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GABA induction of the *Saccharomyces cerevisiae UGA4* gene depends on the quality of the carbon source: Role of the key transcription factors acting in this process

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ABSTRACT

Yeast cells are able to adapt their metabolism according to the quality of both carbon and nitrogen sources available in the environment. *Saccharomyces cerevisiae UGA4* gene encodes a permease capable of transporting γ-aminobutyric acid (GABA) into the cells. Yeast uses this amino acid as a nitrogen source or as a carbon skeleton that enters the tricarboxylic acid cycle.

The quality of the carbon source modulates *UGA4* expression through two parallel pathways, each one acting on different regulatory elements, the UAS_{GATA} and the UAS_{GABA}. In the presence of a fermentable carbon source, *UGA4* expression is induced by GABA while in the presence of a non-fermentable carbon source this expression is GABA-independent.

The aim of this work was to study the mechanisms responsible for the differences in the profiles of *UGA4* expression in both growth conditions.

We found that although the subcellular localization of Gln3 depends on the carbon source and *UGA4* expression depends on Tor1 and Snf1, Gln3 localization does not depend on these kinases. We also found that the phosphorylation of Gln3 is mediated by two systems activated by a non-fermentable carbon source, involving the Snf1 kinase and an unidentified TORC1-regulated kinase.

We also found that the activity of the main transcription factors responsible for *UGA4* induction by GABA varies depending on the quality of the carbon source. In a fermentable carbon source such as glucose, the negative GATA factor Dal80 binds to *UGA4* promoter; only after the addition of the inducer, the positive factors Uga3, Dal81 and Gln3 interact with the promoter removing Dal80 and leading to gene induction. In contrast, in the non-fermentable carbon source acetate the negative GATA factor remains bound to *UGA4* promoter in the presence or absence of GABA, the positive factors are not detected bound in any of these conditions and in consequence, *UGA4* is not induced.

1. Introduction

The budding yeast *Saccharomyces cerevisiae* cells sense the amount and quality of external nutrients through multiple interconnected signaling networks, which allow them to adapt readily and appropriately to changes in nutritional states. Carbon and nitrogen are the two most basic nutrient sources for cellular organisms. In response to the quality of carbon and nitrogen, cells can regulate the expression of genes involved in different metabolic pathways, particularly those involved in utilization and transport of the available nutrients.

The *UGA4* gene that encodes the γ-aminobutyric acid (GABA) and δ-aminolevulinic acid permease in *S. cerevisiae*, is regulated by both nitrogen [1,2] and carbon [3] sources and is inducible by GABA [4,5]. Expression of this permease requires at least two positive acting proteins, the specific Uga3 factor and the pleiotropic Dal81 factor [6,7]. These factors interact with *UGA4* promoter in response to GABA through a 19 bp CG-rich upstream activating sequence, named UAS_{GABA} [8,9]. The promoter region of *UGA4* also contains four adjacent repeats of the heptanucleotide 5′-CGAT(A/T)AG-3′, which constitute a UAS_{GATA} element. The GATA factors Gln3, Gat1 and Dal80 are known to act on this element [1,2,6,10–12]. The role of the other negative GATA factor Gzf3 has not been demonstrated yet on *UGA4* regulation.

We previously demonstrated that the quality of the carbon source modulates *UGA4* expression through two parallel pathways: one of them acting on the Gln3 target sequence, the UAS_{GATA} element, and the other acting on the UAS_{GABA} element of *UGA4* gene [3].

Gln3 activity depends on both its subcellular localization and its phosphorylation [13] and this phosphorylation depends on TORC1

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(target of rapamycin) activity [14]. Moreover, Snf1, a yeast homolog of AMP-activated protein kinase (AMPK), is required for metabolic adaptation in response to reduced levels of available glucose, the preferred carbon source. When glucose is unavailable, alternative carbon sources are used for the production of metabolic energy [15]. Snf1 is activated by glucose limitation by phosphorylation of the threonine 210 and by inactivation of Tor kinases [16,17]. Gln3 phosphorylation and subcellular localization are regulated by glucose availability via the SNF1/AMPK pathway [18].

Based on these findings, the aim of this work was to establish the molecular mechanisms of the two proposed pathways involved in carbon regulation of *UGA4* gene. We found that in glucose Uga3 and Dal81 factors are recruited to *UGA4* promoter in response to GABA and that Gln3 and Dal80 binding to this promoter is also modulated by the inducer. In contrast, under non-fermentable conditions, these factors do not respond to GABA and, in consequence, no induction of *UGA4* is observed. Results here presented suggest that the two pathways regulating *UGA4* expression by carbon source act on two different DNA elements but in a coordinated way.

