At-GDI1 from *Arabidopsis thaliana* encodes a rab-specific GDP dissociation inhibitor that complements the *sec19* mutation of *Saccharomyces cerevisiae*

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Abstract Rab GTPases play a central role in the control of vesicular membrane traffic. These proteins cycle between cytosolic and membrane-bound compartments in a guanine nucleotide-dependent manner, a process that is regulated by several accessory proteins. Of particular interest are the Rab guanosine nucleotide diphosphate dissociation inhibitor proteins (Rab-GDI) which bind to prenylated Rab GTPases, slow the rate of GDP dissociation and escort GDP bound Rab proteins to their target membranes and retrieve them after completion of their catalytic cycle. We have cloned from *Arabidopsis thaliana* a cDNA coding for the Rab guanosine diphosphate dissociation inhibitor (*AtGDI1*) by functional complementation of the *Saccharomyces cerevisiae sec19-1* mutant. The *Arabidopsis* cDNA potentially encodes a 49 850 Da protein which is homologous to yeast GDI (49%) and to other members of the Rab-GDI family (49–63%). Northern blot analysis indicates that the mRNA is expressed in all tissues examined. The existence of a plant homologue of the Rab-GDI family indicates that the basic vesicle traffic control machinery may be highly conserved in plants as it is in yeast and mammals.

Key words: GDP dissociation inhibitor; Plant secretion; *SEC19*; Complementation; *Saccharomyces*; *Arabidopsis*

1. Introduction

Low molecular weight GTPases constitute a family of proteins that participate in a wide range of functions [1,2]. In plants the most well characterized are small GTPases of the Rab family [3–5] which are thought to act in a similar way to their yeast and mammalian orthologues in the control of protein transport and secretion [6–8]. In performing their switch function they cycle between GTP-bound 'on' and GDP-bound 'off' states [9]. It has been observed that in yeast and mammalian cells several accessory proteins can either accelerate or decelerate the GTP hydrolysis several orders of magnitude and thereby regulate the time for which the switch is active. It will therefore be important to obtain detailed information as to the role of related proteins from higher plants in vesicular traffic regulation.

The proteins that affect this switch are (i) the GTPase activating proteins (GAP [10]), (ii) the guanine nucleotide exchange factors (GEF [11]), and (iii) in the case of the Rab and the Rho subfamilies the GDP dissociation inhibitors (GDI [12]). A Rab3a-GDI was first isolated from bovine cytosol [13] and found to inhibit the dissociation of GDP from, and the subsequent binding of GTP to Rab3a. Thus, this enzyme negatively regulates members of the Rab GTPase family antagonizing the activity of the GDP/GTP exchange factor [14]. In addition to their inhibitory action on GDP dissociation, Rab-GDIs form a stable complex with GDP-bound Rab proteins. They thereby prevent in vitro binding of Rab3a to membranes and promote the dissociation of GDP-bound Rab3a from membranes [15,16]. Based on these observations Rab-GDIs are considered as general regulators of Rab function acting as GDP release inhibitors and as chaperones of Rab proteins during their cycle between cytosol and membranes.

Our goal is to analyze systematically Rab proteins from higher plants. To this end we decided to isolate genes encoding GDI-related proteins from the 'model' plant *Arabidopsis thaliana*. To do so, we exploited the secretory yeast mutation (*sec19-1*) that was recently demonstrated to be allelic to *GDI1* [17] and which inhibits protein transport at multiple stages along the secretory pathway [17]. Complementation of this mutant with an *Arabidopsis* expression library resulted in isolation of a higher plant cDNA encoding a GDP-dissociation inhibitor.

2. Materials and methods

2.1. Yeast strain, cDNA library, and growth conditions

The *Saccharomyces cerevisiae sec19-1* strain RSY273 (MATα, *ura3-52*, *his4-619*, *sec19-1*) was a gift from R. Schekman (University of California, Berkeley, CA). The *A. thaliana* λYES cDNA expression library, containing cDNAs under the control of the *GAL1*-inducible promoter [18], was provided by R.W. Davis (Stanford University, Stanford, CA). Standard media such as the complex medium YEPD with glucose/dextrose and the complex medium YEPG with galactose as carbon sources were used for *S. cerevisiae* cultivation [19].

