Endosomal trafficking pathway regulated by ARA6, a RAB5 GTPase unique to plants

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Lineage-specific expansion, followed by functional diversification of key components that act in membrane trafficking, is thought to contribute to lineage-specific diversification of organelles and membrane trafficking pathways. Indeed, recent comparative genomic studies have indicated that specific expansion of RAB and SNARE molecules occurred independently in various eukaryotic lineages over evolutionary history. However, experimental verification of this notion is difficult, because detailed functional analyses of RAB and SNARE proteins uniquely acquired by specific lineages are essential to understanding how new membrane trafficking pathways may have evolved. Recently, we found that a plant-specific RAB GTPase, ARA6, and a plant-unique R-SNARE, VAMP727, mediate a trafficking pathway from endosomes to the plasma membrane in *Arabidopsis thaliana*. Although a similar endosomal trafficking pathway was also reported in animals, the molecular machineries acting in these trafficking systems differ between animals and plants. Thus, trafficking pathways from endosomes to the plasma membrane appear to have been acquired independently in animal and plant systems. We further demonstrated that the ARA6-mediated trafficking pathway is required for the proper salt-stress response of *A. thaliana*. These results indicate that acquisition of a new membrane trafficking pathway may be associated with maximization of the fitness of each organism in a lineage-specific manner.

Membrane trafficking is a key mechanism of protein transport among organelles in all eukaryotic organisms. In this system, sorting the correct cargo proteins into forming transport vesicles and accurately determining the target organelle are critical to maintaining efficient trafficking activities. RAB GTPase and SNARE are key components in regulating tethering and/or fusion between trafficking carriers and target membranes. GTP-bound (activated) RAB interacts with the effector proteins, some of which tether transport vesicles or organelles to the target membrane. Once tethered, one R-SNARE and three Q-SNAREs, which localize on transport carriers and the target membrane, respectively, form a tight complex to execute membrane fusion between the two membranes.[1] Each trafficking pathway involves a specific set of RAB and SNARE molecules, which confers specificity to the trafficking pathway. Some groups of RAB GTPases that function in core trafficking pathways (core RABs) are highly conserved among eukaryotic lineages,[2-4] suggesting that the trafficking pathways in which these RABs function may have existed in the common ancestor of all eukaryotic organisms. On the other hand, distribution of some groups of RAB members is sporadic or specific to certain lineages, suggesting lineage-specific secondary loss of the descendants of the common RAB, or acquisition of new RAB groups by gene duplication followed by functional diversification.[5-9]

It has been suggested that the lineage-specific expansion of RAB GTPase is tightly associated with the acquisition of

new trafficking pathways during evolution,[2] although how newly acquired RAB GTPases pioneer new trafficking pathways is almost completely unknown. It has been reported that plant RAB GTPases are diversified in a unique way. Among the distinguishing features in the organization of land plant RAB GTPases (which include an extraordinarily expanded number of *RAB11* members and no *RAB4* homolog), the existence of a plant-unique group of RAB GTPases, the ARA6 group, is a definitive characteristic. ARA6 group members harbor several characteristic structural features, although their primary amino-acid sequence is similar to metazoan RAB5 members.[1] For example, ARA6 members lack the carboxyl-terminal hypervariable region and the Cys motif that is known to be essential for membrane binding and correct localization of conventional RAB GTPases. Instead, ARA6-type RAB GTPases are anchored via fatty acylation at the N-terminus.[10] One ARA6-type (ARA6/RABF1) and two conventional-type (ARA7/RABF2b and RHA1/RABF2a) RAB5-related GTPases are encoded in the *A. thaliana* genome. Thus far, orthologs of *ARA6* have been found in all land plants for which the genomes have been sequenced, including bryophytes and lycophytes. However, we did not find ARA6 homologs in unicellular green algae or other lineages outside of Plantae (some protists belonging to Apicomplexa also harbor specialized RAB5 homologs with characteristics somewhat similar to ARA6, but monophyly with the plant ARA6 group has not been supported by phylogenetic analyses). Thus, the ARA6-type RAB5 seems to have been newly acquired by the ancestor of land plants. However, it was unknown whether the two types of plant RAB5, the ARA6 group and conventional RAB5, are functionally differentiated and why only plants harbor these two RAB5 groups.

