The Regulatory RAB and ARF GTPases for Vesicular Trafficking[1][W]

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While highly conserved in structure and in fundamental regulatory aspects for their activities, the RAS superfamily of monomeric GTP-binding proteins, or small GTPases, comprise a large family of regulatory molecules that collectively regulate diverse and critical cellular processes in eukaryotes. The RAB and ARF GTPases are members of two of the RAS-related subfamilies that function in regulating vesicle trafficking, starting from regulating the formation of vesicles on donor membranes and directing trafficking specificity to and facilitating vesicle docking on target membranes (Zerial and McBride, 2001; Gillingham and Munro, 2007). Studies in yeast (*Saccharomyces cerevisiae*) and mammalian systems have shown that successive steps in endomembrane trafficking, from the endoplasmic reticulum (ER) to the Golgi, intra-Golgi, post-Golgi, and endocytic trafficking, are mediated by subfamilies of RAB and ARF GTPases. The fundamental roles that these small GTPases play in membrane trafficking are indicated by their conservation throughout eukaryotes and their proliferation in the more complex animal and plant systems. Structural conservation among these GTPases has facilitated a rather comprehensive identification of the corresponding gene families in plants with complete genome information, in particular those for the Arabidopsis (*Arabidopsis thaliana*) RABs and ARFs (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003; Zhang et al., 2007). In most cases examined, sequence conservation among yeast, mammalian, and plant RAB GTPases has correlated with similar localization on discrete subcellular compartments of the endomembrane trafficking systems in these diverse organisms. However, while the most closely related RAB GTPases from diverse organisms appear to hold analogous positions within the endomembrane system, it is clear that experimental strategies directly targeted at examining the specific functions of these small GTPases in plants will be required to determine distinctions in how various membrane compartments are utilized and how plant-specific cargos are sorted and trafficked through these compartments. We refer readers to earlier reviews (Rutherford and Moore, 2002; Vernoud et al., 2003) and focus our discussion here on recent studies that examine the biological role for these small GTPases in specific cellular context as well as emerging studies that explore their broader regulatory networks.

NOMENCLATURE AND THE RESEARCH TOOL BOX

A comprehensive gene/protein nomenclature system that accurately reflects phylogenetic relationships as well as functional specialization is a moving target, as genomic sequences and functional information are continuously being added and refined. This is particularly the case for the plant RAB GTPases, which, like their yeast and mammalian counterparts, constitute the largest of the RAS-related superfamilies of small GTPases. Based on sequence similarity among themselves, and with their yeast and mammalian orthologs, a nomenclature system for the 57 Arabidopsis *RABs* that places them into eight distinct subfamilies (A to H, corresponding to the mammalian RAB GTPase classes of 11, 2, 18, 1, 8, 5, 7, and 6, respectively), each regulating distinct paths in the membrane trafficking systems, has been proposed (Pereira-Leal and Seabra, 2001). The use of numerical designation to reflect closest analogy with mammalian orthologs, e.g. RAB2 and RAB11, has been adopted for some of the studies, especially for RABs from plants other than Arabidopsis. This has the clear advantage for cross-referencing but runs the risk of obscuring aspects of these plant RAB GTPases that have evolved to best serve vesicular trafficking needs in plant cells. Development of a nomenclature system that incorporates the numbering system of the closest mammalian orthologs and at the same time preserves the designation of the eight

distinct subgroups previously defined for the Arabidopsis *RAB*s is currently under way (I. Moore, personal communication). We will use both systems, e.g. RAB11/RABA, for general discussions and retain use of the original nomenclature in most of our discussions of specific studies. We opt to test the use, however, of the system under development in the section where we discuss the RAB5/RABF and RAB7/RABG class of GTPases. Perhaps response from readers will help guide the development of a system that best serves this complex protein family.

Like all RAS-related GTP-binding proteins, RAB GTPases utilize guanine nucleotide exchange and GTP hydrolysis to switch between active (GTP-bound) and inactive (GDP-bound) conformations (Fig. 1). One of the features that distinguish the plant RAB GTPase family is the size of the protein family. To date, knockout mutations have not yielded functional insight for these small GTPases, most probably because of overlapping functions among members of the same subfamily or between closely related subfamilies. On the other hand, replacement of specific amino acid residues in various functional domains of RAS-related GTPases results in defined effects on guanine nucleotide binding and GTP hydrolysis. Mutations that stabilize the GTP-bound activated state, thus up-regulating their regulatory activity, are referred to as constitutively active (CA); conversely, those that render the GDP-bound inactive state to be more predominant confer a dominant negative (DN) effect on the small GTPase-regulated pathways. Much of the functional insight for the RABs and ARFs discussed below and summarized in Figure 2 is derived from studies based on these CA and DN mutations in transformed plant or cell systems.

