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An Arabidopsis Gene Isolated by a Novel Method for Detecting Genetic Interaction in Yeast Encodes the GDP Dissociation Inhibitor of Ara4 GTPase

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The Arabidopsis Ara proteins belong to the Rab/Ypt family of small GTPases, which are implicated in intracellular vesicular traffic. To understand their specific roles in the cell, it is imperative to identify molecules that regulate the GTPase cycle. Such molecules have been found and characterized in animals and yeasts but not in plants. Using a yeast system, we developed a novel method of functional screening to detect interactions between foreign genes and identified this Rab regulator in plants. We found that the expression of the *ARA4* gene in yeast *ypt* mutants causes exaggeration of the mutant phenotype. By introducing an Arabidopsis cDNA library into the *ypt1* mutant, we isolated a clone whose coexpression overcame the deleterious effect of *ARA4*. This gene encodes an Arabidopsis homolog of the Rab GDP dissociation inhibitor (GDI) and was named *AtGDI1*. The expression of *AtGDI1* complemented the yeast *sec19-1* (*gdi1*) mutation. *AtGDI1* is expressed almost ubiquitously in Arabidopsis tissues. The method described here indicates the physiological interaction of two plant molecules, Ara4 and GDI, in yeast and should be applicable to other foreign genes.

INTRODUCTION

A great number of small GTPases, which constitute the so-called Ras superfamily, have been isolated from all of the kingdoms of living organisms and have been shown to be involved in a wide variety of important intracellular events. Rab/Ypt proteins represent the largest subfamily in this superfamily. The biological importance of Rab/Ypt proteins was first elucidated with the conditional lethal yeast mutants *sec4* (Salminen and Novick, 1987) and *ypt1* (Schmitt et al., 1988; Segev et al., 1988). Under restrictive conditions, the cells of *ypt1* and *sec4* mutants accumulate secretory proteins in the endoplasmic reticulum (ER) and secretory vesicles, respectively, indicating that the products of the *YPT1* and *SEC4* genes are required for ER–to–Golgi apparatus and Golgi–to–plasma membrane transport. Later studies identified a large number of similar proteins in various eukaryotic organisms from fungi to mammals. Most of these proteins from higher eukaryotes are collectively referred to as Rab. Rab/Ypt proteins have been implicated in many steps in intracellular vesicular traffic. In addition to Ypt1 and Sec4, Ypt3 appears to regulate transport within the Golgi apparatus in yeast (D. Gallwitz, personal communication), and Ypt51, Ypt52, Ypt53, and Ypt7/Vam4 seem to be involved in endocytosis and morphogenesis of vacuolar compartments (Wichmann et al., 1992; Singer-Krüger et al., 1994; Wada et al., 1996).

The most popular and prevailing idea regarding the common function of Rab/Ypt proteins is that they regulate the commitment of transport vesicles for targeting and/or fusion to the specific acceptor membrane through a conformational change between the GTP and GDP forms (reviewed in Ferro-Novick and Novick, 1993; Nuoffer and Balch, 1994). As with Ras proteins, the GTPase cycle of Rab/Ypt proteins must be strictly controlled for the intracellular transport pathways to operate properly. Several classes of regulator molecules have been found and characterized in animal and yeast cells. These include guanine nucleotide exchange factors (GEFs), which facilitate the exchange of GDP for GTP, GTPase-activating proteins (GAPs), which stimulate intrinsic GTP hydrolysis activity, and GDP dissociation inhibitors (GDIs), which regulate both the nucleotide state and the subcellular localization of Rab/Ypt proteins. With regard to the yeast Ypt proteins, for example, Dss4 has been shown to act as a GEF toward the Sec4 protein (Moya et al., 1993), Gyp6 has been identified as a GAP for Ypt6 (Strom et al., 1993), and Gdi1 is thought to act as a GDI for all of the Ypt proteins in the yeast cell (Garrett et al., 1994).

In plants, >30 members of the Rab family have been identified (Terryn et al., 1993a; Ma, 1994; Verma et al., 1994). Among

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the more interesting features of plant Rab proteins are strict tissue specificities and stringent regulation of gene expression. For example, *RHA1*, a *RAB5* homolog isolated from Arabidopsis, is expressed in the guard cells of stomata, stipules, and the root tip of young plants (Terryn et al., 1993b). Yoshida et al. (1993) have shown that the expression of the pea *PRA2* and *PRA3* genes is markedly repressed by light. Sano et al. (1994) reported that the artificial overexpression of the rice *RGP1* gene in a tobacco plant causes elevation of the cytokinin level and an abnormal induction of salicylic acid in response to wounding. Cheon et al. (1993) have shown that the expression of the antisense RNA of *SRAB1* (a *RAB1* homolog in soybean) and *VRAB7* (a *RAB7* homolog in *Vigna aconitifolia*) interferes with root nodule development. All of these observations imply that plant Rab proteins play important roles in development and morphogenesis.

Matsui et al. (1989) and Anai et al. (1991) from our group have identified a Rab family of proteins from Arabidopsis and named them Ara proteins (Ara, Ara2, Ara3, Ara4, and Ara5). We have been characterizing these Ara proteins (Anai et al., 1994, 1995) and have recently shown that the Ara4 protein is localized in Golgi apparatus–derived vesicles, Golgi stacks, and the *trans*-Golgi network in germinating pollen cells by immunoelectron microscopy (Ueda et al., 1996). This observation is consistent with the fact that the Ara4 protein is highly homologous to the fission yeast Ypt3 and mammalian Rab11, which are also implicated in Golgi functions.

Mutational analysis of the Ara proteins has demonstrated that their biochemical properties are similar to those of the Rab/Ypt proteins from yeast and animal cells. For example, the replacement of Gln by Leu at codon 71 in the Ara4 protein ($Ara4^{Q71L}$), which is equivalent to the Gln-to-Leu exchange at codon 61 in mammalian Ras^{H}, reduces intrinsic GTPase activity but not the ability to bind GTP (Anai et al., 1994), as with Ras^{H} (Der et al., 1986). Similar inhibition of GTPase activity has been demonstrated with yeast $Sec4^{Q79L}$ (Walworth et al., 1992) and mammalian $Rab5^{Q79L}$ (Stenmark et al., 1994). On the other hand, the Asn-to-Ile mutation at codon 125 in Ara4 ($Ara4^{N125I}$) decreases the ability to bind GTP (Anai et al., 1994), again as with the N116I mutation of Ras^{H}. The equivalent mutation in A.t.Rab6 (N122I), another member of the Arabidopsis Rab family, gives a similar result (Bednarek et al., 1994). In mammals and yeast, the corresponding mutations in Rab1, Rab2, Rab5, Ypt1, and Sec4 have also been shown to abolish GTP binding activities (Wagner et al., 1987; Walworth et al., 1989; Bucci et al., 1992; Tisdale et al., 1992).

To understand the precise role of each of these plant Rab proteins, it is important to clarify the regulation of their GTPase cycle. As mentioned above, GEFs, GAPs, and GDIs play critical roles in such regulation in mammalian and yeast cells. In the case of plant Rab proteins, however, little information is available on these regulator molecules, with the exception of one report from our group describing the existence of GAP-like activities for Ara2 and Ara4 in Arabidopsis (Anai et al., 1994).

