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# Common features and differences in the expression of the three genes forming the *UGA* regulon in *Saccharomyces cerevisiae*

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ABSTRACT

The three genes that form the *UGA* regulon in *Saccharomyces cerevisiae* are responsible for the transport and degradation of γ-aminobutyric acid (GABA) in this organism. Despite the differences in the sequence of their promoters, these genes similarly respond to GABA stimuli. The expression of *UGA1*, *UGA2* and *UGA4* depends on GABA induction and nitrogen catabolite repression (NCR). The induction of these genes requires the action of at least two positive proteins, the specific Uga3 and the pleiotropic Uga35/Dal81 transcription factors. Here we show that all the members of the *UGA* regulon, as was already demonstrated for *UGA4*, are negatively regulated by extracellular amino acids through the SPS amino acid sensor. We also show that this negative effect is caused by a low availability of Uga35/Dal81 transcription factor and that Leu3 transcription factor negatively regulates *UGA4* and *UGA1* expression but it does not affect *UGA2* expression.

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## 1. Introduction

The yeast *Saccharomyces cerevisiae*, as many other organisms, has the ability to use a variety of nitrogen sources to synthesize all the nitrogen-containing cellular components. Nitrogen transport, anabolism and catabolism are subject to a tight control at the level of gene transcription and enzyme synthesis, activity and degradation that depends on the nitrogen sources available [1]. This organism is able to use γ-aminobutyric acid (GABA) as a source of cellular nitrogen [2]. GABA uptake is mediated by three permeases: the general amino acid permease Gap1, the proline specific permease Put4 and the GABA specific permease Uga4. Cells deficient in *GAP1*, *PUT4* and *UGA4* are unable to grow in the presence of GABA as a sole nitrogen source due to their inability to transport it into the cell [3]. Once GABA is incorporated into the cell, it can be degraded to produce glutamate and succinate.

The first reaction in the GABA degradation pathway is catalyzed by the product of the *UGA1* gene, the enzyme GABA transaminase (4-aminobutyrate aminotransferase), which converts GABA into succinate semialdehyde (SSA) and glutamate. Then, SSA is converted to succinate by the enzyme succinate semialdehyde dehydrogenase encoded by the *UGA2* gene [2,4,5].

*UGA1*, *UGA2* and *UGA4* expression depends on GABA induction and nitrogen catabolite repression (NCR) [5–7]. Induction of these genes requires the action of at least two positive proteins, the specific Uga3 and the pleiotropic Uga35/Dal81 transcription factors [7]. These factors act through a 19 bp GC-rich upstream activating sequence named $UAS_{GABA}$ present in the *UGA1* and *UGA4* promoters [8–10], while a single consensus binding site for the transcription factor Uga3 has been identified in the 5′-untranslated region of *UGA2* gene [11,12].

Yeast cells rely on the SPS-sensing pathway to respond to extracellular amino acids. Ssy1, Ptr3 and Ssy5 proteins form a plasma membrane complex, named SPS, that functions as a sensor of extracellular amino acids [13]. Stp1 and Stp2 transcription factors are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains. Upon induction by extracellular amino acids, the plasma membrane SPS-sensor catalyses an endoproteolytic processing event that cleaves away the regulatory N-terminal domains [14]. The shorter forms of Stp1 and Stp2 efficiently target to the nucleus, where they bind and activate transcription of genes encoding a subset of amino acid permeases [15–17]. Uga35/Dal81 factor is required for full induction of SPS sensor-dependent expression of the *AGP1*, *PTR2*, and *BAP2* genes [15,18,19] and also increases the efficiency of Stp1 binding to the *AGP1* promoter [20].

*UGA4* gene induction is negatively regulated by different amino acids through the SPS amino acid sensor [21,22]. In the presence of extracellular amino acids, a signal triggered by the SPS sensor and executed by Stp1 and Stp2 transcription factors impairs *in vivo* GABA-dependent binding of Uga35/Dal81 and Uga3 to *UGA4* promoter what correlates with the low induction levels observed in the presence of amino acids [22].

A hierarchy has been proposed for different induction processes mediated by the Uga35/Dal81 factor [15,22], i.e., SPS amino

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acid-regulated genes, GABA-induced genes and allophanate-inducible *DUR* and *DAL* genes. We proposed that the signal triggered by the SPS sensor in response to extracellular amino acids activates Stp1/Stp2 transcription factors, which would be recruiting Uga35/Dal81 to promote transcriptional induction of the amino acid-induced genes and consequently decreasing the availability of Uga35/Dal81 and Uga3 for GABA induction of the *UGA4* gene [22].