Studies during the last decade have provided some partial answers to these questions. As expected from the absence of the C-terminal Cys motif, ARA6 has been shown to detach from the membrane independently of the RAB GDP dissociation inhibitor (RAB GDI),[10] while ARA7 and RHA1 require RAB GDI for recycling between the membrane and the cytosol. On the other hand, all three RAB5-related
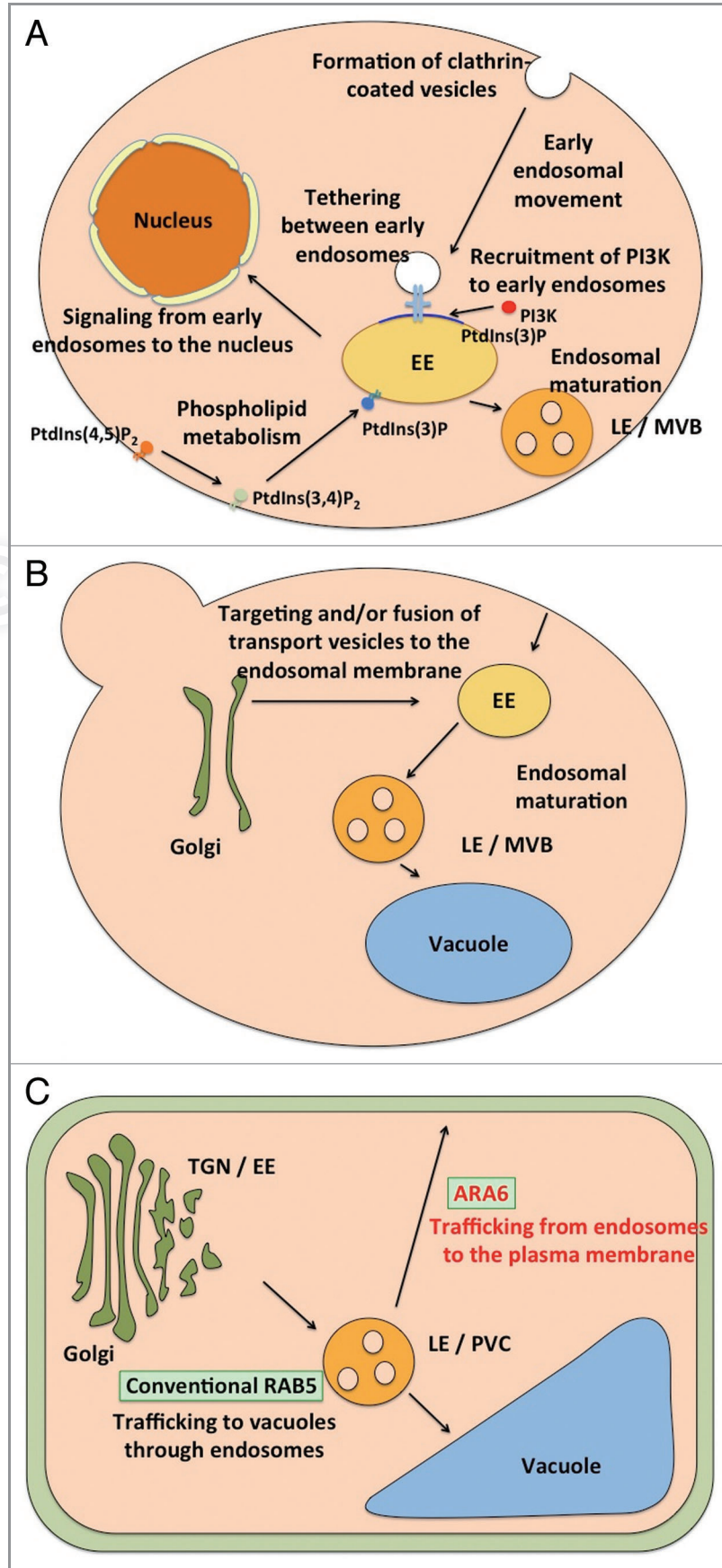
GTPases in *A. thaliana* share a common activating factor, VPS9a.[11] Immuno-electron microscopy studies indicated that both ARA6 and conventional RAB5s localize on the multivesicular endosomes (MVEs),[12] and direct comparison of their subcellular localization in the same cell using different colors of fluorescent proteins further demonstrated that ARA6 and conventional RAB5 localize on different populations of endosomes with considerable overlap.[13] The difference in localization of the two types of RAB5 should reflect differences in their functions. Overexpression of ARA6 and ARA7 conferred different effects on a partial loss-of-function mutant of *VPS9a* in a consistent manner.[11] Functional analysis by over-expressing dominant-negative mutants of conventional RAB5s indicated that ARA7 and RHA1 function in endocytic and vacuolar trafficking pathways.[14-16] However, similar experiments using mutant ARA6 yielded varying results,[16,17] and the precise function of ARA6 is still not clear.