2.2. Isolation of an A. thaliana cDNA which complements the yeast sec19-1 mutation

Competent yeast cells (strain RSY273) were prepared from cultures grown in YEPD media at 25°C and transformed with the λYES cDNA expression library as described [20]. Transformed yeast cells were plated on plates supplemented with uracil and galactose at 25°C to allow cells to recover and to express the cDNAs; after 24 h plates were transferred to the restrictive temperature (37°C) for selection. The transformation efficiency was determined from cells grown at 25°C. After 3 days all colonies grown at the restrictive temperature were replated on YEPD and YEPG plates at 37°C. Colonies growing only on YEPG were further analyzed. DNA was isolated from these transformants and the cDNA insert was subcloned into pUC plasmids.

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2.3. DNA sequence analysis

cDNAs were sequenced using a Perkin Elmer automated sequencer (model 377). Amino acid sequence alignments were generated with the Blast search tool [21]. Multiple alignment of selected members of the gene family and dendrogram analysis was performed using the Clustal program [22].

2.4. Nucleic acid blots

A. thaliana ecotype Columbia (Col-0) were grown as described in the Nottingham Arabidopsis Stock Center catalogue (Internet http://nasc.life.nott.ac.uk). Genomic DNA was isolated from young leaves [23]. RNA was isolated from leaves, inflorescence stems, flowers, and roots from *A. thaliana* ecotype Col-0 as described [24]. Southern and Northern blots were prepared [25] and probed with a ^{32}P-labeled DNA fragment containing the complete *AtGDI1* coding region. The probe was synthesized with [α-^{32}P]dATP (Amersham) by using the Klenow fragment of DNA polymerase (Boehringer) and hexanucleotide primers. After hybridization Hybond N$^+$ membranes (Amersham) were washed three times in 0.5×SSC at room temperature (Southern) or twice in 2×SSC at 42°C and once for 10 min in 2×SSC at room temperature (Northern). Blots were either autoradiographed using Kodax X-Omat X-ray film or subjected to phosphoimage analysis using a STORM phosphoimager (Molecular Dynamics).

3. Results

3.1. Isolation of an A. thaliana cDNA by complementation of a yeast sec19-1 mutant

The yeast *S. cerevisiae* contains in its genome a single copy gene (*GDI1*) encoding a Rab-GDP dissociation inhibitor. This gene was shown to be allelic to the previously identified *SEC19* gene [17,26]. Mutations in the *SEC19* gene (i.e. the *sec19-1* mutation) cause a thermosensitive growth defect, thus allowing the mutant strain to grow at the permissive temperature of 25°C but not at the restrictive temperature of 37°C. Thus, restoration of *SEC19* activity should provide a convenient assay to search for plant cDNAs complementing this function. To test this proposal we transformed a yeast strain containing the *sec19-1* mutation with an *A. thaliana* cDNA expression library [18]. The colonies generated were transferred to plates containing galactose to induce expression of the cDNAs from the *GAL1* promoter. After screening 80 000 colonies we obtained 21 transformants which complemented the GDI-deficient phenotype of the *sec19-1* mutant.