Leu3 is a transcriptional regulator that controls the expression of genes involved in the biosynthesis of branched-chain amino acids [23,24] and is also involved in the transcriptional regulation of *BAP2* and *GDH1* genes, responsible for branched-chain amino acid transport and ammonia utilization, respectively [25,26]. It has also been reported that this factor acts as a repressor on *UGA4* transcription although the mechanism by which it regulates *UGA4* remains unclear [22]. Despite there is a putative Leu3 binding site within the $UAS_{GABA}$ element in *UGA4* promoter, *in vivo* binding of this factor to this promoter was not detected, suggesting an indirect mechanism of regulation [22].

The aim of this work was to determine whether the other members of the *UGA* regulon, *UGA1* and *UGA2*, are subject to amino acid regulation as it occurs for *UGA4*, and to gain further insights into the hierarchical role of Uga35/Dal81 in this regulation. Here we demonstrated that, as already found studying *UGA4* regulation, the induction of *UGA1* and *UGA2* genes is negatively affected by leucine in an SPS-dependent manner, and that this negative effect is caused by the low availability of Uga35/Dal81 transcription factor produced by pre-incubation with leucine. We also showed that Leu3 transcription factor negatively regulates *UGA1* and *UGA4* expression, but it does not regulate *UGA2*.

## 2. Materials and methods

### 2.1. Strains and media

The *S. cerevisiae* strains used in this study, isogenic to the wild type strain Σ1278b, are listed in Table 1. Prototrophic strains were used to avoid the addition of amino acids during growth. Cells were grown in minimal buffered (pH 6.1) medium [27], with 3% glucose as the carbon source and 10 mM proline as the nitrogen source.

### 2.2. Plasmids

The pSBC-*HA-UGA35* plasmid was constructed cloning the *UGA35/DAL81* coding region with six copies of the HA epitope fused to its 5′ end into the p426-GPD plasmid [28]. *HA-UGA35/DAL81* DNA fragment was amplified from genomic DNA of the SBCY10 strain (Table 1) using the primers F-Hind-HA-UGA35 (5′-CCGAA GCTTATGTGCAGGTCGACAACCCTT-3′) and R-Hind-HA-UGA35 (5′-CCGAAGCTTTCCATTTTTACTTATGTGCTA-3′). The functionality of HA-Uga35/Dal81 protein was confirmed by complementation of the inability of *uga35Δ* cells to grow using 10 mM GABA as the sole nitrogen source.

### 2.3. RT-qPCR

Total RNA was extracted from 4-ml cultures as described previously [29]. Genomic DNA was eliminated after incubating RNA with DNAse RQ1 (Promega) for 60 min at 37 °C. cDNA was generated from 1 to 4 μg of total RNA using a RevertAid™ Reverse Transcriptase (Fermentas) with hexa-random primers following the manufacturer's recommended protocol. cDNAs were subsequently quantified by RT-PCR using an Opticon Monitor 3 (Bio-Rad) with the primers F-qRT-UGA4 (5′-CTGCTGCTGTCACATTAACC-3′)/R-qRT-UGA4 (5′-AATACACATAACCACCACTGC-3′), F-UGA1 RT qPCR (5′-GTTCCACGGTAGATTGTTTGC-3′)/R-UGA1 RT qPCR (5′-GTCATCCTCT TTACGGTTTGC-3′), F-UGA2 RT qPCR (5′-AAGCGATTGATGTTGCC-TATG-3′)/R-UGA2 RT qPCR (5′-GCGTATTTGATTTCTCCTTTAGC-3′) and F-TBP qPCR (5′-TATAACCCCAAGCGTTTTGC-3′)/R-TBP qPCR (5′-GCCAGCTTTGAGTCATCCTC-3′). Expression values correspond to the ratio of concentrations of *UGA1, UGA2* and *UGA4* over *TBP1* specific mRNAs determined in each sample and represent the mean ± SEM of at least three independent experiments. Statistical analysis was performed using a block design ANOVA followed by Tukey test.

