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RAB GTPases and their effectors in plant endosomal transport

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The plant endomembrane system comprises distinctive membrane-bounded organelles connected with one another by the membrane trafficking system. The RAB GTPase is a key component of the membrane trafficking machinery that regulates the targeting and tethering of trafficking vesicles to target compartments by acting as a molecular switch cycling between active and inactive states. The functions of RAB GTPases are fulfilled through their interactions with several classes of interacting factors, including guanine nucleotide exchange factors (GEFs) and effector proteins. Effector proteins for plant RAB GTPases consist of evolutionarily conserved and plant-unique factors, which are involved in various membrane trafficking events in plant cells in ways unique to plants. In this review, we summarize recent findings on the functions of endosomal RAB GTPases that underwent unique diversification during plant evolution, with a special focus on RAB5/RABF and RAB11/RABA.

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Introduction

Plant cells comprise various single membrane-bounded organelles, including the endoplasmic reticulum (ER), Golgi apparatus, *trans*-Golgi network/early endosome (TGN/EE), multivesicular endosome (MVE), and vacuole. Membrane trafficking is a fundamental system for transporting proteins and lipids among these organelles and across the plasma membrane (PM), which plays pivotal roles in basic cellular activities and various higher-order physiological functions. A single round of membrane trafficking between donor and target organelles consists of four sequential processes: (1) the formation of vesicles from a donor membrane, (2) transport of vesicles, (3) the tethering of vesicles to a target membrane, and (4) the fusion of vesicles to the target membrane. These processes involve conserved machinery components, one of which is RAB GTPase in the Ras super family [1].

RAB GTPase acts as a molecular switch in membrane trafficking in eukaryotic cells by cycling between GTP-bound active and GDP-bound inactive states. Active RAB GTPase evokes various downstream reactions, including the tethering of transport vesicles to the target membrane, organelle movement, and organelle maturation. The activation of RAB GTPase is mediated by guanine nucleotide exchange factors (GEFs), which are crucial for the spatiotemporal regulation of RAB GTPase activation and the induction of downstream reactions. These downstream reactions are triggered by nucleotide state-dependent interactions with specific factors collectively called effector proteins [1].

Arabidopsis thaliana harbors 57 RAB GTPases classified into eight groups (RABA–RABH) according to their similarity to animal RAB GTPases (RAB1/RABD, RAB2/RABB, RAB5/RABF, RAB6/RABH, RAB7/RABG, RAB8/RABE, RAB11/RABA, and RAB18/RABC) [2,3]. These eight groups are mostly conserved in green plants, with basal plants also harboring additional members, such as RAB23 [4–6]. Among the eight core groups of plant RAB GTPases, RAB5/RABF and RAB11/RABA bear distinctive features unique to plants. In addition to canonical RAB5/RABF2, the green plant lineage possesses a plant-specific type of RAB5, the ARA6/RABF1 group [7–9]. The extremely expanded nature of the RAB11/RABA group is another distinctive feature of plant RAB GTPases. 26 RAB11/RABA members are encoded in the Arabidopsis genome, whereas only two or three RAB11 members exist in yeast and mammalian genomes [2]. Recent studies have unveiled the molecular functions of these RAB GTPases with plant-specific characteristics and their effectors, which we summarize in this review.

