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Characterization of Membrane-Bound Small GTP-Binding Proteins from *Nicotiana tabacum*[1]

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We have cloned nine cDNAs encoding small GTP-binding proteins from leaf cDNA libraries of tobacco (*Nicotiana tabacum*). These cDNAs encode distinct proteins (22–25 kD) that display different levels of identity with members of the mammalian Rab family: Nt-Rab6 with Rab6 (83%), Nt-Rab7a-c with Rab7 (63–70%), and Nt-Rab11a-e with Rab11 (53–69%). Functionally important regions of these proteins, including the "effector binding" domain, the C-terminal Cys residues for membrane attachment, and the four regions involved in GTP-binding and hydrolysis, are highly conserved. Northern and western blot analyses show that these genes are expressed, although at slightly different levels, in all plant tissues examined. We demonstrate that the plant Rab5, Rab6, and Rab11 proteins, similar to their mammalian and yeast counterparts, are tightly bound to membranes and that they exhibit different solubilization characteristics. Furthermore, we show that the yeast GTPase-activating protein Gyp6, shown to be specifically required to control the GTP hydrolysis of the yeast Ypt6 protein, could interact with tobacco GTP-binding proteins. It increases in vitro the GTP hydrolysis rate of the wild-type Nt-Rab7 protein. In addition, it also increases, at different levels, the GTP hydrolysis rates of a Nt-Rab7m protein with a Rab6 effector domain and of two other chimaeric Nt-Rab6/Nt-Rab7 proteins. However, it does not interact with the wild-type Nt-Rab6 protein, which is most similar to the yeast Ypt6 protein.

In eukaryotic cells a superfamily of small GTP-binding proteins has been described. The members of this family are thought to regulate diverse cellular responses, including signal transduction (Ras subfamily), cytoskeletal organization (Rho subfamily), and vesicle trafficking (Rab/Ypt subfamily; Hall, 1990). A number of genes encoding small GTP-binding proteins have been isolated from a variety of plant species. Except for the pea *Rho1* gene (encoding a Rho-like protein; Yang and Watson, 1993) and the tomato and tobacco *Ran* genes (encoding nuclear localized, soluble proteins; Ach and Gruissem, 1994; Merkle et al., 1994), all other plant genes encode proteins that show similarity to different members of the mammalian/yeast Rab/Ypt proteins (for a review, see Terryn et al., 1993).

Morphological and biochemical studies indicate that different members of the Rab/Ypt subfamily have a distinct subcellular distribution and each member is associated with a particular intracellular compartment along the exocytic and endocytic pathways. For example, Ypt1 is associated with the Golgi and the ER-Golgi carrier vesicles, Rab5 is associated with early endosomes and the plasma membrane (Chavrier et al., 1990a), and Rab7 and Rab9 are localized to late endosomes (Chavrier et al., 1990a; Lombardi et al., 1993). With the development of in vitro assays, specific functions have been assigned to certain members of the Rab/Ypt proteins. It has been shown that Sar1 is required for vesicle formation, whereas Ypt1 is involved in targeting and/or fusion of ER-derived vesicles with the Golgi (Pryer et al., 1992; Barlowe et al., 1993). Rab5 was found to control early endosome fusion (Gorvel et al., 1991) and has regulatory functions in the early endocytic pathway (Bucci et al., 1992). Rab9 functions in transport between late endosomes and the trans-Golgi network (Lombardi et al., 1993), whereas Rab8 is involved in vesicular traffic between the trans-Golgi network and the basolateral plasma membrane (Huber et al., 1993).

Like all other members of the Ras superfamily, the members of the Rab/Ypt family have the ability to bind guanine nucleotides and to hydrolyze GTP. However, the intrinsic nucleotide exchange and GTP hydrolysis activities of these proteins are very low. It has been shown recently that these activities of the Rab/Ypt proteins are modulated by interaction with other accessory proteins, including GAPs (Strom et al., 1993), guanine nucleotide exchange factors (Burton et al., 1993), and GDP dissociation inhibitors (Soldati et al., 1993). These findings are important since (a) they couple GTP-binding and hydrolysis to membrane trafficking and recycling and suggest that (b) different Rab/Ypt proteins, together with the identified accessory proteins, are essential components of the intracellular vesicle transport (Balch, 1990; Pfeffer, 1992; Novick and Brennwald, 1993).

Our knowledge of the plant Rab/Ypt-like proteins is also rapidly increasing. Nt-Rab5 and Np-Rab11 (formerly designated Np-Ypt3) have been cloned from *Nicotiana tabacum* and *Nicotiana plumbaginifolia* (Dallmann et al., 1992). Furthermore, various members of a multigene family encoding Rab/Ypt-like proteins have been cloned from *Arabidopsis thaliana*, from pea, and from several other species (re-

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Abbreviations: GAP, GTPase-activating protein; GST, glutathione *S*-transferase; NP-40, Nonidet P-40.

viewed by Terryn et al., 1993). Analysis of transgenic plants expressing cDNAs (encoding different plant Rab/Ypt-like proteins) in antisense orientation also resulted in interesting findings. Results reported by Cheon et al. (1993) indicate that two Rab/Ypt-like proteins are essential for the development of the peribacteroid membrane compartment during root nodule symbiosis in soybean. Transgenic tobacco plants expressing *rgp1* (a rice gene encoding a Rab/Ypt-like protein similar to Rab11) in antisense orientation also showed altered phenotypes, including reduced apical dominance and tillering (Kamada et al., 1992). In addition, the identification of a gene from *A. thaliana* homologous to SAR1 from yeast (d'Enfert et al., 1992) and the fact that plant genes similar to the mammalian Rab1 and Rab6 could functionally complement null mutants of the yeast *YPT1* (Palme et al., 1993) and *YPT6* genes (Bednarek et al., 1994) suggest that the mechanism regulating vesicular transport is conserved among these eukaryotes.

