Uga3 and Uga35/Dal81 Transcription Factors Regulate UGA4 Transcription in Response to \(\gamma\)-Aminobutyric Acid and Leucine

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The Saccharomyces cerevisiae UGA4 gene encodes a permease capable of importing \(\gamma\)-aminobutyric acid (GABA) and \(\delta\)-aminolevulinic acid (ALA) into the cell. GABA-dependent induction of this permease requires at least two positive-acting proteins, the specific factor Uga3 and the pleiotropic factor Uga35/Dal81. UGA4 is subjected to a very complex regulation, and its induction is affected by the presence of extracellular amino acids; this effect is mediated by the plasma membrane amino acid sensor SPS. Our results show that leucine affects UGA4 induction and that the SPS sensor and the downstream effectors Stp1 and Stp2 participate in this regulation. Moreover, we found that the Uga3 and Uga35/Dal81 transcription factors bind to the UGA4 promoter in a GABA-dependent manner and that this binding is impaired by the presence of leucine. We also found that the Leu3 transcription factor negatively regulates UGA4 transcription, although this seems to be through an indirect mechanism.

The utilization of nonpreferred nitrogen sources in the absence of preferred sources requires control at the level of transcription for the synthesis of pathway-specific catabolic enzymes and permeases. This transcriptional control requires two positive signals, the first being a global signal indicating nitrogen limitation and the second being a pathway-specific signal that involves the presence of a substrate or intermediate of a metabolic pathway (35). \(\gamma\)-Aminobutyric acid (GABA) can be used as a nitrogen source by the unicellular budding yeast Saccharomyces cerevisiae, being a poor source. The UGA4 gene encodes a permease capable of importing \(\gamma\)-aminobutyric acid (GABA) and \(\delta\)-aminolevulinic acid (ALA) into Uga4 in this organism. Its expression depends on nitrogen catabolite repression (NCR) and GABA induction (2, 5). Induction of this permease requires at least two positive-acting proteins, the specific Uga3 and the pleiotropic Uga35/Dal81 factors (3, 10). These factors act through a 19-bp CG-rich upstream activating sequence named UAS\(_{\text{GABA}}\). The participation of both Uga3 and Uga35/Dal81 in UGA4 induction was demonstrated by genetic analysis (2, 39), and the interaction of Uga3 with the UAS\(_{\text{GABA}}\) region was shown in vitro in terms of binding using electromobility shift assays (25). The promoter region of UGA4 also contains four adjacent repeats of the heptanucleotide 5’-CGAT(A/T)AG-3’, which constitute a UAS\(_{\text{GATA}}\) element (12). This element, together with the GATA transcription factors, is responsible for the effect of NCR on UGA4.

Yeast cells assess the availability of extracellular nutrients through plasma membrane sensors. Ssy1 is a nutrient receptor that functions together with the two peripheral membrane-associated proteins Ptr3 and Ssy5 as a sensor of extracellular amino acids. Ssy1, Ptr3, and Ssy5 constitute a plasma membrane-associated complex named SPS (18). The homologous zinc finger transcription factors Stp1 and Stp2 are downstream effectors of the SPS sensor pathway. These factors are synthesized as latent cytoplasmic proteins with N-terminal regulatory domains crucial for the regulation of their activity (4, 31). In response to amino acids, Stp1 and Stp2 are activated by endoproteolytic removal of their N-terminal domains and act through specific upstream activating sequences named UAS\(_{\text{SPS}}\), present within SPS sensor-regulated promoters (14, 37). Uga35/Dal81 is required for full induction of amino acid-induced UGS sensor-dependent expression of the AGP1, PTR2, and BAP2 genes (1, 7, 26) and increases the efficiency of Stp1 binding to the AGP1 promoter (8).

Using whole-genome expression analysis of amino acid sensing (16, 17, 28), several groups reported that genes encoding amino acid and peptide transporters are induced by amino acids and that genes under NCR are repressed by amino acids and/or are strongly expressed in a sylΔ mutant. The UGA4 gene could be included in both groups since it encodes a transporter and it is under the control of NCR. Previously, we demonstrated that UGA4 induction diminished in the presence of extracellular amino acids (6).

