Sequence and characterization of two *Arabidopsis thaliana* cDNAs isolated by functional complementation of a yeast *gln3 gdh1* mutant

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Abstract **We have isolated two *Arabidopsis thaliana* cDNAs by complementation of a yeast *gln3 gdh1* strain that is affected in the regulation of nitrogen metabolism. The two clones (*RGA1* and *RGA2*) are homologous to each other and to the *SCARECROW* (*SCR*) gene that is involved in regulating an asymmetric cell division in plants. RGA1, RGA2 and SCR share several structural features and may define a new family of genes. *RGA1* and *RGA2* have been mapped, respectively, to chromosome II and I, and their expression in plant is constitutive.**

Key words: Functional complementation; GLN3; Nitrogen metabolism; Scarecrow; Ammonia

1. Introduction

Nitrate is the major N source for most higher plants. Not surprisingly, nitrate assimilation is controlled at multiple levels. For example the N status of the plant regulates the level of expression of genes encoding nitrate reductase (NR) and nitrite reductase (NiR), involved in N assimilation: lower pools of glutamine lead to an increase in the levels of NR and NiR mRNAs [1–3]. However, to date no plant mutants affected specifically in the regulation of nitrate assimilation, and no genes encoding transcriptional regulatory factors of NR and NiR genes have been isolated.

In the yeast *Saccharomyces cerevisiae*, N assimilation is a highly regulated process. When a preferred N source such as glutamine is present, the expression of genes encoding enzymes involved in the utilization of other N sources is repressed. This overall regulation is termed nitrogen catabolic repression (NCR) and key genes involved in this control have been isolated. The *GLN3* gene [4], and more recently the *NIL1* (*GAT1*) gene [5,6] have been cloned and mediate transcriptional activation of NCR-sensitive genes in the absence of glutamine. The *URE2* gene [7] product acts as a negative regulator of the GLN3 protein in the presence of glutamine. Furthermore, a third general regulator, DAL80 [8], represses the transcription of a subset of GLN3-controlled genes. Interestingly GLN3, NIL1 and DAL80 share a common motif, a GATA zinc finger that is also conserved in the NIT2 [9] and AREA [10] proteins that mediate NCR in, respectively, *Neurospora crassa* and *Aspergillus nidulans*. This points to the partial conservation of these regulatory factors between different organisms. Indeed, functional complementation has been observed between AREA and NIT2 [11].

In an attempt to identify genes homologous to *GLN3* and potentially involved in N metabolism regulation in plants, a PCR approach using degenerated oligonucleotides was developed in our laboratory. This led to the isolation of cDNAs coding for proteins with a potential GATA zinc finger [12]. However, to date there is no evidence that these plant proteins are involved in the regulation of N metabolism.

This paper describes another approach used to isolate plant genes homologous to *GLN3* based on functional complementation. Functional complementation has proven to be a powerful tool in many cases for isolating homologs of yeast genes from different organisms [13–15]. We have transformed a yeast *gln3* mutant with an *Arabidopsis thaliana* cDNA expression library. We were hoping thereby to clone plant cDNAs potentially involved in the NR and NiR genes regulation. This paper describes the isolation of 3 cDNAs by this approach, their protein sequence and their characterization in yeast and in *A. thaliana*.

2. Materials and methods

2.1. Yeast strains and growth conditions

The yeast strains 179-2D (*MAT* a, *ade 2-102*, *ura 3-52*, *gln3-1*, *gdh1-6*) [4], PM44 (*MAT* α, *leu2-3,-112*, *ade2-102*, *gdh1-6*, *gln3Δ4::LEU2*) were obtained from B. Magasanik (MIT, Cambridge, MA, USA). The thermosensitive 667-4B yeast strain (*MAT* a *ura3-52*, *gln1-9142* [16]) was given by A.P. Mitchell (Columbia University, New York, NY, USA). Strains were grown at 28°C (except for 667-4B that was grown at 26°C or 36°C) in minimal media defined as 0.34% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose and either 0.5% ammonium sulfate, 0.2% glutamine or 0.12% glutamate as sole nitrogen source supplemented when needed with adenine (20 mg/l) or uracil (20 mg/l).