In addition to RAB GTPases, SNARE genes are also diversified in a unique way in plants.[18] One of the distinguishing features of plant SNAREs is expanded VAMP7 members; of these, *VAMP727* of *A. thaliana* encodes an R-SNARE with unique structural characteristics.[1] VAMP727 has an approximately 20-amino-acid insertion in the N-terminal longin domain and resides on RAB5-positive endosomes under steady-state conditions.[19] Close homologs of VAMP727 have been found only in seed plants, suggesting that it functions in a newly pioneered trafficking pathway in seed-plant cells.

The colocalization of VAMP727 with RAB5 members on endosomes suggests that VAMP727 contributes to transport pathways between endosomes and other organelles, which may be regulated by RAB5 members. We have found that VAMP727 consistently mediates membrane fusion between endosomes and the vacuole by forming a complex with three Q-SNAREs, SYP22, SYP5 and VTI11. This complex formation is essential for the development and desiccation tolerance of seeds.[20] An attractive hypothesis can be made based upon this function of VAMP727: VAMP727 acquired by ancestral seed plants may have allowed for the

selection of vacuolar trafficking pathways that were better able to accommodate the evolution of seeds, which require the transport of vast amounts of proteins to the vacuole.

We expected that the plant-unique RAB GTPase ARA6 would act in trafficking pathways uniquely acquired by plants; thus, we made this our next research target. We first investigated ARA6 functions by genetic analyses using loss-of-function mutants of *A. thaliana*. We collected putative knockout mutants for all three RAB5 members of *A. thaliana* and established homozygous lines after backcrossing several times. We expected that *ara6* mutants would exhibit a visible phenotype; however, all of the single mutants exhibited phenotypes that were indistinguishable from wild-type plants when grown under normal growth conditions. Thus, we decided to look for interesting genetic interactions between *RAB5* genes and other endosomal/vacuolar function-related genes. We constructed various combinations of multiple mutants by crossing *rab5* and endosomal/vacuolar SNARE mutants or RAB-related mutants and found some unexpected and intriguing genetic interactions between *RAB5* and *SYP22,* a *VAM3* homolog encoding a vacuolar Q-SNARE. Mutations in conventional RAB5 genes enhanced *syp22-1* phenotypes, which is consistent with previous results showing that conventional RAB5s function in the transport pathway from endosomes to the vacuole (**Fig. 1**).[15,16] In sharp contrast, the mutations in *ARA6* almost completely suppressed the phenotypes of *syp22-1* (**Fig. 2**). This genetic interaction demonstrates that ARA6 and conventional RAB5 regulate different transport pathways from endosomes; these respective pathways seem to act counter to one another. Constitutively active mutants of RAB5s exhibited different localization patterns in a consistent manner; constitutively active ARA7 and RHA1 accumulated only at vacuolar and endosomal membranes, but constitutively active ARA6 was also targeted to the plasma membrane. Thus, ARA6 may mediate the trafficking pathway from endosomes to the plasma membrane, whereas conventional RAB5 acts only in the pathway to the vacuole. What downstream events might

ARA6 regulate at the plasma membrane? In our search for an answer, we witnessed a dramatic encounter between two plant-unique trafficking proteins, ARA6 and VAMP727.