Additional important biological roles for RAB- and ARF-regulated pathways have emerged recently from studies in knockout plants defective in proteins that
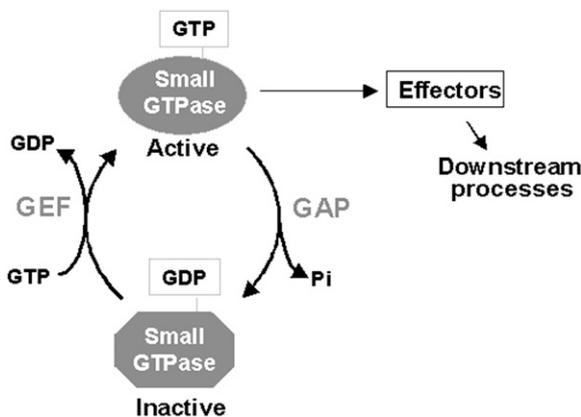

Figure 2. A schematic illustration for the RAB- and ARF-mediated pathways in plant cells known to date. Phragmoplast and polarized outgrowth are depicted as examples of cellular structures that require high levels of polarized secretory activities for their formation. SV, Secretory vesicles; MVE/PVC, multivesiculated endosome/prevacuolar compartment; RE, recycling endosome.

regulate the RAB and ARF GTPase cycle. In particular, knockouts of RAB and ARF guanine exchange factors (GEFs) that stimulate exchange of GDP for GTP, and thereby "turn on" the GTPase by allowing progression into the GTP-bound conformation (Fig. 1), have resulted in observable defects in plant growth and development (Steinmann et al., 1999; Goh et al., 2007; Teh and Moore, 2007). Another class of regulators for these small GTPases are GTPase-activating proteins (GAPs), which stimulate hydrolysis of the GTP bound in the RAB or ARF GTPases and thus "turn off" the GTPase by returning it to its GDP-bound conformation. Mutations in ARF GAP have been shown to result in abnormal vein pattern formation in Arabidopsis (Koizumi et al., 2005; Sieburth et al., 2006). Finally, some success has been gained by analysis of knockouts of downstream effector proteins that are recruited to the GTP-bound, active GTPases and are responsible for carrying out the various biological functions that these small GTPases regulate (Preuss et al., 2006; Stefano et al., 2006).

Development of fluorescent protein-labeled RABs and ARFs and cargo molecules has accelerated the ability to assign subcellular locations for these proteins within the endomembrane system (Fig. 3). Studies of these small GTPases in cellular and developmental processes that rely on high levels of secretion or polar-

Figure 1. The small GTPase regulatory cycle. RAS-related GTPases cycle between an active GTP-bound and an inactive GDP-bound state. GEFs activate these small GTPases, which in turn interact with specific effectors to mediate downstream pathways. GAPs stimulate the inherent GTPase activity of these small G proteins, accelerating the inactivation of their regulatory activity.
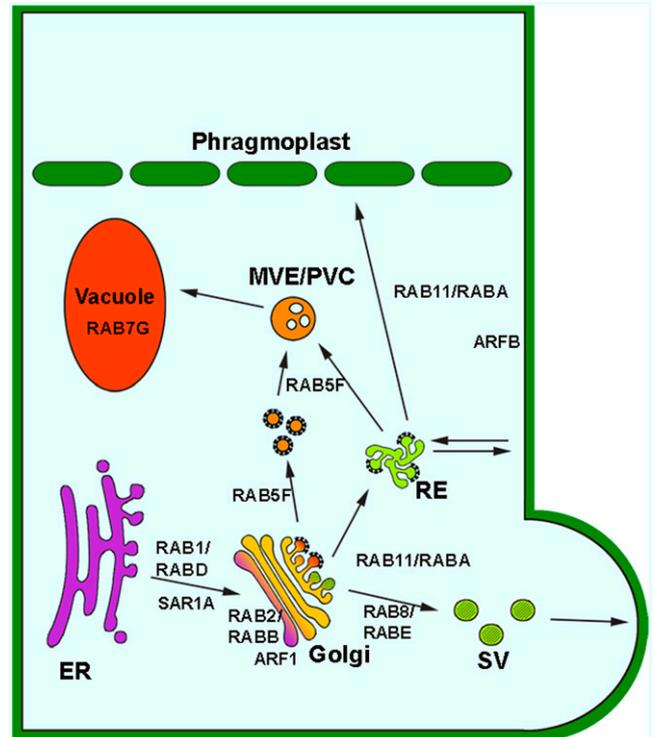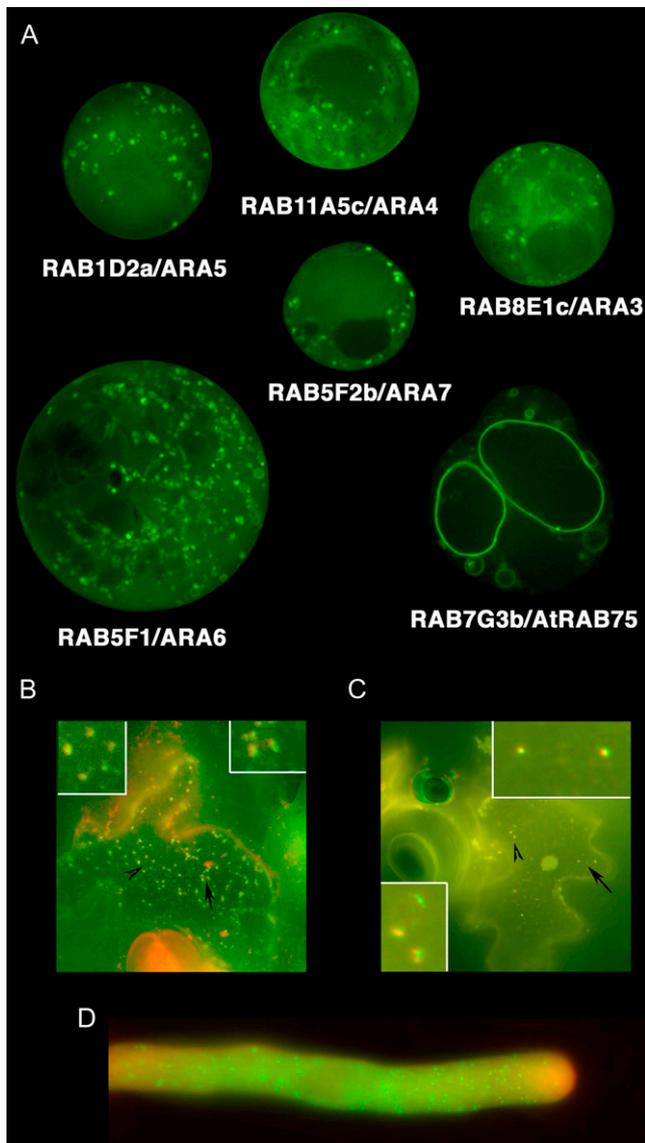
Figure 3. Some model cell systems for the studies of regulatory small GTPases for vesicular trafficking. A, Localization of various GFP-labeled RAB GTPases in transiently transformed Arabidopsis protoplasts. B and C, Localization of NtRAB5 to endosomal compartments in tobacco epidermal cells. B, GFP-NtRAB5 (green) colocalizes on yellow punctate structures with the endocytic marker FM4-64 (red) in epidermal cells of CaMV35S-GFP-NtRab5 transformed tobacco seedlings. C, CFP-NtRAB5 (red) colocalizes with a GFP-labeled auxin efflux protein AtPIN1 (green) known to be recycled through the endosomes (see Geldner and Jürgens, 2006) on yellow punctate structures. The insets in B and C are enlarged from the structures indicated by arrows and arrowheads. The tobacco epidermal cell was transiently cotransformed by agroinfiltration (see Batoko et al., 2000). D, Colocalization of a GFP-NtRAB5 (green) with a CFP-labeled NtRAB11b (red) in an elongating pollen tube showing the polarized concentration of Rab11-labeled transport vesicles in the apex and the localization of Rab5-labeled endosomes in the subtending cytoplasm (B–D; T. Andreyeva and A.Y. Cheung, unpublished data).