2.2. Transformations

Yeast transformation was achieved using lithium acetate [17]. When needed, yeast plasmids were analyzed in the *Escherichia coli* XL1 strain after transformation by electroporation with the Gene-Pulser apparatus (Bio-Rad, Richmond, CA, USA).

2.3. Enzyme assays

Yeast cells were grown in 25-ml cultures at 28°C until they reached mid-log phase. Cells were harvested by centrifugation (30 s, 3000×*g*) and the pellet was washed with water and frozen at −80°C. Extracts were prepared by suspending the frozen cells in extract buffer (0.1 M Tris hydrochloride pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 10% glycerol) and vortexing 4 times for 30 s with glass beads (diameter, 425–600 μm). After 15 min centrifugation (10 000×*g*), the supernatant was used for enzyme assays.

GS synthetase activity was measured according to Mitchell and Magasanik [18]. NAD-GDH and NADP-GDH activities were measured as reported by Doherty [19].

2.4. Plant material

Plants of *A. thaliana* ecotype Columbia were used as the source of RNA and DNA. They were either grown in the greenhouse or in soil (or sand when roots were used as RNA source) and watered with the standard nutrient solution of Coïc and Lesaint [20] or in vitro for 3 weeks on solid medium [21] containing either nitrate (9 mM), glutamate (10 mM), urea (10 mM) or glutamine (5 mM) as sole nitrogen

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source with a photoperiod of 16 h of light (250 μmol photons/m^2 per s) at 20°C and 8 h dark at 15°C.

2.5. Preparation and analysis of DNA and RNA

DNA was prepared from yeast and plants as described by Hoffman and Winston [22] and Dellaporta [23], respectively. RNA was isolated as described by Verwoerd et al. [24]. The probes used for hybridization were the *Not*I inserts of pRGA1 and pRGA2. Southern and Northern blots, hybridizations and probe labelling were performed as described by Dorbe et al. [25]. Stringent washes were performed by washing the membranes at 65°C once in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7), 0.5% sarkosyl, followed by two washes in 0.2×SSC, 0.5% sarkosyl. For non-stringent washes, membranes were rinsed only in 2×SSC and 0.5% sarkosyl at 60°C. Filters were autoradiographed at −80°C using intensifying screens.

2.6. Mapping of RGA1 and RGA2

Oligonucleotides specific of *RGA1* or *RGA2* were used for mapping the genes on YAC clones. For *RGA1* the two oligonucleotides were the following: 5′-CTCTCCGATACTCTTCAGATG-3′ where the underlined C corresponds to the nucleotide 990 of the coding strand of *RGA1*, and the oligonucleotide complementary to the coding strand 5′-CAAGCCACCAGATTACAA-3′ starting at position 1648. For *RGA2* the oligonucleotide 5′-GTTGTGGCTTGTGATGGA-3′ starting at position 1543 of the coding strand and the 18-mer 5′-GGATTTAGTTTGGCTTCG-3′ complementary to the coding strand, starting at position 1866 of *RGA2* were used for the PCR reactions. PCR screening of the CIC library was performed as described by Creusot et al. on three-dimensional YAC pools [26], in a MJ Research PTC100 thermal cycler. The following conditions were used: after 3 min denaturation at 94°C, amplification was achieved with 35 cycles of 15 s denaturation at 94°C, 30 s annealing at 50°C for (*RGA1*) or 55°C (*RGA2*), and 1 min of extension by *Taq* polymerase at 72°C. After the 35 cycles a final 10 min of extension at 72°C was performed.

2.7. DNA sequencing and analysis

For sequencing, the *Not*I inserts of pRGA1, pRGA2 and pRGA3 were cloned in the *Not*I site of the pBluescript KS^- vector (Stratagene, La Jolla, CA, USA). After detailed restriction analysis of the resulting plasmids, sequencing was pursued on overlapping subclones using the reverse and forward primers of pBluescript. When needed, oligonucleotides were designed for sequencing *RGA1* and *RGA2*.