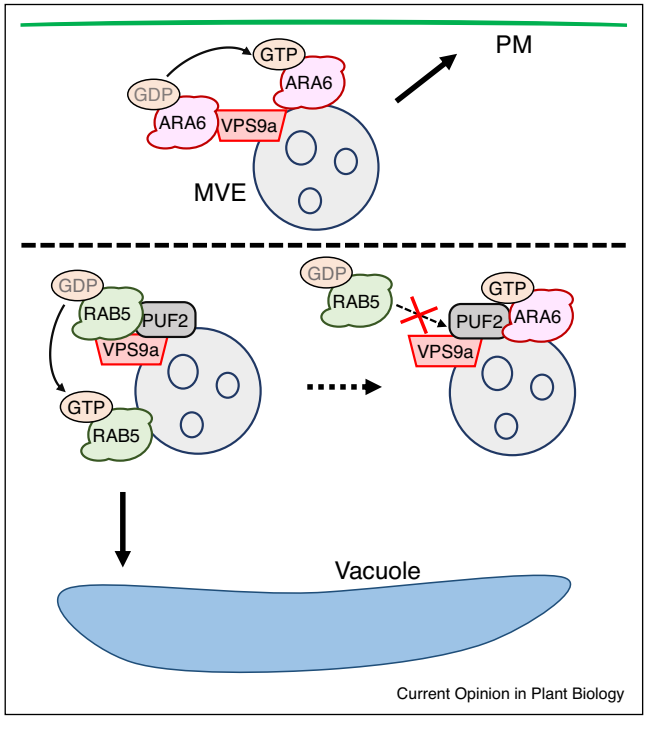
RAB5/RABF-mediated endosomal trafficking

In Arabidopsis, canonical RAB5/RABF2 and plant-specific ARA6/RABF1 are localized to different populations of MVEs with partial overlap and were shown to fulfill distinct functions in endosomal trafficking [7,10]. Canonical RAB5/RABF2 plays critical roles in endocytic and vacuolar transport pathways in Arabidopsis and tobacco, and ARA6/RABF1 mediates trafficking between the MVE and the PM in Arabidopsis [7,11•,12–14] (Figure 1). Despite their

different functions, these RAB5/RABF members share a common activating factor, VPS9a, which mediates the exchange from GDP to GTP on these RAB5/RABF members in Arabidopsis [15] (Figure 1). The *vps9a* mutants exhibit a wide spectrum of developmental phenotypes, indicating the pivotal roles of RAB5/RABF-mediated trafficking pathways in plant development [15,16]. *VPS9a* is also required for normal penetration resistance against powdery mildew fungi, whereas its close homolog, *VPS9b*, seems to have only minor roles, if any, in development and immunity of Arabidopsis [15,17,18]. Although the mechanism by which two RAB5/RABF groups with distinct functions are regulated by the same activating factor remains obscure, it was recently shown that the C-terminal region of VPS9a is involved in the activation of ARA6/RABF1 but not for canonical RAB5/RABF2 in Arabidopsis [19]. This region could be important for the distinct regulation of two groups of plant RAB5/RABF.

Mutations in *ARA6/RABF1* and canonical *RAB5/RABF2* have been shown to have counteracting effects on the *vps9a-2* mutant [7], the molecular mechanism of which remains unsolved. Recently, a key factor that orchestrates the two groups of plant RAB5/RABF was identified. PLANT-UNIQUE RAB5 EFFECTOR 2 (PUF2) was shown to interact with constitutively active ARA6/RABF1. PUF2 contains four coiled-coil domains, and the fourth coiled-coil region is responsible for the interaction with ARA6/RABF1. Intriguingly, subcellular localization and genetic interaction experiments indicated that PUF2 is involved in the vacuolar trafficking pathway mediated by canonical RAB5/RABF2, although PUF2 was isolated as an effector of ARA6/RABF1. Further genetic and biochemical analyses demonstrated that the titration of PUF2 with activated ARA6/RABF1 negatively affected the activation of canonical RAB5/RABF2 [20••] (Figure 1). Given that *PUF2* homologs have not been detected in nonplant systems, plants developed a unique vacuolar/endosomal transport system involving plant-specific factors such as ARA6/RABF1 and PUF2 during evolution.

