**Arabidopsis** Rab Geranylgeranyltransferases Demonstrate Redundancy and Broad Substrate Specificity in Vitro*

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Posttranslational lipid modifications mediate the membrane attachment of Rab GTPases, facilitating their function in regulating intracellular vesicular trafficking. In *Arabidopsis*, most Rab GTPases have two C-terminal cysteines and potentially can be double-geranylgeranylated by heterodimeric Rab geranylgeranyltransferases (Rab-GGTs). Genes encoding two putative α subunits and two putative β subunits of Rab-GGTs have been annotated in the *Arabidopsis thaliana* genome, but little is known about Rab-GGT activity in *Arabidopsis*. In this study, we demonstrate that four different heterodimers can be formed between putative *Arabidopsis* Rab-GGT α subunits RTGTA1/RTGTA2 and β subunits RTGB1/RTGB2, but only RTGTA1/RTGB1 and RTGTA1/RTGB2 exhibit *bona fide* Rab-GGT activity, and they are biochemically redundant in *vivo*. We hypothesize that RTGTA2 function might be disrupted by a 12-amino acid insertion in a conserved motif. We present evidence that *Arabidopsis* Rab-GGTs may have preference for prenylation of C-terminal cysteines in particular positions. We also demonstrate that *Arabidopsis* Rab-GGTs can not only prenylate a great variety of Rab GTPases in the presence of Rab escort protein but, unlike Rab-GGT in yeast and mammals, can also prenylate certain non-Rab GTPases independently of Rab escort protein. Our findings may help to explain some of the phenotypes of *Arabidopsis* protein prenyltransferase mutants.

Small GTPases serve as molecular switches that shuttle between active GTP-bound and inactive GDP-bound forms, providing transient signals to downstream effectors (1, 2). In plants, many membrane-localized small GTPases are important regulators of vesicular trafficking (1). They are typically anchored to membranes via posttranslational lipid modifications (3).

Rab GTPases constitute the largest family of the Ras superfamily of small GTPases (3–5). They are involved in regulating trafficking processes, such as vesicle formation, transport, membrane targeting, and docking (1, 5). The diversity and specific localization of Rab GTPases not only determine membrane identity, but also reflect the complexity of vesicle trafficking (1, 6).

Phylogenetic analysis suggests that the 57 *Arabidopsis* Rab-encoding sequences fall into just eight subfamilies (3, 6), in contrast to ~40 Rab subfamilies in mammals (7). Accordingly, the size of each *Arabidopsis* Rab subfamily is expanded. The distinct composition of the *Arabidopsis* Rab GTPase family suggests plant-specific functions (1, 6). Of these 57 members, 54 include two cysteines that are near the C terminus and are candidate prenylation sites (Table 1). At least one of the non-prenylated Rabs, RABF1/ARA6, is N-myristoylated and palmitoylated (8).

Protein prenylation irreversibly adds one 15-carbon isoprenoid (farnesylation), one 20-carbon isoprenoid (geranylgeranylation), or two 20-carbon isoprenoids (double geranylgeranylation) to one or two C-terminal cysteine residues of target proteins, by forming thioether bonds (9, 10). These three types of prenylation are respectively catalyzed by three distinct heterodimeric enzymes, collectively called protein prenyltransferases (11, 12). Protein farnesyltransferase (PFT)3 and protein geranylgeranyltransferase type I (PGGT-I) target a C-terminal CaaX box, in which C is the cysteine residue to be prenylated, and a is usually an aliphatic amino acid residue. For PFT, X is usually alanine, cysteine, glutamine, methionine, or serine; for PGGT-I, X is almost always leucine (10–12). In *Arabidopsis*, >250 proteins are predicted to be ideal targets for prenylation (10, 13). Among them, type I Rop GTPases and heterotrimeric G protein γ subunits AGG1 and AGG2 have a C-terminal CaaL box and can be geranylgeranylated by PGGT-I (9, 14, 15). Rab geranylgeranyltransferase (Rab-GGT, or protein geranylgeranyltransferase type II) has a broader spectrum of target sequences, most with two cysteine residues, including XCC, XCXc, XCCX, CCXX, and CCXXX (10, 12), where C is a cysteine residue that potentially can be prenylated, and X is a non-specific amino acid residue. It is believed that Rab-GGT only prenylates Rab GTPases; hence the name (16).

All of the known protein prenyltransferases are heterodimeric enzymes that consist of a regulatory α subunit and a catalytic β subunit. PFT and PGGT-I share a common α subunit but have unique β subunits (10, 17). Complete loss of PFT and PGGT-I activities leads to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethality in yeast and animals (17–19). In contrast, mutations in the *Arabidopsis* PFT/PGGT-I activities lead to lethal...
PGGT-I α subunit PLP (pluripetala) cause significant developmental defects, but the mutants are viable and fertile (20). The viability of plp mutants suggests that additional prenylation mechanisms or other types of lipid modification in Arabidopsis might compensate for the loss of PFT/PGGT-I activities (10, 20). Mutations in the Arabidopsis PFT β subunit ERA1 (enhanced response to abscisic acid 1) result in only mild phenotypes (21–24), whereas mutations in the PGGT-I β subunit GGB (geranylgeranyltransferase-I β subunit) result in no detectable phenotypes under normal conditions (25), suggesting considerable target cross-specificity between Arabidopsis PFT and PGGT-I (10, 25).