Leu3 has been described as a regulator of five genes that belong to the branched-chain amino acid synthesis pathway (LEU1, LEU2, LEU4, ILV2, and ILV5), one gene (BAP2) which belongs to a family of permeases involved primarily with the uptake of branched-chain amino acids, and one gene (GDH1) mainly responsible for the assimilation of ammonia. Leu3 activity depends on the presence of \(\alpha\)-isopropylmalate (\(\alpha\)-IPM), an early intermediate in leucine biosynthesis (29). Leu3 acts both as a repressor and as an activator of transcription in the absence or in the presence of \(\alpha\)-IPM, respectively. \(\alpha\)-IPM synthesis is highly regulated, since \(\alpha\)-IPM synthase encoded by LEU4 is feedback inhibited by leucine and reversibly inactivated by coenzyme A (CoA) (29). On the basis of the transcriptional responses and in vivo binding of Leu3, Boer and...
Experiments showed that the Zn(II) 2 Cys6 cluster-type DNA recognizes targets of Uga3 and vice versa, since additional spec- everted CGG repeat spaced by 4 bp but that Leu3 does not them (34). The orientation of these triplets, and the spacing between determinants of DNA binding specificity of the zinc binuclearcluster proteins are the nucleotides surrounding the CGG trip- dets of DNA binding specificity of the zinc binuclear cluster proteins are the nucleotides surrounding the CGG triplets, the orientation of these triplets, and the spacing between them (34).

It has been proposed that Leu3 and Uga3 recognize an everted CGG repeat spaced by 4 bp but that Leu3 does not recognize targets of Uga3 and vice versa, since additional specificity is provided by nucleotides located between the two CGG triplets (38). In addition to the CGG-N4-CGG motif, the nu- cleotides flanking this everted repeat are also essential for Uga3 in vitro binding and activation of transcription (25).

The target sequence of Uga3/Dal81 is controversial (34). Experiments showed that the Zn(II)2 Cys6 cluster-type DNA binding domain of Uga3/Dal81 is not required for its role in allophanate-induced transcription (10), as was described for tamA, an Aspergillus nidulans gene encoding a protein highly similar to Uga3/Dal81 (13).

The UASGABA element of the UGA4 promoter includes 19 bp, 5'-AAAAACCGCCCGCCGCAAT-3', with the central core of this sequence being a GC-rich region that contains a perfect 10-bp palindrome, 5'-CCGGCGGCGG-3' (39).

This work focuses on the interplay of global and specific factors and their influence on the regulation of the catabolic pathway-specific gene UGA4. In order to elucidate the molecular mechanisms of the regulation by amino acids of UGA4 transcription, we demonstrate herein, for the first time, the increased in vivo binding of the Uga3 and Uga35/Dal81 transcription factors to the UGA4 promoter in response to the inducer GABA. We also find that this binding is impaired in cells preincubated with leucine prior to GABA addition in an SPS-dependent manner. Moreover, we show that UGA4 is also strongly regulated by Leu3. Altogether, our results show the relevance of the transcription factors Uga35/Dal81, Uga3, and Leu3 as responsible for the regulation of UGA4 by amino acids.

### MATERIALS AND METHODS

**Strains and media.** The Saccharomyces cerevisiae strains used in this study, isogenic to the wild-type strain Σ178b, are listed in Table 1. It was necessary for this work to use prototrophic strains 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.

**Strain construction.** All the strains generated in this study except for the SBCY08 strain were constructed using the PCR-based gene deletion strategy described by Wach et al. (44, 45) or modified versions of it. All the parental strains are listed in Table 1, and all primers used for PCRIs are listed in Table 2. The len4Δ deletion was generated using the pUG6 plasmid (23) to amplify theloxP-KanMX-loxP cassette. After the strain was generated, the KanMX cassette was excised by recombination mediated by Cre recombinase (pRS457 plasmid).