2. Methods

2.1. Strains and media

The *S. cerevisiae* strains used in this study, isogenic to the wild type Σ1278b, are listed in Table 1. All the strains generated in this study were constructed using the PCR-based gene-deletion strategy [19,20] or modified versions of it [21]. All the parental strains are listed in Table 1 and all primers used for PCR reactions are listed in Supplementary material Table S1.

Cells were grown in minimal medium containing 0.17% Difco yeast nitrogen base (YNB without amino acids and ammonium sulfate) containing 2% glucose or 2% potassium acetate as carbon source and 10 mM proline as nitrogen source.

All yeast transformations were carried out using the lithium method [22].

2.2. Cell extracts and immunoblotting

Cells expressing tagged versions of Uga3 or Dal81 proteins or wild type cells transformed with a plasmid from the Movable ORFs collection containing the open reading frame of *GLN3* gene [23] were grown on the indicated media and were harvested by centrifugation. Protein extraction was immediately carried out as already described [24]. Briefly, total proteins were prepared by lysing yeast cells in 1.85 N NaOH-7.5% β-mercaptoethanol on ice for 10 min, followed by precipitation with trichloroacetic acid (TCA) at a final concentration of 8%. The TCA pellets were neutralized with 1 M unbuffered Tris and resuspended in sodium dodecyl sulfate (SDS) loading buffer. Proteins were separated on a 7% SDS–PAGE, transferred to PVDF membranes and proteins were detected using the rat monoclonal anti-HA antibody (anti-HA high affinity 3E10 Roche) or the rat monoclonal anti-α-tubulin (YOL1/34 Santa Cruz) and with the secondary goat anti-rat antibody IgG conjugated with horseradish peroxidase (Santa Cruz). Chemiluminescence immunodetection was performed (FUJIFILM LAS-1000 Reader) and immunoreactive bands were analyzed by digital imaging.

2.3. Fluorescence microscopy

Cells transformed with the CEN-based plasmid pRS416-GLN3-GFP (kindly provided by Dr. T. Cooper, University of Tennessee, Memphis, USA) [25], containing the full-length *GLN3* gene fused to GFP were fixed with 70 % ethanol in 50 mM Tris/HCl pH 7.5 for 60 min. Nuclei were stained incubating cells with 50 μg/ml DAPI (4′-6-Diamidino-2-phenylindole) in 50 mM Tris/HCl pH 7.5 for 60 min. Cells were washed twice with 50 mM Tris/HCl pH 7.5. Analysis was carried out by using fluorescence microscopy. Gln3-GFP localization was manually scored in 200 or more cells in multiple randomly chosen fields from each image taken [26].

2.4. Quantitative RT-PCR

RT-qPCR experiments were performed according to Cardillo et al. [27]. cDNAs were quantified by RT-PCR using an Opticon Monitor 3 (Bio-Rad) with the primers F-qRT-UGA4 and F-TBP qPCR. Expression values correspond to the ratio of concentrations of *UGA4* over *TBP1* specific mRNAs determined in each sample and represent the mean ± SEM of three independent experiments.

2.5. Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) experiments were performed according to Cardillo et al. [9]. 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 (BioRad) with primers that amplified promoter regions of *UGA4* (F/R-*UGA4*qPCR). A pair of primers that amplified a region located 2.5 Kb downstream of *UGA4* promoter was used as an unbound control. 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 [28]. Results are expressed as the mean ± SEM of three independent experiments.

3. Results and discussion

To further analyze the mechanisms of the regulation of *UGA4* by carbon source proposed by Luzzani et al. [3], in the first place we studied the phosphorylation state and subcellular localization of Gln3. The subcellular localization of the fusion protein Gln3-GFP determined by fluorescence microscopy was classified in three categories, cytoplasmic, nuclear-cytoplasmic and nuclear, according to Tate et al. [26]. A representative collection of standard images

Table 1
Strains used in this work.