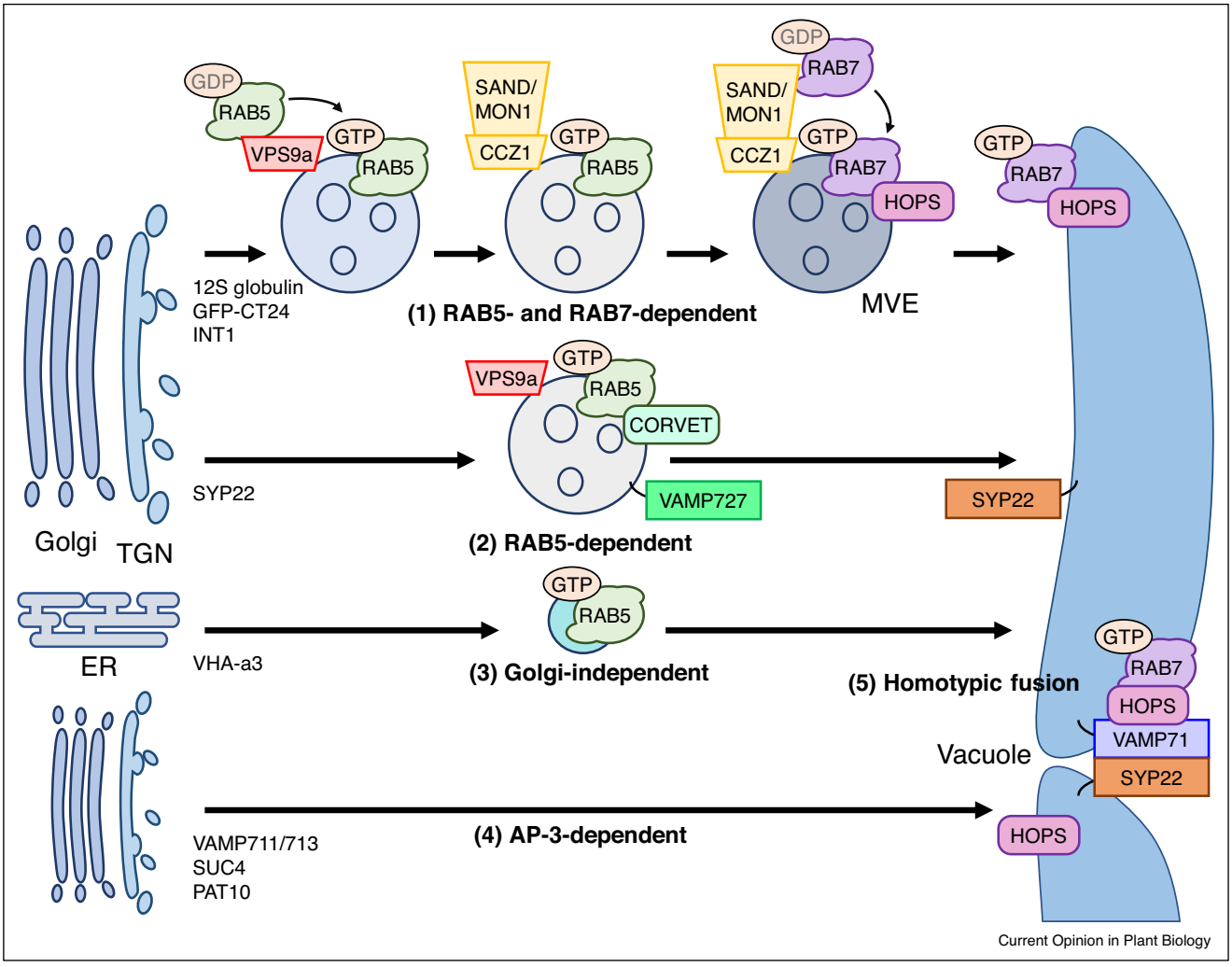
Figure 1



Endosomal trafficking involving the RAB5/RABF group in Arabidopsis cells. ARA6/RABF1 mediates trafficking between the MVE and the PM, whereas canonical RAB5/RABF2 acts in endocytic and vacuolar transport pathways. In the trafficking pathway from the MVE to the vacuole, ARA6/RABF1 negatively regulates canonical RAB5/RABF2 to attenuate vacuolar transport. PUF2 captures GDP-bound canonical RAB5/RABF2 and VPS9a on the MVE to promote transport from the MVE to the vacuole, and ARA6 inhibits this process by titrating PUF2 in its GTP-bound state.