The lenΔ and lenΔ strains were constructed using the pFA6a-KanMX4 plasmid as a template for PCR (45). Strains with a C-terminal tag were generated using the pFA6a-3HA-KanMX6 plasmid (32).

**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 (20), with posterior Cre-mediated excision of the loxP-KanMX-loxP cassette. After the strain was generated, the KanMX cassette was excised by recombination mediated by Cre recombinase (pRS457 plasmid).**

### TABLE 1. Strains used in this work

| Strain      | Genotype | Parent | Primer | Source or reference |
|-------------|----------|--------|--------|---------------------|
| 23344c      | mata ura3 |        |        | M. Grenson          |
| 30995b      | mata ura3 ssy1Δ::KanMX2 | 23344c | F/R-leu3 | This study          |
| KW018       | mata ura3 sst1Δ | 23344c | F/R-leu4 | This study          |
| KW021       | mata ura3 sst2Δ | 23344c | F/R-leu5 | This study          |
| KW023       | mata ura3 sst1Δ/st2Δ | 23344c | F/R-ME | This study          |
| FA050       | mata ura3 uga35Δ::KanMX2 | 23344c | F/R-Tag-UGA35 | This study |
| SBCY01      | mata ura3 leu3Δ::KanMX4 | 23344c | F/R-Tag-UGA3 | This study |
| SBCY02      | mata ura3 LEU3-3HA-kanMX6 | 23344c | F/R-LEU3-Tag | This study |
| SBCY04      | mata ura3 leu4Δ::lox | 23344c | F/R-leu4 | This study          |
| SBCY05      | mata ura3 leu4Δ::lox leu5Δ::KanMX4 | SBCYO4 | F/R-leu5 | This study |
| SBCY08      | mata ura3 his3Δ::KanMX leu4Δ::lox/pSBC-LEU4Δ | SBCYO4 | Plasmid M9299 | This study |
| SBCY10      | mata ura3 6HA-UGA3 | 23344c | F/R-Tag-UGA3 | This study |
| SBCY13      | mata ura3 6HA-UGA35 | 23344c | F/R-Tag-UGA3 | This study |
| SBCY17      | mata ura3 uga35Δ::natMX4 | 23344c | F/R-Tag-UGA3 | This study |
| SBCY18      | mata ura3 sst1Δ::natMX4 | 30995b | F/R-ME | This study          |
| SBCY20      | mata ura3 uga35Δ::natMX4 leu3Δ::KanMX4 | SBCY17 | F/R-leu3 | This study |
| SBCY22      | mata ura3 leu3Δ::KanMX4 6HA-UGA3 | SBCY13 | F/R-leu3 | This study |
| SBCY23      | mata ura3 sst1Δ::natMX4 6HA-UGA35 | SBCY10 | F/R-leu3 | This study |
| SBCY24      | mata ura3 sst1Δ::natMX4 6HA-UGA3 | SBCY18 | F/R-Tag-UGA35 | This study |
| SBCY26      | mata ura3 sst1Δ::natMX4 6HA-UGA3 | SBCY18 | F/R-Tag-UGA35 | This study |
| XK14-15D    | mata LEU4Δhis4 |       |        | G. B. Kohlhaw*       |

* Gently provided by Anders Brandt (Carlsberg Laboratory, Copenhagen Valby, Denmark).
Transformants were selected on rich medium containing 200 μg/ml G418 or 100 μg/ml nourseothricin (ClonNat; Werner BioAgents). The plasmids used to analyze promoter activities were derived from the YEp357 plasmid (36). The CEN3/LEU2 plasmid was used to amplify the LEU5 fbr gene was amplified (F/R-LEU5fbr), and the UASGATA reporter gene were also used. The UASGABAmut-lacZ fusion gene carries the 5′ noncoding region (positions 30 to 15 with respect to the ATG initiation codon, with the sequence GCCGGCGGC deleted from the UAS GABA element. The U/H11012 fusion genes contain the sequence GCCGGCGGC was replaced by ATTAGTAAT (the changed positions are underlined). All these constructions were previously described by Luziani et al. (33). The constructions were verified by DNA sequence analysis. The deviation of these values from the mean was less than 15%.