Strain	Genotype	Parent	Primer	Source or reference
23344c	Matα *ura3*	–	–	M. Grenson
TOY01	Matα *ura3 tor1Δ::KanMX4*	23344c	F/R-TOR1	This study
TOY02	Matα *ura3 tor1Δ::hph*	TOY01	F/R-ME	This study
CELY01	Matα *ura3 snf1Δ::KanMX4*	23344c	F/R-SNF1	This study
CELY02	Matα *ura3 tor1Δ::hph snf1Δ::KanMX4*	TOY02	F/R-SNF1	This study
SBCY41	Matα *ura3 DAL80–3HA-KanMX6*	–	–	[9]
TOY03	Matα *ura3 GLN3–3HA-KanMX6*	–	–	[9]
SBCY10	Matα *ura3 6HA-DAL81*	–	–	[8]
SBCY13	Matα *ura3 6HA-UGA3*	–	–	[8]

demonstrating the differences in these categories is shown in Fig. S1. We found that while in more than half of the cells incubated in the fermentable carbon source, glucose, Gln3-GFP had nuclear localization (52%), when cells were incubated in the non-fermentable carbon source, acetate, the percentage of cells with nuclear Gln3-GFP was significantly lower (26%) (Fig. 1A). We also found that this localization pattern did not depend on the activity of neither Tor1 nor Snf1, since Gln3-GFP localization was similar in wild type cells and in *tor1Δ*, *snf1Δ*, and double mutant *snf1Δ tor1Δ* cells (Fig. 1A).

Since phosphorylation status and localization pattern of Gln3 do not always correlate [13,29,30] indicating that Gln3 activity could vary even when similar localization patterns are observed, we next analyzed the phosphorylation status of Gln3. To do this, we performed western blot analysis since the electrophoretic mobility of Gln3 changes with its phosphorylation [18,30,31].

In wild type cells, mobility of Gln3 in acetate was slower than in glucose (Fig. 1B, lanes 1 and 2) indicating that more phosphorylation was occurring in the non-fermentable carbon source as it was reported earlier for other growth conditions, like glucose-starvation, ethanol, and raffinose [18]. Comparing Gln3 mobility patterns observed in all the strains tested, we found that these patterns did not change in glucose while in acetate did (Fig. 1 B and C). In the non-preferred carbon source, Gln3 seemed to be more phosphorylated in wild type and *tor1*Δ cells than in *snf1*Δ and *tor1*Δ *snf1*Δ cells (Fig. 1B and C, compare lanes 2 and 6 to lanes 12 and 16). This result indicated that the phosphorylation of Gln3 observed in acetate was Snf1-dependent. It must be noticed that after treatment with rapamycin of both *snf1*Δ mutants grown on acetate, Gln3 mobility decreased, indicating a more phosphorylated status (Fig. 1C, lanes 14 and 18). This observation suggested that in these mutants, where Snf1 was absent, there was another kinase that phosphorylated Gln3. This kinase appeared to be active in the non-fermentable carbon source and inhibited by the complex TORC1, since its activity was observed only in cells from acetate treated with rapamycin. In the *tor1*Δ mutants some effect of rapamycin was observed indicating that in this strain there was an active TORC1, which probably contained Tor2 protein (Fig. 1B, lanes 6 and 8 and Fig. 1C, lanes 12 and 14).

In summary, the non-fermentable carbon source, acetate, elicited at least two different mechanisms for Gln3 phosphorylation. One of them is Snf1-dependent as was already reported [13,18,30,31]; the other involves an Snf1-independent kinase as already proposed Tate et al. [30]. Here we demonstrated that this kinase is negatively regulated by TORC1.

Since *UGA4* expression is affected by Gln3 and different phosphorylation levels of this factor could result in different activities, we decided to measure the expression of *UGA4* in wild type, *tor1Δ*, *snf1Δ* and *snf1Δ tor1Δ* cells grown in both carbon sources. Basal levels of expression in glucose were very low and GABA induction levels were similar in all the strains (Fig. 2). These results were in agreement with similar phosphorylation levels of Gln3 (Fig. 1B and C). On the other hand, in the wild type strain, basal expression levels were higher in acetate than in glucose while induction was significantly weaker (Fig. 2A) as we already reported [3]. Low induction in the non-fermentable carbon source correlated with less cells with nuclear Gln3 (Fig. 1A) and a more phosphorylated form of this factor (Fig. 1B). In acetate, the expression pattern of *UGA4* in *tor1Δ* cells (Fig. 2B) was similar to that observed in the wild type, according to similar localization (Fig. 1A) and phosphorylation (Fig. 1B) of Gln3. However, in the two mutant strains *snf1Δ* and *snf1Δ tor1Δ*, where we detected less phosphorylated forms of Gln3 than in the wild type, induction of *UGA4* was not detected (Fig. 2C and D).