In animal and yeast cells, the SAND/MON1–CCZ1 complex, which is responsible for endosomal maturation from RAB5-positive early to RAB7-positive late endosomes, mediates the sequential action of RAB5 and RAB7 by acting as a RAB5 effector and a RAB7 GEF [21–23]. Recent studies revealed that Arabidopsis SAND/MON1–CCZ1 also acts as an effector of RAB5/RABF2 and a GEF for RAB7/RABG, suggesting that the molecular function of this complex is conserved among eukaryotes [24••,25••,26••]. However, Arabidopsis SAND/MON1–CCZ1 is not required for MVE formation but is essential for the fusion of MVEs with the vacuole [24••,26••]. The different requirements of this complex in plant and animal cells could reflect the distinct timing of RAB5 action. Animal RAB5 is required for early endosomal trafficking events, whereas plant canonical RAB5 mainly mediates late endosomal transport. 12S globulin, an artificial vacuolar cargo GFP-CT24, and the inositol transporter INT1 are transported through this RAB5-dependent and RAB7-dependent pathway involving SAND/MON1–CCZ1 in Arabidopsis [24••,25••,26••,27,28•] (Figure 2). In addition to this pathway, there are also vacuolar transport pathways distinctly regulated by canonical RAB5/RABF2 and RAB7/RABG in plant cells [11•,25••]. In Arabidopsis cells, there is a RAB5-dependent and RAB7-independent trafficking pathway for the vacuole. The *vps9a-2* mutation hampered the transport of the soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) SYP22 to the vacuolar membrane, although the loss of *SAND* or *CCZ1* function did not exhibit impairment in SYP22 transport [25••] (Figure 2). This type of trafficking pathway has not been reported in animal and yeast cells, suggesting that Arabidopsis uniquely developed this pathway during evolution. Several vacuolar proteins, including a subunit of vacuolar H^+-ATPase, VHA-a3, are reportedly transported directly from the ER to the vacuole, independent of the Golgi apparatus [29] (Figure 2). The overexpression of dominant-negative canonical RAB5/RABF2 impairs the vacuolar targeting of VHA-a3 [28•] (Figure 2). These

Figure 2



A schematic of vacuolar trafficking pathways in Arabidopsis cells. (1) The pathway dependent on the sequential action of canonical RAB5/RABF2 and RAB7/RABG. Canonical RAB5/RABF2 is activated by VPS9a, to which the SAND/MON1–CCZ1 complex is recruited, which in turn activates RAB7/RABG. This conversion from RAB5/RABF2 to RAB7/RABG mediates maturation of the MVE from RAB5/RABF-positive to RAB7/RABG-positive compartments. 12S globulin, GFP-CT24, and INT1 are transported through this pathway. The HOPS complex also acts in this pathway, whereas the contribution of the CORVET complex remains to be verified. (2) The pathway dependent on RAB5/RABF2 but not RAB7/RABG. SYP22 is transported through this pathway. The CORVET complex mediates membrane fusion between the MVE and the vacuole with the VAMP727-containing SNARE complex under the regulation of canonical RAB5/RABF2. (3) The Golgi-independent pathway responsible for VHA-a3 transport. Canonical RAB5/RABF2 could also function in this pathway. (4) The AP-3-dependent pathway. VAMP711, VAMP713, SUC4, and PAT10 are transported via this pathway. The HOPS complex is also required in this pathway. (5) Homotypic fusion between vacuoles mediated by HOPS, RAB7/RABG, and the VAMP71-containing SNARE complex.

lines of evidence could indicate that the canonical RAB5-dependent and RAB7-independent pathway is equivalent to the Golgi-independent pathway, otherwise they merge on the way to the vacuole.