RGA1 and *RGA2* were totally sequenced on both strands using an Applied Biosystems model 373A automated sequencer and the PRIZM sequencing system (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed with the Wisconsin Genetics Computer Group software [27].

3. Results and discussion

3.1. Isolation of RGA1, RGA2 and RGA3 by complementation of a yeast gln3 gdh1 mutant strain

The *S. cerevisiae* 179-2D strain (*MAT* a, *ade 2-102*, *ura 3-52*, *gln3-1*, *gdh1-6*) is mutated in both the *GDH1* gene encoding the NADP-glutamate dehydrogenase (NADP-GDH) and the *GLN3* gene. Ammonia in yeast is assimilated via NADP-GDH and the glutamine synthetase/glutamate synthase pathways. As a result of these mutations that affect the expression of both the NADP-GDH and the GLN3-dependent glutamine synthetase (GS) gene, the 179-2D strain does not grow on ammonia and requires glutamate or glutamine as nitrogen source for growth. Since this strain was used for the cloning of the *S. cerevisiae GLN3* gene by selecting for restoration of growth on ammonia [4], the same approach was developed to isolate potential plant cDNAs encoding GLN3-like proteins.

An unamplified aliquot of the *A. thaliana* cDNA yeast expression library, constructed by Minet et al. in the yeast vector pFL61 [28], was used to transform the yeast strain 179-2D. The transformation mixture was plated either on minimal glutamine media for determining the efficiency of transformation or on minimal ammonia media to select directly for plant cDNAs rescuing the recipient strain on ammonia. Three clones out of approximately 7000 transformants were thus isolated independently after incubation on selective plates for one week at 28°C. The plasmids present in the three clones were named pRGA1, pRGA2 and pRGA3 and their inserts, respectively, *RGA1*, *RGA2* and *RGA3* (for restoration of growth on ammonia). Complementation was confirmed by isolating the plasmids and transforming again the 179-2D strain. In all three cases the mutant yeast strain was restored for growth on ammonia upon transformation with the *A. thaliana* cDNA clones (see Fig. 1).

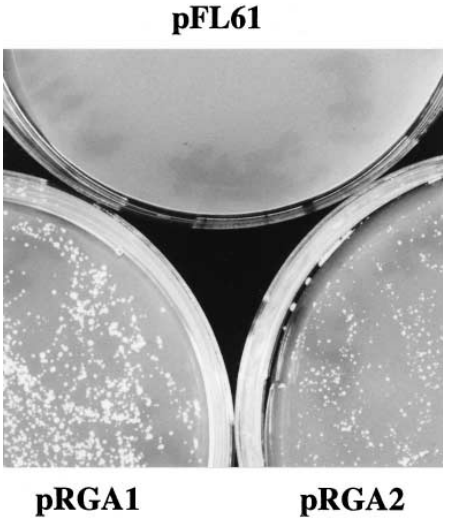

Fig. 1. Restoration of the 179-2D strain by pRGA1 and pRGA2. Transformants were plated on minimal ammonia media. As a control, absence of growth on ammonia is observed for 179-2D transformed by the library vector pFL61.

Detailed restriction analysis of the three clones as well as partial 5′ and 3′ sequencing of the inserts showed that pRGA2 and pRGA3 were identical clones. So only *RGA1* and *RGA2* were retained for further characterization and complete sequencing.

3.2. Characterization of RGA1 and RGA2 in yeast

The PM44 strain (*MAT* α, *leu2-3,-112*, *ade2-102*, *ura3-52*, *gdh1-6*, *gln3Δ4::LEU2*) is mutated in both the *GLN3* and *GDH1* genes just as the 179-2D strain. However the genetic background of this strain is different from that of 179-2D and the *GLN3* gene is disrupted in PM44 and not just point-mutated as in the *gln3-1* allele of 179-2D. Complementation of PM44 by pRGA1 and pRGA2 was observed, indicating that restoration of the 179-2D strain does not involve suppression of the amber-suppressible *gln3* allele present in the latter strain [29].

