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Tomato Rab1A Homologs as Molecular Tools for Studying Rab Geranylgeranyl Transferase in Plant Cells[1]

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Rab proteins attach to membranes along the secretory pathway where they contribute to distinct steps in vesicle-mediated transport. To bind membranes, Rab proteins in fungal and animal cells must be isoprenylated by the enzyme Rab geranylgeranyl transferase (Rab GGTase). We have isolated three tomato (*Lycopersicon esculentum*, M.) cDNAs (*LeRab1A, B,* and *C*) encoding Rab-like proteins and show here that all three are substrates for a Rab GGTase-like activity in plant cells. The plant enzyme is similar to mammalian Rab GGTase in that the plant activity (a) is enhanced by detergent and (b) is inhibited by mutant Rab lacking a prenylation consensus sequence. LeRab1B contains a rare prenylation target motif and was the best substrate for the plant, but not the yeast, Rab GGTase. LeRab1A, B, and C are functional homologs of the *Saccharomyces cerevisiae* Rab protein encoded by *YPT1* and are differentially expressed in tomato. *LeRab1A* mRNA, but not that of *LeRab1B* or *C*, is induced by ethylene in tomato seedlings and is also upregulated in ripening fruit. The increase in *LeRab1A* mRNA expression in ripe fruit may be linked to increased synthesis and export of enzymes like polygalacturonase, pectin esterase, and other enzymes important in fruit softening.

Rab proteins are small GTP-binding proteins that help regulate distinct steps in vesicle-mediated transport (Ferro-Novick and Novick, 1993; Zerial and Stenmark, 1993; Pfeffer, 1994). Members of the Ras superfamily, Rab proteins alternate between GTP- and GDP-bound states and possess weak, intrinsic GTPase activity. Most of the Rab protein in cells is membrane bound, with a small fraction present in the cytosol complexed with RabGDI, a Rab regulatory protein that binds Rab-GDP more efficiently than Rab-GTP (Matsui et al., 1990; Regazzi et al., 1992; Ullrich et al., 1993). The current model of Rab protein function suggests that membrane-bound Rab-GTP controls docking of transport vesicles with cognate receptor membranes (Lian et al., 1994; Søgaard et al., 1994; Stenmark et al., 1994). When transport and target compartments fuse, Rab-GTP is hydrolyzed to Rab-GDP and can be removed from the target membrane by forming a complex with RabGDI. RabGDI maintains Rab in the inactive, GDP-bound state before delivering it to an appropriate cognate donor membrane. This return to the membrane is accompanied by exchange of GTP for GDP and presumably primes Rab to participate in the next round of vesicle-mediated transport (Soldati et al., 1994; Ullrich et al., 1994).

Genes encoding Rab-like proteins have been cloned from a variety of different plant species during the past 4 years (Cheon et al., 1993; Palme et al., 1993; Terryn et al., 1993a, 1993b; Yoshida et al., 1993; Bednarek et al., 1994), but for most, little is known about which steps in secretion these plant Rabs may help perform. Some of these plant Rabs have been shown to be localized to membranes, suggesting that like their counterparts in other organisms they may be involved in regulating the flow of membrane materials and secretory proteins through cells (Bednarek et al., 1994; Haizel et al., 1995). In yeast and mammalian cells, Rab proteins must be isoprenylated by Rab GGTase, also called GGTase type II, to bind membranes, as well as be complexed to RabGDI (Khosravi-Far et al., 1991; Rossi et al., 1991; Kinsella and Maltese, 1992). No such activity has yet been reported in the plant kingdom, although plant Rabs without exception contain C-terminal double-Cys motifs typical of Rab GGTase substrates (Terryn et al., 1993a, 1993b; Ma, 1994).

Rab GGTase transfers C_{20} isoprenoid all-*trans*-geranylgeranyl groups from GGPP, an intermediate in isoprenoid biosynthesis, onto conserved Cys residues at the Rab C terminus via a thioether linkage. The modified Cys's appear within CC, CCXX, and CXC amino acid motifs, where C is Cys and X is variable. Rab GGTase has been purified and cloned from mammalian cells (Seabra et al., 1992a, 1992b; Andres et al., 1993) and *Saccharomyces cerevisiae* (Jiang et al., 1993; Li et al., 1993; Fujimura et al., 1994; Jiang and Ferro-Novick, 1994). In yeast and mammals, Rab GGTase comprises a catalytic α/β heterodimer and a third subunit named REP. REP presents nascent Rab protein to the catalytic heterodimer, which by itself has negligible activity (Seabra et al., 1992a, 1992b; Andres et al., 1993). Two different human REPs have been identified. Both

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Abbreviations: EE-tag, MEYMPME monoclonal antibody tag; GGPP, all-*trans*-geranylgeranylpyrophosphate; GGTase, geranylgeranyl transferase; GST, bacterial glutathione *S*-transferase; MS, Murashige-Skoog; NP-40, Nonidet P-40; RabGDI, Rab GDP-dissociation inhibitor; REP, rab escort protein; URA, uracil.

REP-1 and REP-2 have high homology to RabGDI (Andres et al., 1993; Cremers et al., 1994), and REP-1 has recently been shown to have RabGDI-like activity (Alexandrov et al., 1994). Unlike RabGDI, REP-1 interacts with nonprenylated as well as prenylated Rab. REP is also thought to deliver prenylated Rab to the appropriate membrane-bound compartment and thus may regulate its initial interaction with the secretory apparatus.

One other type of protein GGTase has been described. Type I GGTase, or GGTase I, attaches geranylgeranyl groups to proteins containing the prenylation motif C*aa*L, where *a* is usually an aliphatic amino acid. Its substrates include other members of the Ras superfamily and some trimeric G-protein γ subunits (Schafer and Rine, 1992; Sinensky and Lutz, 1992; Casey, 1994; Omer and Gibbs, 1994). GGTase I and Rab GGTase have distinct biochemical properties. GGTase I is able to modify short peptides containing the C*aa*L consensus motif (Moores et al., 1991). Rab GGTase, however, requires sequences remote from the C terminus to isoprenylate its protein substrate (Moores et al., 1991; Beranger et al., 1994; Sanford et al., 1995), reflecting the need for REP to bind and present Rab to the Rab GGTase catalytic α/β heterodimer. Purified and crude Rab GGTase is inhibited by mutant Rab proteins (RabmCC) in which the prenylation motif Cys residues have been converted to other amino acids (Andres et al., 1993; Sanford et al., 1993; Yalovsky et al., 1996). GGTase I, however, is unaffected by proteins that do not contain its prenylation motif. Finally, in vitro prenylation by Rab GGTase, but not GGTase I, is enhanced by micellar concentrations of detergent (Seabra et al., 1992a, 1992b). Following prenylation, REP remains bound to prenylated Rab (Andres et al., 1993; Alexandrov et al., 1994). Detergent promotes dissociation of REP from prenylated Rab, liberating REP for further rounds of catalysis in the in vitro reaction (Andres et al., 1993).