ized secretory activities facilitate functional association with specific cellular processes and physiological phenomena. Therefore, embryogenesis, cell plate forma-

tion, and the polar growth cells such as root hairs and pollen tubes are popular biological systems for the functional dissection of these small GTPases.

RAB1/RABD AND RAB2/RABB

In mammalian cells, the early secretory pathway of ER to Golgi trafficking and intra-Golgi trafficking are mediated by RAB1 and RAB2 (Zerial and McBride, 2001). Whereas mammalian RAB1 regulates anterograde ER to Golgi trafficking, RAB2 may be more important in intra-Golgi and retrograde Golgi to ER trafficking. While homologs of both RAB1 and RAB2 are found in plants, RAB2 homologs are not found in yeast, where RAB1 alone sufficiently supports the early secretory pathway (Segev et al., 1988). Similar to mammalian *RAB1* (Haubruck et al., 1989), plant *RAB1/RABDs* are able to complement the yeast *ypt1* mutation. On the other hand, a *RAB2* from soybean (*Glycine max*) failed to complement ypt1 (Kim et al., 1996; Park et al., 1997). The functional divergence between RAB1D and RAB2B implicated by these studies remains to be vigorously examined in plant cells.

In Arabidopsis, the RAB1/RABD family is comprised of five subspecies (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003). One of these, RAB1b, has been localized to the ER and Golgi in transiently transformed epidermal cells expressing the GFP-labeled protein (Batoko et al., 2000). Expression of a DN RAB1b induced the accumulation of a secreted form of GFP and a Golgi-targeted *N*-glycosylated GFP variant in a membrane reticulate reminiscent of the ER. This and the accumulation of an endoglycosidase H-sensitive population of the Golgi-targeted GFP confirmed that trafficking from ER to the Golgi was inhibited in cells compromised in their RAB1-regulated trafficking activity as a result of DN RAB1b overexpression. Developmentally, a recent study in maritime pine (*Pinus pinaster*) revealed high levels of expression of a RAB1, PpRAB1, in early zygotic development followed by decline as embryos matured, suggesting a role for this small GTPase in early embryogenesis (Goncalves et al., 2007).