Several groups have reported that Rab/Ypt proteins of higher plants and green algae have the ability to complement *ypt* mutations of budding yeast (Cheon et al., 1993; Bednarek et al., 1994; Park et al., 1994; Fabry et al., 1995; Loraine et al., 1996). In this study, to identify the functional homologs of Ara proteins in yeast, we introduced the *ARA2*, *ARA3*, and *ARA4* genes into several yeast *ypt* mutants and examined whether the expression of each individual *ARA* gene could complement the defect of the mutant cells. To our surprise, none of the *ARA* genes that we tested complemented yeast *ypt* mutants, and the expression of *ARA4* even enhanced the phenotypes of the mutants. Based on this finding, we designed a screening method to search for plant genes that would suppress the harmful effect of *ARA4* expression in yeast. Using this method, we isolated an Arabidopsis gene that encodes plant Rab-GDI. This study not only reports the molecular identification of a regulator molecule for a plant Rab protein but also describes a novel and powerful methodology for identifying and analyzing interacting molecules by using yeast cells as a model system.

RESULTS

Expression of Arabidopsis *ARA* Genes in Yeast *ypt* Mutants

The wild-type *ARA2*, *ARA3*, and *ARA4* genes; Q72L and N126I mutant versions of *ARA2* ($ARA2^{Q72L}$ and $ARA2^{N126I}$); and the Q71L and N125I mutant versions of *ARA4* ($ARA4^{Q71L}$ and $ARA4^{N125I}$) were placed downstream of the *GAL1* promoter on a yeast single-copy plasmid. This promoter is derived from the yeast galactokinase gene, and its expression is stringently regulated (Oshima, 1982): it is highly induced in the presence of galactose and is almost completely repressed in the presence of glucose. Plasmids containing the wild-type and mutant versions of the *ARA* genes under the control of the *GAL1* promoter were introduced into cells of yeast $ypt1^{ts}$, $ypt3^{cs}$, $sec4^{ts}$, *ypt6*, and *ypt7* mutants. These transformed yeast cells were transferred from glucose to galactose medium to induce expression of the *ARA* gene. The phenotype of each *ypt* mutant (temperature-sensitive [ts] growth for *ypt1* and *sec4*, cold-sensitive [cs] growth for *ypt3*, and fragmentation of vacuoles for *ypt6* and *ypt7*) was examined. To our disappointment, none of the *ARA* genes that we tested complemented these *ypt* mutations. However, we realized that the *ARA4* gene aggravated the growth defect of *ypt1*, *ypt3*, and *sec4* cells. The results are summarized in Table 1, where ++ indicates that a significant growth delay or inhibition was observed when the *ARA4* gene was expressed. This effect of *ARA4* expression was more obvious when the mutant Q71L version of the *ARA4* gene ($ARA4^{Q71L}$) was introduced. Even at a permissive temperature (23°C), the growth of *ypt1*, *ypt3*, and *sec4* cells was severely inhibited when $ARA4^{Q71L}$ was expressed (Figures 1A to 1F). $ARA4^{N125I}$ did not affect the growth of any of these yeast mutant cells. The wild-

Table 1. Negative Effects of *ARA* Genes on the Growth of *ypt* Mutants[a]

Yeast Strain	Temp. (°C)	*ARA2* WT	*ARA2* Q72L	*ARA2* N126I	*ARA3* WT	*ARA4* WT	*ARA4* Q71L	*ARA4* N125I
ypt1	15	–	–	–	–	+	+ +	–
	23	NT	NT	NT	NT	+ +	+ +	–
	30	–	–	–	–	+ +	+ +	–
	35.5	–	–	–	–	+ +	+ +	–
ypt3	15	–	+	–	+	+ +	+ +	–
	23	–	–	–	–	+ +	+ +	–
	30	–	–	–	–	+ +	+ +	–
	35.5	NT	NT	NT	NT	+ +	+ +	–
sec4	15	–	+	–	–	+	+ +	–
	23	–	+	–	–	+ +	+ +	–
	30	–	+	–	+	+ +	+ +	–
ypt6	23	NT	NT	NT	NT	–	+	NT
	30	NT	NT	NT	NT	–	+	NT
	35.5	NT	NT	NT	NT	–	+	NT
ypt7	15	–	–	–	–	–	–	–
	23	–	–	–	–	–	–	–
	30	–	–	–	–	–	+	–
	35.5	–	–	–	–	–	+	–
WT	15	–	–	–	–	–	–	–
	23	–	–	–	–	–	–	–
	30	–	–	–	–	–	–	–
	35.5	–	–	–	–	–	–	–

[a] –, no growth defect; +, growth defect; + +, significant growth defect; NT, not tested. The strains are *ypt1*, TSU3-5D; *ypt3*, YTH12; *sec4*, ANS4-8C; *ypt6*, GL72; *ypt7*, VAQ204-9C; WT (wild type), ANY21.

type and mutant alleles of *ARA4* did not markedly affect the growth of *ypt6*, *ypt7*, or wild-type yeast cells. With *ARA2* and *ARA3*, slight growth inhibition was observed in *ypt3* and *sec4*, especially with $ARA2^{Q72L}$, but this effect was not as significant as with $ARA4^{Q71L}$.

Electron Microscopy

To analyze the effect of $Ara4^{Q71L}$ expression in *ypt1*, *ypt3*, and *sec4* cells in more detail, we observed ultrastructural alterations by electron microscopy. The rapid-freezing and freeze–substitution method was used for sample preparation to preserve the fine structures as much as possible. Cells were cultured at 23°C in minimal glucose medium to the logarithmic phase, transferred to minimal galactose medium, and then further cultured at 23°C for another 24 hr. Expression of the Ara4 protein was confirmed by protein blot analysis (data not shown).

Electron microscopic observation revealed that the induction of $ARA4^{Q71L}$ affected a specific step in the secretory pathway in each mutant. As shown in Figures 2A to 2C, the original *ypt1*, *ypt3*, and *sec4* mutants that harbored the vector alone displayed normal cell morphology at the permissive temperature (23°C). Under the same conditions, *ypt1* cells expressing $ARA4^{Q71L}$ accumulated a network of ER (Figures 3A and 3B). In *ypt3* cells transformed with $ARA4^{Q71L}$, strange membrane structures were built up in the cytoplasm (Figures 3C and 3D). These swollen and sometimes multilamellar structures are reminiscent of "Berkeley bodies" (Novick et al., 1980), which were observed to be the result of irreversible accumulation and deformation of Golgi cisternae. In the *sec4* mutant, $ARA4^{Q71L}$ caused the proliferation of numerous vesicles of 70 to 80 nm in diameter. These vesicles looked like Golgi-derived secretory vesicles (Figures 3E and 3F).

Ypt1p, Ypt3p, and Sec4p are involved in ER-to-Golgi transport (Schmitt et al., 1988; Segev et al., 1988; Baker et al., 1990), in intra-Golgi transport (D. Gallwitz, personal communication), and in transport from the Golgi apparatus to the plasma membrane (Novick et al., 1980; Salminen and Novick, 1987), respectively. The electron microscopic observations described above strongly suggest that the expression of $ARA4^{Q71L}$ exaggerates the distinctive transport defect of each mutant. We examined whether protein transport was in fact affected by the expression of $ARA4^{Q71L}$. We used protein gel blot analysis with

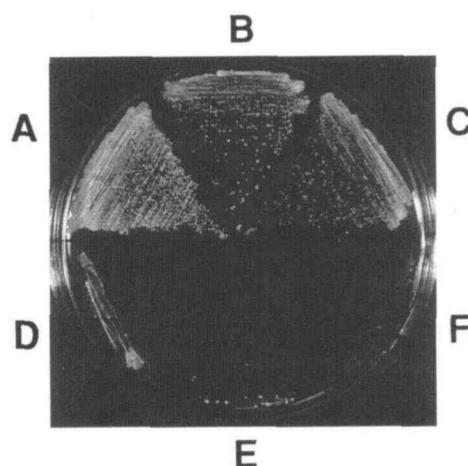

Figure 1. Growth Inhibition of Yeast *ypt* Mutants by the Expression of $ARA4^{Q71L}$.