To study Rab GGTase activity in plants, we isolated three cDNAs (*LeRab1A, B,* and *C*) from tomato (*Lycopersicon esculentum,* M.) encoding potential substrates for Rab GGTase. *LeRab1A, B,* and *C* encode homologs of mammalian Rab1A and are differentially expressed in roots, leaves, and fruit pericarp. We show here that these tomato proteins can substitute for YPT1p, the *S. cerevisiae* Rab1A homolog required in ER-to-Golgi transport in yeast (Segev et al., 1988; Baker et al., 1990). We also show that protein extracts from yeast, tomato, and tobacco tissue-culture cells contain a Rab GGTase-like activity that uses [^{3}H]GGPP to modify recombinant LeRab1A, B, and C protein. The activity from plant cells shares biochemical characteristics with both human and yeast Rab GGTases and therefore is likely to be distinct from GGTase I. And despite their high degree of similarity, LeRab1A, B, and C differ in their relative abilities to serve as substrates for the Rab GGTase in yeast and plant cells.

MATERIALS AND METHODS

Plant Materials

RNA samples were prepared from greenhouse-grown *Lycopersicon esculentum* cv VFNT Cherry LA1221. Mature green fruit were green, full-sized fruit. Breaker fruit were full sized with a slight, external color change at the blossom end of the fruit. Roots were from hydroponically grown plants. Tobacco BY-2 suspension-culture cells (Nagata et al., 1992) were grown in medium containing 4.3 g L^{-1} MS salts (Murashige and Skoog, 1962), 100 mg L^{-1} inositol, 1 mg L^{-1} thiamine, 0.2 mg L^{-1} 2,4-D, 255 mg L^{-1} $KHPO_4$, and 30 g L^{-1} Suc.

Tomato suspension-culture cells were grown in 4.3 g L^{-1} MS salts, 1 mL L^{-1} Linsmaier and Skoog Minors (1000-fold), 5 mL of 20 mM Gresshoff Doy iron chelate, 1 mL of Nitsch's vitamins (1000-fold), 2 mg L^{-1} 2,4-D, 1 mg L^{-1} isopentenyl adenine, 30 g L^{-1} Suc, and 0.1 g L^{-1} *myo*-inositol. Linsmaier and Skoog Minors (1000-fold) contains 0.1 M H_3BO_3, 0.1 M $MnSO_4 \cdot H_2O$, 37 mM $ZnSO_4 \cdot 7\ H_2O$, 5 mM KI, 1 mM $Na_2MoO_4 \cdot 2\ H_2O$, 0.1 mM $CuSO_4 \cdot 5\ H_2O$, and 0.1 mM $CoCl_2 \cdot 6\ H_2O$. Tobacco and tomato cell lines were grown at 25°C with constant shaking (120 rpm) in the dark and were subcultured weekly. All chemicals used for plant tissue-culture cell medium were purchased from either GIBCO-BRL or Sigma.

Isolation of *LeRab1A, B,* and *C* cDNAs

A tomato young-fruit cDNA expression library (Ach and Gruissem, 1994) was screened using polyclonal *YptV1* antibody (Fabry et al., 1992) with goat anti-rabbit alkaline phosphatase (Bio-Rad) as the secondary antibody. Fifteen cDNAs encoding *LeRab1B* and one encoding *LeRab1C* were isolated. A cDNA encoding a tomato homolog of *myo*-inositol monophosphatase (*LeIMP1*) was also isolated (Gillaspy et al., 1995) but was subsequently shown to encode a protein with alkaline phosphatase activity and contained no epitopes in common with Rab proteins (data not shown). The single *LeRab1C* cDNA (with a DNA insert of 1.080 kb) and the largest *LeRab1B* cDNA (0.864 kb) were sequenced by dideoxy sequencing (United States Biochemical). One clone with a DNA insert of 0.803 kb encoding a partial LeRab1A protein was also isolated. This cDNA was used to screen a red fruit cDNA library and 13 cDNAs encoding *LeRab1A* were isolated, 3 of which were sequenced. Each of the *LeRab1* cDNAs sequenced was found to encode full-length LeRab protein as judged by comparison with other Rab proteins. Sequence comparisons were done using GCG sequence analysis software (Genetics Computer Group, Madison, WI).

Tagged Wild-Type and Mutant *LeRab1A, B,* and *C* Constructs

A PCR-based strategy was used to add nucleotides encoding the oligopeptide EE-tag, for which a monoclonal antibody is available (Schaffhausen et al., 1982; Rubinfeld et al., 1991), at the 5′ end coding region of the *LeRab1A, B,* and *C* cDNAs. Sense primers (A1, 5′-CGGGATCCATGGAATACATGCCAATGGAAATGAATCCAGAATAA-3′; B1, 5′-CGGGATCCATGGAATACATGCCAATGGAAATGAGCAACGAATAC-3′; and C1, 5′-CGGGATCCATGGAATACATGCCAATGGAAATGAATCCCGAATAT-3′) containing sequences corresponding to the EE-tag and to the first

four amino acids of *LeRab1A, B,* and *C* were used in combination with antisense primers (A2, 5′-GCCGGATCCTCAAGATGAGCAGCAACC-3′; B2, 5′-GCCGGATCCA GCAGTTAACCACAACAG-3′; and C2, 5′-GCCGGATCCTAAGAAGAACAGCAGCCG-3′) corresponding to DNA sequences encoding the C terminus of each protein. In the case of mutant *LeRab* constructs, antisense primers were used that converted *LeRab1A, B,* and *C* C-terminal prenylation motifs from CCSS, CCG, and CCSS to SGSS, SSG, and GGSS, respectively (A3, 5′-GCGGATCCTCAAGATGAGCCGGAACCGCTC-3′; B3, 5′-GCGGATCCTTAACCAGAAGAGTTGCCC-3′; C3, 5′-GCGGATCCTAAGAAGAACCGCCGCCGTTTT-3′). All primers contained *Bam*HI restriction sites to facilitate cloning of PCR products. PCRs were performed with either *Taq* polymerase (United States Biochemical) or VENT DNA polymerase (New England Biolabs, Beverly, MA). PCR products were digested with *Bam*HI (GIBCO-BRL), gel-purified, and inserted into the *Bam*HI site of pBluescript (Stratagene) for restriction enzyme analysis and dideoxy sequencing to rule out sequence errors introduced by PCR.