Rab-GGT has a distinct set of subunits, and no shared subunits between Rab-GGT and PFT/PGGT-I have been reported. Despite sharing only 20–30% amino acid sequence similarity with their counterparts in PFT and PGGT-I, Rab-GGT subunits structurally resemble PFT and PGGT-I subunits (10, 16). The N-terminal helical domain of the Rab-GGT α subunit is structurally very similar to the PFT/PGGT-I α subunit, whereas the Rab-GGT β subunit forms an α-α barrel structure, as does the PFT β subunit (16, 26, 27). Mammalian and plant Rab-GGT α subunits have an additional immunoglobulin (Ig)-like domain and a leucine-rich repeat (LRR) domain, both of which all known PFT/PGGT-I α subunits and yeast Rab-GGT α subunits lack (12, 26). However, it appears that neither of these two domains is required for Rab-GGT activity (28). Remarkably, rather than recognizing target proteins by itself as PFT and PGGT-I do, Rab-GGT relies on a cofactor protein called Rab escort protein (REP) to recognize Rab GTPases (26, 29). According to different models, REP binds to unprenylated Rab GTPase, either before or after forming a complex with the Rab escort protein (REP) to recognize Rab GTPases (26, 29).

Indirect evidence that RGTB1 is a bona fide Rab-GGT subunit comes from studies of rgtb1 mutants; specifically, the level of prenylated RABA2A is reduced in rgtb1 mutants, and rgtb1 total extracts cannot efficiently prenylate recombinant RABA2A in vitro (37). However, the biochemical activity of the other putative subunits and possible target specificity differences among different αβ combinations remain unknown. It is also unclear whether AtREP is required for Arabidopsis Rab-GGT activity. In this study, we used an isotope-based in vitro prenylation assay to address these questions. Here we report that RGTB1 and RGTB2 are biochemically redundant Rab-GGT β-subunits in Arabidopsis, whereas RGT A1 is the only active α subunit. Arabidopsis Rab-GGT also appears to show a preference for prenylation of cysteines in particular positions at the C terminus. Arabidopsis Rab-GGT not only prenylates a vast variety of Rab GTPases with various C-terminal sequences in vitro in an REP-dependent manner, but, unlike what has been reported for other eukaryotic Rab-GG Ts, can also prenylate certain non-Rab small GTPases in vitro in an REP-independent manner. Our results help partially explain the survivability of Arabidopsis mutants lacking PFT/PGGT-I activity, the lack of phenotypes in PGGT-I mutants, and the observation of partial membrane localization of PGGT-I targets in PFT/PGGT-I mutants (15).

**Experimental Procedures**

**Protein Sequence Analysis**—The sequences of Arabidopsis genes and proteins were acquired from the Arabidopsis Information Resource (TAIR) online database (41). The sequences of yeast genes and proteins were acquired from the Saccharomyces Genome Database (42). The sequences of P. patens proteins were acquired from PlantGDB (43). The rice protein sequence was acquired from the Rice Genome Annotation Project website (44). The Drosophila melanogaster PTA3 protein sequence was acquired from FlyBase (45). The sequences of genes and proteins of rat and human were acquired from the NCBI Reference Sequence (RefSeq) database (46). The pairwise alignments were performed with EMBOSS Needle (47). The multiple sequence alignments were performed with Clustal Omega (48). The conserved motif predictions were performed by InterPro version 51.0 (49) and Motif Scan (50). The alignment of RGT A1 and RGT A2 with rat RABGGTA (Protein Data Bank entry 1LTX) (31) was performed with the NCBI Cn3D application (51).

**Expression of Rab-GGT in Yeast**—The coding sequences of RGT A1, RGT A2, RGT B1, and RGT B2 were amplified by high-fidelity PCR using cDNA from Arabidopsis Col-0 wild-type plants as a template. The resulting PCR products were cloned into the yeast expression vector pESC-HIS (Agilent Technologies, Santa Clara, CA) in two steps. First, the coding sequences of RGT B1 and RGT B2 were cloned into MCS1 (multiple cloning site 1) of pESC-HIS by double digestion with EcoRI and ClaI (New England Biolabs), followed by ligation with T4 DNA ligase (Promega, Madison, WI) to generate in-frame C-terminal fusions with the FLAG epitope tag. The resulting pESC-HIS-RGT B1-FLAG and pESC-HIS-RGT B2-FLAG plasmids were sequenced to verify the absence of PCR-induced mistakes and were used to separately express FLAG-tagged Arabidopsis Rab Geranylgeranyltransferases
RGTB1 and RGTB2 proteins, respectively. Then the coding sequences of RGTA1 and RGTA2 were cloned into MCS2 of pESC-HIS-RGTB1-FLAG and pESC-HIS-RGTB2-FLAG by single digestion with Xmal (New England Biolabs), followed by calf intestinal phosphatase treatment (New England Biolabs) and ligation with T4 DNA ligase to generate in frame C-terminal fusions with the c-Myc epitope tag. The direction of the insert was checked by colony PCR using a GAL1 forward sequencing primer and RGTA1/2 gene-specific reverse primers. The resulting pESC-HIS-RGTB1-FLAG-RGTA1-c-Myc, pESC-HIS-RGTB1-FLAG-RGTA2-c-Myc, pESC-HIS-RGTB2-FLAG-RGTA1-c-Myc, and pESC-HIS-RGTB2-FLAG-RGTA2-c-Myc constructs were verified by sequencing and were used to co-express one α subunit with one β subunit.