(A) and **(D)** $ypt1^{ts}$.
(B) and **(E)** $ypt3^{cs}$.
(C) and **(F)** $sec4^{ts}$.
Yeast mutants transformed with either vector alone (**[A]** to **[C]**) or $ARA4^{Q71L}$ under the control of the *GAL1* promoter (**[D]** to **[F]**) were streaked on galactose medium (YPGS) to induce expression and incubated at 23°C for 4 days.

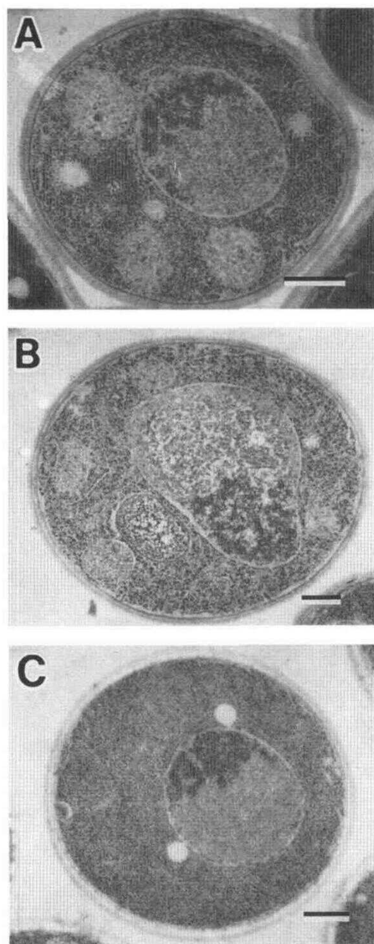

Figure 2. Electron Microscopy of Yeast *ypt* Mutants under Permissive Conditions.

(A) *ypt1*^{ts} (TSU3-5D).
(B) *ypt3*^{cs} (YTH12).
(C) *sec4*^{ts} (ANS4-8C).
Cells harboring the vector alone were cultured at 23°C in MCD medium to the logarithmic phase, washed, and transferred to MCGS medium. Incubation was at 23°C for an additional 24 hr. The cells were harvested and prepared for electron microscopy by the freeze–substitution fixation method. Bars = 0.5 μm.

an anti-carboxypeptidase Y antibody to show that *ypt1* mutant cells expressing *ARA4*^{Q71L} accumulated the ER precursor form of carboxypeptidase Y (data not shown). Thus, the expression of *ARA4*^{Q71L} indeed enhances the transport defect of the mutant. These results imply that the Ara4 protein competes with the Ypt proteins for some common factors in the regulation of this Ypt family of proteins in yeast cells. The depletion of such factor(s) appears to emphasize the weak point of each mutant strain.

Screening of Multicopy Suppressors of the Ara4^{Q71L}-Induced Growth Defect

This interpretation encouraged us to find the molecule(s) that interacts with the Ara4 protein through the functional screening of plant genes. A cDNA library from 10-day-old Arabidopsis seedlings was constructed on a yeast 2-μm–based multicopy plasmid, pYES2. This cDNA library can be expressed in yeast under the control of the *GAL1* promoter. The *ARA4*^{Q71L} gene, again under the control of the *GAL1* promoter, was integrated at the *TRP1* locus on the chromosome of the *ypt1* mutant strain to ensure stable inheritance. Integration was confirmed by DNA gel blot analysis (data not shown). This *ypt1 ARA4*^{Q71L} strain was transformed with the above-mentioned Arabidopsis cDNA expression library. Because both the *ARA4*^{Q71L} and Arabidopsis cDNAs are simultaneously expressed by the *GAL1* promoter, a gene whose product interacts with the Ara4 protein may counteract the harmful effect of *ARA4*^{Q71L} on *ypt1*.

We screened 2 × 10^{5} clones of cDNAs for suppression of the Ara4^{Q71L}-induced growth defect of *ypt1 ARA4*^{Q71L} strain on galactose medium at 30°C. Candidate clones were further tested for plasmid linkage of suppression activity. Among the dozens of candidates that we examined, one cDNA clone (pYES2T1) showed a reproducible remedial effect on the expression of *ARA4*^{Q71L}. The *ypt1 ARA4*^{Q71L} cells harboring this plasmid grew better on galactose medium at 23°C (Figures 4A to 4D) and 15°C (data not shown) than did those containing the vector alone. pYES2T1 also suppressed the growth defect caused by the wild-type *ARA4* that was integrated at the *TRP1* locus but not the growth defect of the *ypt1* mutant at 37°C (data not shown).

Sequence of the Suppressor Gene

Determination of the nucleotide sequence of this clone indicated that the 1622-base cDNA insert contained an open reading frame encoding a protein of 445 amino acid residues (Figure 5). The predicted molecular mass is 49.8 kD. A data base search for similar proteins revealed that this amino acid sequence is highly homologous to the Rab GDIs identified in the animal and fungal kingdoms. The highest identity was found with the GDI for Rab3A from bovine brain (57%; Matsui et al., 1990). Thus, we named this Arabidopsis gene *AtGDI1*. The product of *AtGDI1* showed 52.5% identity with yeast GDI, the gene product of *GDI1/SEC19* (Figure 6; Garrett et al., 1994). An identical but partial sequence was present in the data base of Arabidopsis expressed sequence tags, and similar sequences were found in the rice data base of expressed sequence tags. Homology with AtGDI1 was also detected for Rab escort proteins of mammals and yeast (Waldherr et al., 1993; Fujimura et al., 1994), although the similarity scores were lower.