Yeast Transformation

The *LeRab1B* cDNA was inserted in the *Bam*HI site of the yeast expression plasmid pRH98–3, a 2μ-based, *URA3* yeast-*Escherichia coli* shuttle plasmid in which the glyceraldehyde-3-phosphate dehydrogenase promoter and 3-phosphoglycerate kinase terminator flank a *Bam*HI/*Sal*I cloning site. This plasmid was made from YEplac195 (Bitter and Egan, 1984) by the same protocol used (R. Hampton, personal communication) to construct integrating plasmid pRH98–2 from YIplac211 (Geitz and Sugino, 1988; Hampton and Rine, 1994). *LeRab1C* and *LeRab1A* cDNAs were digested with *Bam*HI and *Xho*I (GIBCO-BRL) and subcloned into the *Bam*HI/*Sal*I-digested pRH98–3 plasmid derived from pRH98–3*LeRab1B*. Tagged mutant and tagged wild-type *LeRab1A, B,* and *C* cDNAs were inserted into the *Bam*HI site of pRH98–3.

Saccharomyces cerevisiae GFUI-6D (*Mata* GAL10-*YPT1*::*HIS3 ura3 leu2 his3 trp1*) cells (Ossig et al., 1995) were transformed using the pRH98–3 expression plasmids and plated onto selective medium containing 2% (w/v) Gal (Schiestl and Gietz, 1989). For complementation testing, URA^+ transformants were streaked onto minimal medium containing either 2% (w/v) Glc or 2% Gal. pAAH5, containing *LEU2* as the selectable marker (Ammerer, 1983), and pAAH5-YPT1 (Schmitt et al., 1986) were also used to transform GFUI-6D. Leu prototrophs were selected on minimal medium containing 2% Gal and then were plated onto minimal medium containing either 2% Glc or 2% Gal. Amino acids and other supplements for yeast medium were purchased from BIO 101 (Vista, CA).

Production of Recombinant Rab

The three mutant and three wild-type tagged *LeRab Bam*HI cDNA fragments used in making the yeast expression plasmids were inserted into the *Bam*HI site of bacterial expression plasmid pRSETA (Invitrogen, San Diego, CA), which introduces a six-His biochemical tag at the N terminus of the cloned protein. *E. coli* strain BL21DE3 was transformed with expression plasmids and cultures were induced by adding 1 mM isopropylthio-β-galactoside (Sigma), followed by incubation at 25°C for 3 to 6 h. The His-tagged Rab proteins were purified as described by Nuoffer et al. (1994) but without the final Sephacryl chromatography step. Recombinant LeRab proteins were concentrated and dialyzed using a collodion apparatus (Schleicher & Schuell) against 50 mM Tris-Cl, pH 7.8, 5 mM $MgCl_2$, and 1 μM GDP. Concentrations and purity of recombinant proteins were determined by densitometric scanning (Molecular Dynamics, Sunnyvale, CA) of Coomassie-stained bands resolved by SDS-PAGE using known amounts of chicken egg lysozyme (Sigma) as standards. Each preparation of recombinant LeRab protein contained less than 5% contaminating protein.

Plant and Yeast Extracts

Tissue-culture cells were collected from 3-d-old cultures by filtration. Cells were washed with buffer A (100 mM Tris, pH 7.8, 5 mM EGTA, 5 mM EDTA, 10 mM β-mercaptoethanol, 10% [w/v] glycerol) and then ground in a mortar with acid-washed, silanized sand in buffer A containing 2 μg mL^{-1} leupeptin, antipain, and aprotinin (Sigma) at 4°C. All subsequent steps were also done at 4°C. Lysates were centrifuged for 10 min at 800*g* and recentrifuged for 30 min at 30,000*g* and again for 2 h at 100,000*g*. The 100,000*g* supernatant was collected and concentrated in a Centriprep-10 device (Amicon, Beverly, MA), dialyzed against buffer B (50 mM Tris, pH 7.8, 10% glycerol, 1 mM DTT), aliquotted, and stored frozen at −80°C until use. Crude extracts from *S. cerevisiae* strain JRY1550 (*Mata leu2 pep4–3 trp1 ura3–52*) were made as described by Moores et al. (1991) except that extracts were centrifuged at 100,000*g* for 2 h. The supernatant from this step was dialyzed against buffer B, aliquotted, and stored at −80°C until use. Protein concentrations were determined using Protein Assay Reagent (Bio-Rad) and BSA (Sigma) as the standard.

Rab Prenyl Transferase Assays

All assays were performed at either 30 or 37°C in 0.05 mL per reaction for the times specified in the text. Unless otherwise indicated, reactions included 50 mM Tris, pH 7.8, 2.5 mM DTT, 5 mM $MgCl_2$, 0.3 mM NP-40, 0.5 mM α,β-methylene ATP (Sigma), 0.5 μM [^{3}H]GGPP of specific activity 15 or 52 Ci/mmol (American Radiolabeled Chemicals, St. Louis, MO), and 2.5 μg of recombinant LeRab, together with varying amounts of plant or yeast protein extract. Reactions also contained 2 μg mL^{-1} leupeptin, antipain, and aprotinin (Sigma). For SDS-PAGE analysis, reactions were stopped by addition of SDS-PAGE sample buffer, fractionated by SDS-PAGE, fixed for 30 min in 10% acetic acid, soaked in the fluorographic reagent Amplify (Amersham) for 30 min, dried onto filter paper, and exposed to Kodak XAR film for 2 to 5 d at −80°C. Exposures were done along with standards of known radioactivity to

ensure that all measurements were made in the linear exposure range of the film.

For scintillation analysis, reactions were performed as above but were stopped by addition of 1 mL of 1 N HCl in ethanol and incubated for 15 min at room temperature to ensure hydrolysis of unbound [^{3}H]GGPP (Moores et al., 1991; Pompliano et al., 1992). Reactions were diluted with 2 mL of 100% ethanol, filtered under vacuum onto Whatman GF/C glass fiber filters, washed four times with 2 mL of ethanol, dried, added to 4.5 mL of scintillation cocktail (Amersham), and counted in a Beckman 6800 scintillation counter.