In animal and yeast cells, the sequential action of RAB5 and RAB7 also involves two tethering complexes, the class C core vacuole/endosome tethering (CORVET) and homotypic fusion and protein sorting (HOPS) complexes. The CORVET and HOPS complexes share core subunits composed of VPS11, VPS16, VPS18, and VPS33 in addition to specific subunits (VPS3 and VPS8 for CORVET and VPS39 and VPS41 for HOPS) [30]. Arabidopsis harbors homologs of all of these subunits that are essential for embryo development and/or the growth of pollen tubes [31••,32–34,35••,36]. Biochemical and genetic analyses revealed that CORVET and HOPS also function as effectors of canonical RAB5/RABF2 and RAB7/RABG, respectively, in plant cells [32,35••]. The *vps41-1* mutation affected the vacuolar targeting of RAB5-dependent and RAB7-dependent cargo, INT1, in the pollen tube [27,28•], which also supports the

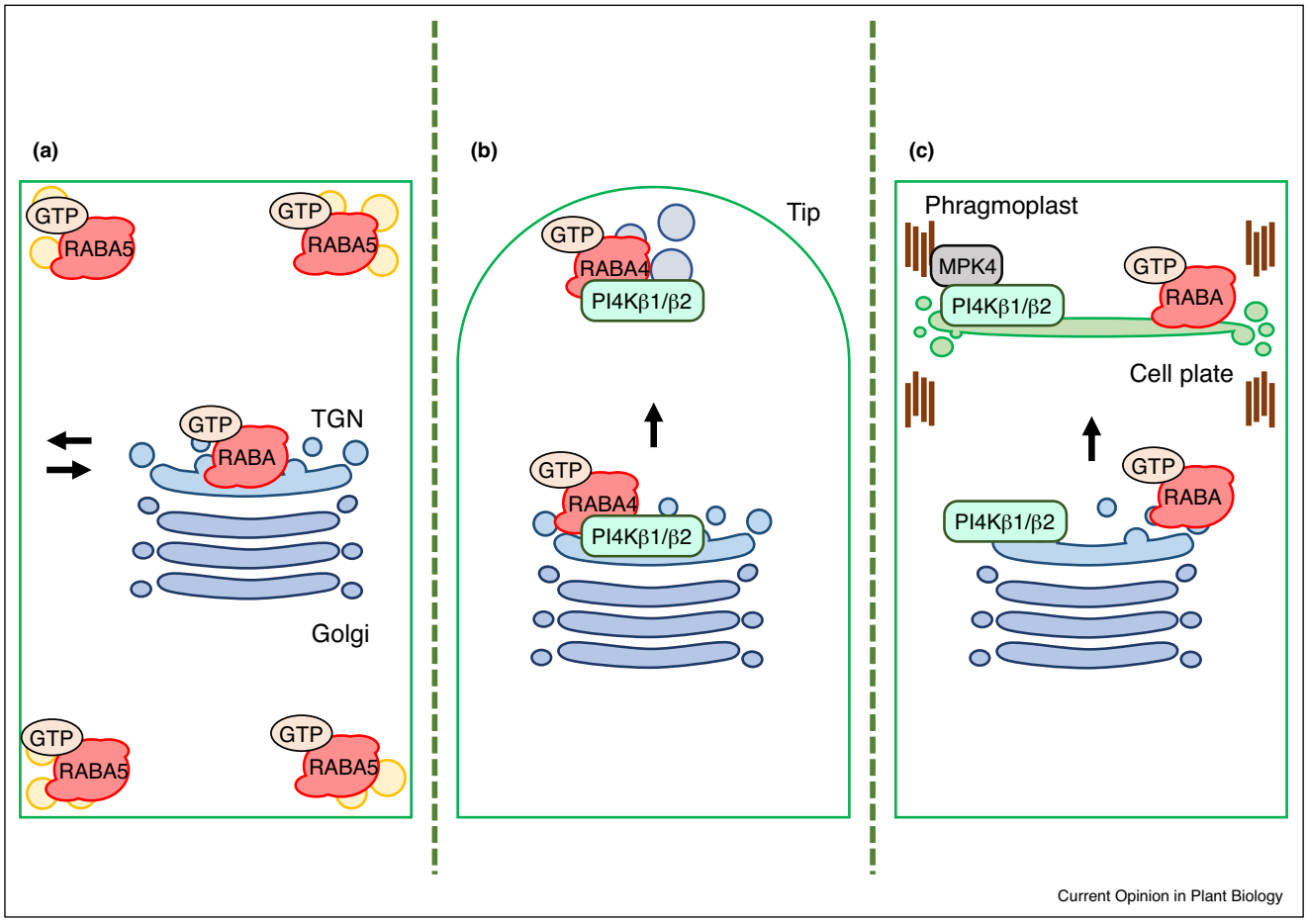
function of HOPS in the RAB5/RABF2-and RAB7/RABG-dependent pathway (Figure 2). Conversely, the vacuolar transport of SYP22 in developing embryos was impaired in the *vps3* mutant but not in the *vps39* mutant [35••], indicating that CORVET but not HOPS is involved in the RAB5-dependent and RAB7-independent pathway (Figure 2). CORVET and HOPS are also known to mediate distinct vacuolar fusion events with different sets of RAB GTPase and SNARE proteins. CORVET cooperates with RAB5/RABF2 and the VAMP727-containing SNARE complex during membrane fusion between the MVE and the vacuole. Conversely, HOPS mediates homotypic vacuolar membrane fusion with RAB7/RABG and the VAMP71-containing SNARE complex [31••,34,35••,37] (Figure 2).