The pESC constructs were transformed into S. cerevisiae YPH499 competent cells using a LiAc/SS carrier DNA/PEG preparation of YPH499 competent cells has also been described previously with overnight incubation at room temperature. The YPH499 competent cells using a LiAc/SS carrier DNA/PEG buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl$_2$, 0.4 mM. After a 6-h induction at 25 °C, the cells were harvested and resuspended in SG medium with 2% galactose and 1% raffinose as carbon sources. The cells were then pelleted by centrifugation, washed with sterile water, and resuspended in SG media supplemented with anti-mouse IgG (Fab-specific)-peroxidase (Sigma-Aldrich) at 1:6500 dilution in 1× PBST while shaking at room temperature for 1 h. We used Pierce ECL Western blotting substrate (Thermo Scientific) for final detection, following the manufacturer's instructions. The chemiluminescence image was taken by an ImageQuant LAS4000 mini imager (GE Healthcare).

Expression of GST-tagged Rab GTPases and Substitution of C-terminal Cysteines—The coding sequences of selected Arabidopsis Rab GTPases (Table 1) were amplified by PCR using cDNA from Arabidopsis Col-0 wild-type plants as a template. The coding sequences were cloned into the pGEX-4T-1 vector (GE Healthcare) to generate in frame GST fusions by a single digest with BamHI or EcoRI, followed by calf intestinal phosphatase treatment and ligation with T4 DNA ligase. Three of the constructs, pGEX-4T-1 RABA4B, pGEX-6P-1 RABF2A, and pGEX-6P-1 RABG3C, were kindly provided by Dr. Erik Nielsen (University of Michigan) (53). All clones were sequenced to verify that they encoded wild-type proteins.

The pGEX RAB constructs were transformed into chemically prepared Escherichia coli BL21 competent cells using a heat-shock method. To express N-GST-tagged Rab GTPases, the BL21 cells containing the expression construct were grown in LB medium with 100 μg/ml ampicillin to an OD$_{600}$ of 0.6–0.8. The expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside into the culture to a final concentration of 0.4 mM. After a 6-h induction at 30 °C, the cells were harvested and resuspended in ice-cold 1× PBS with 100 μg/ml lysozyme, 10 μg/ml DNase I, and 5 mM DTT. After a 5-min incubation at room temperature, the cells were lysed by sonication. The GST-tagged proteins in the supernatant of the cell lysate were purified with GST SpinTrap columns (GE Healthcare), following the manufacturer’s instructions.

The C-terminal cysteine substitution mutant proteins were generated by introducing point mutations into reverse primers for amplifying the coding sequence from the pGEX constructs of the corresponding wild-type proteins. The cloning, expression, and purification procedures were the same as those for wild-type proteins. The presence of the introduced point mutations was verified by DNA sequencing.

We used EZ-View Red FLAG M2 affinity gel beads (Sigma-Aldrich) to pull down FLAG-tagged RGTB1 and RGTB2, along with their respective interacting proteins. We followed the manufacturer’s protocol for the equilibrating, binding, washing, and elution procedures, using 3× FLAG peptide to elute bound proteins.

To detect the tagged proteins by Western blot, protein samples (e.g. soluble fractions from cell extracts and pull-down eluates) were boiled with 2× Laemmli sample buffer (Bio-Rad) and then loaded onto 10% SDS-polyacrylamide gels. The separated proteins were transferred to a Whatman Protran nitrocellulose membrane (GE Healthcare). The membrane was incubated in blotto (1× PBS, pH 7.4, 0.05% Tween 20, 5% nonfat dry milk) at room temperature with shaking for 1 h. For detecting FLAG-tagged RGTB1/2, the washed membrane was incubated with monoclonal anti-FLAG M2-HRP antibody (Sigma-Aldrich) at 1:1000 dilution in 1× PBST (1× PBS, pH 7.4, 0.05% Tween 20), shaking at room temperature for 1 h. For detecting c-Myc-tagged RGTA1/2, the washed membrane was incubated with monoclonal anti-c-Myc antibody (clone 9E10, Sigma-Aldrich) at 1:5000 dilution in 1× PBST and shaken at room temperature for 2 h, followed by another round of washes and incubation with anti-mouse IgG (Fab-specific)-peroxidase (Sigma-Aldrich) at 1:6500 dilution in 1× PBST while shaking at room temperature for 1 h. We used Pierce ECL Western blotting substrate (Thermo Scientific) for final detection, following the user manual. The chemiluminescence image was taken by an ImageQuant LAS4000 mini imager (GE Healthcare).

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We used EZ-View Red advanced protein assay reagent (Cytoskeleton, Denver, CO) to quantify all protein concentrations.

In Vitro Prenylation Assay—The preparation of AtREP protein, procedures of desalting and concentrating the purified recombinant proteins, and procedures of the isotope-based in vitro prenylation assays have been described previously in great detail (54). Unless specified, we generally followed this protocol with one modification that added 0.2–0.3 μl, instead of 1 μl, of tritium-labeled geranylgeranyl diphosphate ($^3$H-GGPP; Amer-
ican Radiolabeled Chemicals, St. Louis, MO) per reaction. We minimized the volume of the \(^{1}H\)-GGPP added to the reactions to minimize inhibition of the prenylation reactions by isopropyl alcohol and ammonia present in the solvent.

**Results**

**RGTA1/2 and RGTB1/2, Encoded by Two Pairs of Paralogous Genes, Are Putative \(\alpha\) and \(\beta\) Subunits of Arabidopsis Rab-GGT**—Based on the annotated full-length coding sequences in the TAIR database and our cDNA sequencing result, 75% of the aligned nucleotides are identical between RGTA1 (At4g24490) and RGTA2 (At5g41820), whereas 85% of the aligned nucleotides are identical between RGTB1 (At5g12210) and RGTB2 (At3g12070), suggesting that RGTA1/2 and RGTB1/2 are two pairs of paralogous genes.