It is generally accepted that GTP-bound RAB promotes tethering of transport carriers to the target organelles, after which SNARE proteins execute the membrane fusion. Thus, we expected that the amount of SNARE complex functioning downstream of ARA6 would be decreased in the *ara6* mutant. In the course of endosomal SNARE pairing by coimmunoprecipitation, we found that VAMP727 also forms a complex with a plasma membrane Q-SNARE, SYP121, indicating participation of VAMP727 in membrane fusion at the plasma membrane in addition to the vacuolar membrane. The function of VAMP727 at the plasma membrane was also demonstrated by total internal reflection fluorescence (TIRF) microscopy and pharmacological analysis; inhibition of endocytosis by wortmannin or Tyrphostin A23 resulted in the accumulation of VAMP727 at the plasma membrane. In the *ara6* mutant, the amount of SNARE complex containing VAMP727 and SYP121 was specifically reduced, whereas the vacuolar SNARE complex containing VAMP727 and SYP22 was unaffected. Thus, ARA6 is required for efficient SNARE complex formation (and membrane fusion) at the plasma membrane. Overexpression of constitutively active ARA6 increased the amount of VAMP727-SYP121 complex in a consistent manner. Thus, the plant-unique RAB5 ARA6 and plant-unique R-SNARE VAMP727 act in the same trafficking pathway in *A. thaliana*: a trafficking pathway from endosomes to the plasma membrane (**Fig. 1**). Currently available genome information suggests that these two molecules were likely acquired independently during plant evolution; ARA6 seems to have emerged in the common ancestor of land plants, while VAMP727 homologs have been identified only in

seed plants thus far. An interesting question is how these molecules were recruited to the same trafficking pathway during land plant evolution (it is still possible that future genome analyses will reveal a more ancient origin for VAMP727).

The plant-unique nature of ARA6 and VAMP727 suggests that the trafficking pathway mediated by these proteins is involved in a plant-unique function. What, then, might this plant function be? A close homolog of ARA6 was isolated as a salt-responsive gene in the common ice plant *Mesembryanthemum crystallinum*.[21] Furthermore, ARA6-type RAB5 in rice root cells was reported to be secreted to the apoplast in response to salinity stress.[22] In *A. thaliana*, we found that loss of function of *ARA6* impaired normal salt-stress tolerance and overexpression of constitutively active ARA6 elevated resistance to salinity stress (**Fig. 3**).[23] Recently, we found that transgenic plants expressing constitutively active ARA6 also exhibited improved tolerance to high-sorbitol stress (**Fig. 3**). Thus, the ARA6 pathway is not specifically required for the salinity-stress response, but it could play more general roles in response to abiotic stresses, which plants encounter in terrestrial environments.

An important future question to consider is what cargos the ARA6-dependent pathway transports under salinity- and/or osmotic-stress conditions. Under salinity-stress conditions, constitutively active ARA6 forms aggregates at subdomains in the plasma membrane (**Fig. 4**). This distinctive localization strongly suggests that ARA6 is involved in the formation of specialized subdomains in the plasma membrane in response to stress conditions. These ARA6-positive domains could be the interface responsible for sensing or detoxification of osmotic and/or salinity stress. Purification of this specialized plasma membrane domain followed by identification of the other resident proteins would be an effective strategy for identifying cargos of the ARA6-mediated trafficking pathway. Known salt stress-related proteins could be also candidates for the cargos. For example, *salt overly sensitive 1* (*SOS1*) encodes a plasma membrane Na$^+$/H$^+$ antiporter, which is closely associated with salt resistance; *SOS1* expression is elevated by salt stress and overexpression