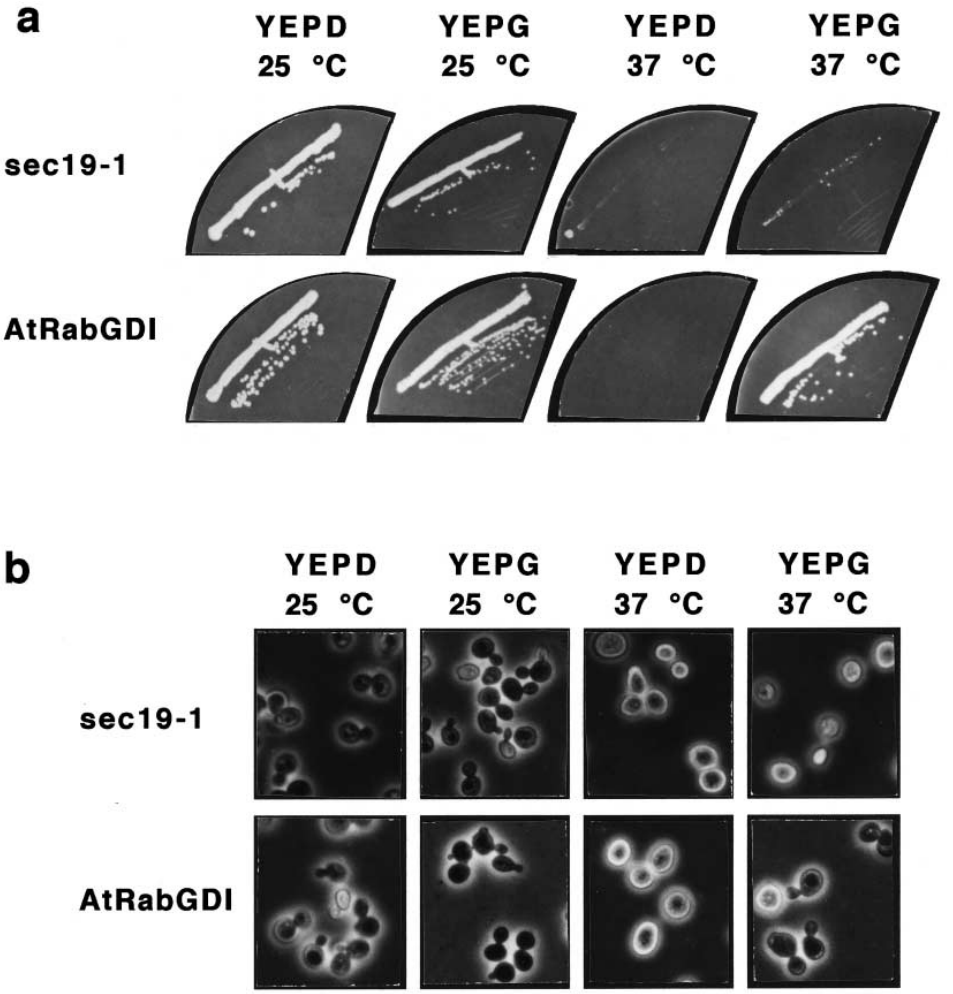

Fig. 1. Complementation of a yeast *sec19-1* mutant by an *A. thaliana* cDNA. The upper row of (a) shows the *sec19-1* yeast strain after growth for 3 days on glucose (YEPD) or galactose (YEPG) media at either the permissive (25°C) or restrictive temperature (37°C). The bottom row shows the growth pattern of a yeast strain transformed with an *Arabidopsis* cDNA (p*ATGDI1*) complementing the *sec19-1* mutant. (b) Phase-contrast microphotographs of cells of the same strains grown in liquid YEPD or YEPG medium for 18 h.

To test whether this complementation resulted from the presence of an *Arabidopsis* cDNA containing plasmid, we grew yeast cells under non-selective conditions to allow for plasmid loss. In addition, we plated the complementing colonies on media containing 5-fluoroorotic acid. This treatment allows selection against the uracil marker on the plasmid to obtain plasmid free segregants. For three clones, *sec19-1* like phenotypes were obtained suggesting that the *sec19-1* phenotype complementing function was provided by an *Arabidopsis* cDNA. We therefore isolated the cDNAs from complemented colonies and retransformed these plasmids into the *sec19-1* mutant. We found that two different cDNAs were apparently able to complement the *sec19-1* mutant. However, only one of the cDNAs complemented the mutant in all respects of colony growth and cell morphology (Fig. 1). This cDNA was chosen for further analysis.

3.2. Sequence analysis of a complementing cDNA

The complementing *A. thaliana* cDNA had an insert size of approx. 1.8 kb. The DNA sequence of this cDNA was determined by the chain termination method [27]. The sequence is available from the EMBL data base – accession number Y07961. An open reading frame potentially encoding a 49 850 Da protein of 445 amino acid residues with a calculated isolelectric charge of p*I* 5.3 was identified. An alignment of the predicted amino acid sequence with several Rab GDP dissociation inhibitors from yeast and animals (Fig. 2) indicated that the proteins share more than 50% similarity when conservative substitutions were taken into account. The *Arabidopsis* cDNA was designated *AtGDI1*. We performed a phylogenetic analysis to determine the relationship of the *Arabidopsis AtGDI1* gene product with other related eukaryotic gene products. The resulting tree (Fig. 3) shows that the *Ara-*

