATTAAGTTGATC
F-*UGA4* DP	GACAATTTCTTCAATCATTG
R-*UGA4* DP	CTTTGGGTTAGAATCTTGATGTG
F-*GLN3*-Tag	TCAGCAATTGCTGACGAATTGGATTGGTTAAAATTTGGTATACGGATCCCCGGGTTAATTAA
R-*GLN3*-Tag	TATTAACATAATAAGAATAATGATAATGATAATACGCGGTCAGAATTCGAGCTCGTTTAAAC
F-*UGA43*-Tag	GAACTACACTCCAGAGCTTCAGTACAATCTCACCCACAACATCGGATCCCCGGGTTAATTAA
R-*UGA43*-Tag	CCTGTTATATTTATACTGTCATGAACGCTACATCCTTTCTTAGAATTCGAGCTCGTTTAAAC
Oligonucleotides for RT-qPCR	
F-qRT-*UGA4*	CTGCTGCTGTCACATTAACC
R-qRT-*UGA4*	AATACACATAACCACCACTGC
F-*UGA1* RT qPCR	GTTCCACGGTAGATTGTTTGC
R-*UGA1* RT qPCR	GTCATCCTCTTTACGGTTTGC
F-*UGA2* RT qPCR	AAGCGATTGATGTTGCCTATG
R-*UGA2* RT qPCR	GCGTATTTGATTTCTCCTTTAGC
F-*TBP* qPCR	TATAACCCCAAGCGTTTTGC
R-*TBP* qPCR	GCCAGCTTTGAGTCATCCTC
Oligonucleotides for qChIP	
F-*UGA1*-qChIP	ACAATCTTTCCAACTATCC
R-*UGA1*-qChIP	TGTTCTATTATCTATCTCTTCC
F-*UGA2*-qChIP	CGGCGTGGTGGTGATAAAGG
R-*UGA2*-qChIP	TGATGGTGATGTGATGTGATGTGG

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 *dal81*Δ strain. In the absence of Dal81, no interaction between HA-Uga3 and the *UGA* promoters was detected (Fig. 2c). Moreover, when *DAL81* expression was

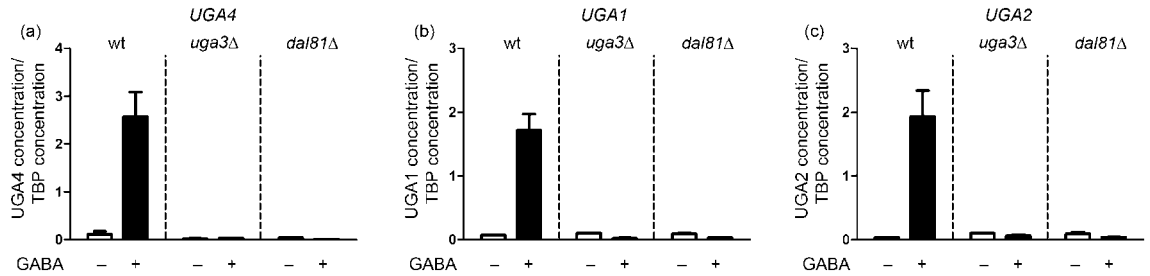

Fig. 1. The induction of *UGA* genes depends on GABA and on Uga3 and Dal81 transcription factors. mRNA levels of *UGA4* (a), *UGA1* (b) and *UGA2* (c) were determined in wild-type (23344c strain), *uga3*Δ (26790a strain) and *dal81*Δ (SBCY17 strain) cells treated (black bars) or not (white bars) with 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.