Figure 2. Genetic interaction between *rab5* and *syp22-1*. *ara7* and *rha1* mutations aggravated abnormal morphological phenotypes of *syp22-1*, whereas *ara6* suppressed *syp22-1* phenotypes. Plants were grown for 30 d.
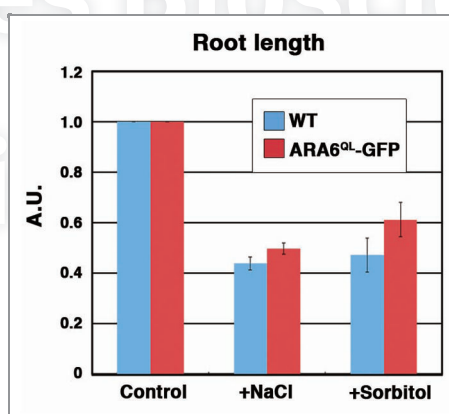
Figure 3. Effect of overexpression of constitutively active ARA6. Overexpression of ARA6^{Q93L}-GFP increased abiotic stress tolerance. WT and transgenic plants expressing ARA6^{Q93L}-GFP were grown on Murashige and Skoog (MS) plates for 4 d, then transferred to MS (control), MS + 100 mM NaCl, or MS + 200 mM sorbitol plates and cultivated for an additional 12 d. The root length of four or five plants grown under each condition was measured, and the average root length under each stress condition was compared with the root length of plants grown on the control medium [n = 4; bars, standard error (SE); A.U., arbitrary units].
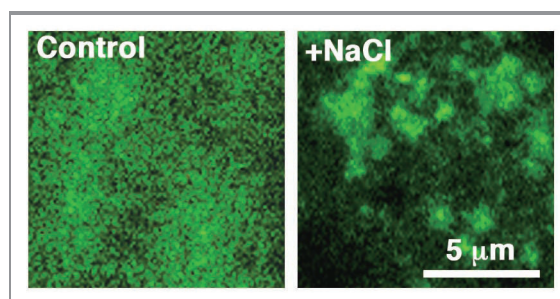
Figure 4. ARA6^{Q93L}-GFP formed discrete speckles on the plasma membrane under stress conditions. Root epidermal cells grown under control or salinity-stress conditions were observed by TIRFM.

of *SOS1* confers salinity-stress tolerance.[24,25] Another *SOS* gene, *SOS6*, encodes a cellulose synthase-like protein, AtCSLD5; the *atcsld5* loss-of-function mutant exhibits phenotypes similar to those of the *ara6* mutant.[26] The interactions between these proteins and the ARA6-dependent trafficking pathway should be explored. ARA6 could also fulfill some regulatory roles at the plasma membrane in a more direct way. SYP121, a binding partner of VAMP727 whose assembly is regulated by ARA6, has been shown to interact with a regulatory K⁺

channel subunit to regulate K⁺ flux.[27] A modulatory role of ARA6 in the assembly of SYP121 and VAMP727 into the complex could have a regulatory effect upon the stress response by controlling K⁺ channel activity at the plasma membrane.[27-29]

As mentioned above, ARA6, a uniquely acquired RAB GTPase, was recruited to the endosome-to-plasma membrane trafficking pathway in plant cells. Similar trafficking pathways also exist in animal cells, but the machinery components involved in these pathways differ between plant and animal

systems.[30] Thus, similar trafficking pathways seem to have been acquired independently in these lineages. Further analysis of ARA6 function, as well as identification and functional analysis of effector molecules of ARA6 and comparative functional analysis using other land plant species, including crops and basal land plant lineages, will reveal how this newly acquired RAB GTPase pioneered a new trafficking pathway and how novel physiological roles were assigned to this new trafficking pathway during land plant evolution.