RNA Analysis

Total RNA was isolated (Ach and Gruissem, 1994) from mature (fully expanded) and immature (not fully expanded) tomato leaves, roots, whole 0.8-cm young fruit, and fruit pericarp from later fruit stages. RNA concentrations were calculated from A_{260}. Samples of 2.5 μg of total RNA were fractionated on 1.0% agarose-formaldehyde gels and transferred to Qiabrane (Qiagen, Chatsworth, CA). DNA probes were labeled by random priming (Stratagene), and all hybridizations were carried out in 5× SSC/5× Denhardt's solution/0.5% (w/v) SDS/100 mg mL^{-1} herring sperm DNA (denatured) for 12 to 15 h at 60 or 65°C. Filters were washed once with 5× SSC, once with 2× SSC, and twice with 0.2× SSC (all with 0.1% SDS) for 20 min per wash at the hybridization temperature. Blots were exposed to Kodak XAR film for several days at −80°C with a Dupont intensifying screen.

For experiments in which expression of *LeRab1B* and *LeRab1C* was analyzed, probes corresponding to the putative 3′ untranslated region of the *LeRab1B* and C cDNAs were used. For analysis of *LeRab1A* expression, the partial cDNA isolated in the young fruit library screen was used. For ethylene treatments, 3-week-old seedlings (cv VFNT cherry) grown in MS agar (GIBCO-BRL) were exposed to humidified ethylene (20 mL L^{-1}) or to air alone for 24 h as described by Lincoln et al. (1987). Total RNA was extracted from seedling shoots.

RESULTS

Cloning and Sequence Analysis of *LeRab1A, B,* and *C*

To isolate genes encoding potential substrates for a tomato Rab-specific GGTase, we screened a tomato young fruit cDNA expression library using a polyclonal antibody against the *Volvox carteri YPT1* homolog *YptV1* (Fabry et al., 1992). We isolated Rab cDNAs representing three different genes, which we designated *LeRab1A, B,* and *C* (*Lycopersicon esculentum* Rab). The deduced translation products of the *LeRab1A, B,* and *C* cDNAs are shown in Figure 1A. The proteins encoded by *LeRab1A* and *C* are more similar to each other than to LeRab1B and each terminates with CCSS, a prenylation motif also found in the mammalian Rab5 protein, which was shown to be prenylated both in intact cells and in vitro by mammalian cell extracts (Kinsella and Maltese, 1991, 1992). LeRab1B, however, terminates with the double-Cys motif CCG, a motif that until now has not been identified as a signal for prenylation by Rab GGTase but which has been reported in at least one other plant Rab-like protein (Nagano et al., 1993). Because of its similarity to other Rab GGTase motifs, it is a likely candidate for modification by this enzyme. All three tomato LeRab proteins share more than 75% identity at the amino acid level to Rab1A (Fig. 1B), a mammalian homolog of the YPT1 protein (Segev et al., 1988; Haubruck et al., 1989; Nuoffer et al., 1994). It was on the

A

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          1                                                  39
LeRab1A   ...MNPEYDY LFKLLLIGDS GVGKSRLLLR FADDSYLESY
LeRab1C   ...------- ---------- -----C---- -------D--
LeRab1B   ...-SN---- ---------- S----C---- ------V-SY
 Rab1A    MSS------- ---------- -----C---- ----T--ESY
YPT1sc    ...--S---- --------N- -----C---- F-DD-Y---Y

          40                                                 79
LeRab1A   ISTIGVDFKI RTVDQDGKTI KLQIWDTAGQ ERFRTITSSY
LeRab1C   ---------- ---E------ ---------- ----------
LeRab1B   ---------- ---E------ ---------- ----------
 Rab1A    ---------- --IEL----- ---------- ----------
YPT1sc    ---------- K--EL----V ---------- ----------

          80                                                 119
LeRab1A   YRGAHGIIVV YDVIDQESFN NVKQWLSEID RYASDNVNKL
LeRab1C   --------I- --IT------ ---------- ----E-----
LeRab1B   --------I- ----EM---- ------N--- ---NES-C--
 Rab1A    ---------- ---------- ------Q--- ----E-----
YPT1sc    ---S----I- ---------- G--M--Q--- ---TS--L--

          120                                                159
LeRab1A   METSAKNATN LVGNKCDLTA QKVVSTETAQ AFADEIGIPF
LeRab1C   --A---S--- -----S--ND NRA--YD--K ----------
LeRab1B   L-----DSI- --------VE N---D-QMGK -L---L----
 Rab1A    L------EK- ---------T K---DYT--K E---SL----
YPT1sc    L----LDS-- --------KD KR--EYDV-K E---ANKM--

          160                                                199
LeRab1A   VEQAFMAMA. ASIKNRMASQ ....PASNNA RPPTVQIRGQ
LeRab1C   ---------. -E------T- ....------ K---------
LeRab1B   -----LT--. GE--KK-GN- ....--GAKR TGS----K--
 Rab1A    ---S--T--. -E--K--GPG ....ATAGG- EKSN-K-QST
YPT1sc    --D--LT--R QU--QS-SQ- NLNET-QKKE DKGN-NLK--