However, our search using the Plant Genome Duplication Database (55) does not map any of these four genes to chromosome regions that were duplicated during the most recent Arabidopsis whole genome duplication (56), contrary to a previous report that these two duplications resulted from the whole genome duplication (57).

Pairwise alignment of the protein sequences shows that RGTA1 (321 aa) and RGTB2 (317 aa) are almost identical to each other, with 85% amino acid identity and 91% similarity. Both RGTA1 and RGTB2 are highly conserved with mammalian Rab-GGT \(\beta\) subunits (RABGGTA) in protein sequences, sharing 72 and 70% similarity to rat (Rattus norvegicus) RABGGTA, respectively, suggesting that they are paralogous putative \(\beta\) subunits of Arabidopsis Rab-GGT.

The protein sequences of RGTA1 (678 aa) and RGTA2 (687 aa) are also highly similar to each other, although the similarity is not as high as that of the two putative \(\beta\) subunits, with 68% amino acid identity and 76% similarity (Fig. 1A). RGTA1 and RGTA2 share 39 and 41% similarity to rat RABGGTA, respectively (Fig. 1A).

A search for conserved domains in RGTA1 predicts five protein prenyltransferase \(\alpha\) subunit (PPTA) repeats in the N-terminal region based on both the Prosite (58) profile (PS51147) and the Pfam (59) profile (PF01239), whereas a similar search for RGTA2 predicts five PPTA repeats based on the Prosite profile but only three based on the Pfam profile. The first and third PPTA repeats in RGTA2 predicted by the Prosite profile are not recognized by the Pfam profile. The alignment of RGTA1, RGTA2, and rat RABGGTA shows high similarity in the N-terminal helical domain consisting of the PPTA repeats (Fig. 1A, I–V). The most noticeable difference within this domain is a 12-aa insertion in the middle of the third (III) PPTA repeat of RGTA2 predicted by the Prosite profile (RGTA2 aa 137–148, Fig. 1A), which apparently disrupts this very conserved motif and may be responsible for the discrepancy between the predictions by the Prosite and Pfam profiles for the third repeat. PPTA repeats have been identified only in known protein prenyltransferase \(\alpha\) subunits (27, 60). Similar to RGTA1, both the Prosite and Pfam profiles recognize five PPTA repeats in most protein prenyltransferase \(\alpha\) subunits, including mammalian and yeast Rab-GGT \(\alpha\) subunits (not shown). With predicted PPTA repeats highly similar to mammalian RABGGTA, RGTA1 and RGTA2 appear to be paralogous putative \(\alpha\) subunits of Arabidopsis Rab-GGT. Disruption in the third PPTA repeat is unique to RGTA2 among the Rab-GGT \(\alpha\) subunits of various eukaryotic species from yeast to humans (Fig. 1B), and it might result in some variation in the secondary structure and possibly also in the biochemical activity of RGTA2.

The alignment also shows that RGTA1 and RGTA2 have extended C-terminal regions compared to rat RABGGTA that contain conserved LRR motifs (Fig. 1A). The intermediating regions of RGTA1 and RGTA2 are much less similar to the Ig-like domain of mammalian RABGGTA, which lies between the helical domain and the LRR domain, despite a few patches of similar sequences found in this region (Fig. 1A). However, previous studies in mammals have shown that the Ig-like domain and the LRR domain are not involved in prenyltransferase activity (28, 61). Therefore, the differences in these regions of RGTA1 and RGTA2 are not likely to affect their putative function as Rab-GGT \(\alpha\) subunits.

**Putative Rab-GGT \(\alpha\) Subunits Form Heterodimers with Putative \(\beta\) Subunits**—All known protein prenyltransferases function as heterodimers consisting of one \(\alpha\) subunit and one \(\beta\) subunit (10, 12). To examine the hypothesis that putative Rab-GGT \(\alpha\) subunits in Arabidopsis partner with putative \(\beta\) subunits as functional Rab-GGTs, we first performed a pull-down experiment to test all four combinations between RGTA1/2 and RGTB1/2 for physical interactions. Each of the subunit combinations was co-expressed in a yeast strain in which the two subunits in all of the four combinations physically interact with each other (Fig. 2). Thus, there are four putative Rab-GGT heterodimers in Arabidopsis: RGTA1-RGTB1, RGTA1-RGTB2, RGTA2-RGTB1, and RGTA2-RGTB2.

However, we noticed that RGTA1 and RGTA2 behaved differently in these experiments. We reproducibly obtained lower amounts of RGTA2 protein than RGTA1 protein in the eluates from pull-down experiments (Fig. 2). There are two possible explanations, which are not mutually exclusive, for this result: 1) the interaction between RGTA2 and either putative \(\beta\) subunit is weaker than that between RGTA1 and either putative \(\beta\) subunit, and 2) RGTA2, in our observation, is less stable than the other tested subunits during the processes of protein expression and purification (data not shown). These findings, together with the observation that a conserved PPTA motif in RGTA2 is disrupted by an insertion (Fig. 1), suggest that heterodimers with RGTA2 might be destabilized by the additional amino acids and/or might show altered biochemical activity in vitro. Given that RGTA1 better meets the criteria of a functional Rab-GGT \(\alpha\) subunit compared with RGTA2 and that biochemical evidence has shown that RGTB1 is involved in Rab geranylgeranylation activity (37), we decided to use...
### Arabidopsis Rab Geranylgeranyltransferases