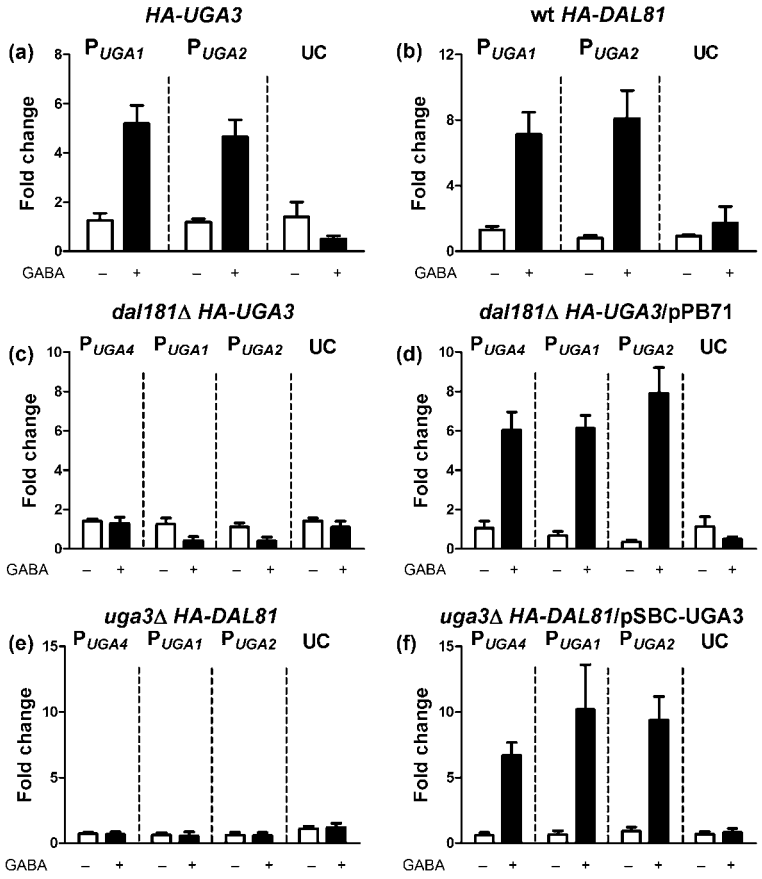

Fig. 2. Uga3 and Dal81 interact with *UGA* promoters in a GABA-dependent manner and they depend on each other for the interaction with their target promoters. (a, b) Wild-type cells expressing the HA-Uga3 (SBCY13 strain; a) or the HA-Dal81 (SBCY10 strain; b) fusion proteins. (c, d) *dal81*Δ cells expressing the HA-Uga3 fusion protein (SBCY25 strain) carrying (c) or not carrying (d) the pPB71 plasmid that encodes the complete *DAL81* gene. (e, f) *uga3*Δ cells expressing the HA-Dal81 fusion protein (SBCY27 strain) carrying (e) or not carrying (f) the pSBC-*UGA3* plasmid that encodes the complete *UGA3* gene. Cells were treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of the *UGA1* promoter (P_{UGA1}), a region of the *UGA2* promoter (P_{UGA2}) and a region 2.5 kb downstream of the *UGA4* promoter (UC) used as a negative control. Results are expressed as the fold change of binding to the promoter of interest and are the mean ± SEM of three independent experiments.

restored by transforming the *dal81*Δ strain with the plasmid pPB71 that encodes the full-length *DAL81* gene, HA-Uga3 recovered its ability to interact with *UGA* promoters in response to GABA (Fig. 2d). So, in *dal81*Δ cells, neither of the two transcription factors responsible for GABA induction, Uga3 and Dal81, bound to the *UGA* promoters, which correlated with the extremely low levels of induction of *UGA* genes in these cells (Fig. 1).

The Dal81 polyglutamine domain spanning residues 73–94, but not the $Zn(II)_2$-Cys_6 domain, is necessary for its activity

It has been demonstrated that the $Zn(II)_2$-Cys_6 domain of the Dal81 protein (residues 150–179) is not essential for the induction of *DUR1/2* gene, measured indirectly through the enzymic activity of urea amidolyase (Bricmont *et al.*, 1991). Similar observations were made for the *A. nidulans* protein TamA, a protein closely related to Dal81 that is involved in nitrogen utilization (Davis *et al.*, 1996). So, we wondered whether the $Zn(II)_2$-Cys_6 domain and also the polyglutamine streeches (residues 73–94 and 227–237) present in the Dal81 protein were required for GABA induction of *UGA* regulon genes. For this purpose, cells deficient in *DAL81* were transformed with plasmids carrying different mutated versions of *DAL81* (Bricmont *et al.*, 1991) and mRNA levels of *UGA4* were measured (Fig. 3). In *dal81*Δ cells, GABA induction of the *UGA4* gene was almost undetectable, but this deficiency was complemented by the complete Dal81 protein (*DAL81*). Dal81 proteins lacking the $Zn(II)_2$-Cys_6 domain [$Zn(II)_2$-Cys_6 150-179Δ] or the polyglutamine stretch between residues 227 and 237 (PolyGln 227-237Δ) also complemented the *dal81* deletion. However, the protein without the polyglutamine stretch between residues 73 and 94 (PolyGln 73-94Δ and PolyGln 73-94Δ 227-237Δ) was not able to restore GABA induction of *UGA4*. When mRNA levels of the other members of the *UGA* regulon, *UGA1* and *UGA2*, were measured, similar results were obtained (Supplementary Fig. S1, available with the online version of this paper). These data clearly indicate that the $Zn(II)_2$-Cys_6 domain and the polyglutamine stretch between residues 227 and 237 of Dal81 are not essential for GABA induction of *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 *UGA4* (Cardillo *et al.*, 2010), *UGA1* and *UGA2* promoters (Fig. 2b). However, the $Zn(II)_2$-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 *UGA* genes (Fig. 3 and Supplementary Fig. S1). These results