          200  210
LeRab1A   PVNQKSGCCSS
LeRab1C   -----N-CCSS
LeRab1B   -IE--GNCCG
 Rab1A    --K-SG-GCC
YPT1sc    SLTNTG-GCC
```

B

	L1A	L1B	L1C	YPT1	Rab1A
L1A	---	75.2	89.7	64.6	75.1
L1B	75.2	---	76.2	67.0	81.7
L1C	89.7	76.2	---	67.0	76.2
YPT1	64.6	67.0	67.0	----	71.8
Rab1A	75.1	81.7	76.2	71.8	----

Figure 1. Predicted amino acid sequences for LeRab1A, B, and C compared to YPT1p and Rab1A. A, Prenylation target sequences are underlined. Dashes represent conserved amino acids. The amino acid sequences for rat Rab1A (accession No. P057111) and *S. cerevisiae YPT1* (accession No. P01123) were obtained from the SwissProt data base. B, Percentage identity of the five Rab proteins. Sequence comparisons were done using GCG sequence analysis software.

basis of their homology to Rab1A that the tomato cDNAs were named.

LeRab1A, B, and *C* Are *YPT1* Homologs

S. cerevisiae YPT1 encodes a Rab-like protein that is essential for growth and is required for ER-to-Golgi transport in yeast (Segev et al., 1988). Mouse Rab1A was first proposed to be a mammalian homolog of YPT1p on both the basis of its sequence similarity with YPT1p (>70% identity) and its ability to replace YPT1p in yeast when expressed at high levels (Haubruck et al., 1987, 1989). Subsequent studies have confirmed the role of Rab1A in ER-to-Golgi transport in mammalian tissue-culture cells (Nuoffer et al., 1994). To test whether *LeRab1A, B,* and *C* might also encode tomato homologs of *YPT1*, the tomato *LeRab* cDNAs were subcloned into yeast expression plasmid pRH98–3 and then used to transform *S. cerevisiae* GFUI-6D cells. This strain's only functional copy of *YPT1* is controlled by the Gal-inducible *GAL10* promoter that permits growth only on Gal-containing medium, since *YPT1* is an essential gene in yeast (Ossig et al., 1995).

For complementation testing, URA^+ GFUI-6D transformants were plated onto selective medium containing Glc or Gal as the sole carbon source. As shown in Figure 2A, GFUI-6D cells containing pAAH5 with *YPT1*, but not pAAH5 with no insert, are able to grow well on minimal medium containing Glc. Similarly, cells transformed with pRH98–3 containing any one of the three tomato genes, but not with pRH98–3 with no insert (Fig. 2B), were able to grow well on Glc-containing medium, demonstrating that the tomato proteins are able to substitute for YPT1p in this test. Thus *LeRab1A, B,* and *C* are functional homologs of *YPT1*.

An epitope tag of amino acid sequence MEYMPME (EE-tag), for which a monoclonal antibody is available (Schaffhausen et al., 1982; Rubinfeld et al., 1991), was introduced at the N terminus of each *LeRab*-coding region, and the tagged proteins were also tested for their ability to complement *YPT1*. It was found that this sequence did not interfere with the ability of the proteins to substitute for YPT1p (Fig. 2B). This experiment was done to test whether this epitope tag might be useful for future immunolocalization and biochemical studies of transgenic plants expressing tagged LeRab1A, B, and C protein. The fact that the EE-tag did not affect the ability to complement also demonstrates that the tag does not significantly interfere with prenylation, since prenylation is necessary for biological function of these proteins. GFUI-6D cells were also transformed with tagged LeRab1A, B, and C constructs in which the C terminal Cys's had been converted to other amino acids (LeRab1AmCC, BmCC, and CmCC). Transformants expressing these mutant proteins lacking the prenylation motif were unable to grow on medium containing Glc (Fig. 2B). Thus complementation of *ypt1* by *LeRab1A, B,* and *C* requires an intact prenylation motif, a finding that is consistent with the belief that prenylation is necessary for Rab protein function.

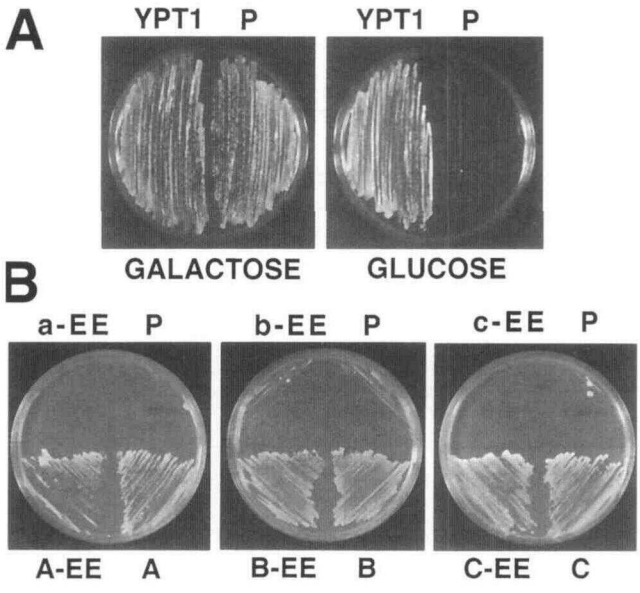

Figure 2. *LeRab1A, B,* and *C* are *YPT1* homologs. A, GFUI-6D transformed with pAAH5 (P) or pAAH5-YPT1 (YPT1) were streaked onto selective media containing Gal or Glc as indicated. B, GFUI-6D transformed with tagged and untagged wild-type *LeRab1A, B,* or *C* and tagged mutant *LeRabs* were streaked onto Glc-containing minimal medium. P, pRH98–3, no insert; A, B, C, pRH98–3-*LeRab1A*, pRH98–3-*LeRab1B*, and pRH98–3-*LeRab1C* without N-terminal EE-tag; A-EE, B-EE, C-EE, pRH98–3-*LeRab1A*, pRH98–3-*LeRab1B*, and pRH98–3-*LeRab1C* with EE-tag; a-EE, b-EE, c-EE, pRH98–3-*LeRab1AmCC*, pRH98–3-*LeRab1BmCC*, and pRH98–3-*LeRab1CmCC* with EE-tag.

Tobacco and Tomato Tissue-Culture Cells Contain Rab GGTase-Like Activity

To study Rab GGTase activity in plant cells, we used poly(His)-tagged LeRab protein produced in bacteria as a substrate in reactions containing yeast and plant-cell protein and [^{3}H]GGPP. Previous experiments showed that GST-tagged LeRab fusion protein was modified by a Rab GGTase-like activity from plant cells (Yalovsky et al., 1996). To characterize this activity in greater detail, we decided to use His-tagged recombinant LeRab proteins, as was done in studies of mammalian Rab GGTase (Seabra et al., 1992a, 1992b; Andres et al., 1993; Cremers et al., 1994), to avoid the possibility that the 26-kD GST moiety of recombinant GST::LeRab might affect the Rab GGTase enzyme's ability to interact with its protein substrate. To test whether the His-tagged LeRabs could serve as substrates for the plant Rab GGTase, we assayed protein extracts prepared from tomato and tobacco tissue-culture cells with a protein extract from yeast as a positive control.

Rab protein substrates used included recombinant LeRab1A, B, and C protein with mutant and wild-type prenylation motifs. Figure 3 shows the labeled protein products separated by SDS-PAGE from prenylation reactions done with crude protein extracts from yeast, tobacco BY-2, and tomato tissue-culture cells. In all three protein extracts, labeling of recombinant LeRab required the presence of an intact prenylation target sequence in