In addition to these pathways, plant cells are equipped with another vacuolar transport pathway mediated by the adaptor protein complex 3 (AP-3). VAMP711, VAMP713, vacuolar sucrose transporter SUC4, and PROTEIN S-ACYL TRANSFERASE10 (PAT10) are cargos transported via the AP-3-dependent trafficking pathway [25••,27,38] (Figure 2). The AP-3-dependent pathway requires HOPS activity, whereas mutants defective in canonical RAB5/RABF2 or RAB7/RABG do not exhibit defects in the transport of AP-3 pathway cargos [25••,27,35••] (Figure 2). It has also been reported that the plant-specific effector of canonical RAB5/RABF2, ENDOSOMAL RAB EFFECTOR WITH PX-DOMAIN (EREX), is involved in the vacuolar transport of seed storage proteins [39]. These lines of evidence indicate that plants developed a unique vacuolar trafficking system by using both evolutionarily conserved and newly acquired machinery components during evolution, which underpins unique and complex plant vacuolar functions.

RAB11/RABA-mediated trafficking pathways

Twenty-six members of the Arabidopsis RAB11/RABA group are divided into RABA1–RABA6 subgroups according to their sequence similarity [2]. RAB11/RABA members

Figure 3



Schematic models of cellular events involving RAB11/RABA members. **(a)** Most RAB11/RABA members are partly associated with the TGN/EE. RABA5c is localized to distinctive compartments aligned at the geometric edges of Arabidopsis root cells. **(b)** RABA4 members and PI4Kβ1/β2 localize at the TGN/EE and tip-localized compartments and function in tip growth. **(c)** PI4Kβ1/β2 also localize to the cell plate, where they interact with MPK4, and function in phragmoplast organization and cell plate formation. Several RABA members are also localized to the forming cell plate.

were shown to be associated and/or partially colocalized with TGN/EE markers and to act on the secretory and endocytic pathways in Arabidopsis and tobacco cells [40,41,42•,43–48] (Figure 3a). Recently, RABA5c was shown to localize to distinct compartments from other RABA-positive domains; RABA5c localized to compartments aligned along the geometric edges of Arabidopsis cells (Figure 3a). A dominant-negative form of RABA5c did not localize to this compartment and affected cellular geometry in lateral organs, suggesting that the localization of RABA5c at the geometric edge is required for proper cell patterning in roots [49••].