**Table 1**
Strains used in this work.

| Strain | Genotype | References |
|---|---|---|
| Σ1278b | *Matα* | [32] |
| 23344c | *Matα ura3* | [3] |
| 30995b | *Matα ura3 ssy1Δ::KanMX2* | [18] |
| SBCY01 | *Matα ura3 leu3Δ::KanMX4* | [22] |
| SBCY10 | *Matα ura3 6HA-UGA35* | [22] |

## 3. Results and discussion

Since GABA-dependent induction of *UGA4* gene is affected by the presence of extracellular amino acids [21,22], we decided to test if the other genes that form the *UGA* regulon were subject to the same regulation. For this purpose, we measured *UGA4*, *UGA1* and *UGA2* mRNA levels in wild type and *ssy1Δ* cells treated or not with leucine, a commonly used inducer of the SPS sensor, prior to the addition of GABA. GABA induction of *UGA1* and *UGA2* was inhibited by leucine (Fig. 1A–C); similar results were obtained studying *UGA4* regulation [22]. This inhibition was caused by a signal triggered by the SPS amino acid sensor since cells deficient in the Ssy1 protein, a core SPS sensor component, were insensitive to leucine (Fig. 1D–F). These results suggest that all *UGA* genes are target of the signal triggered by the SPS sensor.

It has been proposed that Uga35/Dal81 transcription factor could act as a hierarchical regulator of different induction processes depending on the inducers present in the culture media [15,22]. This proposal was based on the evidences that both GABA-induced *UGA4* gene expression and allophanate-induced *DUR* genes expression are inhibited by amino acids through the SPS sensor. We also demonstrated that leucine inhibits GABA-induced recruitment of Uga35/Dal81 and Uga3 transcription factors to *UGA4* promoter in an SPS-dependent manner, correlating with the low induction levels observed in those conditions [22]. Uga35/Dal81 was detected bound to the promoters of many SPS-regulated genes [30]. According to the model proposed, upon activation of the SPS pathway by extracellular amino acids, Uga35/Dal81 might be recruited specifically to the promoters of genes whose expression is induced by external amino acids rather than to those responding to GABA or allophanate, leading to a low availability of this factor for its recruitment to the *UGA, DUR* or *DAL* promoters even when GABA or allophanate are added to the media, respectively. According to our proposal, an excess of Uga35/Dal81 protein would prevent the negative effect of leucine on *UGA* genes induction. To test this hypothesis we decided to over-express Uga35/Dal81 protein and to analyze the effect of leucine on the induction of *UGA* genes. To do this, we measured *UGA1*, *UGA2* and *UGA4* mRNA levels in *UGA35/DAL81* deficient cells carrying the pSBC-*HA-UGA35* plasmid, treated or not with leucine before the addition of GABA. Results (Fig. 2) showed that the over-expression of Uga35/Dal81 transcription factor prevented the amino acid inhibition of *UGA* genes induction. Although our results showing no negative effect of leucine on *UGA* genes induction in cells over-expressing Uga35/Dal81 support the idea of a lower availability of Uga35/Dal81 to act on *UGA* genes induction, the possibility of a differential post-translational modification causing higher affinity of Uga35/Dal81 for a certain induc-