The 667-4B strain (*MAT* a *ura3-52*, *gln1-9142* [16]) carries a thermosensitive mutation in the *GLN1* gene that encodes GS. This strain grows on minimal ammonia media at 26°C, but not at 36°C where glutamine is necessary for growth. No complementation was observed when 667-4B was transformed by pRGA1 or pRGA2 and plated on minimal ammonia media at 36°C, indicating that the plasmids do not compensate for a total lack of GS activity and so probably do not code for GS (see below).

We tested whether complementation of the 179-2D strain

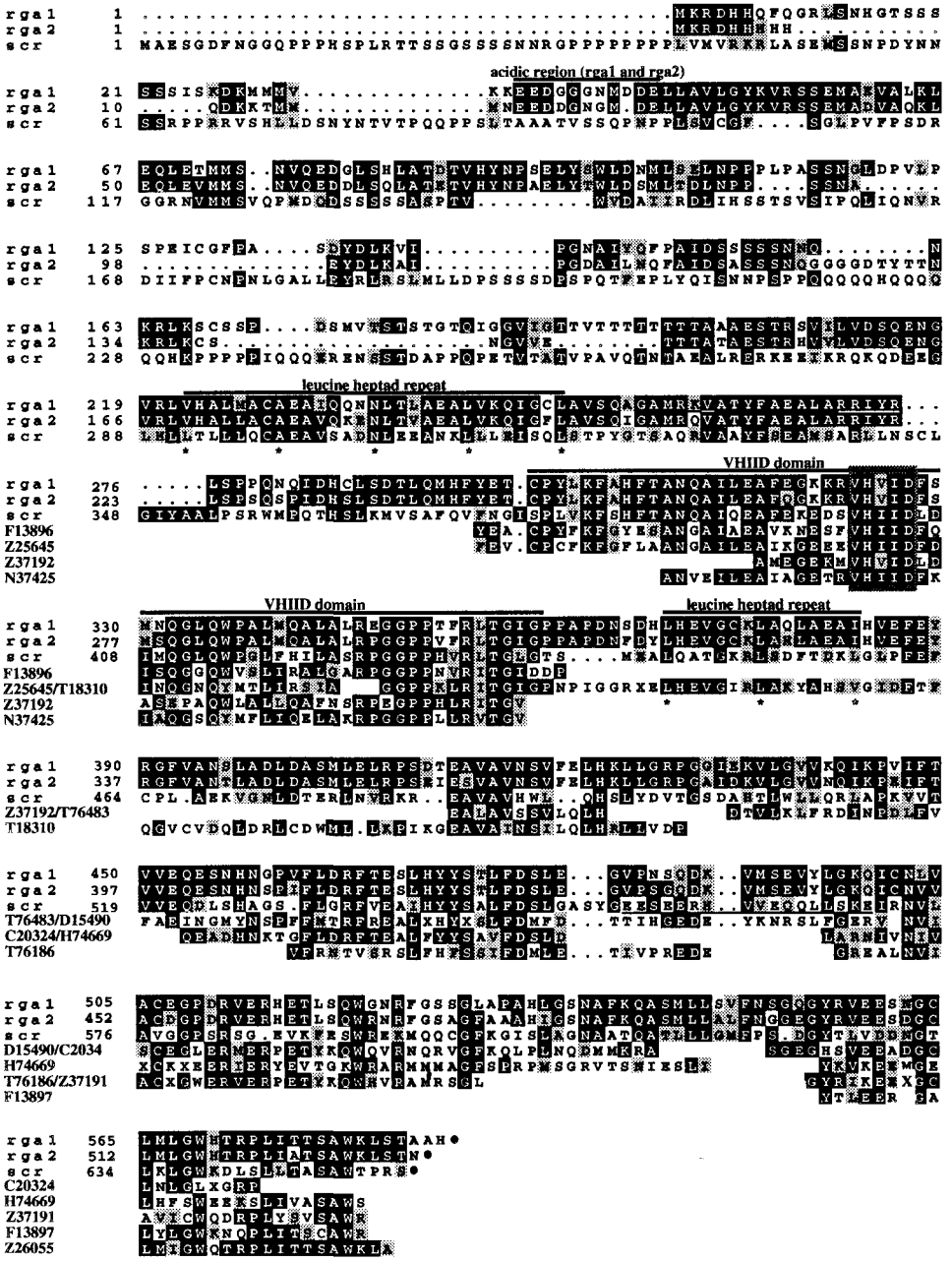

Fig. 2. Alignment of the protein sequences of RGA1, RGA2, SCR and translated regions of ESTs showing homologies with RGA1. Alignments were performed using the Pileup program from the Wisconsin Genetics Computer Group software [27]. For a given EST, only homologies with the same reading frame were kept. Blacked circles indicate the stop codons of RGA1, RGA2 and SCR. Dots stand for gaps, whereas blanks serve as spacers between the translated regions from different ESTs. Residues conserved between at least two sequences are boxed with a black background when they are identical and with a gray background when similar. The two potential leucine heptad repeat regions, the VHIID domain and the acidic regions (of RGA1 and RGA2) are indicated. The d residues of the potential leucine zippers are marked with an asterisk and the VHIID sequence is boxed in gray. The