Although these results provide circumstantial evidence for the possible function of certain small GTP-binding proteins, they yield only limited information about (a) their expression levels, (b) their localization within the plant cell, and (c) their interaction with accessory proteins like GAPs and GDP dissociation inhibitors. It should be noted in this respect that a characteristic feature of Rab/Ypt proteins is the posttranslational prenylation, which anchors these proteins to different membranes in mammalian and yeast cells (Magee and Newman, 1992). Plant Rab/Ypt-like proteins, similar to their mammalian/yeast counterparts, contain the CXC domain shown to be essential for membrane attachment (Terryn et al., 1993). In addition, Randall et al. (1993) have demonstrated the activity of a geranylgeranyl:protein transferase in tobacco cells.

Here we report the isolation of nine cDNA clones that encode proteins displaying high similarity to the mammalian Rab6, Rab7, and Rab11 small GTP-binding proteins. Our results show that (a) the tobacco Rab5 protein (the cDNA of which was cloned earlier; Dallmann et al., 1992) and the tobacco Rab6 and Rab11 proteins are membrane bound, (b) they have different solubilization properties, and (c) the tobacco Rab7 but not the tobacco Rab6 protein can interact with the yeast Ypt6-specific GAP, Gyp6.

MATERIALS AND METHODS

cDNA Library Screening, DNA Sequencing

Two 24-mer degenerate oligonucleotides corresponding to the highly conserved IWDTAGQE motif of small GTP-binding proteins were chemically synthesised using an Applied Biosystems 380A DNA synthesizer and oligomer A, 5'-AT(ACT) TGG GAT AC(AGCT) GC(AGCT) GG(AGCT) CAA GAA-3'; and oligomer B, 5'-AT(ACT) TGG GAC AC(AGCT) GC(AGCT) GG(AGCT) CAG GAG-3'. *Nicotiana tabacum* leaf cDNA library constructed in λZAPII was purchased from Stratagene. Recombinant clones (2.5×10^8) were screened by plaque hybridization using a 1:1 ratio of the two degenerate oligonucleotides as probes, which were end labeled with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase. Filters were prehybridized for 6 h at 52°C in 6× SSC, 5× Denhardt's solution, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA. Hybridization was performed in the same solution supplemented with 2.5×10^6 cpm/mL of labeled probe for 20 h at 52°C. Filters were washed with 6× SSC, 0.1% SDS for 1 h at room temperature and twice for 10 min at 52°C with 1× SSC, 0.1% SDS. After the filters were washed, they were air dried and autoradiographed. Positive λZAPII clones were isolated and processed to Bluescript phagemids. Nucleotide sequences of the isolated cDNA clones were determined by the dideoxy chain termination method (Sanger et al., 1977).

Recombinant DNA Manipulation, Mutagenesis

All standard DNA techniques were performed according to the method of Sambrook et al. (1989) unless otherwise indicated. We used two successive rounds of PCR and specific overlapping oligonucleotides to produce the mutant Nt-Rab7m cDNA and the chimaeric clones containing different segments of the Nt-Rab7a and Nt-Rab6 cDNAs. In the first PCR round cDNA fragments coding for the N-terminal and the C-terminal parts of the mutant or chimaeric proteins were amplified and isolated. A second PCR round was then performed by using these various fragments (containing a 15-bp-long overlapping region at the site of mutation/fusion) and primers specific for the 5' and 3' borders of the corresponding cDNAs. The isolated PCR products encoding the complete mutated or chimaeric proteins were cloned in pQE expression vectors (Qiagen, Chatsworth, CA) and sequenced.

RNA Isolation and Northern Hybridization

Total RNA was isolated, as described by Nagy et al. (1987), from different tissues of mature flowering tobacco plants grown in a greenhouse under 16-h light/8-h dark cycles. For northern hybridization experiments, 20 μg of total RNA was denatured and fractionated on 1% agarose gels containing formaldehyde. Gels were blotted onto nylon membrane filters (Hybond-N, Amersham). We determined the quality of the isolated total RNA and the loading of gels by visualizing the separated RNAs by ethidium bromide fluorescence and hybridizing the filters with labeled rRNA probe. After UV cross-linking and prehybridizing, the filters were hybridized using probes labeled with [α-^{32}P]dATP (Amersham) by random priming as described by Dallmann et al. (1992). The different probes used represented the 3' terminal variable regions of the isolated cDNAs. Filter washing was performed for 1 h at room temperature and subsequently twice at 65°C with 0.1× SSC, 0.1% SDS.

Expression of Proteins in *Escherichia coli*, Immunization

The GST Gene Fusion System pGEX-2T (Pharmacia) was used to express the proteins encoded by the full-length *Nt-Rab5* and *Np-Rab11* (formerly called *Np-Ypt3*) cDNAs as described by Dallmann et al. (1992). Growth, induction, preparation of cell extracts, purification of overexpressed proteins by affinity chromatography on GSH Sepharose 4B

(Pharmacia), and cleavage of the GST fusion proteins with thrombin were performed according to the manufacturers' protocols. The GST fusion part was removed by another affinity chromatography on GSH Sepharose 4B.

For overexpression in *E. coli*, the tobacco cDNA clones *Nt-Rab6*, *Nt-Rab7*, *Nt-Rab7m*, *Nt-Rab76m*, and *Nt-Rab67m* were subcloned into the pQE9 expression vector (Qiagen) and expressed in the M15[pREP4] strain. The overexpressed proteins were purified by Ni-chelate affinity chromatography. The recombinant yeast Ypt6 protein was a generous gift from M. Strom. The yeast Gyp6 cDNA was cloned by PCR from yeast DNA, subcloned into the pET 11 expression vector (Novagen, Madison, WI), and expressed in the BL21[DE3] strain.

The overexpressed plant proteins were purified by preparative SDS-PAGE and injected into mice with Freund's complete adjuvant. The antisera obtained were purified by affinity chromatography using the purified plant GTP-binding proteins covalently coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia).