#### A

| Sequence | Alignment |
|----------|-----------|
| RnRABGGA 1 |           |
| ATRGTA1 1 |           |
| ATRGTA2 1 |           |
| consensus 1 |           |
| RnRABGGA 61 |           |
| ATRGTA1 57 |           |
| ATRGTA2 55 |           |
| consensus 61 |           |
| RnRABGGA 119 |          |
| ATRGTA1 117 |          |
| ATRGTA2 114 |          |
| consensus 121 |          |
| RnRABGGA 165 |          |
| ATRGTA1 162 |          |
| ATRGTA2 174 |          |
| consensus 181 |          |
| RnRABGGA 224 |          |
| ATRGTA1 218 |          |
| ATRGTA2 231 |          |
| consensus 241 |          |
| RnRABGGA 273 |          |
| ATRGTA1 276 |          |
| ATRGTA2 291 |          |
| consensus 301 |          |
| RnRABGGA 311 |          |
| ATRGTA1 334 |          |
| ATRGTA2 348 |          |
| consensus 361 |          |
| RnRABGGA 341 |          |
| ATRGTA1 390 |          |
| ATRGTA2 418 |          |
| consensus 421 |          |
| RnRABGGA 398 |          |
| ATRGTA1 450 |          |
| ATRGTA2 467 |          |
| consensus 481 |          |
| RnRABGGA 441 |          |
| ATRGTA1 510 |          |
| ATRGTA2 523 |          |
| consensus 541 |          |
| RnRABGGA 498 |          |
| ATRGTA1 563 |          |
| ATRGTA2 576 |          |
| consensus 601 |          |
| RnRABGGA 557 |          |
| ATRGTA1 604 |          |
| ATRGTA2 618 |          |
| consensus 661 |          |
| RnRABGGA 668 |          |
| ATRGTA1 661 |          |
| ATRGTA2 721 |          |

#### B

| Sequence | Alignment |
|----------|-----------|
| ATRGTA1 116 |           |
| ATRGTA2 124 |           |
| ScBEV4 117 |           |
| PpRGA1 119 |           |
| Cs06q0677500 116 |       |
| DmPTAR3 150 |           |
| RnRABGGA 118 |          |
| HsRABGGA 118 |          |
| consensus |           |

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**Note:** The alignment and diagram provide a visual representation of the sequences and their alignment, showcasing the evolutionary relationships and conserved motifs among the Arabidopsis Rab geranylgeranyltransferases.
Rab-GGT activity, and there is no detectable background activity from yeast Rab-GGT subunits. RGTB1-RGTA1 prenylates C-terminal Cysteine Residues at Different Positions in Vitro and May Exhibit Preference for Particular Positions—Unlike PFT and PGGT-I, which target more specific C-terminal CaaX sequences, Rab-GGT targets a wide variety of C-terminal sequences in the CaaX consensus C-Xaa-Cys, where Xaa can be any amino acid.

### Table 1

| C-terminal consensus | Name | C-terminal sequence | C-terminal consensus | Name | C-terminal sequence |
|----------------------|------|--------------------|----------------------|------|--------------------|
| RabA1a               | CCSN | RabA2a             | CCSS                 |
| RabA1b               | CCSN | RabA4b             | CCSS                 |
| RabA1c               | CCSD | RabA4c             | CCSS                 |
| RabA1d               | CCSD | RabA4d             | CCSS                 |
| RabA1e               | CCSD | RabA5b             | GCGG                 |
| RabA1f               | CCSD | RabA5e             | GCGG                 |
| RabA1g               | CCSS | RabE1d             | CCSS                 |
| RabA1h               | CCAT | RabE1e             | CCSS                 |
| RabA1i               | CCSD | RabE1f             | CCSS                 |
| RabA2b               | CCST | RabB1b             | GCGG                 |
| RabA2c               | CCSS | RabC2a             | GCGG                 |
| RabA2d               | CCST | RabC2b             | GCGG                 |
| RabA3a               | CCSS | RabA3             | SCSC                 |
| RabA3b               | CCSD | RabA3             | SCSC                 |
| RabA5c               | CCSS | RabG1             | RCSC                 |
| RabA6a               | CFK  | RabG2             | GCAC                 |
| RabA6b               | CCYK | RabG3a             | GCAC                 |
| RabC1                | CCSS | RabG3b             | GCAC                 |
| RabC2b               | CCSS | RabG3c             | GECG                 |
| RabD1                | CCQG | RabG3d             | GECG                 |
| RabD2a               | CCST | RabG3e             | GECG                 |
| RabD2b               | CCSS | RabG3f             | GECG                 |
| RabD2c               | CCSS | RabH1a             | NCSC                 |
| RabE1a               | CGGT | RabH1b             | GCSC                 |
| RabE1c               | CGGT | RabH1c             | GCSC                 |
| RabE4e               | (pseudogene) | RabH1d | ACSC                |
| RabE1b               | TILE | RabH2             | GCAC                 |
| RabF1                | APSS | RabH3             | GCAC                 |