potential bipartite nuclear localization signal of RGA1 and the acidic region of SCR are underlined. All ESTs are from *Arabidopsis* except C20324, D15490, D39460 that are from rice, T18310 from maize and H74669 from *Brassica napus*.

was due to the restoration of NADP-GDH activity or to a GLN3-like activity (by measuring the activities of GS and NAD-GDH whose genes are GLN3-dependent). Activity measurements were performed on the 179-2D strain transformed with pRGA1 or pRGA2 or with the control vector pFL61. No significant difference in the activities of any of these three enzymes was detected between the former transformants and the control transformant, when grown on glutamate or glutamine (data not shown). This indicates that RGA1 and RGA2 likely do not encode a plant GS or GDH or GLN3-homolog.

3.3. Nucleotide sequence of RGA1 and RGA2

The complete sequences of*RGA1* (EMBL accesssion No. Y11336) and *RGA2* (EMBL accession No. Y11337) were determined. The *RGA1* cDNA is 2210 nucleotides long, whereas

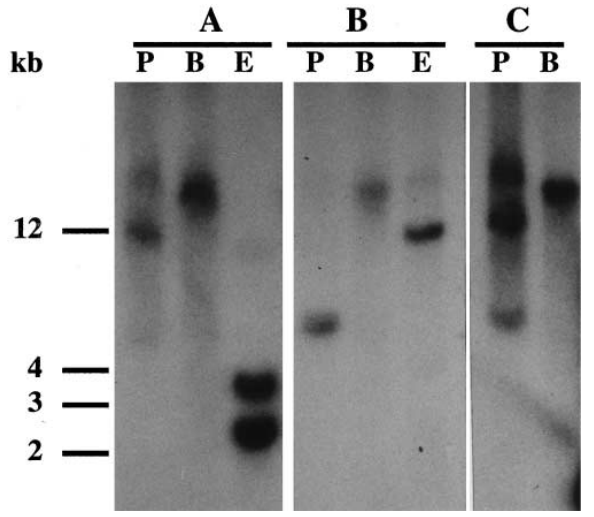

Fig. 3. Genomic organization of the *RGA1* and *RGA2* genes. Blots containing DNA (1 μg) from *A. thaliana* was digested by *Pst*I (P), *Bam*HI (B) or *Eco*RI (E) and probed with *RGA1* (A) or *RGA2* (B) and washed under stringent conditions before autoradiography. In (C) are shown the hybridization bands revealed after probing with *RGA1* and washing the blot non-stringently.