To further investigate the role of Gln3 in the regulation by the carbon source, we measured the binding of this factor to *UGA4* promoter. In glucose, Gln3-HA bound to DNA in response to GABA (Fig. 3A), as we already reported [9]. On the contrary, in acetate, no binding of Gln3-HA was detected (Fig 3A) correlating with the very low induction observed (Fig 2A).

It is well known that the GATA factors Gln3 and Dal80 compete with each other for the DNA binding sites present in the promoters

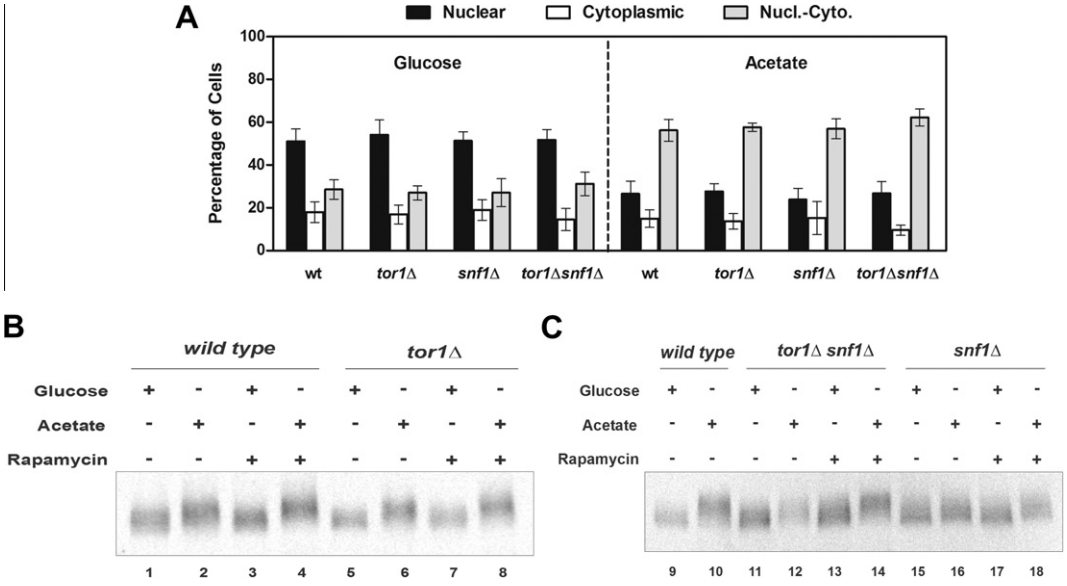

Fig. 1. Effect of the carbon source on the subcellular localization and on the phosphorylation levels of Gln3. (A) Wild type (23344c), *tor1Δ* TOY01, *snf1Δ* (CELY01) and *snf1Δ tor1Δ* (CELY02) cells transformed with the plasmid pRS416-GLN3-GFP, were grown on minimal medium with glucose as the carbon source. Harvested cells were transferred to fresh medium containing glucose or acetate and incubated for 30 min. Then, cells were fixed and nuclei were stained with DAPI. Images were processed and colored using the Image J software. (B) and (C) Wild type (23344c), *tor1Δ* TOY01, *snf1Δ* CELY01) and *snf1Δ tor1Δ* (CELY02) cells transformed with the plasmid carrying the fusion gene *GAL1-GLN3-HA* from the Movable ORFs collection were grown on minimal medium with 2% raffinose, induced with 1% galactose for 1 h and incubated in minimal medium with glucose or acetate in the presence or absence of 200 ng/ml rapamycin for 30 min. Total cell extracts were prepared and immunoblotting was carried out with anti-HA antibody.