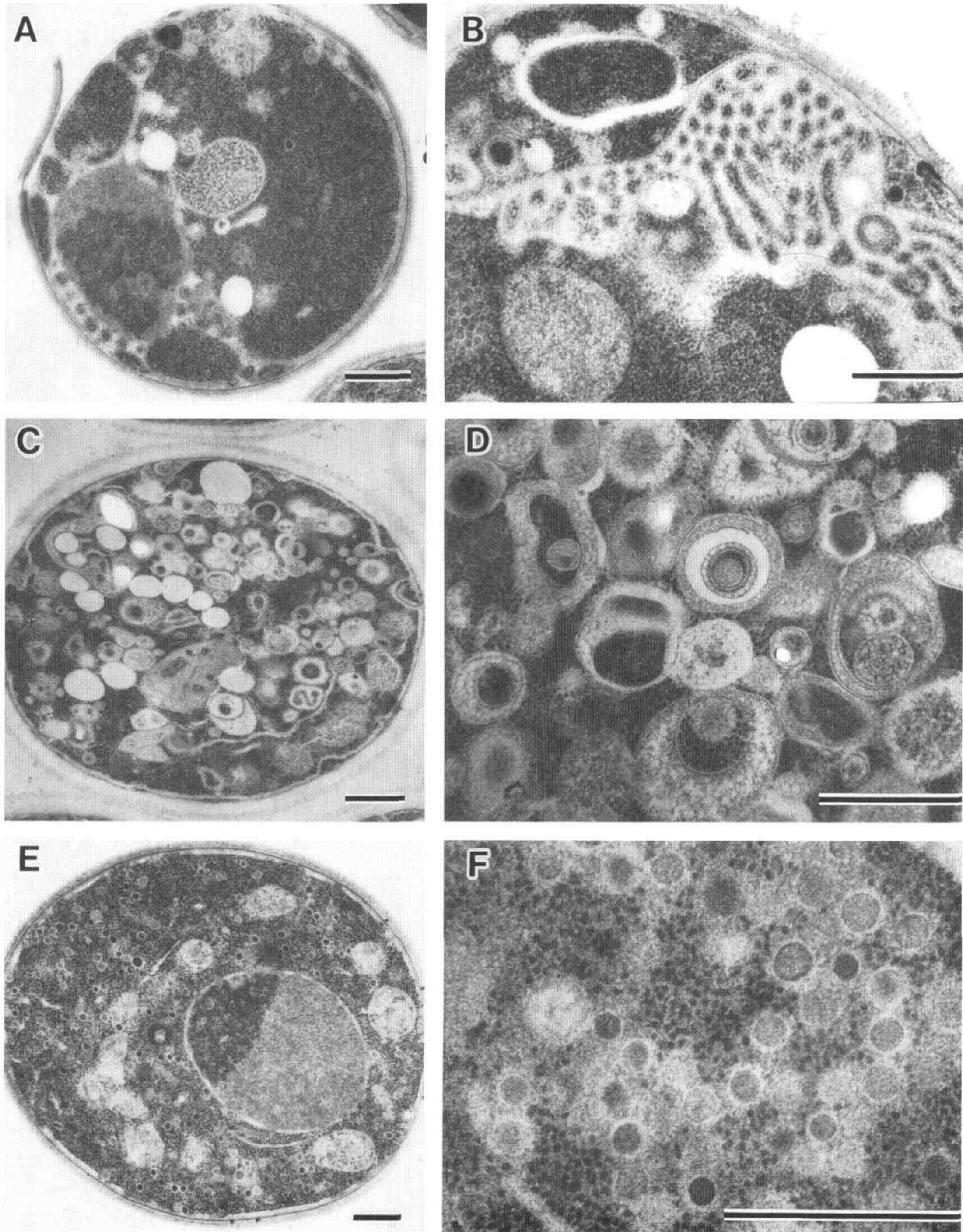

Figure 3. Electron Microscopy of Yeast *ypt* Mutants Expressing *ARA4*^{Q71L}.

(A) and **(B)** *ypt1*^{ts} (TSU3-5D).
(C) and **(D)** *ypt3*^{cs} (YTH12).
(E) and **(F)** *sec4*^{ts} (ANS4-8C).
Cells harboring the plasmid that contained *ARA4*^{Q71L} under the control of the *GAL1* promoter were cultured and subjected to induction of *ARA4*^{Q71L} expression at 23°C by the same procedure as described in the legend to Figure 2. Bars = 0.5 μm.

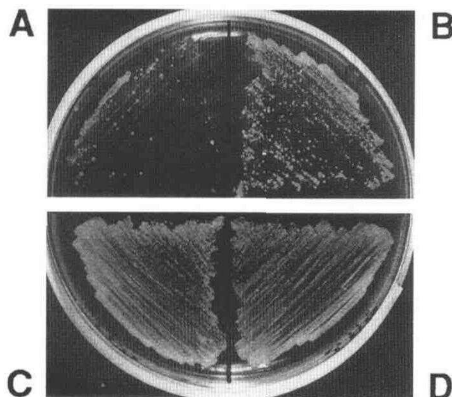

Figure 4. Suppression of the *ARA4*^{Q71L}-Induced Growth Defect by the Arabidopsis cDNA Clone pYES2T1.

(A) *ypt1*^{ts} *ARA4*^{Q71L} cells transformed with vector pYES2.
(B) The same cells as shown in **(A)** transformed with pYES2T1 (*AtGDI1*).
(C) *ypt1*^{ts} (TSU3-5D).
(D) Wild type (ANY21).
Cells in **(A)** to **(D)** were incubated at 23°C for 5 days.

Complementation of a Yeast *sec19* (*gdi1*) Mutant with *AtGDI1*

To examine whether the *AtGDI1* gene product could function as GDI in yeast, we introduced the *AtGDI1* cDNA under control of the *GAL1* promoter (pYES2T1) into the yeast *sec19-1* mutant, which harbors a *ts* allele of *GDI1* (Novick et al., 1980). At the restrictive temperature (37°C), the original *sec19-1* mutant could not grow on either glucose or galactose. However, *sec19-1* cells that had been transformed with pYES2T1 did grow when the expression of *AtGDI1* was induced on galactose (Figure 7). Thus, the *AtGDI1* gene complemented the yeast *sec19-1* *ts* mutation very well, indicating that it can replace the function of yeast GDI in the regulation of yeast Ypt proteins.

Characterization of the *AtGDI1* Gene in Arabidopsis

DNA gel blot analysis was performed with the *AtGDI1* gene. Genomic DNA of Arabidopsis (ecotype Columbia) was digested with EcoRI, BamHI, HindIII, and XhoI, separated on an agarose gel, transferred to a nylon membrane, and hybridized with the full-length *AtGDI1* cDNA and the 0.2-kb PstI-XhoI cDNA fragment, containing mainly the 3′ untranslated region (UTR). The cDNA sequence of *AtGDI1* contains one restriction site for HindIII and one for XhoI. There are no restriction sites for EcoRI or BamHI.

As shown in Figure 8A, the full-length *AtGDI1* cDNA probe hybridized with several fragments under a low-stringency condition. With a high-stringency wash, only one band remained in the lanes of EcoRI and XhoI, but two to three bands were still observed in the lanes of BamHI and HindIII (Figure 8B). These results suggest that in addition to the authentic *AtGDI1* gene that we identified, one or more other genes encode GDI homologs in the genome of Arabidopsis. GDI appears to constitute a small family of genes in Arabidopsis. To confirm this, we repeated the experiment with the 3′ UTR fragment as a probe, which was expected to be more specific to the *AtGDI1* gene. As shown in Figure 8C, two major bands were still seen in all lanes under the low-stringency condition. This finding indicates that a gene closely related to *AtGDI1* exists in Arabidopsis. After the high-stringency wash, single bands remained in all lanes (Figure 8D), indicating that these bands represent the authentic *AtGDI1* gene.