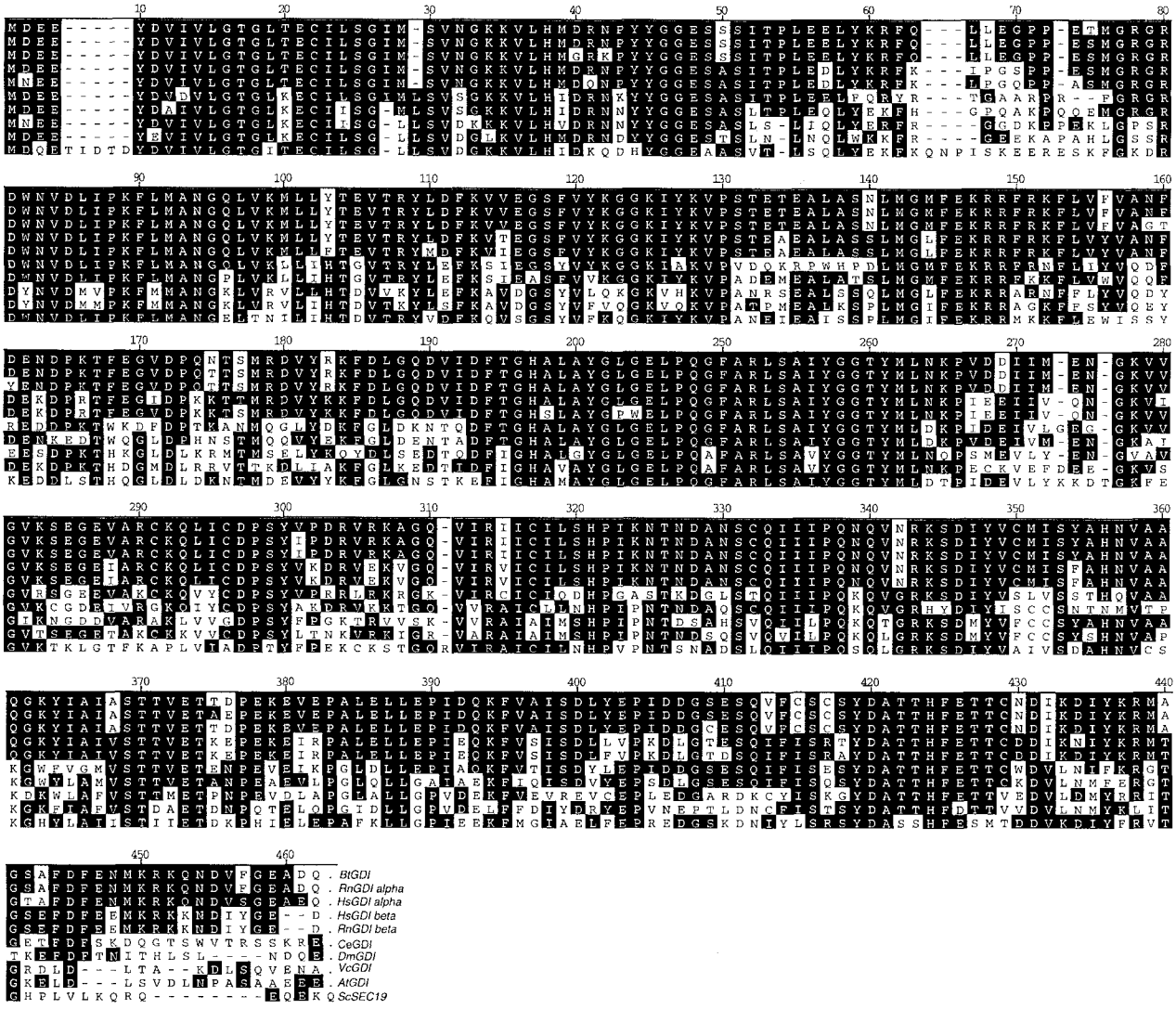

Fig. 2. Comparison of AtGDI1p with various Rab-GDIs. Sequences were aligned using the Clustal method from the MegAlign program, part of the Laseragene sequence analysis software program available from DNASTAR Inc. Amino acid identity is denoted by a black background. Dashed lines indicate gaps. *BtGDI*, *Bos tauri* Rab-GDI (accession number D90103); *RnGDIα*, *Rattus norvegicus* Rab-GDI (accession number X74401); *HsGDIα*, human Rab-GDI (accession number D45021); *HsGDIβ*, human Rab-GDI (accession number D13988); *RnGDIβ*, *Rattus norvegicus* Rab-GDIβ (accession number X74402); *DmGDI*, *Drosophila melanogaster* Rab-GDI (accession number L03209); *CeRabGDI*, *Caenorhabditis elegans* Rab-GDI (accession number U00002); *VcRabGDI*, *Volvox carteri* Rab-GDI (accession number U62866); *AtGDI1*, *Arabidopsis thaliana* Rab-GDI (accession number Y07961); *ScSEC19*, *Saccharomyces cerevisiae* SEC19p (accession number S69371).