The RAB2/RABB GTPase family is relatively small, represented by two to four members in Arabidopsis, maize (*Zea mays*), and rice (*Oryza sativa*; Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003; Zhang et al., 2007). The functional significance of the RAB2/RABB-regulated pathway has been examined in pollen tubes, which represent one of the most dramatic polar growth cell types in nature (Cheung et al., 2002). In addition to an extensive ER system that spans the entire pollen tube cytoplasm except at the extreme apex, pollen tubes maintain a highly polarized organelle distribution pattern with an extremely high density of transport vesicles segregated to the apical cytoplasm, while larger organelles, including the Golgi bodies and the endosomes, are largely confined to the subapical and

distal regions of the tube (Cheung and Wu, 2008). For a majority of the secreted proteins that have been examined, vesicles loaded with these proteins accumulate in the apical zone, presumably either in transit to fusion with the plasma membrane or as recycled vesicles after endocytosis (Parton et al., 2001). Growth perturbation unfailingly associates with disruption of the polarized organellar distribution pattern, in particular dissipation of the apical collections of vesicles. In tobacco (*Nicotiana tabacum*) pollen tubes, GFP-NtRAB2 associates efficiently with Golgi bodies. Expression of DN NtRAB2s substantially reduced the delivery of GFP-labeled Golgi-located cargo proteins to their destination, while cytoplasmic and a reticular membrane signal were augmented, supporting a role for NtRAB2 in the early secretory pathway. While not prohibiting cell membrane protein localization, their delivery was compromised as the normal accumulation of vesicles loaded with these cargo molecules in the tube apex and pollen tube growth rate were both reduced. In maize, hypermorphic mutations in one of its *RAB2*s, *ZmRAB2A1*, induces wart-like structures on leaf surfaces, suggesting a role in cell wall secretion in expanding leaf cells (Zhang et al., 2007).

RAB11/RABA

In both yeast and animal systems, the number of RAB11/YPT31/32-like Rab GTPases are relatively small in comparison to the entire RAB GTPase family complement, three of approximately 66 in *Homo sapiens* and two of 11 in yeast (Pereira-Leal and Seabra, 2001; Stenmark and Olkkonen, 2001). In contrast, 26 of the 57 RAB GTPases present in Arabidopsis are RAB11/YPT31/32-related GTPases. Intriguingly, the large number of RAB11/RABA GTPases seem to be a conserved feature in most plants, as large numbers of this class of proteins have also been identified in legumes and monocots (Borg et al., 1997; Zhang et al., 2007).

In animals, members of the RAB11 family have been found to regulate trafficking through specialized endosomal compartments called recycling endosomes (Ullrich et al., 1996), while in yeast, YPT31/32 regulate exit of secretory and endocytic cargo from the trans-Golgi cisterna (Benli et al., 1996; Jedd et al., 1997; Chen et al., 2005). More recently, roles for these RAB GTPases in aspects of yeast and animal cell cytokinesis have been identified as well (Pelissier et al., 2003; Riggs et al., 2003; Ortiz and Novick, 2006).

What role do Rab11/RABA GTPases play in growth and development of plants? Several lines of evidence suggest that at least some members of this RAB GTPase family play important roles in secretion and/or recycling of cell wall components in plants. Several RAB11/RABA GTPases have now been localized either as fluorescent fusion proteins or by cell fractionation techniques and appear to localize to compartments that partially overlap with trans-Golgi elements and endosomal compartments (Inaba et al., 2002; Preuss

et al., 2004; de Graaf et al., 2005; Chow et al., 2008). Antisense inhibition of RAB11 GTPases in tomato (*Solanum lycopersicum*) results in complex developmental abnormalities and delayed fruit ripening, which could be attributed to impaired cell wall deposition (Lu et al., 2001). In Arabidopsis, RABA4b displays polarized distribution to membrane compartments that accumulate at the tips of growing root hair cells (Preuss et al., 2004) and, through its interaction with a pair of phosphatidylinositol 4-OH kinases, regulates proper root hair growth (Preuss et al., 2006). Additionally, in tobacco pollen, NtRAB11b localizes to vesicular congregates that, while rapidly cycling in and out of the apical zone of growing pollen tubes, concentrate at the tips of these polar growth cells (Supplemental Movie S1). Overexpression of DN and CA mutant forms of this RAB GTPase interfered with pollen tube expansion, in particular with focused growth at the tip (de Graaf et al., 2005). These results suggest a common role for members of the RAB11/RABA GTPases in the regulation of tip growth, possibly through polarized secretion of new cell wall components or through control of polar recycling events during pollen tube and root hair expansion. More recently, examination of the subcellular distribution of two other Arabidopsis RAB11/RABA, RAB-A2 and RAB-A3, has shown that these Rab GTPases localize to membranes that specifically label the margins of growing phragmoplasts. Intriguingly, the membrane compartments to which these two RAB11/RABA family members were localized could be labeled with the bulk-flow endocytic marker, FM4-64, implying that at least some members of the RAB11/RABA compartments are reached by endocytic membrane traffic. Further, in this study, inducible expression of a DN form of RAB-A2 resulted in formation of multinucleate cells and cell wall stubs consistent with a role in polarized delivery of cargo to sites of new cell wall synthesis (Chow et al., 2008).

Taken together, these results highlight a role for members of the RAB11/RABA GTPase family in trafficking events between the plant trans-Golgi network and the plasma membrane. Major challenges going forward in the study of these membrane compartments will be to determine the extent to which this large family of RAB11/RABA GTPases resides on similar or distinct compartments and to determine to what extent these compartments are involved in trafficking of secretory or endocytic cargo.