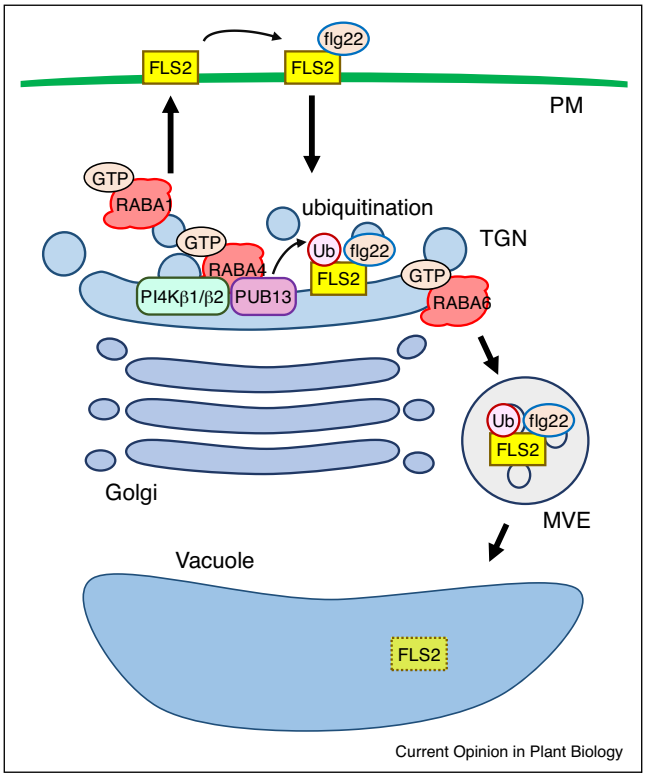
Whereas effector molecules responsible for fulfilling the RABA5c function remain to be identified, some effector proteins of RABA4 members have been reported thus far. Phosphatidylinositol 4-kinases (PI4K) β1 and PI4Kβ2 act as effectors of the RAB11/RABA group member RABA4b in tip-growing cells. PI4Kβ1/β2 localize on the TGN/EE with RABA4b and coordinate the polarized secretion of cell wall components during tip growth in root hairs [46,50••,51] (Figure 3b). The phosphatidylinositol 4-phosphate (PI4P) phosphatase ROOT HAIR DEFECTIVE 4 (RHD4) is required for the proper localization of RABA4b at the tips of root hairs [52]. These lines of evidence indicate that regulation of the PI4P level is critical for proper polarized root hair growth. PI4Kβ1 also interacts with RABA4d, which is specifically expressed in pollen. The loss of RABA4d or PI4Kβ1/β2 function resulted in defective pollen tube growth, suggesting that PI4Kβ1/β2 could also act as an effector of RABA4d in growing pollen tubes [53•] (Figure 3b). PI4Kβ1/β2 are also required for proper organization of the phragmoplast and cell plate formation through regulating and/or interacting with MAP65-3 and the MAP kinase MPK4 [54] (Figure 3c). Several RAB11/RABA members involved in cytokinesis are also localized to the cell plate [40,41,43,46,47] (Figure 3c). The functional interplay between RAB11/RABA and PI4Kβ1/β2 in cell plate formation should be verified in future studies. Intriguingly, RABA1e and RABA2a on forming cell plates exhibit different sensitivities to a cytokinesis inhibitor, Endosidin 7, which may suggest that each RAB11/RABA member plays a distinctive role in cell plate formation [55].