References

1. Ebine K, Ueda T. Unique mechanism of plant endocytic/vacuolar transport pathways. J Plant Res 2009; 122:21-30; PMID:19082690; http://dx.doi.org/10.1007/s10265-008-0200-x

2. Dacks JB, Field MC. Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode. J Cell Sci 2007; 120:2977-85; PMID:17715154; http://dx.doi.org/10.1242/jcs.013250

3. Elias M. Patterns and processes in the evolution of the eukaryotic endomembrane system. Mol Membr Biol 2010; 27:469-89; PMID:21067450; http://dx.doi.org/10.3109/09687688.2010.521201

4. Pereira-Leal JB, Seabra MC. Evolution of the Rab family of small GTP-binding proteins. J Mol Biol 2001; 313:889-901; PMID:11697911; http://dx.doi.org/10.1006/jmbi.2001.5072

5. Langsley G, van Noort V, Carret C, Meissner M, de Villiers EP, Bishop R, et al. Comparative genomics of the Rab protein family in Apicomplexan parasites. Microbes Infect 2008; 10:462-70; PMID:18468471; http://dx.doi.org/10.1016/j.micinf.2008.01.017

6. Nielsen E, Cheung AY, Ueda T. The regulatory RAB and ARF GTPases for vesicular trafficking. Plant Physiol 2008; 147:1516-26; PMID:18678743; http://dx.doi.org/10.1104/pp.108.121798

7. Pereira-Leal JB, Seabra MC. The mammalian Rab family of small GTPases: definition of family and subfamily sequence motifs suggests a mechanism for functional specificity in the Ras superfamily. J Mol Biol 2000; 301:1077-87; PMID:10966806; http://dx.doi.org/10.1006/jmbi.2000.4010

8. Saito-Nakano Y, Loftus BJ, Hall N, Nozaki T. The diversity of Rab GTPases in Entamoeba histolytica. Exp Parasitol 2005; 110:244-52; PMID:15955319; http://dx.doi.org/10.1016/j.exppara.2005.02.021

9. Saito-Nakano Y, Nakahara T, Nakano K, Nozaki T, Numata O. Marked amplification and diversification of products of ras genes from rat brain, Rab GTPases, in the ciliates Tetrahymena thermophila and Paramecium tetraurelia. J Eukaryot Microbiol 2010; 57:389-99; PMID:20738463; http://dx.doi.org/10.1111/j.1550-7408.2010.00503.x

10. Ueda T, Yamaguchi M, Uchimiya H, Nakano A. Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. EMBO J 2001; 20:4730-41; PMID:11532937; http://dx.doi.org/10.1093/emboj/20.17.4730

11. Goh T, Uchida W, Arakawa S, Ito E, Dainobu T, Ebine K, et al. VPS9a, the common activator for two distinct types of Rab5 GTPases, is essential for the development of Arabidopsis thaliana. Plant Cell 2007; 19:3504-15; PMID:18055610; http://dx.doi.org/10.1105/tpc.107.053876

12. Haas TJ, Sliwinski MK, Martinez DE, Preuss M, Ebine K, Ueda T, et al. The Arabidopsis AAA ATPase SKD1 is involved in multivesicular endosome function and interacts with its positive regulator LYST-interacting protein. Plant Cell 2007; 19:1295-312; PMID:17468262; http://dx.doi.org/10.1105/tpc.106.049346

13. Ueda T, Uemura T, Sato MH, Nakano A. Functional differentiation of endosomes in Arabidopsis cells. Plant J 2004; 40:783-9; PMID:15546360; http://dx.doi.org/10.1111/j.1365-313X.2004.02249.x

14. Dhonukshe P, Baluska F, Schlicht M, Hlavacka A, Samaj J, Friml J, et al. Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. Dev Cell 2006; 10:137-50; PMID:16399085; http://dx.doi.org/10.1016/j.devcel.2005.11.015

15. Kotzer AM, Brandizzi F, Neumann U, Paris N, Moore I, Hawes C. AtRabF2b (Ara7) acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells. J Cell Sci 2004; 117:6377-89; PMID:15561767; http://dx.doi.org/10.1242/jcs.01564

16. Sohn EJ, Kim ES, Zhao M, Kim SJ, Kim H, Kim YW, et al. Rha1, an *Arabidopsis* Rab5 homolog, plays a critical role in the vacuolar trafficking of soluble cargo proteins. Plant Cell 2003; 15:1057-70; PMID:12724533; http://dx.doi.org/10.1105/tpc.009779