- **Chromatin immunoprecipitation (ChIP) assays.** Cells (a 100-ml culture) were grown to an optical density at 600 nm (OD600) of 0.8 and after different treatments were fixed for 20 min at room temperature in the presence of 1% formaldehyde. Glycine was then added to give a final concentration of 125 mM and frozen for 20 min at room temperature in the presence of 1% formaldehyde. Glycine was then added to give a final concentration of 125 mM and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3 × 10 s at 15% amplitude) and clarified by centrifugation (17,000 × g for 30 min) and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3 × 10 s at 15% amplitude) and clarified by centrifugation (17,000 × g for 30 min) and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3 × 10 s at 15% amplitude) and clarified by centrifugation (17,000 × g for 30 min) and resuspended in 0.4 ml of fresh FA lysis buffer. Samples were sonicated to obtain DNA fragments with an average size of 500 bp (Branson Sonifier; 3 × 10 s at 15% amplitude) and clarified by centrifugation (17,000 × g for 30 min). Protein content was measured using the Bradford assay, and 1 mg of protein was used for each immunoprecipitation. Samples were stored at −80°C. Normal mouse IgG (Santa Cruz) or monoclonal antihemagglutinin (anti-HA) antibody (12CA5 Roche) were added to 25 μl of preblocked (1 mg/ml salmon sperm DNA and 1 mg/ml bovine serum albumin) magnetic beads coupled to protein G (Dynal). After a 2-hour incubation, beads were added to each lysate and were incubated overnight at 4°C in a rotator. Immune complexes were sequentially washed five times with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride) and the samples were subjected to SDS-PAGE. Normal mouse IgG (Santa Cruz) or monoclonal antihemagglutinin (anti-HA) antibody (12CA5 Roche) were added to 25 μl of preblocked (1 mg/ml salmon sperm DNA and 1 mg/ml bovine serum albumin) magnetic beads coupled to protein G (Dynal). After a 2-hour incubation, beads were added to each lysate and were incubated overnight at 4°C in a rotator. Immune complexes were sequentially washed five times.
times with FA lysis buffer; four times with FA lysis buffer containing 500 mM NaCl, five times with wash buffer (10 mM Tris-Cl, pH 8, 0.25 mM L-Cys, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and two times with Tris-EDTA (TE) buffer. Bound proteins were eluted from the beads by adding 150 µl elution buffer (50 mM Tris-Cl, pH 8, 10 mM EDTA, 1% SDS) and incubating for 15 min at 65°C. Cross-linking was reversed by an overnight incubation at 65°C in the presence of protease K (0.25 mg/ml). DNA was purified using a QIAquick PCR purification kit (Qiagen). Real-time quantitative PCR (qPCR) was carried out with an Opticon Monitor 3 (Bio-Rad) with primers that amplified promoter regions of the UGA4 (F/R-UGA4qPCR) and LEU2 (F/R-LEU2qPCR) genes (Table 2). A pair of primers that amplify a region located 2.5 kb downstream of the UGA4 promoter was used as an unbound control (F/R-UGA4UCqPCR).

ChIP DNA was normalized to input DNA and calculated as a signal-to-noise change of binding to the promoter of interest (30). Propagation of error was handled using standard root mean square methods.

Nucleotide sequence accession number. The sequence determined for the present study has been deposited in GenBank under accession no. GU598519.

RESULTS

Previous studies have shown that the induction of UGA4 was inhibited by the addition of a mix of amino acids to the culture medium and that this effect was mediated by the SPS amino acid sensor system (6). To test if individual amino acids known to be SPS activators have the same negative effect on UGA4 expression, wild-type and syvΔ cells were incubated with leucine, phenylalanine, tryptophan, or methionine or not incubated (Table 3). In a wild-type strain, GABA induction of the UGA4-lacZ fusion gene was significantly reduced by the addition of the amino acids tested, whereas in a syvΔ mutant, this effect was not observed, indicating that the treatment with each amino acid was sufficient to reduce UGA4 expression and that this decrease was dependent on the activity of the SPS sensor. Similar results were obtained using ptr3Δ and syv5Δ cells (data not shown).