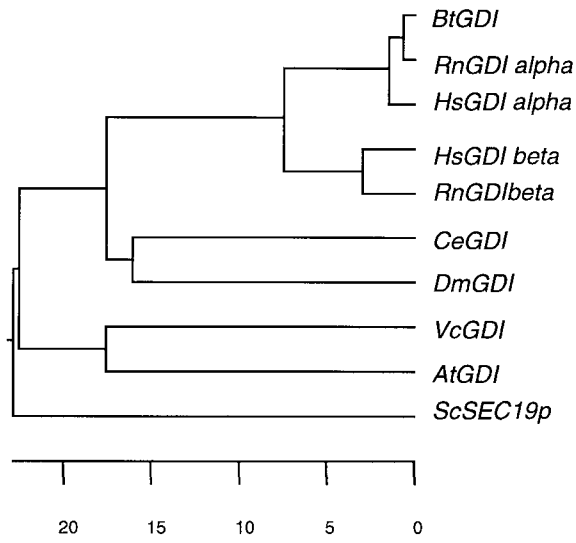

Fig. 3. Dendrogram representing the evolutionary relationships in the Rab-GDI family. The predicted protein products were analyzed by pairwise alignment using the Genetics Computer Group Inc., programme 'PileUp'. The figure shows a dendrogram of the sequence relationships with the percentage similarity below the dendrogram. The species and gene abbreviations are indicated on the right and refer to GenBank accession numbers as indicated in Fig. 2.

bidopsis AtGDI1 is more closely related to the *VcGDI* from the green alga *Volvox* than to animal *GDIs* or to the yeast *GDI1/SEC19* gene product. While the yeast *SEC19* gene seems to have branched off very early in evolution and thus forms a separate branch, the surprising functional complementation of the *sec19-1* mutant points to a very high conservation of functional important motifs in the AtGDI1 protein sequence (Fig. 4).

3.3. Southern analysis of the A. thaliana AtGDI1 gene

To investigate the number of *AtGDI* related genes in *Arabidopsis*, a Southern analysis was performed with a radiolabeled full length cDNA as a probe (Fig. 5). Only a single hybridizing band under stringent hybridization conditions was found after digestion of genomic DNA with *Eco*RI, *Hin*dIII or *Xba*I. This indicates that there is a single Rab-GDI gene present in the *Arabidopsis* genome which is sufficiently distinct from other *Arabidopsis* GDI genes, particularly those that regulate small *Arabidopsis* GTPases like Rho, Ral, or Rac. The presence of other bands seen under less stringent hybridization conditions indicates that additional *GDIs* are present in the *Arabidopsis* genome probably forming a small gene family.

3.4. mRNA distribution of AtGDI1

To determine the pattern of *AtGDI1* expression in different organs of *Arabidopsis* we performed RNA hybridization analysis. We hybridized a Northern blot of total RNA (20 μg) from various *Arabidopsis* tissues with a radiolabeled probe containing a *Sac*I-*Xba*I 3′ fragment of the *AtGDI1* coding region. A single band of approx. 1.8 kb and similar intensity was detected in all tissues tested (Fig. 6). Quantitative analysis using a phosphoimager revealed small differences of less than 2-fold in expression levels between the tissues tested. The expression of *AtGDI1* throughout all plant tissues is consistent with a general function for this GDI homolog in plant cells.