RAB8/RABE

The yeast and mammalian RAB8 homologs show functional association with polarized secretion of proteins from the Golgi apparatus to the plasma membrane, such as regulating polarized secretion during the budding process in yeast (Salminen and Novick, 1987; Goud et al., 1988) and post-Golgi basolateral membrane trafficking in mammalian epithelial cells (Huber et al., 1993b). Misregulating RAB8 activity in

fibroblasts resulted in cellular protrusions and relocalization of actin to membrane protrusion sites and mislocalization of apical proteins (Peranen et al., 1996; Hattula et al., 2006; Sato et al., 2007). In immature and fully polarized neurons in culture, RAB8 preferentially localizes to axons and dendrites; antisense suppression impairs anterograde trafficking in these neurites (Huber et al., 1993a, 1995). Rab8-mediated vesicle trafficking is also critical for membrane assembly in the primary cilium, a hair-like organelle with sensory function on the surface of most vertebrate cells (Nachury et al., 2007). The Arabidopsis RAB8/RABE family is comprised of five closely related members (Rutherford and Moore, 2002; Vernoud et al., 2003). So far, functional studies have been reported only for RABE1d in transiently transformed epidermal cells. Studies based on a DN form of RABE1d in conjunction with similar mutations in the RAB5 and RAB11 class of GTPases, RABD2 and RABF2, respectively, and a large number of differentially labeled marker proteins or indicator dyes showed that the Arabidopsis RABE-regulated step is post-Golgi and that DN RABE1d inhibits anterograde trafficking to the plasma membrane and diverts secretory activity to the vacuolar pathway (Zheng et al., 2005). In tomato, a RAB8 GTPase has been identified as interacting with the avirulence factor avrPto from the pathogen *Pseudomonas* sp., and this interaction was dependent on the presence of the host cell resistance protein, implying a possible role in vesicular activity associated with the launch of a defense response (Bogdanove and Martin, 2000). An ethylene-induced RAB8 homolog has also been identified from tomato, suggesting a role in ethylene-regulated cell wall dissolution processes that are dependent on enhanced secretion of hydrolytic proteins (Moshkov et al., 2003). It will be important to dissect how the RAB8/RABE- and RAB11/RABA-regulated pathways intercept and diverge in the post-Golgi steps of membrane trafficking.

RAB5/RABF

The best-characterized RAB GTPase in animals is RAB5, which was initially demonstrated to regulate homotypic early endosomal fusion and fusion between plasma membrane-derived endocytic vesicles and early endosomes (Gorvel et al., 1991; Bucci et al., 1992). Later, this small GTPase was shown to be involved in a wide spectrum of endocytic events, including endosome motility along microtubules (Nielsen et al., 1999; Hoepfner et al., 2005), compartmentalization of membrane domains on endosomes through the regulation of phospholipid contents (Christoforidis et al., 1999; Miaczynska and Zerial, 2002), and direct signaling between the endosomes and nucleus (Miaczynska et al., 2004). In contrast, the physiological significance of RAB5 members in plants was not described until several years ago, though the first plant *RAB5* homolog was isolated

over 15 years ago (Terryn et al., 1992). This could be due to the skepticism surrounding the occurrence of endocytosis in plant cells (Aniento and Robinson, 2005). Today, however, constitutive recycling and ligand- or substrate-induced endocytosis of plasma membrane proteins and lipids have been firmly demonstrated. Endocytosis in plant cells is now recognized as an essential part of plant life (Geldner and Jürgens, 2006), which brought attention to the mechanism involving RAB5.

There are three RAB5 homologs in the Arabidopsis genome: *RAB5F2a/RHA1*, *RAB5F2b/ARA7*, and *RAB5F1/ARA6* (Ueda et al., 2001; Figs. 2 and 3). The two *RAB5F2* genes seem to be orthologous to animal *RAB5*s, because they encode GTPases with high overall similarity to animal RAB5s. In contrast, RAB5F1 harbors unique structural features, such as N-terminal fatty acylation sites instead of a C-terminal Cys motif. This particular RAB5 has not yet been identified in organisms other than plants, making its presence one of the more remarkable features of the organization of plant RAB GTPases in addition to an extremely expanded RAB11A family.

Several lines of evidence indicate that plant RAB5s function in the endocytic pathway. All three Arabidopsis RAB5F proteins localize on punctate organelles, which are labeled by the endocytosis tracer FM4-64 (Ueda et al., 2004; Takano et al., 2005; Jaillais et al., 2008; see also Fig. 3B). The internalization of FM4-64 was inhibited by overexpressed DN RAB5F2b, and endocytosed BOR1, a boron transporter whose endocytosis from the plasma membrane is induced by an elevated boron concentration, passed through the RAB5F2b-positive endosomes en route to the vacuoles (Takano et al., 2005). These results indicate the presence of an endosomal property for RAB5F-positive organelles.

It was recently reported that the plant trans-Golgi domain also harbors endosomal property. RAB11A2a- and RAB11A3-positive compartments are stained by FM4-64 before this dye reaches endosomes bearing RAB5F2 or GNOM (Chow et al., 2008). These RABA compartments partially overlap with VHA-A1-positive compartments, which are also stained by FM4-64 after a shorter period of incubation than RAB5F2 endosomes (Dettmer et al., 2006). These results likely indicate that RAB11A compartments function as earlier endosomes than RAB5F2 endosomes; it is not clear yet, however, whether RAB11A, RAB5F2, and GNOM compartments function in the same endocytic route in a sequential manner or are on different pathways. Time lapse localization of cargo proteins such as BOR1, FLS2, and PIN1, whose endocytosis can be triggered or inhibited by its ligands or substrates (Paciorek et al., 2005; Takano et al., 2005; Robatzek et al., 2006) on distinct endosomes, will be helpful to be more conclusive.