17. Bolte S, Brown S, Satiat-Jeunemaitre B. The N-myristoylated Rab-GTPase m-Rabmc is involved in post-Golgi trafficking events to the lytic vacuole in plant cells. J Cell Sci 2004; 117:943-54; PMID:14762108; http://dx.doi.org/10.1242/jcs.00920

18. Sanderfoot A. Increases in the number of SNARE genes parallels the rise of multicellularity among the green plants. Plant Physiol 2007; 144:6-17; PMID:17369437; http://dx.doi.org/10.1104/pp.106.092973

19. Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K, Sato MH. Systematic analysis of SNARE molecules in Arabidopsis: dissection of the post-Golgi network in plant cells. Cell Struct Funct 2004; 29:49-65; PMID:15342965; http://dx.doi.org/10.1247/csf.29.49

20. Ebine K, Okatani Y, Uemura T, Goh T, Shoda K, Niihama M, et al. A SNARE complex unique to seed plants is required for protein storage vacuole biogenesis and seed development of Arabidopsis thaliana. Plant Cell 2008; 20:3006-21; PMID:18984676; http://dx.doi.org/10.1105/tpc.107.057711

21. Bolte S, Schiene K, Dietz KJ. Characterization of a small GTP-binding protein of the rab 5 family in Mesembryanthemum crystallinum with increased level of expression during early salt stress. Plant Mol Biol 2000; 42:923-36; PMID:10890538; http://dx.doi.org/10.1023/A:1006449715236

22. Zhang L, Tian LH, Zhao JF, Song Y, Zhang CJ, Guo Y. Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. Plant Physiol 2009; 149:916-28; PMID:19036832; http://dx.doi.org/10.1104/pp.108.131144

23. Ebine K, Fujimoto M, Okatani Y, Nishiyama T, Goh T, Ito E, et al. A membrane trafficking pathway regulated by the plant-specific RAB GTPase ARA6. Nat Cell Biol 2011; 13:853-9; PMID:21666683; http://dx.doi.org/10.1038/ncb2270

24. Shi H, Ishitani M, Kim C, Zhu JK. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proc Natl Acad Sci USA 2000; 97:6896-901; PMID:10823923; http://dx.doi.org/10.1073/pnas.120170197

25. Shi H, Quintero FJ, Pardo JM, Zhu JK. The putative plasma membrane Na(+)/H(+) antiporter SOS1 controls long-distance Na(+) transport in plants. Plant Cell 2002; 14:465-77; PMID:11884687; http://dx.doi.org/10.1105/tpc.010371

26. Zhu J, Lee BH, Dellinger M, Cui X, Zhang C, Wu S, et al. A cellulose synthase-like protein is required for osmotic stress tolerance in Arabidopsis. Plant J 2010; 63:128-40; PMID:20409003

27. Honsbein A, Sokolovski S, Grefen C, Campanoni P, Pratelli R, Paneque M, et al. A tripartite SNARE-K+ channel complex mediates in channel-dependent K+ nutrition in Arabidopsis. Plant Cell 2009; 21:2859-77; PMID:19794113; http://dx.doi.org/10.1105/tpc.109.066118

28. Grefen C, Chen Z, Honsbein A, Donald N, Hills A, Blatt MR. A novel motif essential for SNARE interaction with the K(+) channel KC1 and channel gating in Arabidopsis. Plant Cell 2010; 22:3076-92; PMID:20884800; http://dx.doi.org/10.1105/tpc.110.077768

29. Grefen C, Honsbein A, Blatt MR. Ion transport, membrane traffic and cellular volume control. Curr Opin Plant Biol 2011; 14:332-9; PMID:21507708; http://dx.doi.org/10.1016/j.pbi.2011.03.017

30. Lakkaraju A, Rodriguez-Boulan E. Itinerant exosomes: emerging roles in cell and tissue polarity. Trends Cell Biol 2008; 18:199-209; PMID:18396047; http://dx.doi.org/10.1016/j.tcb.2008.03.002