To get further insights into the regulation of UGA4 by amino acids, we tested whether the downstream effectors of the SPS signaling pathway participate in UGA4 regulation by leucine, an amino acid commonly used as an inducer of the SPS sensor. Although in the single mutants stp1Δ and stp2Δ, GABA induction of UGA4 diminished in the presence of extracellular leucine, this effect disappeared in the double mutant stp1Δ stp2Δ (Fig. 1), suggesting that at least one of these two factors is essential for the signaling cascade that is triggered by the extracellular amino acids and that modulates UGA4 expression.

In order to establish the regions of the UGA4 promoter responsible for the effect of leucine on UGA4 transcription, we analyzed the promoter activity of different DNA fragments covering the region comprising positions −583 to +1 of this gene (Fig. 2A). The induction driven by both the complete promoter of UGA4 and the promoter lacking the UAS_GATA element was repressed in the presence of leucine (Fig. 2B and D). In a syvΔ strain, the induction profiles of the UGA4-lacZ fusion gene were similar in the presence and absence of leucine (Fig. 2C). These results indicate that the UAS_GATA element is not a target sequence of the signal triggered by leucine. The construct lacking both the UAS_GATA and the UAS_GABA elements was unable to produce any significant expression (Fig. 2E). The transcription levels directed by the constructs without the central core of the UAS_GABA element were high and independent of GABA, but they were still sensitive to the presence of leucine (Fig. 2F and G). Altogether, these results indicate that neither the UAS_GATA element nor the central core of the UAS_GABA element participates in the regulation by leucine of the UGA4 gene.

In silico analysis using the databases YEASTRACT (http://www.yeastract.com) (41) and SCPD (http://ruhai.csilh.edu/SCPD) revealed that there is a consensus binding site for the transcription factor Leu3 within the UAS_GABA region, as was already mentioned (38, 39). These findings and the whole-genome transcriptional profiles reported by Boer and collaborators showed that the expression of UGA4 is modulated by leucine in a manner that involves the SPS amino acid sensor system and that the UAS_GATA and UAS_GABA elements contribute to the regulation of UGA4 transcription.
orators (9) prompted us to postulate that Leu3 was a putative repressor of the UGA4 gene acting on the UAS GABA region.

In order to determine whether or not Leu3 modulates UGA4 expression, cells deficient in LEU3 were transformed with the plasmid containing the full-length promoter region of UGA4 fused to lacZ. The results depicted in Fig. 3A supported our hypothesis proposing Leu3 as a negative regulator of the UGA4 gene since high levels of UGA4 expression in leu3/H9004 cells were detected. The transcription of UGA4 in the absence of Leu3 did not depend on GABA, suggesting that this factor is involved in the induction process, probably by maintaining low basal levels of UGA4 expression. On the other hand, Leu3 seems to be participating in the regulation of UGA4 by leucine, although it might be remarked that in cells lacking Leu3, some effect of leucine on UGA4 expression was still detectable (Fig. 3A).

Gene regulation by Leu3 depends on the levels of α-IPM (29). To determine whether the negative effect of Leu3 on UGA4 expression is also modulated by intracellular levels of α-IPM, we measured the expression of our UGA4-lacZ fusion gene in strains with different capacities for synthesizing α-IPM (Table 4). In both a wild-type strain and a mutant strain pro-
incubated in minimal medium or in cells treated with leucine (Fig. 3B). Similar results were obtained with the use of a strain that expresses an N-terminal tagged version of Leu3 (data not shown). This result supported the idea that Leu3 acts negatively on UGA4 expression in an indirect way as mentioned above. The LEU2 promoter, a well-known Leu3 target (29), was used as a positive control for Leu3-HA binding. The functionality of both C- and N-tagged fusion proteins was checked by measuring UGA4 expression in these strains (data not shown).