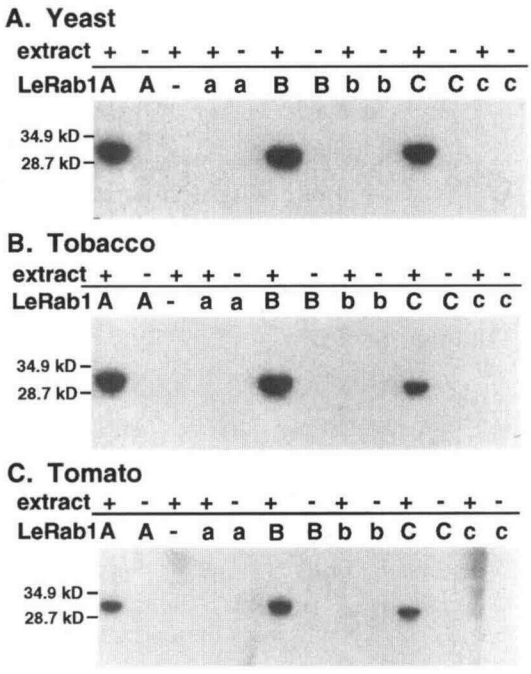

Figure 3. Plant and yeast extracts modify wild-type and not mutant LeRab1A, B, and C. The prenylation reactions were allowed to proceed for 1 h at 30°C with or without extract together with recombinant LeRab as indicated. Uppercase A, B, and C are wild-type LeRab1A, B, and C protein, respectively, and lowercase a, b, and c are mutant LeRab1AmCC, LeRab1BmCC, and LeRab1CmCC protein, respectively. Reactions with extract (+) contained 50 μg of yeast protein (A), 52 μg of tobacco tissue-culture cell protein (B), or 65 μg of tomato tissue-culture cell protein (C).

the protein substrate, suggesting that the plant Rabs, like Rabs from other species, are modified on the Cys residues contained within the prenylation motif. In reactions performed without addition of recombinant Rab (lane 3), no ^{3}H-labeled product is detectable, demonstrating that the labeled proteins present in lanes 1, 6, and 10 were not supplied by the protein extract.

Rab-Modifying Activity in Plant Cells Is Biochemically More Similar to Rab GGTase than GGTase I

Mutant protein substrate lacking the C-terminal prenylation motif competes in vitro with Rab GGTase but not GGTase I (Seabra et al., 1992a, 1992b; Sanford et al., 1993). To test whether the Rab-modifying activity in plant-cell extracts is reduced by addition of mutant Rab, increasing concentrations of mutant LeRab1B protein (LeRab1BmCC) were included in reactions with tobacco tissue-culture cell protein extract together with [^{3}H]G-GPP and 0.5 μM wild-type LeRab1B protein (Fig. 4A). As a control, reactions were also performed with increasing amounts of BSA instead of LeRab1BmCC. Reactions were stopped by addition of 1 mL 1 N HCl in ethanol and processed as described in "Materials and Methods." Assays were performed in triplicate and the mean of values obtained, minus the mean of blank reactions in which recombinant LeRab1B was omitted, are shown as the percentage of labeling obtained in control reactions in which neither BSA nor LeRab1BmCC was included. Figure 4A shows that LeRab1BmCC, but not BSA, reduced labeling of LeRab1B. Similar results were obtained with GST::LeRab1CmCC (Yalovsky et al., 1996) and LeRab1AmCC (data not shown).

We also tested the effect of detergent on labeling in the in vitro assay. Rab GGTase activity in mammals and yeast is enhanced by detergent, which promotes dissociation of REP from prenylated Rab and permits REP to participate in further rounds of catalysis (Seabra et al., 1992a, 1992b). Using wild-type recombinant LeRab1B as the protein substrate in assays with tobacco tissue-culture cell protein extract, we measured Rab modification over time in the presence or absence of 0.3 mM NP-40. Reactions were done in duplicate and the mean of values obtained minus the mean of blank reactions in which LeRab1B was omitted are shown in Figure 4B. As with the mammalian enzyme, NP-40 enhanced modification of recombinant LeRab1B relative to the control reactions in which no detergent was added. These results demonstrate that the modifying activity under investigation is

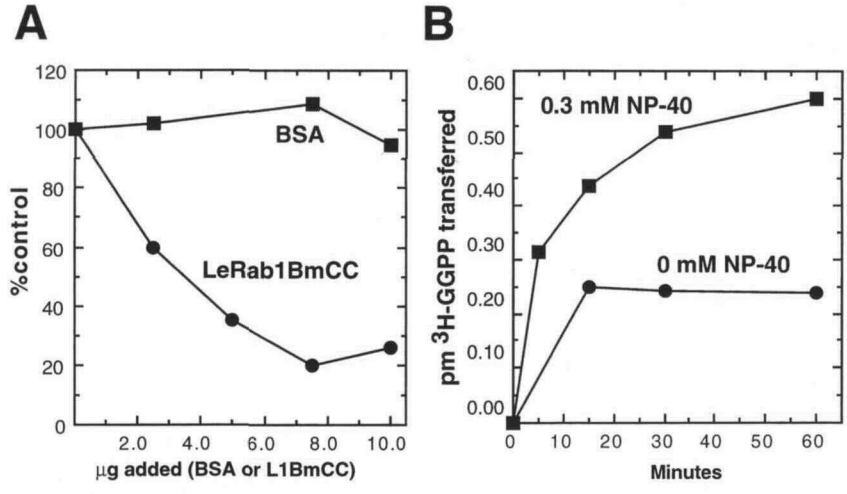

Figure 4. Rab-modifying activity in tobacco cells is inhibited by LeRab1BmCC and enhanced by NP-40. A, Assays contained 30 μg of tobacco tissue-culture cell extract together with increasing amounts of recombinant LeRab1BmCC or BSA and 0.5 μM recombinant LeRab1B. Assays were done in triplicate for 1 h and mean values, shown as percentages of activity obtained when neither BSA nor LeRab1BmCC was added, are shown. Each assay was performed at 30°C and stopped by addition of acid in ethanol and processed as described in "Materials and Methods." B, Assays contained 2.0 μM recombinant LeRab1B and 30 μg of tobacco tissue-culture cell extract with or without 0.3 mM NP-40 as indicated. The means of values obtained from duplicate assays processed as in A at the times indicated on the *x* axis are shown.

likely to be Rab GGTase rather than GGTase I or some other novel protein prenyl transferase.

Plant Extracts Are Most Active when LeRab1B Is Supplied

In humans, two different REPs have been cloned (Andres et al., 1993; Cremers et al., 1994). REP-1 and REP-2 differ in their ability to prenylate Rab3A in combination with the same catalytic heterodimer. Rab3A is about a 3-fold poorer substrate with REP-2 than with REP-1. Rab GGTases containing either REP-1 or REP-2 prenylate Rab1A with about equal efficiency, however (Cremers et al., 1994). To determine whether the plant Rab GGTase-like activity might also distinguish between different Rab proteins, we measured the relative ability of LeRab1A, B, and C to serve as substrates in reactions with yeast and plant-cell protein extracts. Reactions were performed in triplicate for 30 min at 37°C and contained 0.3 mM NP-40. Since the tomato extract contained a background activity that was able to convert the radioactive GGPP precursor to an acid-ethanol-insoluble compound when no recombinant Rab was included (data not shown), we subjected the reactions to SDS-PAGE analysis, followed by densitometric scanning of the resulting autoradiograph to quantitate relative labeling of recombinant LeRab proteins.