Protein Isolation and Immunoblot Analysis

Total protein extracts from different tobacco tissues were prepared in a buffer containing 400 mM mannitol, 25 mM Tris-HCl, 5 mM KCl, 2 mM EDTA, 28 mM 2-mercaptoethanol, 0.1 mM 2-mercaptobenzothiazole, 5 mM 6-aminocaproic acid, 1 mM benzamidine, 2 mM PMSF, 1 μg/mL antipain, and 1 μg/mL leupeptin, pH 7.8. The homogenate was filtered through Miracloth (Calbiochem) and fractionated by successive centrifugation steps at 3,000*g* and 20,000*g* for 10 min at 4°C. The supernatant was further fractionated by centrifugation at 150,000*g* for 1 h at 4°C yielding the microsomal pellet and the cytosolic supernatant fraction. Pellet fractions and the TCA-precipitated supernatant fractions were then dissolved in SDS sample buffer, denatured, and analyzed on 15% SDS-PAGE (Laemmli, 1970). The separated proteins were transferred electrophoretically to polyvinylidene difluoride membranes (Immobilon P, Millipore) and incubated with 500-fold diluted, affinity-purified, antisera against Nt-Rab5, Nt-Rab6, and Np-Rab11 proteins. The immune complexes were detected with 4000-fold diluted, alkaline phosphatase-coupled, goat anti-mouse antibodies (Sigma). To characterize the solubilization features of the membrane-bound plant proteins, microsomal pellet fractions were treated with NaCl, urea, Na_2CO_3, and detergents according to the method of Rodriguez-Rosales et al. (1992).

GTP-Binding Assays, GAP Assays

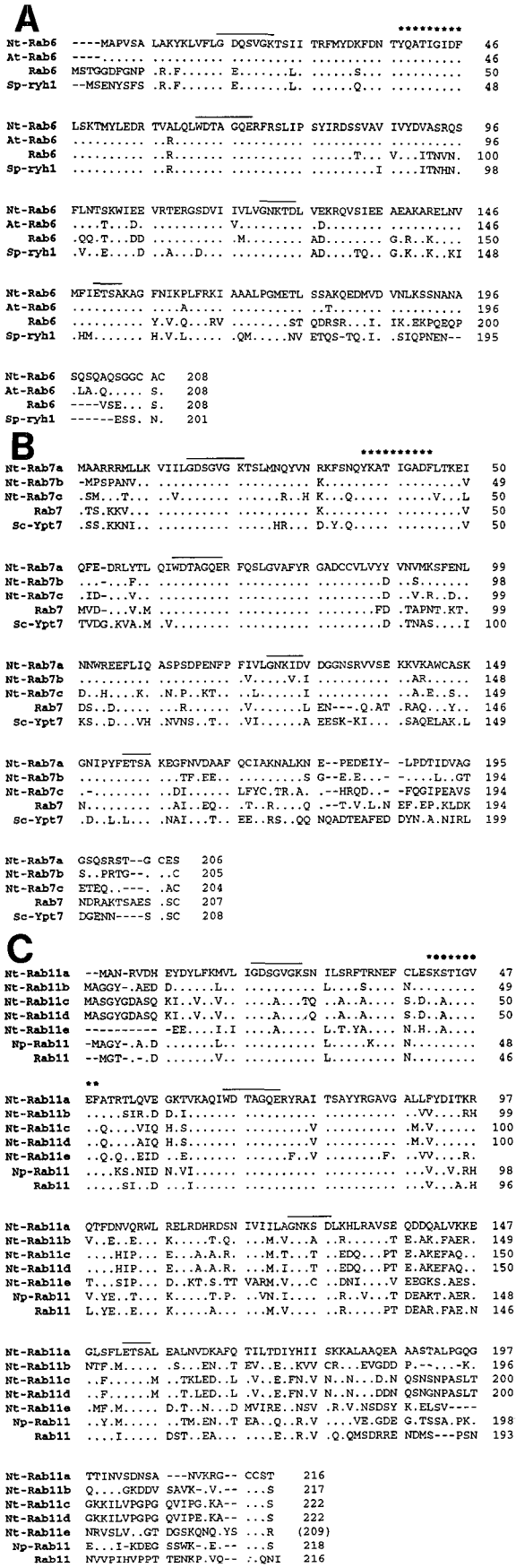
GAP assays, filter-binding assays, and the loading of tobacco small GTP-binding proteins with 5′-[γ^{32}P]GTP were done according to the method of Strom et al. (1993). For the analysis of reaction products by TLC, small GTP-binding proteins were loaded with 5′-[α^{32}P]GTP (Amersham) and purified by passage through Bio-Spin 6 chromatography columns (Bio-Rad). *E. coli* extracts containing Gyp6 GAP activity were isolated as described by Strom et al. (1993). The GAP assay mixture contained 10 μL of the eluate, 25 μL of reaction mixture (50 mM Tris-HCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mg/mL BSA, 2 mM ATP, pH 8), and 15-μL *E. coli* extracts (0.15 μg/μL total protein) with or without Gyp6 GAP activity and was incubated at 30°C. Five-microliter aliquots of the assay mixture were removed 0, 5, and 30 min after the addition of *E. coli* extracts, mixed with 5 μL of stop solution (0.2% SDS, 5 mM EDTA), and further incubated at 70°C for 4 min. The reaction products were then analyzed by TLC. Three-microliter aliquots were spotted onto polyethyleneimine cellulose foils (Merck), and the chromatogram was developed in 1 M potassium phosphate, pH 3.4. The reaction products or the applied GTP and GDP standard were visualized by autoradiography or by UV light, respectively.

RESULTS

Small GTP-binding proteins encoded by the *Rab/Ypt* and *Ran* gene families exhibit a high degree of functional and structural conservation in all eukaryotic cells studied. This conservation is especially high in those four regions that are known to be involved in GTP binding and hydrolysis (Bourne et al., 1991). We have used two sets of degenerate oligonucleotides corresponding to the IWDTAGQE motif as labeled probes in plaque hybridization experiments to isolate tobacco cDNAs encoding small GTP-binding proteins. So far, we have isolated and characterized 11 cDNAs that show considerable similarity to different members of the mammalian/yeast Rab/Ypt gene families.