Considering that the Uga35/Dal81 transcription factor is required for the full induction of several amino acid permeases in response to signals triggered by the SPS sensor (1, 8) and that this factor is also required for the induction of UGA genes by GABA (42), we decided to study the in vivo binding of Uga35/Dal81 to the UASGABA region of the UGA4 promoter. For this, we performed ChIP assays using a strain expressing the HA-Uga35/Dal81 fusion protein. We found that HA-Uga35/Dal81 bound to the UGA4 promoter in a GABA-dependent manner and that this binding was impaired by preincubation with leucine (Fig. 4A). These observations correlate with the low levels of UGA4 induction measured in the presence of leucine (Table 3 and Fig. 2B).

In both inducible processes (transcription of genes controlled by the SPS pathway and induction by GABA of UGA genes), Uga35/Dal81 acts together with an inducer-specific transcription factor (1). In the case of the response to GABA of UGA4, this factor is Uga3. For this reason, we decided to investigate Uga3 binding to the UGA4 promoter in vivo under the same conditions used to test the binding for Uga35/Dal81. Our results showed that HA-Uga3 interacted with the UGA4 promoter similarly to the way that HA-Uga35/Dal81 did (Fig. 4B), suggesting that Uga3 bound to the UGA4 promoter in a GABA-dependent manner and that leucine weakened this interaction.

To determine whether the effect of leucine on HA-Uga3 and HA-Uga35/Dal81 binding to the UGA4 promoter is dependent on the SPS sensor, we performed ChIP assays using strains deficient in SSY1 and expressing tagged versions of Uga3 or Uga35/Dal81. We detected both transcription factors bound to the UGA4 promoter even in the presence of leucine (Fig. 4C and D), confirming that the lower binding capacity of HA-Uga3 and HA-Uga35/Dal81 in the presence of leucine observed in wild-type cells (Fig. 4A and B) was caused by the signal triggered by this amino acid through the SPS sensor system.

### Table 4. Expression of the UGA4-lacZ fusion gene in cells with different α-IPM-synthesizing capacities

| Condition | Value for: |
|-----------|------------|
| Wild type | ssyΔ | leu4Δ | LEU4<sup>str</sup> |
| MM        | 90 ± 3    | 51 ± 7 | 39 ± 2 | 60 ± 5 |
| MM-GABA   | 544 ± 41  | 454 ± 26 | 131 ± 2 | 475 ± 44 |
| MM-Leu (2 mM)-Ile | 64 ± 1 | 18 ± 1 | 14 ± 1 | 76 ± 5 |
| MM-Leu (2 mM)-Ile | 274 ± 13 | 547 ± 9 | 67 ± 5 | 240 ± 3 |

*β-Galactosidase activity was determined for wild-type (23344c), ssyΔ (30995b), leu4Δ leu5Δ (SCBY05), and LEU4<sup>str</sup> (SCBY08) cells carrying the UGA4-lacZ fusion gene. Cells were grown in minimal medium (MM) containing or not containing leucine and isoleucine. Each culture was divided in two, and only one half was induced with 0.1 mM GABA for 60 min. Samples were taken out, and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.

### Table 5. Expression of the UGA35-lacZ and UGA3-lacZ fusion genes in wild type, leu3Δ, and leu4Δ leu5Δ cells

| Gene     | Value for: |
|----------|------------|
| Wild type | leu3Δ | leu4Δ leu5Δ |
| UGA35-lacZ | 47.2 ± 2.8 | ≤10 |
| UGA3-lacZ | 128.3 ± 8.0 | 33.6 ± 1.6 | ≤10 |