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-103                           GGCACGAGCAATCACCGCCGATTCTCTCTCTCTCTCTCTCGAG
 -60  TCTCTCGACGGTCGCATATCAAAAGTCCATTTCTTTCTTCTCTTTGGAGGATCAGCAACT
   1  ATGGATGAAGAGTACGAAGTCATAGTTCTGGGTACTGGTCTCAAGGAGTGCATCCTTAGT
   1  MetAspGluGluTyrGluValIleValLeuGlyThrGlyLeuLysGluCysIleLeuSer

  61  GGTCTTCTCTCCGTCGATGGTCTTAAGGTACTTCACATGGACAGGAATGACTACTACGGT
  21  GlyLeuLeuSerValAspGlyLeuLysValLeuHisMetAspArgAsnAspTyrTyrGly

 121  GGAGAGTCAACTTCTCTTAATCTCAATCAGCTGTGGAAGAAGTTCAGGGGAGAAGAGAAG
  41  GlyGluSerThrSerLeuAsnLeuAsnGlnLeuTrpLysLysPheArgGlyGluGluLys

 181  GCTCCTGCTCATTTAGGTTCTAGCAGAGACTACAATGTTGACATGATGCCAAAGTTTATG
  61  AraProAlaHisLeuGlySerSerArgAspTyrAsnValAspMetMetProLysPheMet

 241  ATGGCTAACGGAAAGCTTGTTCGTGTCCTTATTCATACGGATGTGACCAAGTACTTGTCT
  81  MetAlaAsnGlyLysLeuValArgValLeuIleHisThrAspValThrLysTyrLeuSer

 301  TTTAAAGCTGTTGATGGAAGCTATGTTTTTGTCCAAGGCAAGGTCCAAAAAGTGCCAGCA
 101  PheLysAlaValAspGlySerTyrValPheValGlnGlyLysValGlnLysValProAla

 361  ACTCCCATGGAGGCCCTCAAATCTCCTCTTATGGGTATTTTTGAGAAACGTCGAGCTGGA
 121  ThrProMetGluAlaLeuLysSerProLeuMetGlyIlePheGluLysArgArgAlaGly

 421  AAGTTTTTCAGCTATGTTCAAGAATACGACGAGAAGGACCCAAAAACACATGATGGAATG
 141  LysPhePheSerTyrValGlnGluTyrAspGluLysAspProLysThrHisAspGlyMet

 481  GATTTGAGGAGAGTTACAACTAAGGATTTGATTGCGAAATTCGGTCTTAAAGAAGATACT
 161  AspLeuArgArgValThrThrLysAspLeuIleAlaLysPheGlyLeuLysGluAspThr

 541  ATTGACTTTATTGGTCATGCAGTGGCACTTCACTGTAATGACAATCATCTCCATCAACCT
 181  IleAspPheIleGlyHisAlaValAlaLeuHisCysAsnAspAsnHisLeuHisGlnPro

 601  GCCTATGATACTGTAATGAGAATGAAGCTCTACGCAGAGTCCCTTGCACGTTTTCAAGGA
 201  AlaTyrAspThrValMetArgMetLysLeuTyrAlaGluSerLeuAlaArgPheGlnGly

 661  GGTTCACCATATATCTATCCTCTCTATGGGTTGGGAGAATTGCCTCAGGCATTTGCACGA
 221  GlySerProTyrIleTyrProLeuTyrGlyLeuGlyGluLeuProGlnAlaPheAlaArg

 721  CTTAGTGCTGTCTATGGTGGGACGTATATGTTGAACAAACCTGAGTGCAAGGTAGAGTTT
 241  LeuSerAlaValTyrGlyGlyThrTyrMetLeuAsnLysProGluCysLysValGluPhe

 781  GACGAGGAGGGTAAGGTTTCTGGTGTTACATCTGAGGGAGAGACTGCCAAATGCAAGAAA
 261  AspGluGluGlyLysValSerGlyValThrSerGluGlyGluThrAlaLysCysLysLys

 841  GTTGTCTGCGATCCTTCTTACCTAACCAACAAGGTCAGGAAGATTGGCAGGGTTGCTCGG
 281  ValValCysAspProSerTyrLeuThrAsnLysValArgLysIleGlyArgValAlaArg

 901  GCGATTGCCATTATGAGCCACCCTATTCCAAACACCAATGATTCTCAGTCGGTGCAAGTC
 301  AlaIleAlaIleMetSerHisProIleProAsnThrAsnAspSerGlnSerValGlnVal

 961  ATTCTACCCCAAAAACAATTGGGCCGCAAATCAGACATGTATGTCTTCTGTTGTTCATAT
 321  IleLeuProGlnLysGlnLeuGlyArgLysSerAspMetTyrValPheCysCysSerTyr

1021  TCACACAACGTTGCTCCCAAGGGAAAGTTCATAGCATTTGTGTCAACAGACGCTGAAACT
 341  SerHisAsnValAlaProLysGlyLysPheIleAlaPheValSerThrAspAlaGluThr

1081  GATAACCCTCAAACCGAGCTCCAGCCTGGAATCGACCTTCTTGGTCCTGTTGATGAGCTG
 361  AspAsnProGlnThrGluLeuGlnProGlyIleAspLeuLeuGlyProValAspGluLeu

1141  TTCTTCGACATCTATGATAGATATGAGCCTGTCAACGAACCCACTTTGGACAACTGCTTC
 381  PhePheAspIleTyrAspArgTyrGluProValAsnGluProThrLeuAspAsnCysPhe

1201  ATATCAACGAGTTATGATGCTACAACACACTTTGACACAACTGTTGTTGATGTGTTGAAC
 401  IleSerThrSerTyrAspAlaThrThrHisPheAspThrThrValValAspValLeuAsn

1261  ATGTATAAATTGATCACCGGAAAGGAACTGGATCTAAGTGTGGATCTTAACGCAGCTAGT
 421  MetTyrLysLeuIleThrGlyLysGluLeuAspLeuSerValAspLeuAsnAlaAlaSer

1321  GCTGCAGAGGAGGAATGATGGAAAATATTGCAAGCTCTGTCTCTCTCTGTTAGTCACCAA
 441  AlaAlaGluGluGluEnd
1381  CATGATGTAACCGGTTCTTTTCTCCCTCTTCTTAAAAGTTGTCTCACCAAATGTTCTGTT
1441  AAAATACTAAATCAAACCGATCTCATCATGCGTACCTATTCCCGGTTAAAAAAAAAAAAA
1501  AAAAAAAAAAAAAAAAAA
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Figure 5. Nucleotide Sequence and Deduced Amino Acid Sequence of the *AtGDI1* Gene.

The entire sequence of the cDNA insert of pYES2T1 is shown. The DDBJ, EMBL, and GenBank accession number is D83531.

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AtGDI1          1 MDEE-----YEVIVLGTGLKE[illegible]
GDI for Rab3A   1 MDEE-----YDVIVLGTGL[illegible]
Gdi1            1 [illegible]TIDTD[illegible]DVIVLGTG[illegible]TECIL[illegible]

AtGDI1         46 LN-[illegible]RGE----[illegible]KA[illegible]AHL[illegible]SS[illegible]RV[illegible]
GDI for Rab3A  46 I[illegible]P[illegible]EEL[illegible]QLL----[illegible]GP[illegible]ETM[illegible]RG[illegible]KM[illegible]
Gdi1           51 V[illegible]-[illegible]KQNPISK[illegible]ERESKF[illegible]KD[illegible]TNI[illegible]

AtGDI1         91 [illegible]PME[illegible]AG
GDI for Rab3A  92 LY[illegible]FR
Gdi1          100 [illegible]MK

AtGDI1        141 K[illegible]FSY[illegible]QE[illegible]DEKDPKT[illegible]RRV[illegible]TK[illegible]LIA[illegible]
GDI for Rab3A 142 [illegible]VF[illegible]ANF[illegible]DPKT[illegible]PQNTS[illegible]
Gdi1          150 KF[illegible]EWISS[illegible]LS[illegible]DKN[illegible]

AtGDI1        191 HC[illegible]NH[illegible]YD[illegible]FQ[illegible]
GDI for Rab3A 192 YRT[illegible]CLE[illegible]IN[illegible]IKLY[illegible]
Gdi1          200 WT[illegible]RPSFE[illegible]CQ[illegible]VA[illegible]

AtGDI1        241 LSA[illegible]YGGTYMLNK[illegible]ECK[illegible]EFDE[illegible]LT
GDI for Rab3A 241 LSAIYGGTYMLNK[illegible]DII--M[illegible]
Gdi1          249 [illegible]DT[illegible]LYKKDT[illegible]TKLG[illegible]APL[illegible]