A member of the plant U-box family of E3 ubiquitin ligases, PLANT U-BOX13 (PUB13), was recently identified as another RABA4b effector. PUB13-mediated ubiquitination was required for the ligand-induced degradation of the flagellin receptor FLAGELLIN SENSING 2 (FLS2), which is responsible for downregulating flagellin-induced immune responses [56]. PUB13 interacts with GTP-bound RABA4b and PI4P and colocalizes with RABA4b in leaf tissue cells. Intriguingly, both *pub13* and *pi4kβ1/β2* mutants exhibited enhanced immune responses and elevated resistance against *Pseudomonas syringae* pv. *tomato* DC3000 [56,57••]. These findings suggest that RABA4b, PI4Kβ1/β2, and PUB13 cooperate to attenuate FLS2-dependent immune responses (Figure 4). It has also been reported that PUB13-mediated ubiquitination is involved in endocytosis of another PM-resident receptor-like kinase, BRI1 [58]. It would be interesting to see whether RABA4 and PI4Kβ1/β2 are also involved in this process to verify the universality of the function of RABA4 in the endocytic degradation of receptor kinases. RABA1 and RABA6 are also involved in FLS2 trafficking [42•] (Figure 4). The identification of effector molecules for each group of RAB11/RABA proteins would facilitate our understanding of the detailed mechanisms of secretory/endocytic trafficking pathways in plants, which would also provide important insights into the functional diversification of RAB11/RABA members during plant evolution.

Conclusion and perspectives

We have summarized knowledge of two groups of plant endosomal RAB GTPases, RAB5/RABF and RAB11/RABA, and their specific effector molecules. Several effector proteins have been identified in the last five

Figure 4



Subcellular trafficking of FLS2 mediated by RAB11/RABA members and their effectors. RABA1 mediates the transport of newly synthesized FLS2 to the PM. FLS2 bound with flagellin is rapidly internalized from the PM and ubiquitinated by PUB13 on the TGN/EE, where RABA4 and PI4Kβ1/β2 also reside. Ubiquitinated FLS2 is further transported to the MVE and degraded in the vacuole. RABA6 could be involved in the maturation of the MVE.

years, which improved our understanding of the molecular functions of these RAB GTPases. However, there should be more unknown effectors cooperating with these RAB GTPases in endosomal/secretory trafficking, given that animal RAB5 fulfills its functions through its interactions with dozens of effector proteins [59]. Given the plant-unique regulation of RAB5/RABF-involving and RAB11/RABA-involving trafficking pathways and unique sets of effector proteins acting there, it would be highly likely that other plant RAB groups also fulfill their functions in distinct ways from non-plant systems. To identify novel RAB-effector interactions in plants, emerging techniques such as BioID, which allows to identify weak and/or transient interactions in living cell contexts [60], in combination with widely used methods including yeast tow-hybrid screening, would be effective. Meanwhile, it would also be interesting to examine how plants developed their unique trafficking systems during evolution. Comparative analyses between angiosperms including Arabidopsis and basal plant lineages such as chlorophytes, charophytes, and bryophytes would be efficient to reconstitute how the unique plant membrane trafficking system has been developed during evolution. Genome data from several basal plant lineages recently released [4,6,61–63] have raised new and interesting questions. For example, ARA6/RABF1 members are well conserved among land plants although its distribution is sporadic in algal species [7,8], which may reflect a critical requirement of this RAB GTPase for terrestrial life. Acquisition of a novel RAB GTPase has also been reported recently from a study of the liverwort *Marchantia polymorpha*. In addition to an orthologous protein to animal and plant RAB2/RABB, *M. polymorpha* harbors a unique RAB2/RABB protein comprising the IFT43-like domain at the C-terminus [5]. These RAB GTPases would be good models for studying how and why plants acquired plant-specific RAB GTPases. The study of *M. polymorpha* also suggested that secondary loss of ancient RAB GTPases could have contributed to specialization and/or differentiation of the plant membrane trafficking system. *M. polymorpha* harbors RAB21 and RAB23, which are lost in Arabidopsis, whose physiological significance should be investigated in future studies [5,64,65]. It would be also interesting to ask whether there is any relevance between the distinctive expansion of RAB11/RABA members and other expanded membrane trafficking factors such as the EXO70 group, a subunit of the exocyst complex involved in tethering of secretory vesicles to the PM [66–68] during plant evolution. Further studies would lead to exciting answers to these questions and raise more exciting new questions to address in future studies.

Conflict of interest statement

Nothing declared.

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