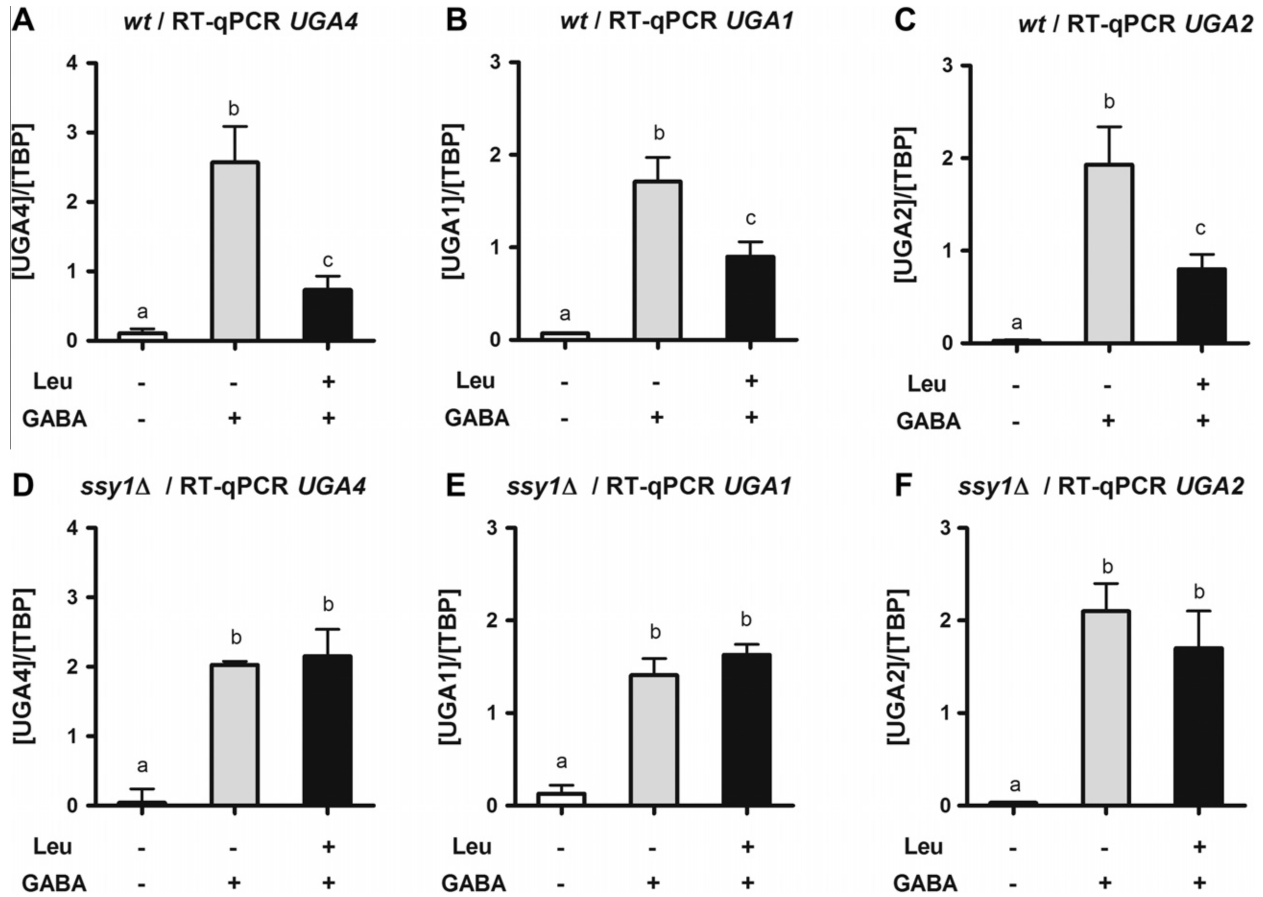


**Fig. 1.** Effect of leucine on *UGA4*, *UGA1* and *UGA2* expression in wild type and *ssy1*Δ cells. mRNA levels of *UGA4* (A, D), *UGA1* (B, E) and *UGA2* (D, F) were determined in wild type (A–C) and *ssy1*Δ cells (D–F) preincubated (black bars) or not (white and grey bars) with 1.3 mM leucine for 30 min before the addition of 0.1 mM GABA (grey and black bars). After a 30-min incubation mRNA levels were measured by RT-qPCR. *UGA1*, *UGA2* and *UGA4* values were normalized with *TBP1*. The bars with different letters (a–c) are significantly different from each other ($p < 0.05$).