4. Discussion

We report the isolation and characterization of an *Arabidopsis* cDNA that encodes a protein that complements the yeast *sec19-1* mutant. This thermosensitive mutant blocks the secretion of invertase and acid phosphatase at the restrictive temperature and leads to an accumulation of carboxypeptidase Y subsequent to the temperature shift [26,28]. This mutant was later shown by complementation to be allelic to *GDI1* in which depletion of the Gdi1p protein leads to a loss of a soluble pool of the Sec4p protein. The loss of the Sec4p protein results in a block of membrane traffic at multiple places along the secretory pathway. Cloning and sequence analysis of the *S. cerevisiae GDI1* gene identified an open reading frame of 455 amino acids with more than 50% identity to Rab GDIs from various organisms [17,29]. By gene disruption it was shown that the yeast *GDI* gene is essential for cell viability [17]. Depletion of the Gdi1p activity using an *GAL1*-inducible *GDI1* replacement vector further demonstrated that loss of *GDI1* function results in (i) a partial block of invertase transport between ER and Golgi, (ii) defects in the transport and processing of carboxypeptidase Y, (iii) an exaggeration of the ER and accumulation of cup-shaped Golgi-related structures, (iv) the build-up of vesicles in the cyto-

Percent Similarity

	1	2	3	4	5	6	7	8	9	10		
1		98.4	96.9	86.8	84.3	63.6	64.0	53.8	53.6	51.3	1	BtGDI
2	1.3		96.9	86.8	84.3	63.4	64.0	53.8	53.6	51.3	2	RnGDI alpha
3	2.9	2.9		85.2	83.0	62.3	62.9	53.6	53.1	50.9	3	HsGDI alpha
4	13.0	13.0	14.6		93.9	63.5	64.5	53.2	53.6	52.0	4	HsGDI beta
5	15.5	15.5	16.9	5.8		60.5	62.0	51.1	50.9	50.2	5	RnGDI beta
6	33.9	34.2	35.3	34.1	37.0		65.2	52.0	51.8	48.6	6	CeGDI
7	34.6	34.6	35.7	33.9	36.4	32.1		52.7	51.9	51.0	7	DmGDI
8	44.4	44.4	44.4	44.4	46.5	44.4	44.6		62.8	50.5	8	VcGDI
9	44.2	44.2	44.2	44.0	46.7	46.2	45.1	35.1		48.9	9	AtGDI
10	44.4	44.4	44.6	43.4	45.1	46.8	45.1	45.7	47.2		10	ScSEC19
	1	2	3	4	5	6	7	8	9	10		

Percent Divergence

Fig. 4. Comparison of predicted protein products pairwise identities between inferred amino acid sequences. Sequences were adjusted to optimize alignments of conserved residues and the percent of aligned identities determined. The Rab-GDIs are listed as in Fig. 2.

sol, and (v) finally in a loss of the soluble pool of the Sec4p protein indicating that the Gdi1p protein functions in the cycling of Sec4p from membranes back into the soluble pool. These data as well as the biochemical demonstration of inhibition of dissociation of [^{3}H]GDP from various Rab proteins but not Ras are consistent with the view that the yeast *GDI1* gene encodes a Rab specific GDP dissociation inhibitor.

We therefore expected that the complementation of the *sec19-1* mutant by *Arabidopsis* cDNAs should provide a means to identify a cognate *Arabidopsis GDI* gene or other plant genes acting as suppressors in this pathway. Indeed, we were able to isolate by expression of an *Arabidopsis* cDNA library in *sec19-1* yeast cells two cDNAs that complemented this mutant to various degrees. Only one clone, however, complemented the mutant in all respects at 37°C, i.e. full stimulation of growth at the restrictive temperature, and absence of unbudded or occasionally deformed cells with altered optical properties which could be caused by the accumulation of vesicles in the cytoplasm. It is possible that the analysis of the other partially complementing clone that was isolated during our complementation experiments may result in identification of a gene that encodes a plant protein that modulates or suppresses other functions along the secretory protein transport pathway.

The fully complementing *Arabidopsis* cDNA was sequenced and found to encode a protein homologous to the yeast GDI1/SEC19 protein. The homology to other Rab-GDIs ranged from 49% to 63%. The closest relative is the recently isolated Rab-GDI from *Volvox carterii* with 63% identity [30].