Isolation and Characterization of the *Nt-Rab6* cDNA

A 760-bp cDNA clone, designated *Nt-Rab6*, encodes a protein of 208 amino acid residues. It shares 73 and 71% amino acid identity (83 and 80% similarity) with the human Rab6 (Zahraoui et al., 1989) and fission yeast Ryh1 proteins (Hengst et al., 1990), respectively (Fig. 1A). The deduced amino acid sequence of this tobacco protein also displays 60% identity (77% similarity) with the yeast Ypt6 and 95% identity (97% similarity) with the recently described At-Rab6 protein from *A. thaliana* (Bednarek et al., 1994). Moreover, the Nt-Rab6 protein also shows some similarity to other members of the Rab/Ypt family, although to a significantly lower extent (30–45%). An amino acid alignment of the Nt-Rab6 protein with its homologs from other species demonstrates that regions shown to participate in GTP binding and hydrolysis are well conserved between these proteins (Fig. 1A, residues marked by a line). Indeed, the G-1 (GDQSVGK, positions 16–22), G-3 (WDTAGQE, positions 63–69), G-4 (NKTD, positions 122–125), and G-5 domains (ETSA, positions 150–153) are almost identical. Moreover, the C-terminal CXC motif shown to be essential for membrane attachment is also conserved. Finally, the "effector" domains YQATIGIDF (positions 38–46; indicated by asterisks in Fig. 1A), which are thought to interact with the GAP specific for the Rab6/Ypt6-like proteins (Strom et al., 1993), are identical in these proteins.



Isolation and Characterization of *Nt-Rab7a-c* cDNAs

Three cDNA clones, designated *Nt-Rab7a*, *Nt-Rab7b*, and *Nt-Rab7c*, encode proteins of 206, 205, and 204 amino acid residues, respectively. The deduced amino acid sequences of these proteins show 56 to 58% identity (72–76% similarity) with the *Saccharomyces cerevisiae* Ypt7 protein (Schimmöller and Riezman, 1993), 63 to 70% identity (76–82% similarity) with the canine Rab7 protein (Chavrier et al., 1990b), and 73 to 84% identity with one another (Fig. 1B). These three tobacco proteins are most closely related to a Ypt7-like protein from pea (Drew et al., 1993; 70% identity) and to the Ara5 protein from *A. thaliana* (Anai et al., 1991; 75% identity). The regions involved in GTP binding and hydrolysis (residues marked by a line in Fig. 1B), as well as the C-terminal CXC motif required for membrane attachment, are highly conserved in these proteins. Amino acid residues that make up the effector domains (YKATIGADF, positions 37–45, indicated by asterisks in Fig. 1B) are identical in these tobacco Rab7-like proteins and in their mammalian and yeast counterparts. Among all known small GTP-binding proteins, the effector domain of the Rab7-like proteins is the most similar to that of Rab6-like proteins. Figure 1, A and B, shows that seven of nine amino acid residues encoding these regions are identical in the Rab6 and Rab7 proteins.

Isolation and Characterization of the *Nt-Rab11a-e* cDNAs

Five cDNA clones, designated *Nt-Rab11a*, *Nt-Rab11b*, *Nt-Rab11c*, *Nt-Rab11d*, and *Nt-Rab11e*, and the recently isolated *Np-Rab11* (formerly named *Np-Ypt3*; Dallmann et al., 1992), encode proteins of 216, 217, 222, and 218 amino acid residues (the *Nt-Rab11e* sequence is partial). These proteins display 54 to 97% identity with each other. Nt-Rab11c and Nt-Rab11d exhibit 97% identity and are thus the most closely related proteins. The tobacco proteins show 53 to 69% identity (71–80% similarity) with the mammalian Rab11 protein (Chavrier et al., 1990b) and 41 to 46% identity (60–64% similarity) with the *Schizosaccharomyces pombe* Ypt3 protein (Miyake and Yamamoto, 1990). Chavrier et al. (1990b) previously reported that the mammalian Rab11 and the Ypt3 proteins are probably homologs, showing 80% similarity. Among all small GTP-binding proteins

Figure 1. The deduced amino acid sequences of tobacco Rab6, Rab7, and Rab11 proteins and comparisons with homologous proteins from other species. Amino acid residues identical with those of the uppermost tobacco Rab-like protein are indicated by dots; gaps inserted by the computer program are indicated by dashes. The numbering of amino acid residues is on the right. The putative effector-binding domains are indicated by asterisks; conserved regions involved in GTP-binding and hydrolysis are indicated by lines above the sequence. A, Alignment of the tobacco Nt-Rab6 protein with *A. thaliana* At-Rab6, human Rab6, and *S. pombe* Sp-ryh1 proteins. B, Alignment of the tobacco Nt-Rab7a, Rab7b, and Rab7c proteins with the canine Rab7 and the yeast Sc-Ypt7 proteins. C, Alignment of the tobacco Nt-Rab11a, Nt-Rab11b, Nt-Rab11c, Nt-Rab11d, and Nt-Rab11e proteins with the *N. plumbaginifolia* Np-Rab11 (formerly designated Np-Ypt3; Dallmann et al., 1992) and the canine Rab11 proteins.

known in plants, these tobacco proteins are most closely related to the *A.* thaliana At-Rab11 and ARA-2 proteins (Anai et al., 1991; Yi and Guerinot, 1994) and to the pea pra6 and pra7 proteins (Nagano et al., 1993). They share 65 to 67% amino acid identity (81–82% similarity) with these proteins. Again, the regions known to participate in GTP binding and hydrolysis, G-1 to G-4, are almost identical in these proteins (Fig. 1C, amino acid residues marked by a line). The C-terminal CCXX motif, which is essential for membrane attachment, is very well conserved in all Rab11-like proteins (as well as in the Nt-Rab5 protein; Dallmann et al., 1992), but it differs characteristically from the CXC motif of Rab6 and Rab7-like proteins. Finally, the effector-binding domain (SKS/ATIGVEF; indicated by asterisks in Fig. 1C), which provides specificity for interaction with GAP activities, is almost identical with and unique to this group of proteins.