### Figure 1

**The protein sequence alignments of Arabidopsis RGTA1, RGTA2, and their orthologs.** A, the alignment of the full-length protein sequences of Arabidopsis RGTA1 (AtRGTA1), Arabidopsis RGTA2 (AtRGTA2), and rat Rab-GGT α subunit (RnRABGGTA). The predicted PPTA repeats are marked as I, II, IV, and V. The 12-aa insertion (HQKQDEKQDDPP) in the third PPTA repeat (III) of RGTA2 is underlined. B, alignment of the third PPTA repeats of Arabidopsis RGTA1, Arabidopsis RGTA2, and Rab-GGT α subunits of yeast (ScBET4), P. patens (PpRGTA1), rice (Os06g0677500), D. melanogaster (DmPTAR3), rat (RnRABGGTA), and human (HsRABGGTA). Black background and asterisk, identical residues; gray background and dot, similar residues.
Arabidopsis Rab Geranylgeranyltransferases

Variety of C-terminal sequences, most of which contain two cysteine residues for double geranylglyceranlylation (4, 16). In Arabidopsis, 54 of 57 identified Rab genes are predicted to encode Rab GTPases that have C-terminal cysteine residues and potentially can be prenylated (Table 1). To investigate which cysteine residues are prenylated by Rab-GGT, we use the same representative Rabs in the above activity assays to generate three representative Rabs in the above activity assays to generate three cysteine residue substituted and one with both cysteine residues substituted. We then performed in vitro prenylation assays using the same quantity of wild-type and corresponding mutant Rab proteins across different reactions, in the presence of AtREP. The presence or absence of individual components in each reaction is indicated by a plus or minus sign, respectively. The reaction mixture was resolved by 10% SDS-PAGE. The x-ray film was exposed to the vacuum-dried SDS-PAGE gel at −80 °C for 48 h to detect radiolabeled 3H-GGPP. The bands of the gels indicate that the target protein specificity of Rab-GGT, it is possible that Arabidopsis Rab-GGT-, Rab-GGT, and 2) artificial target proteins cannot be prenylated by Arabidopsis Rab-GGT, it is possible that Arabidopsis Rab-GGT might have target protein specificity similar to that of their counterparts in animals and yeast. However, contrary to the lethality of PFT/PGTT-I α subunit loss-of-function mutants in animals and yeast, the Arabidopsis PFT/PGTT-I α subunit knockout mutant plp is viable and fertile (20), suggesting that some other prenyltransferase activity, possibly from Rab-GGT, can partially compensate for the loss of PFT/PGTT-I.

To test this hypothesis, we chose several non-Rab GTP-binding proteins reported to be prenylated by PGTT-I in Arabidopsis to perform in vitro prenylation assays: AGG1 and AGG2, which are two γ subunits of Arabidopsis heterotrimeric G proteins (15, 64, 65), and AtROP1, one of the Arabidopsis Rop family GTPases (9, 66). Our results indicate that RGT1-RGTB1 can also prenylate AGG2 and AtROP1, but not

![Figure 3](image-url)
AGG1, in vitro (Fig. 5). Moreover, the cross-specificity of RGTA1/RGTB1 on AGG2 and AtROP1 does not require AtREP, although the presence of AtREP appears to stimulate the prenylation of AtROP1 (Fig. 5). Therefore, RGTA1/RGTB1 can prenylate certain Arabidopsis PGGT-I target proteins in an REP-independent manner.

RGTB1 and RGTB2 Are Redundant Rab-GGT Subunits, whereas RGTA2 Does Not Appear to Be a Functional Rab-GGT α Subunit in Vitro—By using in vitro prenylation assays, we have shown that RGTA1-RGTB1 is a bona fide Rab-GGT. However, as discussed earlier, the other three putative Rab-GGT heterodimers may have altered activities and/or target specificities due to differences between paralogous putative subunits.

To investigate whether RGTA1-RGTB2, RGTA2-RGTB1, and RGTA2-RGTB2 are also functional and, if so, whether they have different target specificities, we performed in vitro prenylation assays using a subset of Arabidopsis Rab GTPases to represent the different subfamilies of the entire Rab family. The representative proteins were chosen based on their phylogenetic relationship (3, 6), expressed sequence tag availability, and C-terminal sequences (Table 1). AGG2 and AtROP1, which we showed above to be prenylated by RGTA1/RGTB1, were also included. For each target protein, four reactions were carried out, using the four possible subunit heterodimers, respectively. The results, which are summarized in Table 2, demonstrate that RGTA1-RGTB1 and RGTA1-RGTB2 can prenylate every target protein tested in the presence of AtREP, suggesting

proteins were chosen to represent the four different types of C-terminal sequences found in Arabidopsis Rab GTPases: RABA1A for -CCXX (A), RABA2A for -CCXXX (B); RABF2A for -XCXX (C), and RABG2 for -XCXC (D). For each representative protein, two single-cysteine substitution mutants and one double-cysteine substitution mutant were generated and tested in the in vitro prenylation assay. Exposure time for autoradiography was 24 h. The Coomassie Blue staining shows that equal amounts of target proteins were used in each reaction within each panel. Faint bands are marked with asterisk to help visualization.
TABLE 2
Target specificities of RGTA1-RGTB1, RGTA1-RGTB2, RGTA2-RGTB1, and RGTA2-RGTB2