RGA2 is 1951 nucleotides long. In both cases a single open reading frame was found which encodes a polypeptide of 587 amino acids (*RGA1*) and 532 amino acids (*RGA2*). The coding regions of both cDNAs are complete since upstream from the initiator ATG stop codons was found in all reading frames. Orientation of both cDNAs was expected, such that the 5′ region lies downstream from the phosphoglycerate kinase promoter of the expression library vector, and the 3′ region lies upstream from the terminator of the phosphoglycerate kinase.

Interestingly, both clones were highly homologous: there is 82% identity at the nucleotide level, and 83% identity (and 91% similarity) at the protein level (Fig. 2). This indicates that the complementation of the 179-2D strain by pRGA1 and pRGA2 is significant. Divergences between both clones reside in the N-terminal region of the corresponding proteins: markedly, RGA1 comprises two serine/threonine-rich regions that are missing in RGA2.

Comparison with sequences in the databases using the BLAST program [30] shows that no homology with GS, GDH or GLN3 can be found, as expected from the activity measurements. RGA1 and RGA2 are however homologous (55% similarity and 32% identity) to the *SCARECROW* gene (*SCR* [31]) that is involved in specifying an asymmetric cell division during plant development. Plants defective in *SCR* show an aberrant root morphology[32]. Significant homology starts near amino acid 220 of RGA1 and includes several regions delineated in the SCR protein: two potential leucine heptad repeats and the VHIID domain defined by Di Laurenzio et al. [31] containing the amino-acid sequence VHIID (see Fig. 2). In this latter domain that is conserved in expressed sequence tags (ESTs) from *Arabidopsis*, rice and maize [31], the homology between RGA1 and SCR raises to 75% similarity and 62% identity on the 60 amino acid-long region. In SCR a small acidic region was found in the C terminal region of the protein. For both RGA1 and RGA2, a small acidic region can also be found but lying in the N terminal portion of the proteins (Fig. 2). Contrary to SCR, no clearcut basic region can be found before the first potential leucine heptad repeat; however, in RGA1, a potential nuclear bipartite localization motif can be found in the region that lies between the two potential leucine zippers. In the N-terminal region of SCR, stretches rich in glutamine and proline can be found that are missing in RGA1 and RGA2.

Alignment of the C terminal protein sequences from RGA1, RGA2 and SCR with translations of ESTs shows that these proteins belong to a same family that includes proteins from rice, maize and *Brassica napus* (Fig. 2 and Di Laurenzio et al. [31]).

3.4. Genomic organization of the RGA1 and RGA2 genes

Southern blots were made using *A. thaliana* DNA and full length probes of *RGA1* or *RGA2*. Under stringent washing conditions, the hybridization patterns of both probes indicate that *RGA1* (Fig. 3A) and *RGA2* (Fig. 3B) are likely to be single-copy genes. Under non-stringent washing conditions, cross-hybridization could be detected between the two genes, using either *RGA1* (Fig. 3C) or *RGA2* (data not shown) as probes. No other new hybridization band could be detected, confirming that RGA1 and RGA2 are more closely related to each other than to other members of the SCR family.

Oligonucleotides specific of RGA1 or RGA2 were designed (see materials and methods) for mapping experiments using the *Arabidopsis* CIC YAC library [26]. The PCR primers of RGA1 identified three YAC clones (CIC7C11, 2F4 and 6G2) located at the top of chromosome II. The YAC 6G2 contains the RFLP marker ve012 (map position 10.5 cM). The PCR primers of RGA2 identify three YAC clones (CIC3G6, 4H9, 11C3) that are part of a contig located on chromosome I between ve006 (18.7 cM) and ve007 (23.7 cM) and containing the marker m219 on CIC3G6.

3.5. Expression of RGA1 and RGA2 in A. thaliana

Northern blots were performed to determine whether the expression of *RGA1* and *RGA2* was regulated by the nitrogen source used in plant growth media, and whether any organ-specific expression could be detected. A single hybridization band can be detected when *RGA1* or *RGA2* were used as probes, and the size of the mRNA was as expected, that is about 2.2 kb for *RGA1* and 2 kb for *RGA2*. Both genes appeared to be highly expressed as they are readily detected using total RNAs.