On the other hand, there are implications that RAB5F2 plays critical roles in the biosynthetic vacuolar transport pathway. Both RAB5F2 proteins colocalize with marker molecules known to be on the prevacuolar compartment or multivesiculated endosomes (Figs. 2

and 3; Sohn et al., 2003; Kotzer et al., 2004; Lee et al., 2004; Ueda et al., 2004), and overexpression of DN RAB5F2 perturbs the transport of soluble vacuolar proteins to vacuoles (Sohn et al., 2003; Kotzer et al., 2004). Thus, RAB5F2 proteins should be involved in both endocytic and vacuolar transport pathways, suggesting that these two transport pathways merge at RAB5F2-positive organelles in plant cells.

RAB5F1 and RAB5F2 localize on different populations of multivesiculated endosomes/prevacuolar compartments with considerable overlap (Ueda et al., 2004; Haas et al., 2007), suggesting that these two RAB5F groups have different functions. This is also supported by different genetic interactions with the gene encoding their common GEF, *VPS9a* (Goh et al., 2007), though further studies are needed. The RAB5F2 homolog is found in almost all plant lineages, which suggests that the critical roles of RAB5 in vesicular trafficking are well conserved in plants. The only known exception is a unicellular red alga with a very small genome, *Cyanidioschyzon merolae*, which does not harbor a *RAB5*-related sequence (Matsuzaki et al., 2004). It is also of interest to ask whether *C. merolae* carries out endocytosis and, if so, how it is performed.

RAB7/RABG

Mammalian RAB7 is known to regulate membrane fusion at the late endosomes, and its yeast counterpart, Ypt7, mediates the fusion of vacuoles. Plants also harbor genes homologous to RAB7 whose functions, however, have not yet been clearly revealed. The Arabidopsis genome encodes eight putative RAB7 proteins, seven of which localize to the vacuolar membrane (Saito et al., 2002; T. Ueda, unpublished data; Fig. 3A). The functions of the RAB7G proteins seem to be highly redundant, because multiple mutants (double, triple, and quadruple mutants) do not show any abnormal phenotype (T. Ueda, unpublished data). On the other hand, the *vacuoleless1* mutant, in which a subunit of a putative downstream effector complex of RAB7G was mutated, exhibits embryonic lethality (Rojo et al., 2001), suggesting the importance of RAB7G function in plant development. RAB7G could also be involved in the stress response, as indicated by the overexpression of RAB7G3e conferring salt and osmotic stress tolerance (Mazel et al., 2004). Further biochemical and genetic analyses, as well as cytological studies, will unveil the function of this group more precisely.

SAR AND ARF GTPASES

The formation of transport vesicles on donor membranes begins with the assembly of several sets of coat protein complexes (COPs) mediated by a class of small GTPases, the SAR/ARF family. Recent studies in *S. cerevisiae* and mammalian cells have revealed that this class of GTPase regulates multiple sequential steps in the formation of transport vesicles, including coat recruitment, cargo sorting, completion of fission, and uncoating transport vesicles. Each transport step seems to employ specific sets of coat proteins and regulatory GTPase, for example, COPII and SAR1 in transport from the ER to the Golgi, COPI and ARF1 in transport from the Golgi to the ER or intra-Golgi traffic, and clathrin-adaptor complexes and ARFs in multiple steps in post-Golgi or endocytic pathways (for review, see Gillingham and Munro, 2007; Sato and Nakano, 2007). Most coat proteins and SAR/ARF GTPases are well conserved in plants, so the molecular framework associated with COPs and SAR/ARF seems to be also.

SAR1, now included in the ARF family, was identified as a multicopy suppressor of *sec12* mutant in yeast. Sec12p was later found to be an activating GEF for Sar1p (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993). Sar1p directly binds to the COPII component, a heterodimer of Sec23p and Sec24p, which further recruits the Sec13p-Sec31p subcomplex. Sec24p also binds exposed cytoplasmic signals of transmembrane cargo, facilitating the sorting of cargo molecules into COPII vesicles. Because plant SAR1 can substitute the function of yeast Sar1p (d'Enfert et al., 1992; Takeuchi et al., 1998), the molecular function of SAR1 should be conserved in plants, whereas the spatial distribution and organization of plant ER export sites (ERES), where SAR1 is expected to act, are regulated in a distinct manner from yeasts or animals (Hanton et al., 2006).