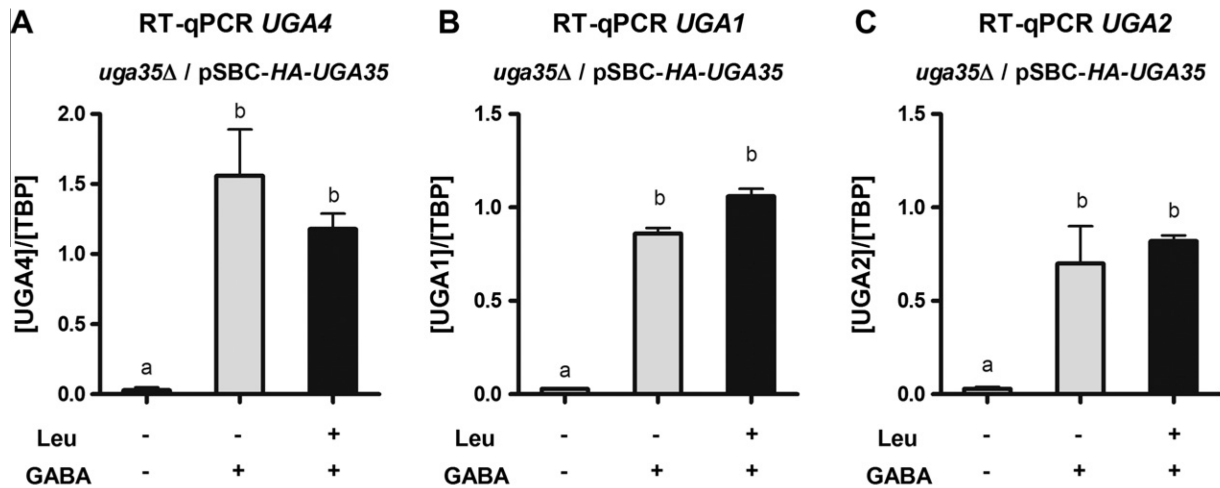


**Fig. 2.** Effect of Uga35/Dal81 in the regulation of *UGA4*, *UGA1* and *UGA2* by leucine. mRNA levels of *UGA4* (A), *UGA1* (B) and *UGA2* (C) were determined in *uga35*Δ cells carrying the pSBC-*HA-UGA35* plasmid. Cells were preincubated (black bars) or not (white and grey bars) with 1.3 mM leucine for 30 min before the addition of 0.1 mM GABA (grey and black bars). After a 30-min incubation mRNA levels were measured by RT-qPCR. *UGA1*, *UGA2* and *UGA4* values were normalized with *TBP1*. The bars with different letters (a–c) are significantly different from each other ($p < 0.05$).

tion process cannot be discarded. Western blots showed very high amounts of Uga35/Dal81 protein synthesized from the pSBC-*HA-UGA35* plasmid (data not shown). So, the machinery producing the post-translational modifications could be saturated, leaving many molecules unmodified allowing the activity of Uga35/Dal81 in *UGA* genes in response to GABA. The mechanisms that lead to Uga3 and Uga35/Dal81 activation in response to GABA are yet unknown, although it has been proposed that this activation could be occurring through a post-translational modification since expression levels of neither of the two factors is affected by the

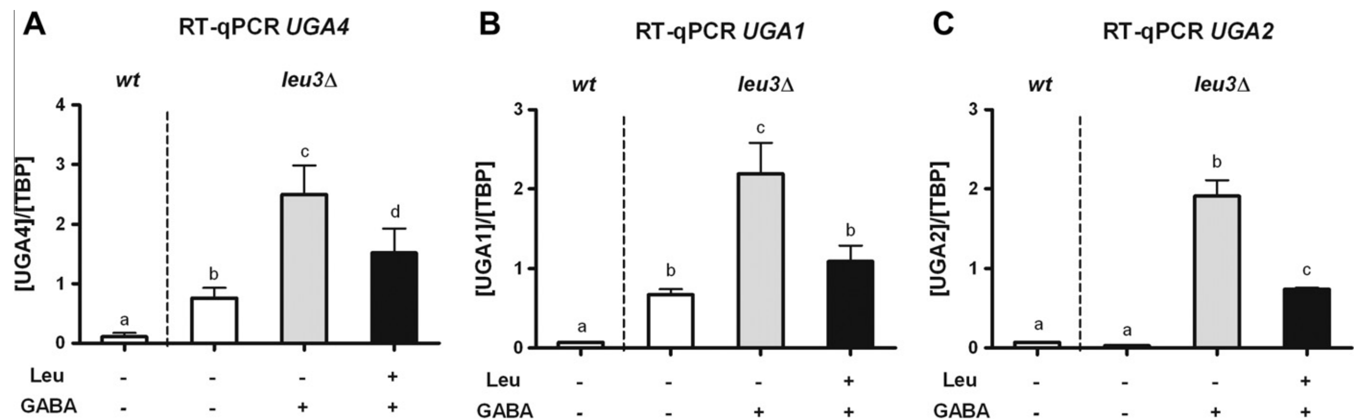


**Fig. 3.** Effect of Leu3 on *UGA4*, *UGA1* and *UGA2* expression. mRNA levels of *UGA4* (A), *UGA1* (B) and *UGA2* (C) were determined in wild type and *leu3*Δ cells preincubated (black bars) or not (white and grey bars) with 1.3 mM leucine for 30 min before the addition of 0.1 mM GABA (grey and black bars). After a 30-min incubation mRNA levels were measured by RT-qPCR. *UGA1*, *UGA2* and *UGA4* values were normalized with *TBP1*. The bars with different letters (a–c) are significantly different from each other ($p < 0.05$).

addition of the inducer [31]. Our results, along with the fact that pre-incubation of wild type cells with GABA prior to the addition of leucine does not affect *AGP1* induction (data not shown), support the idea of a hierarchical mechanism of regulation mediated by Uga35/Dal81 transcription factor.