Expression Pattern of the Tobacco *Rab* Genes

We determined the organ-specific expression of the tobacco *Rab* genes by northern blot analyses. Total RNA from different tissues of 12-week-old and of fully developed, flowering tobacco plants were hybridized with probes corresponding to the 3′ untranslated regions of different cDNAs. These probes were cDNA specific, since under stringent conditions they hybridized to only a single, full-length cDNA clone (except for the *Nt-Rab11d* probe, which weakly cross-hybridized to *Nt-Rab11c*). We found that the expression pattern of all *Nt-Rab7* and *Nt-Rab11* genes examined were similar. Figure 2, B to D, clearly shows that the steady-state mRNA levels of these genes are relatively high in stems and unripe fruit, lower in flower tissues (except for petal), and very low in leaves and roots. A similar pattern was observed for *Nt-Rab5* and *Np-Rab11* genes (Dallmann et al., 1992). The expression pattern of the *Nt-Rab6* gene was different (Fig. 2A). The highest *Nt-Rab6* mRNA level was detected in stamen and it was slightly lower in petals and in unripe fruit. It was further reduced but nearly identical in stems and roots and barely detectable in leaves. To ascertain that the detected organ-specific expression patterns of the tobacco *Rab* genes are mRNA specific, the same filters were hybridized with a probe encoding a Chl *a*/*b*-binding protein (*Nt-Cab21*). Consistent with previous data, the expression pattern of this *Cab* gene was clearly different. Figure 2E illustrates that the *Cab* mRNA abundance was high in Chl-containing organs. It was highest in leaves and stems, lower in sepals and unripe fruit, and below detection level in roots and petals.

Membrane Association and Solubilization Characteristics of Tobacco Rab5, Rab6, and Rab11 Proteins

Antibodies specific for Nt-Rab5, Nt-Rab6, and Np-Rab11 proteins were raised against *E. coli*-produced fusion proteins. Total protein extracts from different organs of tobacco plants were isolated and the abundance of these Rab proteins was characterized by western blot analyses. These proteins were expressed in all tissues examined. However, the relative amounts of the proteins were quite different,

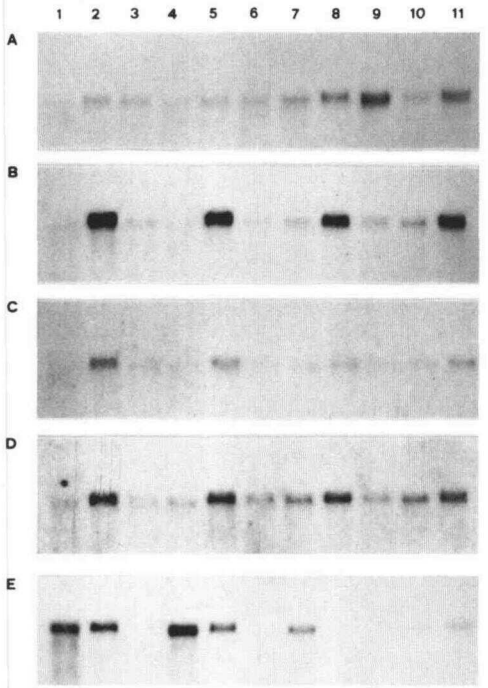


Figure 2. Northern blot analysis of tobacco genes encoding Rab6-, Rab7-, and Rab11-like small GTP-binding proteins. The hybridization probes were short fragments representing the 3′ untranslated regions of the *Nt-Rab6* (A), *Nt-Rab7b* (B), *Nt-Rab11d* (C), *Nt-Rab11e* (D), and *Nt-Cab21* (E) genes. Total RNA from leaves (lane 1), stems (lane 2), and roots (lane 3) of 12-week-old tobacco plants, as well as from leaves (lane 4), stems (lane 5), roots (lane 6), sepals (lane 7), petals (lane 8), stamen (lane 9), carpels (lane 10), and unripe fruit (lane 11) of fully matured, flowering tobacco plants, was analyzed. Each lane contains 20 μg of total RNA. RNAs used in these experiments are from the same preparation.

showing good correlation with their mRNA steady-state levels. Once again we found that the relative levels of these proteins were very low in leaves, in leaf veins, and in seedlings. Higher protein levels were detected in stems and flower tissues, especially in petals and carpels (data not shown).

To assess whether these tobacco proteins, like their mammalian and yeast homologs, are membrane bound, total protein extracts isolated from tobacco petal or carpel tissues were fractionated and analyzed by western blotting. Figure 3 indicates that each of the antibodies raised against these three tobacco small GTP-binding proteins recognized one major band of 22 to 24 kD, mainly in the high-speed pellet fractions containing microsomal membranes (150,000*g*, Fig. 3, lane 3). In the medium-speed pellet fractions (20,000*g*, Fig. 3, lane 2) a significantly lower amount of these proteins was detected. In contrast, the low-speed pellet fractions and the cytosolic supernatant fractions did not contain significant amounts of the Rab proteins analyzed (Fig. 3, lanes 1 and 4).

We also determined the solubilization properties of these membrane-bound proteins. Fractions containing microsomal membranes were incubated with high concentrations of NaCl and urea or at high pH. Proteins that are loosely

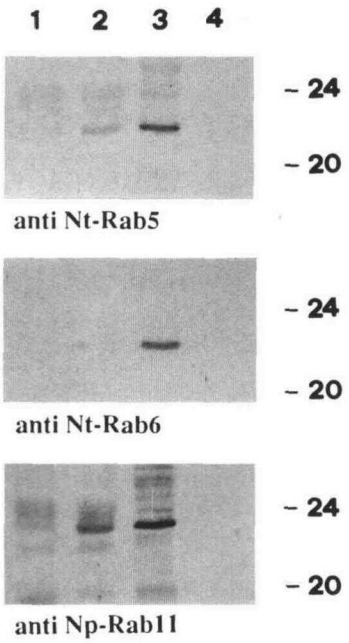


Figure 3. The small GTP-binding proteins Nt-Rab5, Nt-Rab6, and Np-Rab11 are bound to microsomal membranes. Total protein extracts prepared from *N. tabacum* and *N. plumbaginifolia* carpels were isolated and fractionated by differential centrifugation. Fifteen micrograms of protein of each fraction were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and probed with antibodies specific for Nt-Rab5, Nt-Rab6, and Np-Rab11: 3,000*g* (lane 1), 20,000*g* (lane 2), and 150,000*g* (lane 3) pellet fractions and cytosolic supernatant fraction (lane 4). The positions of molecular mass markers (kD) are indicated on the right.