In both plant extracts, LeRab1B was the superior substrate. In Figure 5, B and C, labeling of LeRab1A and C is shown as a percentage of the labeling obtained with LeRab1B. Both plant-cell protein extracts labeled LeRab1C approximately 35% as well as LeRab1B. LeRab1A was labeled about 40% as well as LeRab1B in the tomato protein extract and about 65% as well as LeRab1B in the tobacco protein extract. In contrast, when yeast protein extract was tested, LeRab1A was the best substrate (Fig. 5A). In the yeast protein extract, LeRab1B and LeRab1C were both labeled about 60% as well as LeRab1A. This latter observation argues against the possibility that the difference in labeling seen with the different LeRab proteins in tobacco and tomato protein extracts was due to relative quality of the LeRab preparations used.

LeRab1A, B, and *C* mRNA Expression Pattern

To gain insight into the function of the *LeRab1A, B,* and *C* genes in tomato, we examined their mRNA expression patterns in various organs and fruit stages using gene-specific DNA probes. To confirm that the DNA probes used in these experiments were gene specific, blots containing tomato genomic DNA digested with *Eco*RI were probed under the same conditions used to probe the RNA blots. In all three cases, each *LeRab* probe hybridized to only one band (data not shown), confirming that the probes used were indeed gene specific. We then probed three RNA blots containing total RNA isolated from tomato roots, mature and immature leaves, and fruit pericarp in various stages of fruit development. As shown in Figure 6, all three messages are present at high levels in fruit pericarp. *LeRab1B* and C RNAs are also more abundant in immature leaves than in mature leaves. *LeRab1A* mRNA is more abundant in ripening fruit than in fruit at earlier stages of development.

Fruit ripening in tomato is marked by an increase in respiration known as the climacteric, which is believed to be triggered by a burst of ethylene biosynthesis (Yang and Hoffman, 1984). Following the climacteric, a variety of cellular processes is triggered. Some of these processes include increased activity of cell-wall-modifying enzymes like polygalacturonase and pectin esterase, differentiation of photosynthetic chloroplasts into lycopene-containing chromoplasts, and transcription of ripening-related genes like *E4* and *E8* (Lincoln et al., 1987; Fischer and Bennet, 1991). The observation that *LeRab1A* mRNA is more abundant in ripening fruit than in earlier stages suggested that *LeRab1A* might be partially regulated by ethylene. To test whether accumulation of *LeRab1A* transcript might be controlled in part by ethylene, 3-week-old seedlings were treated for 24 h with 20 mL L^{-1}

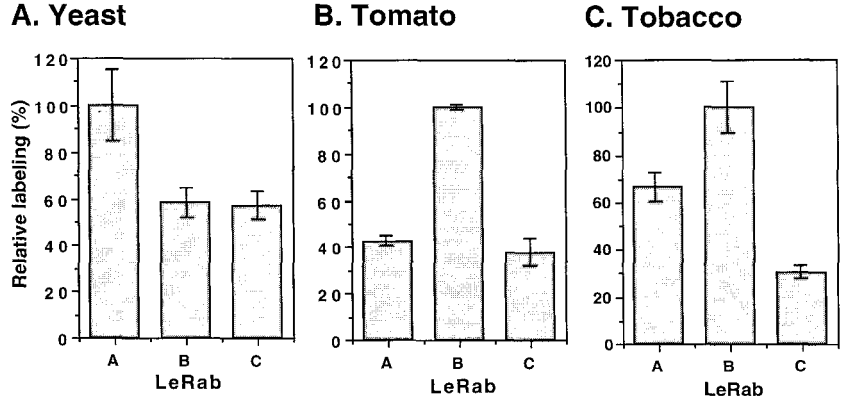

Figure 5. Differential modification of LeRab1A, B, and C protein by yeast and plant tissue-culture cell protein extracts. Assays were performed for 30 min at 37°C and analyzed by SDS-PAGE as described in "Materials and Methods." Bands corresponding to labeled Rab were quantified using densitometry. The means from triplicate assays are shown with error bars indicating SE. A, Labeling of LeRab1B and C is shown relative to LeRab1A labeling in reactions with 50 μg of yeast protein extract. B and C, Labeling of LeRab1A and C is shown relative to LeRab1B labeling in reactions with 65 μg of tomato tissue-culture cell protein (B) and 52 μg of tobacco tissue-culture cell protein (C).

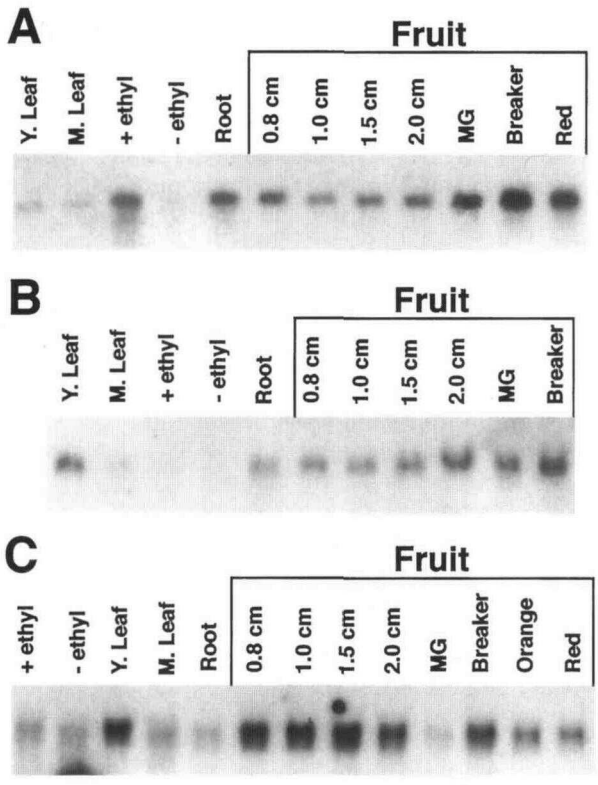

Figure 6. RNA expression analysis. Each blot contained 2.5 μg of total RNA isolated from young leaves (Y. Leaf), mature leaves (M. Leaf), ethylene-treated shoots (+ethyl), nonethylene-treated control shoots (−ethyl), roots (Root), whole 0.8-cm fruit (0.8 cm), pericarp from 1.0-, 1.5-, and 2.0-cm fruit (1.0 cm, 1.5 cm, and 2.0 cm), mature green (MG), breaker, orange, and red fruit pericarp. Blots were probed using DNA probes specific for *LeRab1A* (A), *LeRab1B* (B), and *LeRab1C* (C).

ethylene and total RNA was isolated from the shoots of ethylene-treated plants, along with control plants of the same age. As shown in Figure 6A, *LeRab1A* message, but not that of *LeRab1C* or *LeRab1B*, was more abundant in ethylene-treated tissues relative to the control plants of the same age.

DISCUSSION

Rab proteins in yeast and mammalian cells regulate diverse steps in the vesicle-mediated secretory pathway. Different subclasses of Rab proteins are localized to distinct classes of transport vesicles and target membranes, where they are thought to contribute to the specificity of transport vesicle docking and/or vesicle-target membrane-fusion events. It is possible to group Rab proteins into different subclasses on the basis of their relatedness to each other at the amino acid level. These classifications are significant because related Rab proteins from different species often perform related functions. For example, mammalian Rab1A is associated with ER and *cis*-Golgi membranes and is required for the ER-to-Golgi step of the mammalian secretory pathway, as is the YPT1 protein, its most closely related yeast counterpart (Segev et al., 1988; Haubruck et al., 1989; Nuoffer et al., 1994).

Genes encoding Rab-like proteins have been isolated from *V. carteri*, rice, *Arabidopsis thaliana*, tobacco, pea, maize, soybean, and tomato, but for many of these genes, their precise roles in the secretory pathway of these plants have yet to be identified (Terryn et al., 1993a, 1993b; Ma, 1994). One approach to understanding which secretory steps they might perform is to determine whether they complement mutations in similar genes in yeast. For example, a soybean *YPT1*-like gene was shown to be able to substitute for *YPT1* in yeast and was also shown to be required for formation of the peribacteroid membrane surrounding internalized *Rhizobium meliloti* bacteria in transgenic soybean nodule cells (Cheon et al., 1993). On the basis of these results, it was postulated that the soybean YPT1p-like protein is required in transport between the ER and Golgi in the soybean secretory pathway in nodule cells. Based on their homology to Rab1A as well as on their ability to substitute for YPT1p, we suggest that the closely related proteins encoded by *LeRab1A, B*, and *C* are likely to regulate a similar step in the tomato cells where they are expressed.