AtGDI1        290 N[illegible]SHPIKNTN[illegible]SQ[illegible]FCC
GDI for Rab3A 289 DR[illegible]CMI
Gdi1          299 E[illegible]CKST[illegible]VP[illegible]SN[illegible]AIV

AtGDI1        339 [illegible]
GDI for Rab3A 338 [illegible]
Gdi1          349 [illegible]F

AtGDI1        389 [illegible]VNEPTL[illegible]SYDATTH[illegible]LNM[illegible]KE[illegible]---[illegible]SVD
GDI for Rab3A 388 [illegible]ESQV[illegible]SYDATTHFET[illegible]CN[illegible]SAF[illegible]FENM[illegible]RK
Gdi1          399 [illegible]IYL[illegible]SYDASS[illegible]SMTD[illegible]HP[illegible]V---[illegible]QR

AtGDI1        436 L[illegible]AASAA[illegible]EE
GDI for Rab3A 438 [illegible]DVFGEAD[illegible]
Gdi1          446 [illegible]EQ----[illegible]K[illegible]
```

Figure 6. Amino Acid Sequence Comparison of AtGDI1, GDI for Rab3A, and Yeast GDI (Gdi1).

Black boxes indicate conserved amino acid residues.

To examine the expression of *AtGDI1* in suspension culture and various tissues of Arabidopsis, RNA gel blot analysis was performed. Total RNA was isolated from a suspension culture, roots, cotyledons, seedlings, rosettes, stems, young siliques, and floral buds. Isolated RNA was separated by electrophoresis, transferred to a nylon membrane, and hybridized with the 3′ UTR fragment of *AtGDI1*. The membrane was washed under the same conditions as in the high-stringency wash of the DNA gel blot (Figure 8D). As shown in Figure 9, *AtGDI1* was expressed in almost all of the tissues of Arabidopsis. The length of the mRNA was ~1.8 kb. Expression was high in the suspension culture, roots, rosettes, stems, and floral buds, lower in cotyledons and siliques, and even lower in seedlings. It should be noted here that the probe might be hybridizing with two bands that have slightly different mobilities even under this most stringent condition. Because DNA–RNA hybridization is more stable than DNA–DNA hybridization, we cannot rule out the possibility that the result in Figure 9 represents the expression of two closely related *AtGDI* genes. Even so, a marked difference in the expression pattern was not seen.

DISCUSSION

The discovery of the involvement of small GTPases in the secretory pathway in the late 1980s (Salminen and Novick, 1987; Schmitt et al., 1988; Segev et al., 1988; Nakano and Muramatsu, 1989) introduced the concept that molecular switches regulate membrane traffic. The Rab/Ypt proteins are now understood to be involved in targeting and/or fusion of transport vesicles to the correct acceptor membrane. The conformational change between the GTP- and GDP-bound states is critical in such roles, as with other members of the Ras superfamily. Conversion between these states is not reversible but rather is executed by a cyclic reaction involving guanine nucleotide exchange from GDP to GTP and hydrolysis of GTP. Regulator molecules, such as GAPs, GEFs, and GDIs, are essential for driving this so-called GTPase cycle. Information regarding these regulators has been accumulating from studies with yeast and mammalian cells. In plants, however, although many Rab proteins have been identified, information on their regulator molecules is almost completely lacking. This report

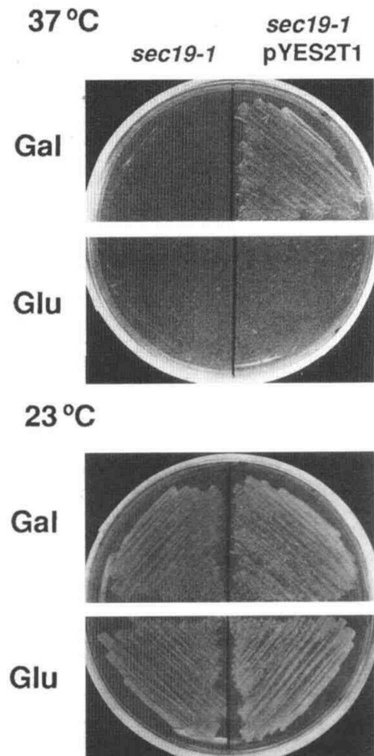

Figure 7. *AtGDI1* Complements the Yeast *sec19-1* Mutant.

sec19-1 (*gdi1*) cells transformed with pYES2T1 (*AtGDI1*) were streaked on galactose (Gal; YPGS) and glucose (Glu; YPD) plates and incubated at 37 and 23°C for 3 days.

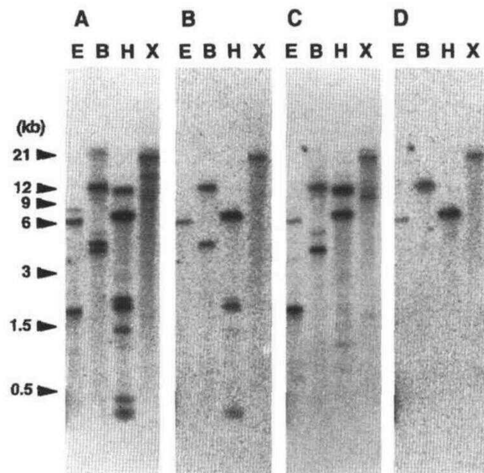

Figure 8. DNA Gel Blot Analysis of *AtGDI1*.

Genomic DNA was prepared from Arabidopsis (ecotype Columbia), digested with EcoRI (E), BamHI (B), HindIII (H), and XhoI (X), and subjected to gel blot hybridization. The full-length *AtGDI1* cDNA (**[A]** and **[B]**) and the 0.2-kb PstI-XhoI fragment, containing mainly the 3' UTR (**[C]** and **[D]**), were used as probes.
(A) The full-length *AtGDI1* cDNA probe: low-stringency condition.
(B) The full-length *AtGDI1* cDNA probe: high-stringency condition.
(C) The 3' UTR probe: low-stringency condition.
(D) The 3' UTR probe: high-stringency condition.
Numbers at left indicate molecular length markers in kilobases.

describes the isolation of Arabidopsis Rab GDI by a novel method that takes advantage of yeast genetics.

Identification of *AtGDI1*

Our hunt for Arabidopsis Rab regulators began with an unexpected finding. In an attempt to test whether any of the Arabidopsis *ARA* genes, members of the *rab*/*YPT* family, could complement yeast *ypt* mutants, we found that *ARA4*, particularly its Q71L mutant version, causes marked aggravation of the mutant phenotypes of *ypt1*, *ypt3*, and *sec4*. For example, *ypt1* cells, which are defective in ER-to-Golgi transport at the restrictive temperature, accumulate the ER membrane even at the permissive temperature when $ARA4^{Q71L}$ is expressed. The accumulation of Golgi membranes and secretory vesicles by $ARA4^{Q71L}$ was also observed in *ypt3* and *sec4* mutant cells, respectively, under permissive growth conditions. Apparently, the weak point of each mutant is exaggerated by the expression of $ARA4^{Q71L}$. We reasoned that this effect could occur because the expression of the foreign Rab protein ($Ara4^{Q71L}$) interferes with or competes for the bona fide Ypt regulator(s) or effector(s) in yeast. If so, coexpression of the target or any other plant molecule that correctly interacts with the Ara4 protein may suppress the growth defect in yeast.

To substantiate this idea, we constructed an Arabidopsis cDNA library, which can be expressed in yeast by using the regulatable *GAL1* promoter, introduced this cDNA library into the *ypt1* mutant expressing $ARA4^{Q71L}$, and screened for clones that remedy the growth defect. Using this screening procedure, we obtained a cDNA clone that encodes an Arabidopsis counterpart of the Rab GDI. That this gene, named *AtGDI1*, is in fact derived from Arabidopsis was confirmed by DNA and RNA gel blot hybridization analyses as well as by detection of the same sequence in the expressed sequence tag data base of Arabidopsis.

Overexpression of Ara4 harms yeast cells, probably by titrating yeast Ypt regulators. Kamada et al. (1992), working with transgenic tobacco plants, reported that tobacco plants expressing a rice Rab protein, Rgp1, showed abnormal phenotypes, including the loss of apical dominance and altered morphology of leaves and flowers. This could be interpreted as a consequence of the competition between Rgp1 and tobacco Rab proteins for Rab regulator(s), which leads to pleiotropic phenotypes.