associated with membranes should be washed away, whereas proteins that are tightly bound to membranes should stay bound under these conditions. Figure 4 shows that the Nt-Rab5, Nt-Rab6, and Np-Rab11 proteins are tightly bound to membranes and they could be solubilized only by detergents. The solubilization characteristics of these proteins, however, were quite different. After treatment with nondenaturing detergents, such as dodecyl maltosid and NP-40 at final concentrations of 2%, almost all of the membrane-bound Rab6 and Rab11 proteins were solubilized and could not be pelleted again (Fig. 4, B and C, lanes 4 and 5). In contrast, the membrane-bound Rab5 protein could only be solubilized to a certain extent with 2% NP-40. Even after treatment with 5% NP-40, a considerable amount of Rab5 protein stays membrane bound (Fig. 4A).

Gyp6, a Yeast Ypt6-Specific GAP, Enhances the GTP Hydrolysis of Wild-Type and Mutant Nt-Rab7 but Not of the Nt-Rab6 Protein in Vitro

We investigated the possibility of whether the yeast GAP Gyp6 (Strom et al., 1993) can regulate GTP hydrolysis of Nt-Rab6 or that of other tobacco small GTP-binding proteins. cDNAs encoding Nt-Rab5, Nt-Rab6, Nt-Rab7, and Np-Rab11 proteins, as well as cDNA clones for the yeast small GTP-binding protein Ypt6 and the yeast GAP Gyp6, were overexpressed in *E. coli*. The bacteria-produced GTP-binding proteins were purified, loaded with radioactively labeled GTP, and incubated with crude extract containing Gyp6 activity or with a control extract. GTPase activity of the individual GTP-binding proteins was measured and the analysis of the reaction products was performed as described by Strom et al. (1993). We found that the intrinsic rate of GTP hydrolysis of all Rab-like proteins was very low (Fig. 5, squares). Incubation with extract containing Gyp6 activity increased the GTP hydrolysis rate of the yeast Ypt6 protein more than 10-fold (Fig. 5G) but did not affect that of the tobacco Nt-Rab5 and Nt-Rab6 proteins at all (Fig. 5, A and B). However, the GTPase activity of the Nt-Rab7 protein was clearly induced (about 3- to 4-fold; Fig. 5C). TLC analysis demonstrated that the reaction product was indeed GDP (Fig. 7B). Therefore, we concluded that the results obtained were not influenced by possible changes in the ratio of GTP/GDP exchange.

The effector-binding domain of the Nt-Rab7 protein is closely related to but not identical with that of the yeast Ypt6 protein (Fig. 1A). To assess the possible contribution of different domains of the Nt-Rab7 protein to the interaction with yeast GAP, we engineered one mutant Nt-Rab7

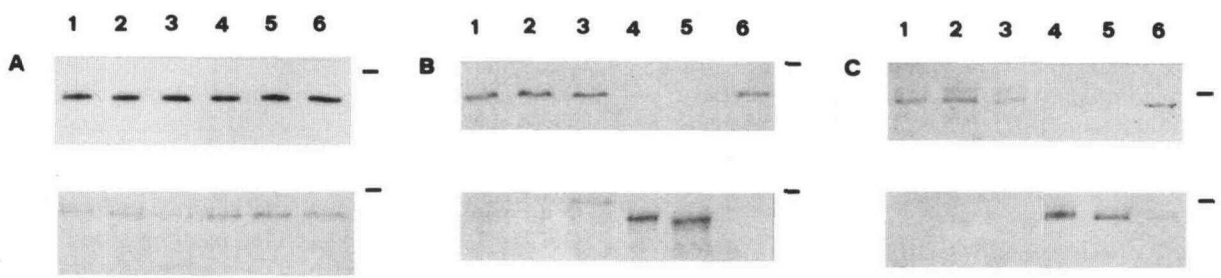


Figure 4. The membrane-bound small GTP-binding proteins show different solubilization characteristics. Microsomal membrane fractions were prepared as described in Figure 3, treated with different compounds on ice for 30 min, and fractionated by centrifugation at 150,000*g* into a pellet and a supernatant fraction. Fifteen micrograms of protein of each pellet (top panels) and supernatant fraction (bottom panels) were then separated by SDS-PAGE, blotted onto membranes, and probed with antibodies specific for Nt-Rab5 (A), Nt-Rab6 (B), and Np-Rab11 (C). Treatments were (A) 0.1% (v/v) (lane 1), 0.2% (lane 2), 0.5% (lane 3), 1% (lane 4), 2% (lane 5), and 5% (lane 6) NP-40; (B and C) Tris buffer for control (lane 1), 1 M NaCl (lane 2), 5 M urea (lane 3), 2% (w/v) dodecyl maltosid (lane 4), 2% (v/v) NP-40 (lane 5), and 0.2 M Na_2CO_3 (lane 6). The position of the 24-kD molecular mass marker is indicated on the right.