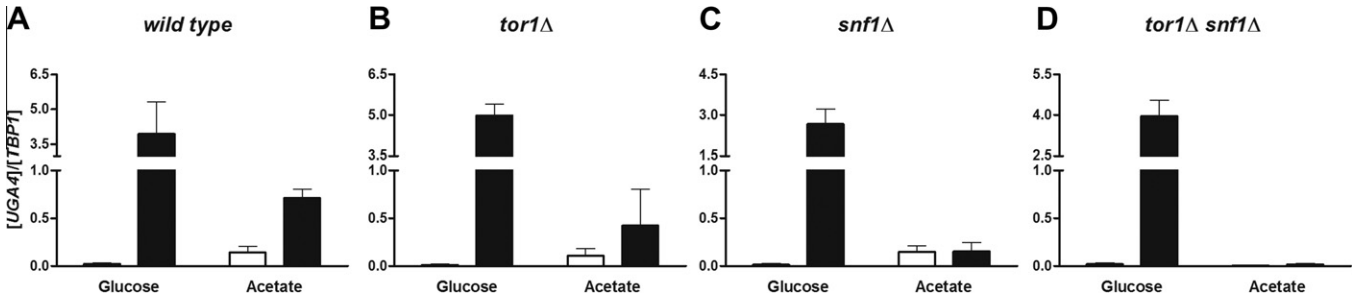

Fig. 2. Expression of *UGA4* gene in wild type, *tor1*Δ, *snf1*Δ and *tor1*Δ *snf1*Δ cells. mRNA levels of *UGA4* were determined in wild type (23344c strain), *tor1*Δ (TOY01 strain), *snf1*Δ (CELY01 strain) and *tor1*Δ *snf1*Δ (CELY02 strain) cells grown on minimal medium with glucose as the carbon source. Harvested cells were transferred to fresh medium containing glucose or acetate, incubated for 30 min, and 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.

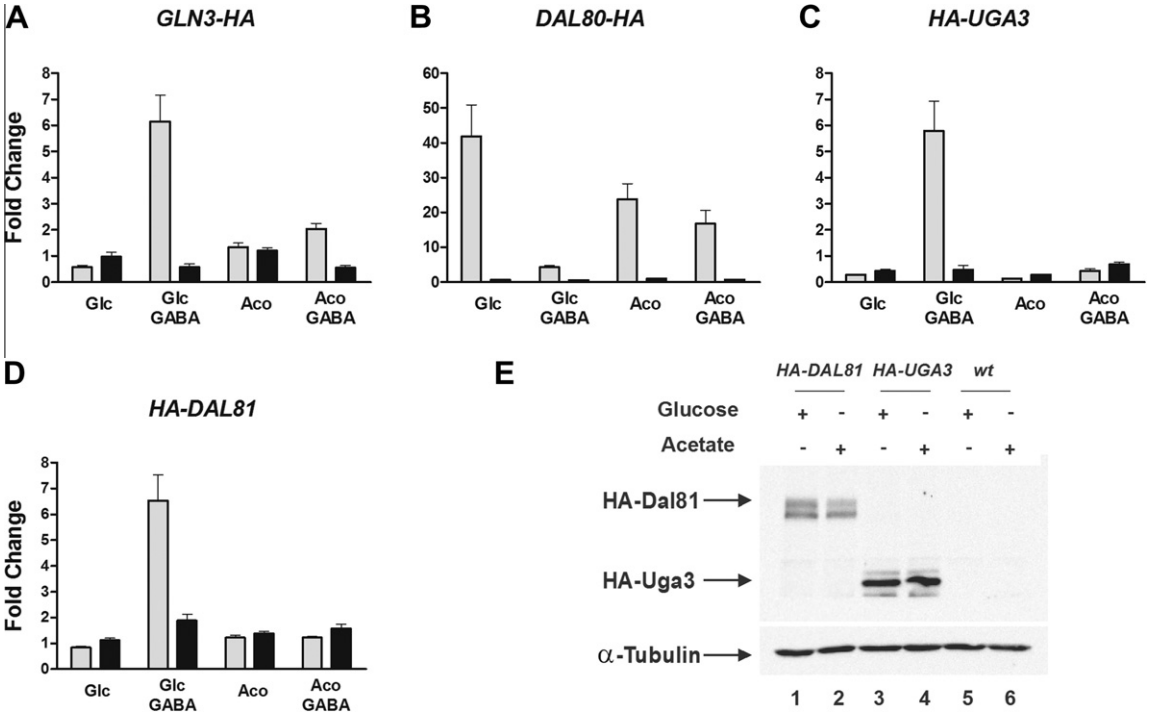

Fig. 3. Interaction between transcription factors and the promoter of *UGA4*. (A)–(D) Cells expressing the Gln3-HA (TOY03) (A), Dal80-HA (SBCY41 strain) (B), HA-Uga3 (SBCY13) (C) or HA-Dal81 (SBCY10 strain) (D) fusion proteins were grown on minimal medium with glucose as the carbon source. Harvested cells were transferred to fresh medium containing glucose or acetate, incubated for 30 min, and treated 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 specific primers that amplify a region of *UGA4* promoter (gray bars) and a region 2.5 kb downstream of *UGA4* promoter (UC) used as a negative control (black bars). Results are expressed as the fold change of binding to *UGA4* promoter and are the mean ± SEM of three independent experiments. (E) Cells expressing the HA-Dal81 (SBCY10 strain) (lanes 1 and 2) or HA-Uga3 (SBCY13) (lanes 3 and 4) fusion proteins were grown on minimal medium with glucose as the carbon source. Harvested cells were transferred to fresh medium containing glucose or acetate, incubated for 30 min, and grown on glucose (lanes 1, 3 and 5) or acetate (lanes 2, 4 and 6) as carbon sources. Total cell extracts were prepared and immunoblotting was carried out with anti-HA antibody or anti-α-tubulin. Lanes 5 and 6 correspond to extracts prepared from wild type cells (23344c).