AtGDI1 Is an Ara GDI

GDI was originally identified as a protein that inhibits the dissociation of GDP from Rab3A (Sasaki et al., 1990). The current understanding is that the function of GDI is to detach the GDP-bound form of a Rab protein from the membrane and provide a cytosolic pool for the next round of the Rab GTPase cycle (Araki et al., 1990; Ullrich et al., 1993, 1994; Dirac-Svejstrup et al., 1994; Soldati et al., 1994). An interesting feature of GDI is its broad specificity. The GDI for Rab3A can act on all of the examined Rab proteins (Rab1A, Rab2, Rab3A, Rab4B, Rab5, Rab6, Rab7, Rab8, Rab9, Rab10, and Rab11) in mammals and also on Sec4 in yeast, and has been renamed "Rab GDI" collectively (Sasaki et al., 1991; Garret et al., 1993; Ullrich et al., 1993). Yeast GDI encoded by *GDI1*/*SEC19* has been iden-

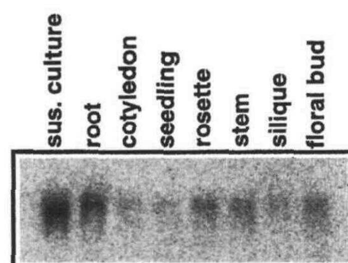

Figure 9. Expression of *AtGDI1* in Arabidopsis tissues.

Total RNA was extracted from a suspension (sus.) culture, roots, cotyledons, seedlings, rosettes, stems, young siliques, and floral buds of Arabidopsis and subjected to gel blot hybridization, with the PstI-XhoI fragment of the *AtGDI1* cDNA used as a probe.

tified by homology. Its mutant phenotype has been shown to be quite pleiotropic; the conditional mutants (*sec19*^{ts} and *GAL1-GDI1* shutoff) accumulate ER, the Golgi apparatus, and secretory vesicles under restrictive conditions (Novick et al., 1980; Garrett et al., 1994). If overproduction of the plant *ARA4* gene in yeast titrates the yeast GDI, it should cause similar pleiotropic defects in multiple steps of vesicular transport. Presumably, the decrease in the functional yeast GDI and one of the *ypt* mutations, *ypt1*, *ypt3*, or *sec4*, produced a specific block of transport in each mutant strain.

The fact that the Arabidopsis *AtGDI1* gene complemented the *ts* mutant allele of yeast *GDI1* indicates that AtGDI1 in fact acts as a GDI in yeast. This functional conservation is another remarkable property of GDIs. It has also been reported that the Rab GDI from bovine and Drosophila interact with the yeast Sec4 protein (Sasaki et al., 1991; Garrett et al., 1993). Thus, GDI must be a very well conserved general machinery of Rab regulation. There are at least three possible reasons why the expression of *AtGDI1* suppressed the deleterious effect of *ARA4*^{Q71L} in *ypt1*. First, *AtGDI1* could suppress the *ypt1* lesion by itself. This was ruled out because *AtGDI1* did not rescue the growth of *ypt1* cells without *ARA4*^{Q71L} at a high temperature. Second, *AtGDI1* might provide functional GDI when the yeast GDI is recruited to cope with the foreign Rab, Ara4^{Q71L}. This explanation is also unlikely because the yeast system would prefer the yeast GDI to the plant GDI, even though they are similar. The third and most likely explanation is that AtGDI1 directly interacts with Ara4^{Q71L} and thus eliminates the cause of the growth inhibition.

A remaining question is whether these two molecules indeed interact physically. The Q71L mutation that we used in this study stabilizes the GTP-bound state of the Ara4 protein (Anai et al., 1994). Biochemical experiments showed that GDI bound only to the GDP form (Araki et al., 1990). However, our observations strongly suggest that AtGDI1 and Ara4^{Q71L} interact directly. In the case of the Rho/Rac family, another subfamily of the Ras superfamily, GDI has been shown to interact not only with the GDP-bound form but also with the GTP-bound form (Hart et al., 1992; Leonard et al., 1992; Chuang et al., 1993; Sasaki et al., 1993). The molecular basis of the interaction between AtGDI1 and Ara4^{Q71L} should be examined further.

The *GDI* Gene Family

The results of DNA gel blot analysis suggested that there are two or three genes for GDI in Arabidopsis. In rat and mouse, there are at least five GDI species (Janoueix-Lerosey et al., 1995), whereas yeast has only one. This may imply that GDI has diverse functions, especially in multicellular organisms (see Pfeffer et al., 1995). RNA gel blot analysis of *AtGDI1* indicates that it is almost ubiquitously expressed in all of the tissues examined. However, there is some difference in the expression level. For example, the amount of *AtGDI1* mRNA is lower in seedlings, cotyledons, and young siliques. These organs contain embryonic or young developing tissues, a fact suggesting that switching of gene expression might take place among the Arabidopsis *AtGDI* gene family. In the future, it will be intriguing to explore their functional differentiation from the perspective of the roles of the Rab proteins in development.

A New Method for Identifying Interacting Molecules by Using Yeast

To identify molecules that react with a particular protein, yeast cells have been successfully used as a test tube in the two-hybrid approach (Fields and Song, 1989). Here, we present another method in which yeast is used to identify interacting molecules. If the expression of a gene of interest gives a growth phenotype in yeast, suppressor genes can be selected by convenient plate work. When the expression of a foreign gene and a yeast mutation gives a synthetic lethal effect, as in this study, the genetic system in yeast provides a powerful screening method to identify interaction. The expression of genes whose products can bind to the gene product that causes the harmful effect should suppress the lethal phenotype. In plant research, it has been practically impossible to knock out a desired gene. Therefore, functional analyses of plant genes depend on in vitro assays and expression systems in other organisms, like yeast. The method that we present here takes advantage of yeast genetics in the search for interacting molecules and is advantageous in that physiological phenotypes can always be monitored during functional screening. This approach to identifying interacting molecules would facilitate the study of plant genes.

METHODS

Yeast Strains and Culture Conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 2. Yeast cells were grown in YPD (1% [w/v] Bacto yeast extract [Difco, Detroit, MI], 2% [w/v] polypeptone [Nihon Seiyaku, Tokyo, Japan], and 2% [w/v] glucose) or in MCD (0.67% yeast nitrogen base without amino acids [Difco], 0.5% casamino acids [Difco], and 2% glucose), supplemented appropriately. For derepression of the galactokinase *GAL1* promoter, glucose in these media was replaced by 5% (w/v) galactose and 0.2% (w/v) sucrose (YPGS or MCGS).