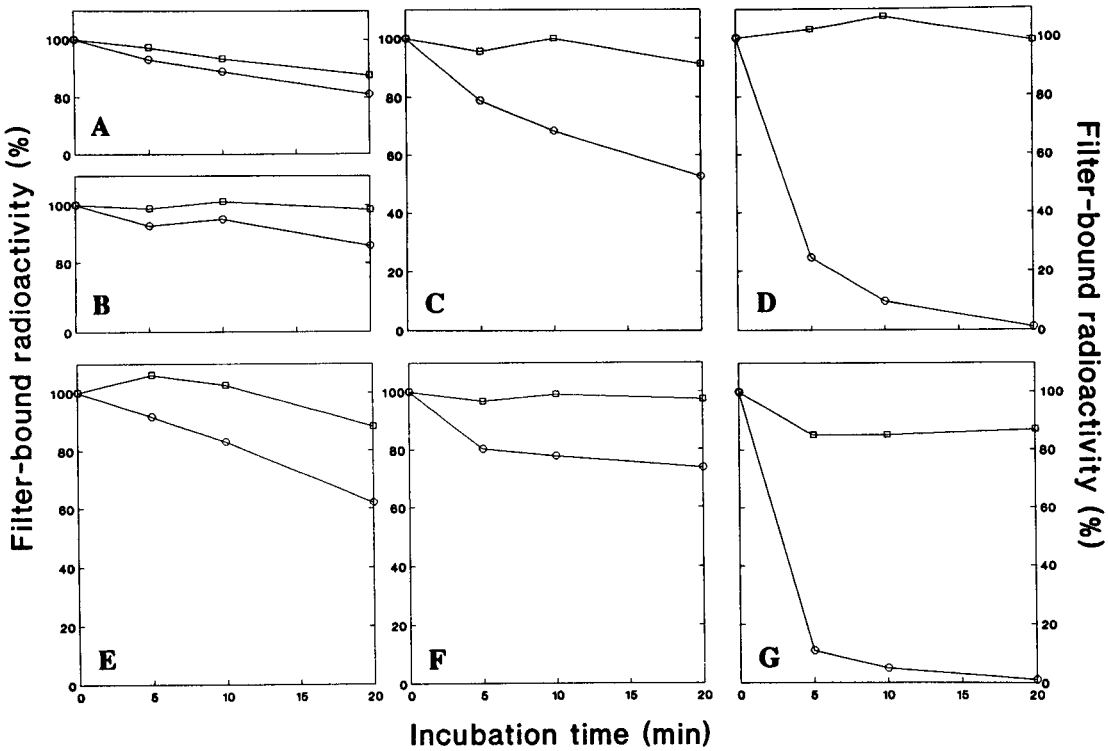


Figure 5. Gyp6, a yeast GAP specific for the yeast Ypt6 small GTP-binding protein regulates, although at different levels, GTP hydrolysis of the wild-type Nt-Rab7a and the mutant Nt-Rab7m, Nt-Rab76m, and Nt-Rab67m proteins but not that of the tobacco Nt-Rab6 and Nt-Rab5 proteins. Small GTP-binding proteins from tobacco Nt-Rab5 (A), Nt-Rab6 (B), Nt-Rab7 (C), Nt-Rab7m (D), Nt-Rab67m (E), and Nt-Rab76m (F) and the yeast Sc-Ypt6 protein (G) were expressed in *E. coli*, purified, loaded with 5′-[γ^{32}P]GTP (660, 000 cpm ± 12%/μg protein), and incubated with *E. coli* extracts with (circles) or without (squares) Gyp6 activity at 30°C. For each GAP assay, equal cpm of the different loaded Rab proteins (160,000 cpm/GAP assay) were added. Aliquots were taken at the times indicated, filtered through nitrocellulose filters, and washed, and the filter-bound radioactivity was measured. Experiments were carried out in triplicate, and the variation was less than 8%.

protein and two chimaeric Nt-Rab7/Nt-Rab6 proteins (Fig. 6). In the mutant Nt-Rab7 protein, designated Nt-Rab7m, the effector-binding region of Nt-Rab7 was replaced by that of Nt-Rab6. The first chimaeric protein, designated Nt-Rab76m, resulted in a protein containing the N terminus of Nt-Rab7 (amino acid residues 1–36 of Nt-Rab7a) followed by the effector-binding domain and the C-terminal part of Nt-Rab6 (amino acid residues 38–208 of Nt-Rab6). The second chimaeric protein, designated Nt-Rab67m, contained the N terminus and the effector-binding domain of Nt-Rab6 (amino acid residues 1–46 of Nt-Rab6) fused to the C-terminal part of Nt-Rab7a (amino acid residues 46–206 of Nt-Rab7a). All three mutant proteins were overexpressed in *E. coli* and their GTPase activity and ability to interact with yeast Gyp6 tested as described above. Again, we found that the intrinsic GTPase activity of these mutant proteins was very low (Fig. 5, D–F, squares). However, Gyp6 increased about 10-fold the GTPase activity of Nt-Rab7m, thus making this protein comparable to the yeast Ypt6 protein (Fig. 5, D and G, open circles). GTP hydrolysis rates of the chimaeric Nt-Rab76m and Nt-Rab67m proteins were not affected: they were similar to that of Nt-Rab7 but significantly higher than that of Nt-Rab6 (Fig. 5, E and F, open circles). TLC analysis again demonstrated that GDP was produced by interaction of Gyp6 with the overexpressed plant proteins (Fig. 7B).

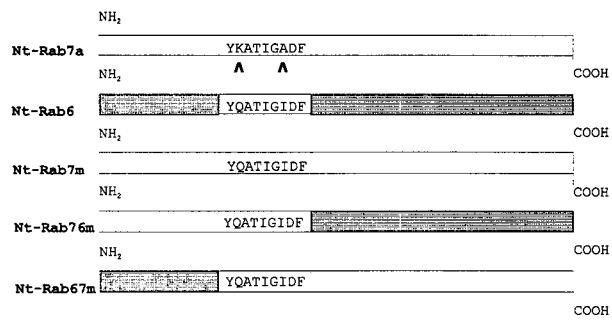


Figure 6. Structure of the mutant tobacco small GTP-binding proteins used in GAP assays. The effector domain sequences are boxed, and arrowheads indicate the mutated amino acid residues. The wild-type Nt-Rab6 sequences are illustrated with shaded boxes.