*β-Galactosidase activity was determined for wild-type (23344c), leu3Δ (SCBY01), and leu4Δ leu5Δ (SCBY05) cells carrying the UGA35-lacZ and UGA3-lacZ fusion genes. Cells were grown in minimal medium. Samples were taken out, and β-galactosidase activity was measured. The results shown, expressed in Miller Units, are the means ± standard deviations for duplicates within a representative assay.
In our attempt to understand the events that caused the high levels of UGA4 expression in a LEU3-deficient strain, we decided to study the recruitment of HA-Uga3 and HA-Uga35/Dal81 to the promoter of UGA4 in the absence of Leu3. Although the expression level of the UGA3-lacZ fusion gene in a leu3/H9004 strain was significantly lower than that in a wild-type strain (Table 5), this transcription factor appeared bound to the UGA4 promoter after the addition of GABA (Fig. 5, left panel), as was already observed in the wild-type strain (Fig. 4B). On the other hand, HA-Uga35/Dal81 binding seemed to be impaired by the leu3 deficiency (Fig. 5, right panel). In this strain, UGA35-lacZ fusion gene expression was almost undetectable, explaining the low level of recruitment observed (Table 5). These results did not explain the high basal levels of UGA4 expression observed in the absence of Leu3 (Fig. 3A); however, the lack of UGA4 induction in this strain would be explained by the low level of availability of Uga35/Dal81. The expression levels of UGA4-lacZ, UASGABAmut-lacZ (Fig. 6), and UASGABAdel-lacZ (data not shown) in a leu3Δ uga35Δ strain were significantly lower than those in a leu3Δ strain, indicating that the Uga35/Dal81 factor is in some way involved in the high levels of expression observed under both conditions (i.e., UGA4-lacZ expression in a leu3Δ strain and expression driven by the UASGABAmut-lacZ construction in a wild-type strain) in the absence of an inducer.

**DISCUSSION**

The aim of this work was to elucidate the mechanisms by which leucine regulates UGA4 induction. Here, we demonstrate for the first time that leucine affects the GABA-mediated binding of the Uga3 and Uga35/Dal81 transcription factors to the UGA4 promoter and that this effect depends on the SPS sensor pathway.
We previously reported that UGA4 expression is regulated by amino acids through the plasma membrane sensor SPS (6). In this paper, we studied the effect of leucine, one of the most potent known elicitors of signaling through SPS (15, 19), on UGA4. The effect of leucine on UGA4 expression was detected shortly after the addition of this amino acid, suggesting a sensor-mediated response that was strictly dependent on the components of the SPS sensor.

In the stp1Δ and stp2Δ single mutants, the effect of leucine was almost indistinguishable from that observed in the wild type, while no effect of leucine was detected in the stp1Δ stp2Δ double mutant, indicating that these two factors are involved in UGA4 regulation by leucine and confirming that they are functionally redundant (31).

On the basis of the proposal of Abdel-Sater and collaborators (1) and the findings of Boban and Ljungdahl (8), demonstrating that the binding of Stp1 to the AGP1 promoter was dependent on the presence of Uga35/Dal81, we studied the participation of Uga35/Dal81 in UGA4 regulation by leucine. In this work, we demonstrated that GABA induced the interaction between HA-Uga35/Dal81 and the UGA4 promoter and that leucine impaired this interaction.

The behavior observed in the binding of HA-Uga3 to the UGA4 promoter in response to leucine was similar to that observed for HA-Uga35/Dal81. This was an unexpected result, since Uga3 is an inducer-specific transcription factor of UGA4 expression observed for HA-Uga35/Dal81. This was an unexpected result, suggesting a sensor-mediated response that was strictly dependent on the components of the SPS sensor. The Uga35/Dal81 factor seems to be participating in two opposite processes. On the one hand, it mediates the negative effect of leucine on UGA4 induction. On the other hand, the high basal levels of UGA4 expression observed in leu3Δ cells and the high levels of expression of the UAS_{GABA}mut-lacZ fusion gene in wild-type cells seem to depend on Uga3 and Uga35/Dal81 factors act.

Bricmont and collaborators (10) have demonstrated that the Zn(II)$_2$Cys$_6$ cluster-type DNA domain of Uga35/Dal81 is not required for its role in allophanate-induced transcription. Our results showed that HA-Uga35/Dal81 bound to the UGA4 promoter, and participated in the interaction between HA-Uga35/Dal81 and the UGA4 promoter and that leucine impaired this interaction.