The up-regulation of *LeRab1A* mRNA accumulation in ripening fruit as well as its regulation by ethylene suggests that this gene, more so than the other tomato Rab1 isoforms described here, may function in a general up-regulation of the secretory pathway in response to ethylene, the plant hormone that initiates fruit ripening. Since fruit ripening involves degradation of cell walls by secreted cell-wall-modifying enzymes like polygalacturonase and pectin esterase, it is possible that *LeRab1A* may be important for fruit softening during ripening. Similarly, higher levels of *LeRab1C* mRNA in young fruit and in developing leaves relative to mature leaves and red fruit may reflect a possible role in up-regulating the secretory pathway of dividing and expanding cells. There is some evidence that the level of Rab protein in cells may be a limiting factor in controlling the rate of vesicle-mediated transport. Rab5, for example, is localized to the plasma membrane and to early endosomes and has been shown to be rate limiting both in vivo and in vitro with respect to endocytosis in mammalian cells (Bucci et al., 1992). Thus, expression of the *LeRab* genes may in part help regulate the rate of secretion in the cells where they are expressed. Although the LeRab1A, B, and C proteins may each regulate the same step in the tomato secretory pathway, as suggested by their common ability to substitute for YPT1p in yeast, their expression in tomato may be linked to mechanisms that control the rate at which membrane materials and secretory proteins are transported through the secretory pathway in the different types of cells where they are expressed.

The precise biochemical mechanism underlying the role of Rab proteins in promoting vesicle targeting to and/or fusion with target membranes is not understood. It is known, however, that their function in secretion is intimately connected with the cycle of GTP binding and hydrolysis and that their localization to intracellular membranes is dependent on their ability to serve as substrates for prenylation. Rab GGTase, the enzyme that catalyzes

this modification, has been extensively characterized in yeast and mammalian cells. We show here that extracts from tobacco and tomato tissue-culture cells contain a Rab-modifying activity that utilizes [^{3}H]GGPP to label recombinant LeRab protein and that this activity shares distinguishing biochemical properties with the mammalian Rab GGTase enzyme. Like Rab GGTase, the activity measured is subject to competition in vitro by the addition of recombinant protein substrate in which the prenylation motif Cys residues have been converted to other amino acids. Second, we show that adding detergent to the Rab GGTase assay increased overall modification of recombinant LeRab1B, another feature the plant activity shares in common with mammalian Rab GGTase. Based on the results presented here and in an accompanying paper (Yalovsky et al., 1996), we believe that the Rab-modifying activity under investigation is due to Rab GGTase and not GGTase I or some other novel protein prenyl transferase.

REP-1 and REP-2, encoded in humans by *choroideremia* and *choroideremia-like*, differ in their ability to present Rab3A to the catalytic portion of the Rab GGTase (Seabra et al., 1993; Cremers et al., 1994). Choroideremia is an inherited form of blindness caused by degeneration of the retina and adjacent tissues (Cremers et al., 1990). The surprising result that choroideremia is caused by mutations in a gene that should be essential in all cells, given that Rab proteins are ubiquitous, was partly explained by isolation of the closely related gene *choroideremia-like*, encoding REP-2. Presumably this and other genes encoding the REP component of Rab GGTase compensate for the lack of REP-1 in most cells but not in cells affected in choroideremia. These observations led to the hypothesis that in mammals different REP genes may be differentially expressed in cell types containing different subclasses of Rab protein (Cremers et al., 1994).

Similarly, our observation that tissue-culture cells from two different plant species contain Rab-modifying activity that is most active when LeRab1B rather than LeRab1A or C is used for the reaction suggests that, like human Rab GGTase, tobacco and tomato Rab GGTases distinguish between Rab protein substrates. These results suggest that plants also may contain multiple REP proteins that differ in their relative abilities to present plant Rab proteins to the catalytic subunits of the enzyme. That this preference for LeRab1B is conserved in tissue-culture cells from tobacco and tomato suggests that plant REPs may be regulated with respect to cell type in a way that is conserved between different plant species. Moreover, the nearly 2-fold difference in activity when LeRab1A versus C was tested using yeast and tobacco protein extract may provide clues as to which amino acid residues in the protein substrate most affect the ability of the enzyme to modify its target, since LeRab1A and C are highly homologous and share almost 90% amino acid identity. Thus our continuing study of Rab GGTase using these three highly homologous tomato Rab proteins as biochemical and molecular tools may ultimately yield greater insight into the mechanisms underlying Rab GGTase function.

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The accession numbers for the sequences reported in this article are U38464 (*LeRab1A*), U38465 (*LeRab1B*), and U38466 (*LeRab1C*).

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