DISCUSSION

The cellular function of monomeric plant Ypt/Rab-like GTP-binding proteins is not known. The fact that *A. thaliana Rab6* (Bednarek et al., 1994) and maize *yptm1* and *yptm2* (Palme et al., 1993) genes are functional homologs of the yeast *YPT6* and *YPT1* genes suggests that these plant pro-

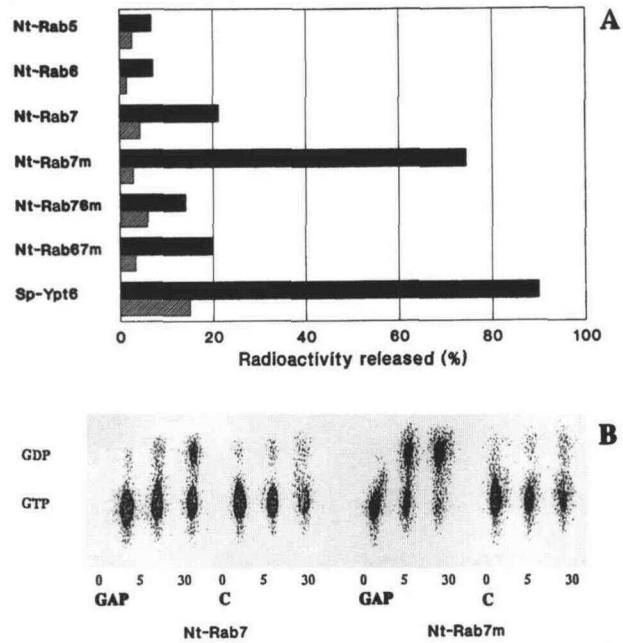


Figure 7. Characterization of the products and quantitation of the initial rates of GTP hydrolysis of tobacco GTP-binding proteins promoted by the *S. cerevisiae* Gyp6 GAP activity. A, Initial rates of GTP hydrolysis (given as percentages of the radioactivity released during the first 5 min of incubation and calculated from experiments shown in Fig. 5) of samples incubated with (black bars) or without (stippled bars) *S. cerevisiae* Gyp6 activity. Experiments were carried out in triplicate, and the variation was less than 8%. B, TLC of guanine nucleotide reaction products using small GTP-binding proteins loaded with 5′-[α^{32}P]GTP reacted with Gyp6 activity (GAP) or control extracts (C). Aliquots were taken at 0, 5, and 30 min after the addition of GAP activity. Results obtained with the wild-type Nt-Rab7a and Nt-Rab7m are shown.

teins may also be involved in regulating secretory pathways. We have isolated nine cDNA clones, all of which encode proteins that contain the conserved sequence motifs characteristic of Rab/Ypt proteins. Furthermore, in good agreement with their postulated function, we have shown that (a) the Nt-Rab5, Nt-Rab6, Nt-Rab7, and Np-Rab11 proteins were able to bind GTP in vitro and (b) they were tightly bound to membranes and seem to be present, at different levels, in all tissues examined. Although these proteins were exclusively associated with the microsomal membrane fractions, they displayed different solubilization characteristics (Fig. 4). This latter observation could be due to different interaction of tobacco Rab-like proteins with other proteins integrated into or attached to membranes or the harboring membrane itself.

The hypervariable C-terminal regions of Rab/Ypt-like proteins were shown to function as signals for the localization of these proteins to appropriate membranes of different subcellular compartments (Chavrier et al., 1991). The C-terminal regions of the characterized tobacco Rab-like proteins varied greatly in both sequence and length, similar to their mammalian homologs (Fig. 1). In contrast to the mammalian Rab proteins, however, neither the subcellular localization nor the function of the C-terminal domains of any plant Rab-like proteins are known. We have raised specific antibodies against Nt-Rab5, Nt-Rab6, and Np-Rab11 proteins. We assume that by immunogold EM we will be able to define the intracellular location of these proteins and to assess the role of the C-terminal regions by determining the subcellular distribution of chimaeric proteins expressed in transgenic plants.

The intrinsic activity of monomeric GTP-binding proteins, including the ones of the Rab/Ypt subfamily, is very low. GAPs can modulate the cellular activity of these GTP-binding proteins by increasing the rate of GTP hydrolysis by several orders of magnitude (Zerial and Stenmark, 1993). Indeed, Strom et al. (1993) reported the isolation of a yeast GAP, termed Gyp6 (GAP of Ypt6), that specifically interacts with the yeast Ypt6 protein. The effector-binding domains, which are thought to be essential for the interaction with specific GAPs, are identical in the yeast Ypt6, *A. thaliana* Rab6, and tobacco Nt-Rab6 proteins (Fig. 1A). Yet, we found that the yeast Gyp6 (GAP) protein cannot regulate, at least in vitro, the GTPase activity of either tobacco Nt-Rab6 (Figs. 5 and 7) or At-Rab6 proteins (data not shown), even though At-Rab6 can functionally complement the yeast YPT6 null mutant (Bednarek et al., 1994). However, we demonstrated that GTPase activity of Nt-Rab7a, which contains only a similar but not identical effector-binding region, was partially induced (about 3- to 4-fold) by Gyp6 and that GTPase activity of the mutant Nt-Rab7m protein containing a Nt-Rab6 effector-binding domain was highly induced (about 10-fold) by the yeast GAP (Fig. 7A). The replacement of either the N- or the C-terminal region of the Nt-Rab7m protein with the corresponding regions of the wild-type Nt-Rab6 protein was inhibitory and reduced the GTPase activity of the respective mutant protein in the presence of Gyp6 to levels similar to that of the wild-type Nt-Rab7 protein (Fig. 7A). It is unlikely that this deleterious effect was caused by expressing these proteins in *E. coli*, since all of these proteins readily bound GTP, were recognized specifically by the produced antibodies, and had the expected mol wt. However, it has been reported recently that cooperation of three well-defined domains is required to distinguish functionally the Ras-related Ypt1 and Sec4 proteins (Brennwald and Novick, 1993; Dunn et al., 1993). Furthermore, it has been demonstrated that the substitution of a 24-residue Ypt1 segment together with 12 residues of Ypt1 corresponding to the effector region transforms Sec4 into a fully functional Ypt1 protein without residual Sec4 function (Dunn et al., 1993). It is not yet proven that the specificity of interaction of GAPs and Ypt/Rab-like proteins is also determined by different domains. Our data, however, suggest that, although the effector regions are important, other domains of these plant Rab-like proteins are also required for optimal interaction. We assume that by using this in vitro system it will be possible to localize precisely these regions and this may facilitate the isolation of plant GAPs.

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