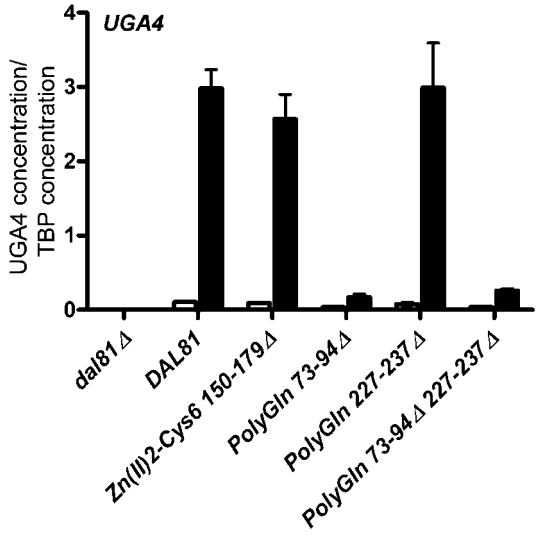

Fig. 3. The Dal81 polyglutamine domain spanning residues 73–94, but not the $Zn(II)_2$-Cys_6 domain, is necessary for its activity in GABA induction of *UGA4* gene. mRNA levels of the *UGA4* gene were determined in *dal81*Δ cells (SBCY17 strain) transformed with the plasmids that express different mutated versions of Dal81 protein (Bricmont *et al.*, 1991). Cells were treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. mRNA levels were quantified by RT-qPCR. *UGA4* values were normalized with *TBP1* and represent the mean ± SEM of three independent experiments.

suggested that Dal81 might interact with *UGA* promoters through another protein. The fact that Uga3 and Dal81 act together through the UAS_{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 *uga3*Δ strain. No interaction between HA-Dal81 and the *UGA* promoters was detected in the absence of Uga3 (Fig. 2e). However, when *UGA3* expression was restored by transforming the *uga3*Δ *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 *uga3*Δ 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_{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_{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_{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_{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_{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 *uga3*Δ and *dal81*Δ, Dal80-HA remained bound to *UGA1* and *UGA4* promoters, showing

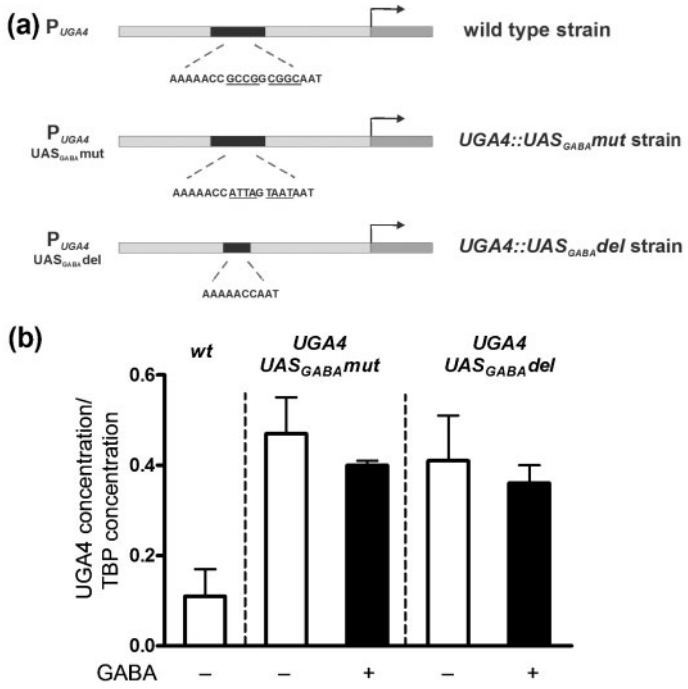

Fig. 4. Mutations in the central core of the UAS_{GABA} element present in *UGA4* promoter cause high basal levels of expression and abolish GABA induction. (a) Schematic representation of the mutation (SBCY29 strain) or deletion (SBCY30 strain) generated on the *UGA4* promoter. (b) mRNA levels of the *UGA4* gene were determined in wild-type (23344c strain) cells and in cells carrying a mutation or a deletion in the central core of the UAS_{GABA} element present in *UGA4* promoter. Cells were treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. mRNA levels were measured by RT-qPCR. *UGA4* values were normalized with *TBP1* and represent the mean ± SEM of three independent experiments.