In plants, organization of membrane trafficking pathways between the ER and Golgi compartments display important differences from animal and yeast systems. Yeast generally do not organize Golgi membranes into stacks (Preuss et al., 1992), and while in animals Golgi stacks are organized in large ribbon-like arrays around the microtubule-organizing center, plants instead contain large numbers of independent mini-stacks that display motility along bundled actin filaments (Boevink et al., 1998; Ladinsky et al., 1999). The distinctions with regard to spatial distributions of ER and Golgi membranes in these different systems have led to interesting questions regarding the roles and placement of elements of the COPII- and COPI-mediated trafficking components (Hawes, 2005). In plants, AtSAR1 proteins associate with ER membranes as well as localizing at Golgi-associated ERES and interference with SAR1 activity results in defects in trafficking from the ER to the Golgi (Bar-Peled and Raikhel, 1997; daSilva et al., 2004; Hanton et al., 2008). On the other hand, some plant COPII components display unique distributions on ER membranes. Unlike other systems, fluorescent protein fusions of several of the COPII coat proteins (Sec13, Sec23, and Sec24) have been found localized at ERES in the vicinity of mobile Golgi stacks (Yang et al., 2005; Matheson et al., 2006; Stefano et al., 2006; Hanton et al., 2007), while Sec12 is distributed uniformly over the ER (daSilva et al., 2004; Yang et al., 2005). This

finding suggests that elements of the COPII vesicle coat may be assembled on areas of the ER besides the ERES at the ER/Golgi interface (Hanton et al., 2008).

ARF was first identified as a cofactor required in the ADP-ribosylation of $G\alpha_s$ by cholera toxin (Enomoto and Gill, 1980; Kahn and Gilman, 1984), but the ADP-ribosylation appears not to be involved in normal cellular activity. The mammalian ARF family (excluding SARs) consists of ARF and ARL (ARF-like). ARF proteins are further classified into three subclasses based on structural and functional criteria. Class-I ARF are reported to facilitate the assembly of COPI and to play a role in effective cargo sorting. However, the precise molecular mechanisms of COPI vesicle formation and cargo selection have not been clearly elucidated compared to COPII vesicles due to the lack of an in vitro reconstitution system for this process. Small GTPases structurally, but not functionally, related to ARF proteins are designated as ARL and are involved in various cellular activities. In plants, ARF1 has been shown to localize to Golgi and endosomes, and interference with this protein affects various plant functions, such as cell polarity determination, cell proliferation, cell elongation, and fertility (Gebbie et al., 2005; Xu and Scheres, 2005). Furthermore, ARFB, which shows significant homology to mammalian ARF6, localizes to the plasma membrane (Matheson et al., 2008).

Plants express a conserved family of ARF proteins consisting of ARF and ARL subgroups (Vernoud et al., 2003). Plant ARFs seem to have critical roles in multiple steps of the membrane traffic pathways, including retrograde and anterograde ER-Golgi trafficking and BP80-dependent vacuolar transport (Lee et al., 2002; Takeuchi et al., 2002; Pimpl et al., 2003). Extensive studies done recently on the ARF GEFs demonstrated that temporally and spatially regulated activation of ARF is critical for both transport through the Golgi and endosomal recycling (Richter et al., 2007; Teh and Moore, 2007). As for ARL proteins, a few reports indicate that this subgroup is also involved in various cellular functions (McElver et al., 2000; Steinborn et al., 2002; Stefano et al., 2006), but the molecular and physiological significance of most ARL members is still to be determined.

THE GTPASE CYCLE IS THE ENGINE THAT DRIVES VECTORIAL TRANSPORT

As described earlier, the molecular switch function of a small GTPase is carried out by cycling between its active GTP-bound form and the inactive GDP-bound form (Fig. 1). Switching from the inactive to active state is accomplished by replacing bound GDP with GTP, which requires GEF. Functional counterparts of Sec12p, the GEF for the yeast Sar1p, are conserved in animals and plants. ARF GEFs are distinguished by a conserved Sec7 domain. Though eukaryotic ARF GEFs are subclassed into eight groups, Arabidopsis contains only two groups of ARF-associated GEF, GBF, and BIG

proteins, suggesting that there was unique evolution of plant ARF GEFs. In fact, recent studies revealed that two GBF-type ARF GEFs, GNOM and GNL1, which are likely to have derived from the common ancestral ARF GEF, are functionally differentiated; GNOM acquired a function in endosomal recycling in addition to the ancestral function on the Golgi (Richter et al., 2007; Teh and Moore, 2007). Our knowledge of the RAB GEF in plants is still limited, but VPS9a from Arabidopsis was recently found to be a specific GEF for RAB5 (Goh et al., 2007). VPS9a seems to be practically the sole RAB5 GEF that activates both plant-unique and conventional RAB5s in Arabidopsis. In contrast, many RAB5 GEFs with divergent domain structures have been shown to activate RAB5 at distinct steps in the endocytic pathway in animal cells (Carney et al., 2006). These results suggest that RAB GEFs have also evolved uniquely from the animal system.

After performing functions in their active form, GTPases are inactivated by hydrolyzing GTP to GDP, which is accelerated by the GAP (Fig. 1). GTP hydrolysis on the yeast Sar1p is facilitated by Sec23p, which also has a homolog in Arabidopsis. There are 15 putative ARF GAPs in Arabidopsis, only some of which have been characterized. For example, *van3* and *scarface* were isolated as mutants associated with abnormal vein patterning in an independent screening and are allelic mutants of an ARF GAP (Koizumi et al., 2005; Sieburth et al., 2006). The previously mentioned *GNOM* was also identified as a gene responsible for a mutated vein patterning (Koizumi et al., 2000). Taken together, this suggests that a correctly regulated ARF GTPase cycle, which is also essential for proper auxin transport, is critical for normal vein patterning. Another ARF GAP, RPA, activates GTPase activity of ARF1. *rpa* mutants are defective in root hair development, indicating that ARF also plays an important role in tip growth (Song et al., 2006). Although RAB GAP-like activity in plant cells was detected earlier (Anai et al., 1994) and a rice RAB-GAP has been isolated (Heo et al., 2005), there is currently little information available on its physiological role.