As *UGA4*, *UGA1* promoter contains a putative binding site for Leu3 transcription factor that is absent in *UGA2* promoter. Previously we demonstrated that Leu3 negatively regulates *UGA4* expression probably in an indirect way [22], but the role of this factor in amino acid regulation of *UGA4* remains unclear. When *UGA4* expression was measured in *leu3*Δ cells carrying a construction that contains the *UGA4* promoter fused to *lacZ* gene (*UGA4–lacZ*), basal expression levels were higher than in wild type cells. This expression seemed to be insensitive to the presence of extracellular amino acids and no induction was observed [22]. To extend these results, we decided to analyze the role of Leu3 in *UGA* genes regulation. We measured mRNA levels of *UGA4*, *UGA1* and *UGA2* in *leu3*Δ cells. In the absence of Leu3, *UGA4* and *UGA1* showed higher basal levels of expression than those observed in wild type cells (Fig. 3A and B), indicating that this factor was acting as a repressor of the expression of both genes. In contrast, basal *UGA2* expression remained unaffected in the absence of Leu3 (Fig. 3C), in agreement with the fact that there is no consensus binding site for Leu3 in the promoter of this gene. On the other hand, GABA induction and leucine inhibition of this induction was observed measuring the three mRNAs (Fig. 3A–C). As we mentioned above, no induction of *UGA4* was detected in *leu3*Δ cells with reporter gene assays using *lacZ* as the reporter [22]; however, when *UGA4* mRNA levels were measured by RT-qPCR, some induction was detected (Fig. 3A). One possible explanation could be that intracellular accumulation of the reporter protein caused by the high basal levels of expression in the absence of Leu3 may mask the induction brought about by addition of the effector due to the stability exhibited by the β-galactosidase enzyme. So, when basal levels of expression are high, induction of *UGA4* cannot be detected using reporter gene assays.

Although leucine negative effect on *UGA4* and *UGA1* mRNA levels seemed to be weaker in *leu3*Δ cells than in wild type cells (compare Figs. 1A and B to 3A and B), it must be noticed that the basal levels of expression were higher in the absence of Leu3. In the case of *UGA2*, where the basal levels of expression remained unaffected, the strength of the inhibition by leucine seemed to be similar to the one observed in the wild type strain (compare Figs. 3C to 1C). Taken together these results suggest that Leu3 transcription factor does not participate in amino acid regulation of *UGA* genes, but it does participate in *UGA1* and *UGA4* regulation.

Although we were not able to detect any interaction between Leu3 and *UGA4* promoter [22], the consensus binding site for this factor present in *UGA4* and *UGA1* promoters points to the regulation of these genes by Leu3. GABA can be degraded in two consecutive reactions mediated by *UGA1* and *UGA2* gene products. The first reaction gives glutamate and SSA as products. SSA must be rapidly and irreversely converted into succinate since it is highly toxic. SSA toxicity makes this last reaction essential and could be the physiological cause of the fact that Leu3 negatively regulates *UGA4* and *UGA1* expression but not *UGA2* expression.

In summary, this work deepens our understanding on *UGA4* gene regulation by amino acids and extends our knowledge to the other members of the *UGA* regulon. Here we demonstrated for the first time that the three members of the *UGA* regulon, *UGA1, UGA2* and *UGA4*, are negatively regulated by extracellular leucine and that this inhibition is mediated by the SPS amino acid sensor. Results here presented, along with those previously published [22], suggest that the signal triggered by the amino acids through the SPS sensor leads to a preferential induction of SPS-target genes over GABA-regulated genes coordinated by Uga35/Dal81 transcription factor. If this coordination is achieved either by sequestration of Uga35/Dal81 transcription factor in the promoters of SPS-regulated genes or by post-translational modification of Uga35/Dal81 transcription factor remains to be elucidated. On the other hand, we found that the transcription factor Leu3 negatively regulates the expression of *UGA1* and *UGA4* but not *UGA2*, according to the physiological necessity of the cells to rapidly catabolyze the toxic product of the first reaction in GABA degradation pathway.

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