Hybridization of RNAs extracted from aerial parts of *Arabidopsis* plantlets with *RGA1* (Fig. 4-IA) or *RGA2* (Fig. 4-IB), showed that no substantial difference in the expression levels of these genes could be detected when plants were grown in vitro on media containing either nitrate, glutamate, glutamine or urea as sole nitrogen source. Thus contrary to the NR, NiR genes, *DAL80* and *NIL1*, the nitrogen source does not affect the expression of *RGA1* and *RGA2*. However this does not rule out that RGA1 and RGA2 are involved in N metabolism, since GLN3 expression is also independent of N source.

RGA1 (Fig. 4-IIA) and *RGA2* (Fig. 4-IIB) appear to be expressed in many plant organs such as roots, rosette leaves, stems and inflorescences of greenhouse grown *A. thaliana* plants.

3.6. Conclusions

By functional complementation of a *S. cerevisiae gln3 gdh1* mutant we have isolated two cDNAs from *A. thaliana*. These

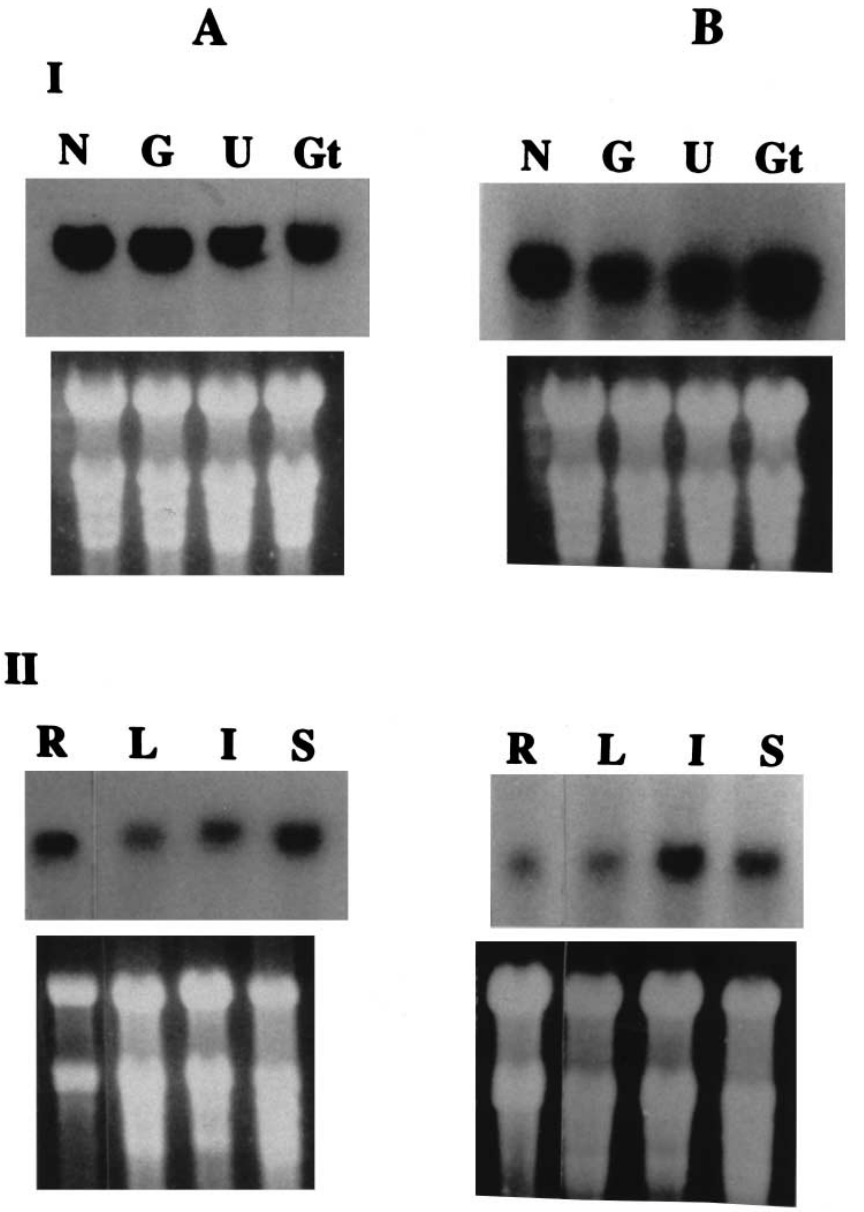

Fig. 4. Northern blots of RNAs extracted from *A. thaliana* using either *RGA1* (A) as probe or *RGA2* (B). In I, RNAs were extracted from aerial parts of plants grown in vitro on media containing either nitrate (N), glutamine (G), urea (U) or glutamate (Gt) as sole nitrogen source. In II, RNAs were extracted from roots (R), rosette leaves (L), stems (S) or inflorescences (I) of *A. thaliana* plants grown in the greenhouse. Under each blot are visualised the rRNAs transferred onto the nylon membrane to compare loading of wells.

cDNAs encode two highly homologous members of the SCR family and help define common motifs shared by members of this family: two potential leucine heptad repeats and the VHIID domain. Members of the family which may include proteins from *Arabidopsis*, rice, maize and *Brassica napus* seem to differ in the N terminal region which may determine the specificity of each protein. How RGA1 and RGA2 enable the 179-2D strain to grow on ammonia is unclear. Ammonia is assimilated in yeast by the NADP-GDH and the GS/Glutamate synthase pathways [33]. Mutants affected simultaneously in both pathways fail to grow on ammonia, showing that in yeast these are the sole physiological routes for ammonia assimilation. However, this does not rule out the possibility that RGA1 or RGA2 somehow favour alternate minor routes for ammonia assimilation. For example, though NADGDH has no physiological role in ammonia assimilation, when