of their target genes and that the balance of this competition leads to different expression levels [12]. We recently reported that the binding of Gln3 and Dal80 to *UGA4* promoter in glucose responds to GABA in an opposite way: Dal80, but not Gln3, interacts with DNA in the absence of GABA while Gln3, but not Dal80, interacts with DNA in the presence of GABA [9]. So, we decided to also compare the binding of Dal80 in both carbon sources. In glucose, Dal80-HA bound to *UGA4* promoter in the absence of the inducer and its binding decreased after the addition of GABA (Fig. 3B) as we already reported [9]. In acetate, this negative GATA factor was detected bound to *UGA4* promoter even in the presence of the inducer (Fig. 3B) in agreement with the fact that Gln3-HA was not detected bound (Fig. 3A).

Luzzani et al. [3] proposed a second mechanism responsible for the regulation by carbon source of *UGA4* gene which would act on the UAS_{GABA} element. *UGA4* induction depends on Uga3 and Dal81 transcription factors that act through this element [5,7]. Since the main difference between expression in glucose and acetate is that the induction in acetate is significantly lower than in glucose (Fig. 2A), we decided to analyze the binding dynamics of Uga3 and Dal81 to *UGA4* promoter in both carbon sources. In glucose, both factors were detected bound to DNA only after the addition of the inducer (Fig. 3C and D) as we already reported [8]. In contrast, HA-Uga3 and HA-Dal81 binding was impaired in acetate (Fig. 3C and D). As this observation could be due to a decrease in the expression of these factors in acetate, we analyzed by Western

blot their expression. We found that Uga3 and Dal81 levels were similar in both carbon sources (Fig. 3E), confirming that the binding ability of these factors depended on the carbon source. These results correlated with the very low induction observed in this condition (Fig. 2A). It must be noted that different forms of both Uga3 and Dal81 proteins were detected. Although the mechanisms that lead to the activation of Uga3 and Dal81 in response to GABA are still unknown, it has been proposed that Uga3 activation may occur through a post-translational modification since levels of expression of this factor remain unaffected in the presence of the inducer [32]. Moreover, *in silico* analysis of *UGA3* and *DAL81* coding sequences yielded many putative phosphorylation sites [33]. Taken together, all this evidences suggest that the different forms of both Uga3 and Dal81 proteins detected could be a consequence of different phosphorylation states. Further research must be done in order to demonstrate this hypothesis.

In summary, in glucose, the negative GATA factor Dal80 is bound to *UGA4* promoter repressing its expression. When GABA is added, the two factors responsible for induction, Uga3 and Dal81, and the non-phosphorylated positive GATA factor Gln3, which competes with Dal80 for binding to the UAS_{GATA} element, bind to the promoter producing the induction of *UGA4*. In acetate, Dal80 interacts with *UGA4* promoter whereas the highly phosphorylated Gln3, Uga3 and Dal81 factors do not. This interaction accounts for the absence of GABA induction.

Results presented here do not allow us to determine whether the phosphorylation of Gln3 in acetate or its less nuclear distribution impairs its interaction with *UGA4* promoter. However, recently we showed that in glucose the binding dynamics in response to GABA of Dal80, and consequently of Gln3, are modulated by Uga3 and Dal81 transcription factors [9]. These findings resemble those observed in acetate where we do not detect binding of Uga3 and/or Dal81 and at the same time we do not detect changes in the binding of the GATA factors after the addition of GABA. All together these observations suggest that the impaired binding of Gln3 in acetate could be a consequence of the lack of function of Uga3 and Dal81, although we can not discard an effect of the phosphorylation status of Gln3 on its binding, and that consequently could affect Dal80 binding.

In conclusion, the quality of the carbon source regulates *UGA4* expression by two parallel but connected pathways. The role in these pathways of other transcription factors known to regulate *UGA4*, such as Gat1 [12] and Leu3 [8], needs to be analyzed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.047.

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