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

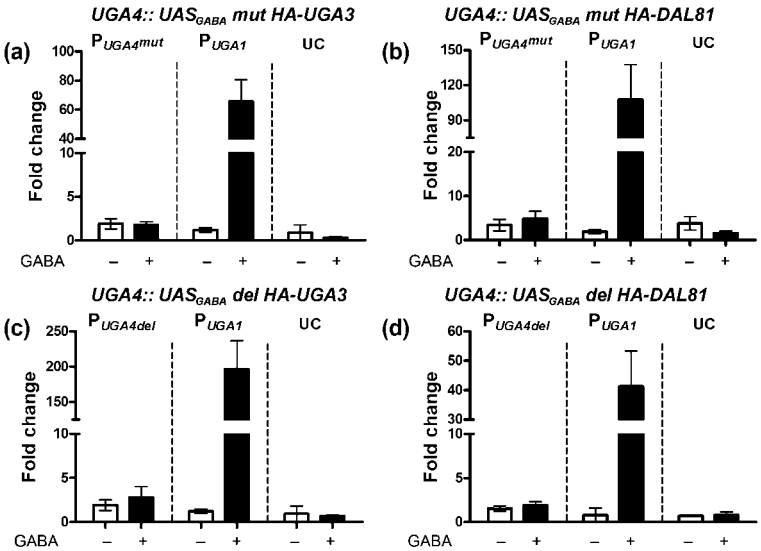

Fig. 5. Uga3 and Dal81 interact with the *UGA4* promoter through the central core of the UAS_{GABA} element. Cells lacking the central core of the UAS_{GABA} element present in *UGA4* promoter and expressing the fusion proteins HA-Uga3 [(a) SBCY33 strain; (c) SBCY34 strain] or HA-Dal81 [(b) SBCY35 strain; (d) SBCY36 strain] were treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of the *UGA4* promoter (P_{UGA4}), a region of the *UGA1* promoter (P_{UGA1}) and a region 2.5 kb downstream of the *UGA4* promoter (UC) used as a negative control. Results are expressed as the fold change of binding to the promoter of interest and are the mean ± SEM of three independent experiments.

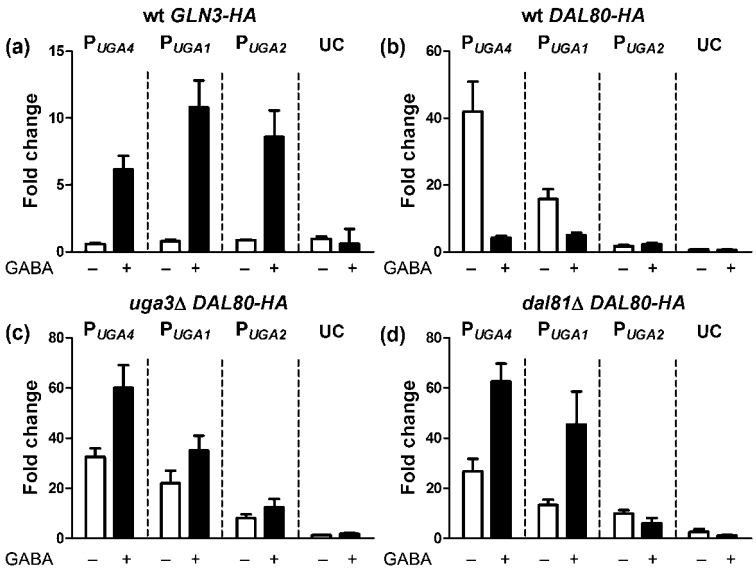

Fig. 6. GABA affects the interaction of Gln3 and Dal80 GATA factors with *UGA* promoters. (a) Wild-type cells expressing the Gln3-HA fusion protein (TOY01 strain). (b) Wild-type cells expressing the Dal80-HA fusion protein (SBCY41 strain). (c) *uga3*Δ cells expressing the Dal80-HA fusion protein (SBCY43 strain). (d) *dal81*Δ cells expressing the Dal80-HA fusion protein (SBCY44 strain). Cells were treated (black bars) or not (white bars) with 0.1 mM GABA for 30 min. ChIP assays were carried out using antibodies against the HA epitope. qPCR was performed with specific primers that amplify a region of the *UGA4* promoter (P_{UGA4}), a region of the *UGA1* promoter (P_{UGA1}), a region of the *UGA2* promoter (P_{UGA2}) and a region 2.5 kb downstream of the *UGA4* promoter (UC) used as a negative control. Results are expressed as the fold change of binding to the promoter of interest and are the mean ± SEM of three independent experiments.

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 UAS_{GABA} 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 *dal81*Δ 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 of Dal81 with the *UGA4* promoter when the consensus binding site for Uga3 was altered (Fig. 5b, d).

Gln3 transcription factor is important for the transactivation 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 *AGP1*, 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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