it is overexpressed it can contribute to ammonia assimilation and restore growth on ammonia of a yeast strain lacking both NADP-GDH and glutamate synthase [33]. It is possible that RGA1 and RGA2 restore growth on ammonia by activating transcriptionally yeast genes involved in these alternate non-physiological routes, as trans-activation of yeast genes has been reported for plant transcription factors [34]. Alternatively, it has been shown that high level of expression of heterologous proteins can interfere with yeast cellular functions leading to a novel phenotype of the transformed yeast strain [35]. Likewise, RGA1 and RGA2 by interacting with yeast proteins could somehow affect the expression of yeast genes leading to ammonia assimilation by a non-physiological route.

Further experiments must be undertaken to define the function of *RGA1* and *RGA2* in plants. For example transgenic plants with anti-sense or over-expressing constructs and in situ hybridizations may help gain an insight on their role. If the transgenic plants are affected in N metabolism, or if RGA1 and RGA2 are found in the same tissues as NR, this could indicate the possible involvement of these proteins in N metabolism. Experiments designed to test whether RGA1 and RGA2 are transcriptional factors could include immunolocalization of the proteins as a nuclear localization would support the fact that they may be transcriptional factors. Also, if RGA1 and RGA2 turn out to be DNA-binding proteins, potential DNA target sequences of RGA1 and RGA2 could be defined by random selection and PCR [36]. Database searching could then uncover the potential genes controlled by these proteins.

At a molecular level, restoration of growth of the yeast *gln3 gdh1* mutant by RGA1 and RGA2 can be used to define important residues for functionality: mutant proteins that are highly expressed but do not restore growth of the mutant yeast strain would point to critical residues. Indeed, a similar approach with the *Arabidopsis* HAT4 protein [37] has led to the isolation of mutations affecting highly conserved residues in homeo domains and leucine zippers. Finally, it would be of interest to test whether SCR complements the yeast *gln3 gdh1* mutant strain, and, since SCR is a regulator of an asymmetric cell division in plants, whether SCR affects cell division in yeast.

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