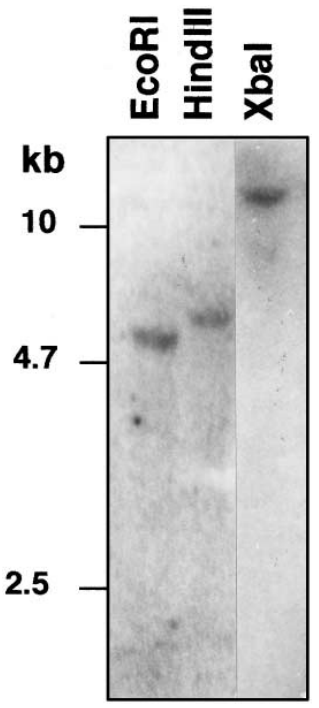

Fig. 5. Southern blot analysis of the *AtGDI1* gene. Genomic DNA (10 μg) from *A. thaliana* was digested overnight with *Eco*RI (lane1), *Hin*dIII (lane 2), and *Xba*I (lane 3). The digestion products were separated by electrophoresis in an agarose gel and transferred onto nylon membrane. The membrane was hybridised with a ^{32}P-labeled cDNA fragment containing the *AtGDI1* coding region. Positions of DNA size markers are indicated on the left.

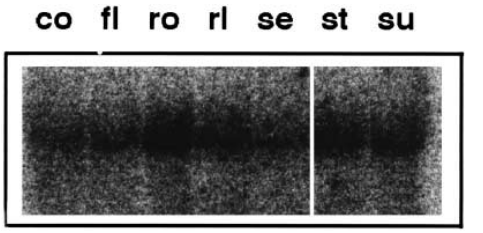

Fig. 6. Northern blot analysis of *AtGDI1* transcripts. Total RNA (20 μg) from cotyledons (co), flowers (fl), roots (ro), rosette leaves (rl), seedlings (se), stem (st), and callus suspension culture (su) was separated in a 1.2% agarose/6% formaldehyde gel and blotted onto nylon membrane. The membrane was hybridised with a ^{32}P-labeled cDNA fragment containing the *AtGDI1* open reading frame.

To explore further the similarity of AtGDI1 with other members of the GDI family we used an algorithm which finds statistically significant blocks of continuous amino acids shared between multiple protein sequences. We found three domains that are highly conserved between the plant and green algae GDIs and that are shared with other GDIs and Rab escort proteins which deliver newly synthesized Rab proteins to the geranylgeranyl transferase for prenylation. These sequence conserved regions (SCR) are located at position 11 to 25 (SCR1), position 33 to 46 (SCR2), and position 244 to 258 (SCR3). These SCR regions were also found in the mammalian *choroideraemia* gene product, a GDI-like protein also referred to as Rab-escort protein [31,32]. A particularly striking feature of these SCRs is revealed by the analysis of the location of these regions in the three-dimensional structure of the recently resolved crystal structure of the bovine α-isoform of Rab-GDI [31]. Residues located in the SCR3 region wind back to the SCR1 region where they form a highly compact structure. In contrast, all non-well conserved residues are located to the opposite face of the molecule. From the conserved residues shown in Fig. 2 it is likely that the plant Rab-GDIs also form a similarly structured molecule, in which, as with the mammalian enzyme, contacts to the Rab proteins will be made via the SCR1 and SCR3 regions [31]. Through this interaction they mask the highly hydrophobic prenylated Rab C-termini and enable them to stay in the cytosol when complexed with GDIs in a stoichiometric ratio. It has been suggested that GDIs function in the vesicular transport like the βγ-subunits of heterotrimeric G-proteins in signalling processes. While the complex consisting of αβγ-subunits serves as a membrane-associated reservoir for activation and release of the GTPase α-subunit from the βγ-subunits in response to a wide range of signals, the Rab-GDI complex may provide a cytosolic reservoir from which specific Rab proteins can be released in response to signals to selectively regulate the endocytic and the exocytic pathway. However, other additional oxidative or hydroxylative functions affecting vesicle transport may be indicated by the striking structural similarity between Rab-GDIs and FAD-containing monoxygenases and oxidases [31].

While we have not yet tested biochemically the plant GDP dissociation inhibition function of our clone, the sequence conservation and the specificity in yeast strongly suggest that the *AtGDI1* will also function biochemically as a GDI. We expect that in line with its role as a negative regulator of rab proteins in vesicle transport our *Arabidopsis GDI* gene product will display an essential function in plant secretion

control. Further description of the function of this protein awaits immunodetection and functional analysis of mutants in transgenic plants.

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