The behavior observed in the binding of HA-Uga3 to the UGA4 promoter in response to leucine was similar to that observed for HA-Uga35/Dal81. This was an unexpected result, since Uga3 is an inducer-specific transcription factor of UGA4 genes and there were no previous reports relating Uga3 with amino acid-regulated genes and GABA-induced genes. Our results showing that the decrease caused by leucine in the recruitment of HA-Uga35/Dal81 to the UGA4 promoter depended on Ssy1 support this hypothesis. The signal triggered by the SPS sensor in response to extracellular leucine activates Stp1/Stp2, which would be recruiting Uga35/Dal81 to promote transcriptional leucine induction of other permeases and decreasing the availability of Uga35/Dal81, and consequently of Uga3, for GABA induction of the UGA4 gene (Fig. 7).

Therefore, the element in the UGA4 promoter involved in the response to leucine seems to be the UAS_{GABA} element, since this element is in this regulatory region where both the Uga3 and the Uga35/Dal81 factors act.

Bricmont and collaborators (10) have demonstrated that the Zn(II)$_2$Cys$_6$ cluster-type DNA domain of Uga35/Dal81 is not required for its role in allophanate-induced transcription. Our results showed that HA-Uga35/Dal81 bound to the UGA4 promoter. Two possible explanations may account for this. The first element is that Uga35/Dal81 directly interacts with the UGA4 promoter. The second one is that Uga35/Dal81 might be associated with the UGA4 promoter via an interaction with another protein, such as Uga3.

The Uga35/Dal81 factor seems to be participating in two opposite processes. On the one hand, it mediates the negative effect of leucine on UGA4 induction. On the other hand, the high basal levels of UGA4 expression observed in leu3Δ cells and the high levels of expression of the UAS_{GABA}mut-lacZ fusion gene in wild-type cells seem to depend on Uga3 and Uga35/Dal81 factors under this condition.

The repression of UGA4 by leucine was barely detected in cells deficient in the LEU3 gene, suggesting that UGA4 regulation by leucine was mediated at least in part by Leu3. However, we were not able to detect significant binding of...
Leu3-HA to the UGA4 promoter. Tang and collaborators, comparing expression and binding under both low and high levels of Leu3 activity, showed no detectable binding of Leu3 to several genes whose expression was affected by LEU3 deletion. They proposed that this could be due to the low sensitivity of the ChIP technique or due to an indirect regulation of these genes by Leu3. Moreover, these authors were not able to detect Leu3 binding to the UGA4 promoter even when experiments were performed under conditions of high levels of activity of Leu3 (40). It is worth remarking that the growth conditions they used are different from those used in the present report. The facts that the effect of Leu3 on UGA4 was negative under all conditions, in contrast to what was described for the BAP2 and LEU2 genes (29, 37), and that we were not able to detect Leu3-HA binding to the UGA4 promoter suggested an indirect regulation of UGA4 by Leu3. Transcription driven by the UASGAB1mut and the UASGAB1del constructs in a uga353/dal81Δ strain was still quite sensitive to leucine, and this effect was not observed when LEU3 was also deleted. This suggests that Leu3 is further regulating UGA4 in response to leucine besides its effect on UGA35/DAL81.

In summary, we demonstrated that the UGA4 gene is under the regulation of the SPS signaling pathway and that its induction was inhibited by amino acids, as was the expression of the other genes under both NCR and SPS regulation (16). Leu3, Uga3, and Uga35/Dal81 play an important role in the regulation of UGA4 transcription, and these three factors are responsible for the negative effect of leucine on GABA induction. The mechanism by which Leu3 negatively regulates UGA4 transcription still remains unclear, although evidence presented here suggests that this effect is indirect rather than caused by the direct interaction of this factor with the UGA4 promoter. Uga3 and Uga35/Dal81 bind to the UGA4 promoter in a GABA-dependent manner, and this binding is impaired by extracellular leucine through the SPS sensor (Fig. 7). Some interplay between SPS signaling molecules and Leu3 has been proposed (37) since Stp1 and Leu3 are both dependent on Ssy1. However, this connection is not completely elucidated yet.

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