| Target protein | C-terminal sequence | RGTA1-RGTB1 | RGTA1-RGTB2 | RGTA2-RGTB1 | RGTA2-RGTB2 |
|----------------|---------------------|-------------|-------------|-------------|-------------|
| RABA1A         | CSN                 | +           | +           | -           | -           |
| RABA1E         | CCS                 | +           | +           | -           | -           |
| RABA1F         | CCS                 | +           | +           | -           | -           |
| RABA2A         | CSS                 | +           | +           | -           | -           |
| RABA2C         | CSS                 | +           | +           | -           | -           |
| RABA3          | SCS                 | +           | +           | -           | -           |
| RABA4B         | CTS                 | +           | +           | -           | -           |
| RABA4D         | CCK                 | +           | +           | -           | -           |
| RABA5A         | CSS                 | +           | +           | -           | -           |
| RABA5C         | CSS                 | +           | +           | -           | -           |
| RAB1           | GGC                 | +           | +           | -           | -           |
| RAB2           | GCS                 | +           | +           | -           | -           |
| RAB3           | CGC                 | +           | +           | -           | -           |
| RAB4           | CCA                 | +           | +           | -           | -           |
| RAB5           | GCQ                 | +           | +           | -           | -           |
| RAB1A          | CSN                 | +           | +           | -           | -           |
| RAB2A          | CCS                 | +           | +           | -           | -           |
| RAB3A          | CCG                 | +           | +           | -           | -           |
| ATROPI         | CSIL                | +           | +           | -           | -           |

that RGTB1 and RGTB2 are biochemically redundant when partnered with RGTA1 in vitro (Table 2 and Fig. 6). However, none of the target proteins tested were prenylated by RGTB1-RGTB1 or RGTB2-RGTB2, indicating that RGTB2 appears not to be a functional Rab-GGT subunit when partnered with either RGTB1 or RGTB2 in vitro (Table 2 and Fig. 6). As discussed earlier, the loss of Rab-GGT subunit function might result from the disrupted third PPTA repeat in the RGTA2 helical domain (Fig. 1).

Discussion

Rab-GGT activity was detected in plants nearly 20 years ago (33–35). Two pairs of paralogous genes in Arabidopsis, RGTA1/2 and RGTB1/2, have long been annotated as genes encoding putative α and β subunits of Arabidopsis Rab-GGTs, respectively, based on homology (36). However, except for studies done with rgtb1 mutant plant extracts (37), the biochemical activities, partner/cofactor requirements, and substrate specificities of those putative Rab-GGT subunits had not been characterized. In this study, we present biochemical evidence that all four α-β combinations among RGTA1/2 and RGTB1/2 form heterodimers. Our assays indicate that RGTA1-RGTB1 and RGTA1-RGTB2 exhibit similar Rab-GGT activity and can prenylate a wide spectrum of Rab GTPases in vitro. In contrast, RGTB1-RGTB1 and RGTB2-RGTB2 did not show detectable Rab-GGT activity in our assays, possibly due to a 12-aa insertion that disrupts the third PPTA repeat in RGTB2. We also demonstrate that AtREP is required for the Rab-GGT activity of RGTA1-RGTB1 and RGTA1-RGTB2 prenylation of Rab GTPases.

By substituting the C-terminal cysteine residues, we demonstrate that the Arabidopsis Rab-GGT RGTA1-RGTB1 can recognize and prenylate all four types of C-terminal sequences found in Arabidopsis Rab GTPases (-CCXX, -CCXXX, -XCCX, -XCCCY), and both cysteine residues in the C-terminal sequences can be prenylated when GGPP is in abundance. However, the single-substitution mutant Rab GT Pases show different degrees of prenylation, suggesting some preference in prenylation of cysteine residues at various positions. The fourth amino acid residue from the C-terminal end appears to be the most favored prenylation site, whereas the fifth and the first appear to be least favored. Previous work in mammals has shown that the double geranylgeranylation of Rab GT Pases occurs in two sequential but independent steps, and the order of the two steps appears to be random (67). The proximal sequences on the N-terminal side of the cysteine residues are flexible in terms of prenylation target specificity (68). Therefore, the preference that we observe may solely rely on the position of the amino acid residue relative to the C-terminal end, although we cannot rule out the possibility that Arabidopsis Rab-GGTs show a greater preference for certain proximal amino acids. We hypothesize that the space limitation in the Rab-GGT catalytic site is responsible for the prenylation preference at different positions because the size or shape of the site might confine the C-terminal sequence in a certain conformation and only allow the cysteine residues close to the catalytic center to be efficiently prenylated.

Several target protein cross-specificities between PFT and PGGT-I have been reported (15, 19, 20, 69), but it has long been believed that Rab-GGT only prenylates Rab GT Pases (16, 26). One of the novel findings in our study is that, in addition to Rab GT Pases, Arabidopsis Rab-GGT can also prenylate certain PGGT-I targets in vitro, including the G-protein γ subunit AGG2 and the Rop family GT Pase ATROPI. This finding may help to explain the viability and fertility of the Arabidopsis PFT/
PGGT-I α subunit mutant plp as well as the mild phenotype of the Arabidopsis PGGT-I β subunit mutant ggb and residual membrane localization of the PGGT-I target AGG2 in plp (15, 20, 25), because Rab-GGT may at least partially compensate for the loss of PGGT-I in Arabidopsis. However, unidentified additional prenyltransferase components as well as other types of lipid modifications, such as S-acylation, myristoylation, and palmitoylation, may also potentially compensate for the loss of PFT/PGGT-I activity (10).

Previous studies of Rab-GGTs in mammals and yeast have shown that Rab-GGTs are completely dependent on AtREP for target specificity (i.e. the recognition and binding of Rab GTPases) (61). However, our results indicate that the prenyltransferase activities of Arabidopsis Rab-GGT in prenylation of AGG2 and AtROP1 are independent of AtREP, suggesting that Arabidopsis Rab-GGT can recognize and recruit certain target proteins, other than Rab GTPases, by itself. One similar case has been observed in C. elegans, in which the prenylation of some specific Rab GTPases is independent of REP (70). It has been proposed that an ancient Rab-GGT, once a PGGT-I-like protein, evolved to interact with an accessory protein over time and eventually gave up the specificity to the accessory protein, thus giving rise to the modern Rab-GGT and REP system (61). It is possible that Arabidopsis Rab-GGT retained or regained some specificity cues from a PGGT-I-like ancestor.