Once inactivated, small GTPases detach from the membrane and are kept in the GDP-bound inactive state until the next round of the GTPase activation cycle begins (Fig. 1). While SAR/ARF members do not require specific factors for this process, dissociation of most RAB GTPases from membranes is mediated by a conserved protein family, the RAB GDP dissociation inhibitor (RAB GDI). The only exception to this is RAB5F1/ARA6, which is recycled from membranes independently of RAB GDI (Ueda et al., 2001). RAB GDI binds only to GDP-bound RABs and keeps them in the GDP-bound state by inhibiting GDP release. Plants also harbor functionally conserved RAB GDIs with broad substrate specificity (Ueda et al., 1996; Zarsky et al., 1997; Ueda et al., 1998), in contrast to GEFs and GAPs, which interact only with specific RAB members. A new round of the GTPase cycle begins with disso-

ciation from the GDI and attachment to the membrane, which is mediated by GDI displacement factor. The Pra/Yip family of proteins, whose homologs are also found in plants, is proposed to undertake this function (Sivars et al., 2003) but has not yet been tested in plants.

EFFECTORS AND DOWNSTREAM FUNCTION

While GTP-binding and hydrolysis are intrinsic to RAB GTPase regulatory function, it is the recruitment and interaction of the RAB GTPases with cytosolic effector proteins that allow these RAB GTPases to carry out their regulatory functions in membrane trafficking. In animal and yeast systems, significant progress has been made in identifying RAB effector proteins (Zerial and McBride, 2001; Grosshans et al., 2006; Pfeffer, 2007). Initial models of RAB GTPase function indicated that these small GTPases played an essential role in regulation of membrane recognition events just prior to membrane fusion (Salminen and Novick, 1987; Novick and Brennwald, 1993). However, as identification of RAB effector proteins has proceeded, the functions of these effectors have highlighted additional roles for RAB GTPases in membrane-trafficking events. As a result, current models of RAB GTPase function involve roles in diverse aspects of membrane trafficking, such as vesicle formation (Carroll et al., 2001), recruitment of cytoskeletal motor proteins (Wu et al., 1998; Echard et al., 1998; Nielsen et al., 1999; Wagner et al., 2002; Hoepfner et al., 2005), and vesicle tethering and fusion (Christoforidis et al., 1999; Guo et al., 1999; Tall et al., 1999; Nielsen et al., 2000; Moyer et al., 2001).

In plants, very few RAB effector proteins have been identified and characterized. The structural heterogeneity of RAB effectors makes it unlikely that plant RAB effectors can be identified by sequence similarity alone (Zerial and McBride, 2001). However, some established RAB effectors, such as lipid kinases (Christoforidis et al., 1999), are present in Arabidopsis. One such lipid kinase, PI-4 Kβ1, has been shown to be an effector of RABA4b and plays important roles in regulation of polarized secretion in plants (Preuss et al., 2006). Additionally, specific lipid-binding domains are associated with RAB effector protein functions (Simonsen et al., 1998; Christoforidis et al., 1999), and GFP-2xFYVE domains have been shown to be selectively recruited to endosomal compartments (Voigt et al., 2005), indicating that plant endosomes also selectively accumulate PI-3P. Clearly, further identification of plant RAB effector proteins is urgently needed to expand our understanding of RAB GTPase functions during membrane trafficking in plants and will be required to elucidate the specifically tailored roles of plant RAB GTPase and their unique functions in plant-specific membrane trafficking.

PROSPECTUS

As the framework for fundamental characterization of the regulatory small GTPases for vesicular trafficking is already quite well established, future challenges lie in obtaining a precise understanding of how these plant RABs and ARFs act on the cellular level and how the processes they mediate impact overall plant growth, development, and response to the environment. How the functions of members within each of the RAB subclasses are conserved or have diverged and to what extent members from different subclasses may overlap in their functional pathways remain largely unexplored in plants. On the cell biological and biochemical levels, identification of effectors is likely to be an effective means to resolve functional divergence among related small GTPases and reveal how specificity between these small GTPases and the trafficking pathway they mediate is established. Imaging approaches that resolve dynamic interactions (see e.g. Held et al., 2008) between these small GTPases with their regulators, effectors, and their target membrane compartments should elucidate transient regulatory and functional events that are most likely to be important aspects of how vesicular trafficking pathways are regulated. While understanding of the functional significance of RAB- and ARF-regulated vesicular trafficking in cell growth and plant development is emerging, how plants rely on a responsive membrane-trafficking system to meet specific biotic and abiotic challenges, such as in response to pathogens and wounding, remains to be explored. These efforts together will reach beyond establishing RABs and ARFs as fundamental regulators of vesicular trafficking in plant cells to elucidate the roles that these small GTPases have evolved to meet needs that are unique to the growth and developmental strategies in plants.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Movie S1. Trafficking of GFP-NtRAB11b-labeled vesicular bodies in elongating tobacco pollen tubes showing the dynamics of vesicular trafficking in these polar growth cell types and differential localization of the RAB11-labeled vesicular structures to the apical cytoplasm. Reproduced from Cheung and Wu (2008).

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