Expression of *ARA4* in *ypt* Mutants

DNA fragments containing the wild-type *ARA2*, *ARA3*, and *ARA4* genes; Q72L and N126I mutant versions of *ARA2* (*ARA2*^{Q72L} and *ARA2*^{N126I}); and the Q71L and N125I mutant versions of *ARA4* (*ARA4*^{Q71L} and *ARA4*^{N125I}) were placed under the *GAL1* promoter in the single-copy plasmid YCpUG-578T (from H. Qadota, University of Tokyo, Tokyo, Japan), a derivative of pRS316 (Sikorski and Hieter, 1989) containing the

Table 2. Yeast Strains Used in This Study

Strain	Genotype	Reference
TSU3-5D	*MATα ypt1*^{ts} *ura3 leu2*	H.D. Schmitt[a]
YTH12	*MAT***a** *ypt31*^{E49Qcs}*-LEU2 ypt32::HIS4 ura3 leu2 his4 lys2*	D. Gallwitz[a]
ANS4-8C	*MATα sec4-2 ura3 leu2 his*	Nakano and Muramatsu (1989)
GL72	*MATα ypt6::LEU2 leu2 his3*	H.D. Schmitt[a]
VAQ204-9C	*MAT***a** *ypt7/vam4-1 ura3 leu2 ade2*	Wada et al. (1996)
ANS19-4A	*MAT***a** *gdi1/sec19-1 ura3 leu2 his*	Nakano and Muramatsu (1989)
ANY21	*MAT***a** *ura3 leu2 trp1 his3 his4*	Nakano and Muramatsu (1989)

[a] Max-Planck Institute for Biophysical Chemistry, Göttingen, Germany.

CMK1 terminator and the *URA3* marker. This plasmid was introduced into TSU3-5D (*ypt1*^{ts}), YTH12 (*ypt31*^{cs} *ypt32Δ*), ANS4-8C (*sec4-2*), GL72 (*ypt6*), VAQ204-9C (*ypt7*), and ANY21 (wild-type) cells. After selection of transformants on minimal glucose medium (MCD minus uracil), cells were streaked on minimal galactose plates (MCGS minus uracil) and incubated at 15, 23, 30, and 35.5°C.

Electron Microscopy

Samples for transmission electron microscopy were prepared as previously described (Sun et al., 1992; Ueda et al., 1996). After centrifugation, pellets of cells were mounted on copper meshes to form a thin layer and plunged into liquid propane. Frozen cells were transferred to 4% OsO_4 in anhydrous acetone that had been precooled in a dry ice/acetone bath and kept at −80°C for 48 hr. Samples were held at −35°C for 2 hr, at 4°C for 2 hr, and then at room temperature for 2 hr. After a wash with anhydrous acetone, samples were embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate.

Construction of an *Arabidopsis thaliana* cDNA Library to Be Expressed in Yeast

Poly(A)$^+$ RNAs were isolated from whole seedlings of 10-day-old Arabidopsis (ecotype Columbia) by using an mRNA separator kit (Clontech, Palo Alto, CA). cDNAs were synthesized and ligated to EcoRI adapters according to the instructions provided with the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). After digestion with XhoI, cDNAs were inserted into the EcoRI-XhoI sites of the yeast expression vector pYES2 (Invitrogen, Leek, The Netherlands), which is a 2-μm–based multicopy plasmid carrying the *GAL1* promoter and a selectable *URA3* marker. These constructs were introduced into strain XL1-Blue MRF′ of *Escherichia coli* (Stratagene) by electroporation. Approximately 1.8 × 10^6 independent clones were pooled and amplified in 250 mL of culture to prepare plasmid DNA.

Screening of the Arabidopsis cDNA That Suppresses *ARA4*^{Q71L} in Yeast

GAL1-ARA4^{Q71L} was integrated with the *HIS3* marker into the *TRP1* locus of the *ypt1*^{ts} cells (TSU3-5D). Integration of this fragment was confirmed by DNA gel blot analysis. The plasmid library was introduced into *ypt1*^{ts} *trp1*::*GAL1*-ARA4^{Q71L} cells. Ura$^+$ transformants were selected on minimal galactose plates at 30°C. Growing colonies were streaked on galactose/FOA plates (MCGS plus 0.1% fluoroorotic acid and uracil), which compelled the loss of cDNA-containing plasmid. Clones that showed significantly slower growth on the FOA medium than on the minimal galactose medium were isolated. cDNA plasmids were isolated from these candidates and reintroduced into *ypt1*^{ts} *trp1*::*GAL1-ARA4*^{Q71L} cells. One plasmid (pYES2T1) reproducibly restored growth on galactose. The cDNA insert was excised with EcoRI and XbaI, subcloned into the pBluescript II SK+ vector (Stratagene), and sequenced with an automated sequencer (model 4000; LICOR, Lincoln, NE) by using a SequiTherm Cycle-Sequencing Kit-LC (Epicenter Technology, Madison, WI). The nucleotide sequence and deduced amino acid sequence were compared with sequences in the GenBank, EMBL, and DDBJ data bases.

DNA Gel Blot Analysis

Genomic DNA was prepared from the Columbia ecotype and digested with EcoRI, BamHI, HindIII, and XhoI. Digested DNA was electrophoresed in 0.7% agarose gel. DNA was denatured and transferred to a nylon membrane filter (Hybond-N$^+$; Amersham), according to the manufacturer's instructions. The full-length *AtGDI1* cDNA and the 0.2-kb PstI-XhoI (the XhoI site in the vector) cDNA fragment mainly containing the 3′ untranslated region (UTR) were used as probe templates. The filter was hybridized with ^{32}P-labeled, random-primed cDNA fragments in a hybridization solution containing 6 × SSPE (1 × SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, and 1 mM Na_2EDTA, pH 7.7), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.5% SDS, and 20 μg/mL of salmon sperm DNA at 65°C for 16 hr. The filter was washed twice with 2 × SSPE and 0.1% SDS for 5 min at room temperature, and twice for 30 min at 65°C (low-stringency condition). After exposure to an imaging plate for appropriate durations, the same filter was washed twice with 0.1 × SSPE and 0.1% SDS for 30 min at 65°C (high-stringency condition). For visualization of the radioautogram, an Imaging Plate Scanner BAS1000 (Fuji Film Co., Tokyo, Japan) was used.

RNA Gel Blot Analysis

Total RNA was isolated from a suspension culture (from M. Umeda, University of Tokyo, Tokyo, Japan), roots, cotyledons, seedlings, rosettes, stems, young siliques, and floral buds of Arabidopsis (ecotype Columbia) by the phenol–SDS method (Palmiter, 1974). After electrophoresis of 10 μg of total RNA per lane in a 1.2% formaldehyde–agarose

gel, RNA was transferred to a Hybond-N+ membrane and hybridized with the ^{32}P-labeled, random-primed PstI-XhoI *AtGDI1* cDNA fragment (3′ UTR) according to the manufacturer's specifications. Hybridization was performed at 42°C. The filter was washed twice with 2 × SSPE and 0.1% SDS for 5 min at room temperature, twice with 2 × SSPE and 0.1% SDS for 30 min at 65°C, and twice with 0.1 × SSPE and 0.1% SDS for 30 min at 65°C. For visualization of the filter, an Imaging Plate Scanner BAS1000 was used.

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