In animals and yeast, generally only one copy of each Rab-GGT subunit gene is present in the genome. In contrast, duplications of Rab-GGT subunits are found in multiple plant species (37, 39, 57). It has been suggested that duplications in different plant species have occurred independently, rather than having been inherited from a common ancestor (37). Some researchers have proposed that the two sets of Rab-GGT subunits in Arabidopsis were duplicated simultaneously in the recent whole genome duplication event (57). However, based on our analysis, none of the Arabidopsis Rab-GGT genes are found in any of the duplicated chromosome regions proposed to be involved in the whole genome duplication. Moreover, the flanking sequences of the Rab-GGT genes are not related to any sequence in other chromosome regions, suggesting that these genes have not been duplicated in large syntenic blocks.

The functional significance of having two copies of Rab-GGT subunits remains unclear (57). In yeast, loss of either of the single-copy Rab-GGT subunits leads to lethality (37). In Arabidopsis and P. patens, in which RGTB is duplicated, the rgtb1 and rgtb2 single knock-out mutants are viable, whereas the rgtb1 rgtb2 double mutants are non-viable, indicating genetic redundancy between the duplicated RGTB genes (38, 39). The duplicated RGTB genes appear to be completely redundant in P. patens, because neither single rgtb knock-out shows a detectable phenotype (39). However, each of the Arabidopsis rgtb single knockouts has a distinct set of mutant phenotypes (37, 38), suggesting that Arabidopsis RGTB1 and RGTB2 are biochemically redundant in vitro, because neither single rgtb knock-out shows a detectable phenotype (39). However, each of the Arabidopsis rgtb single knockouts has a distinct set of mutant phenotypes (37, 38), suggesting that Arabidopsis RGTB1 and RGTB2 are biochemically redundant in vitro, suggesting that there might be additional factors that differentiate RGTB1 and RGTB2 functions in vivo. Alternatively, the partial redundancy of RGTB1 and RGTB2 in Arabidopsis may result from differential expression.

In contrast, no Arabidopsis rgta1 or rgta2 mutants have been reported. Our results indicate that, although RGTA1 partners with both RGTB1 and RGTB2 to form a functional Rab-GGT, RGTA2 seems not to be functional in vitro. If this is also true in

FIGURE 6. Representative in vitro prenylation assays to test target specificities of RGTA1-RGTB1, RGTA1-RGTB2, RGTA2-RGTB1, and RGTA2-RGTB2. The presence or absence of individual components in each reaction is indicated by plus or minus signs, respectively. For the in vitro prenylation reactions testing the same Rab target, a mixture including reaction buffer, AtREP, Rab, and 3H-GGPP was prepared before adding different RGTA-RGTB heterodimers to each aliquot. The results are not quantitative. Exposure time for autoradiography was 24 h.
vivo, rghta1 mutants should be non-viable, similar to P. patens rghta1 mutants (39), whereas rghta2 mutants might exhibit no phenotype. It has been proposed that, despite possible redundancy, the duplicated Rab-GGT subunits in Arabidopsis may result in increased enzyme dosage and differential specificity in order to deal with the large family of Arabidopsis Rab GTPases (38, 57). However, our finding that RGT1A is possibly the only functional Rab-GGT α subunit may make it the limiting factor in forming heterodimeric enzymes. Together with the observation that the transcript level of RGT1A is much lower than that of RGTA2 throughout the plant (37), the dosage effect hypothesis may not be supported in Arabidopsis.

Among numerous variations between the protein sequences of Arabidopsis RGTA1 and RGTA2, probably the strongest explanation for the loss of RGTA2 α subunit function is the 12-aa insertion in the third PPTA repeat, which is the longest stretch of continuous variation in the pairwise alignment between RGT1A and RGTA2. This insertion may be unique to the Arabidopsis lineage, based on our search for RGTA homologs in various plant species (data not shown). We also noticed that some of the nucleotide sequences encoding the inserted amino acids contain some repetitive sequences (data not shown), suggesting that the insertion might have been introduced by replication slippage after RGTA was duplicated. By aligning RGTA2 protein sequence to the structure of rat RAB-GGTA in the Rab-GGT-REP complex (Protein Data Bank entry 1LTX) (31), we located the insertion at the C-terminal end of the aα helix. The insertion might result in an extended linker between aα and aβ helices or even more significant changes in the structural conformation that could impair function. For example, the C-terminal end of the aα helix is facing and close to the Rab-GGT αβ interface; thus, the additional amino acids might interfere with dimerization, consistent with our observation that the interactions between RGTA2 and RGTB1/2 are weaker than that of RGT1A. It is also possible that the extension of the linker between helices caused by the insertion might block the access of Rab GTPase to the enzyme’s catalytic center. It would be interesting to see whether removing the insertion from RGTA2 rescues its interaction with β subunits and its α subunit function.

Author Contributions—W. S. designed experiments, performed all experiments, prepared all figures and tables, and wrote the manuscript. Q. Z. provided select constructs and helped to develop in vitro prenylation assay protocols. M. P. R. provided intellectual framework and input, guided experimental design and direction, and revised the manuscript. B. N. K. provided intellectual and experimental input and laboratory facilities and revised the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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