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SHORT COMMUNICATION

Proportional subcellular localization of *Arabidopsis thaliana* RabA1a

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ABSTRACT

Subcellular localization of trafficking proteins in a single cell affects the assembly of trafficking machinery between organelles and vesicles throughout the targeting pathway. RabGTPase is one of the regulators to direct specific targeting of cargo molecules depending on GDP/GTP bound status. We have recently determined the crystal structures of GDP-bound inactive and both GTP- and GppNHp-bound active forms of *Arabidopsis* RabA1a. It is notable that the switch regions of RabA1a exhibit conformational changes derived by GDP or GTP binding. However, it was not clear that where the GDP- or GTP-bound RabA1a is localized at the subcellular level in a cell. Here we demonstrate that the distinct proportion of subcellular localization of RabA1a depends on its site-specific mutation as the GDP- or GTP-bound form. RabA1a proteins located at the plasma membrane, endosomes, and cytosol. While the GDP-bound form of RabA1a^{S27N} located more at endosomes than the plasma membrane compared to the proportions of RabA1a wild-type, and the GTP-bound RabA1a^{Q72L} located mainly at the plasma membrane in comparison to RabA1a wild-type and RabA1a^{S27N}. These distinct proportional localizations of RabA1a enable a cognate interaction between inactive/active RabA1 and effector molecules to direct specific targeting of its cargo molecules.

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Subcellular organelles in eukaryotes compartmentalize cellular processes and interplay to maintain cellular homeostasis. Membrane trafficking via vesicle transport is the most fundamental mechanism for communication between organelles for various cellular functions. RabGTPases are the key players that act as molecular switches in budding, moving, docking, and fusion of vesicles during membrane trafficking.[1] RabGTPases undergo conformational changes upon binding to GDP or GTP. Thus, RabGTPases employ specific effector repertoires such as GTPase activating proteins (GAPs), GDP/GTP exchange factors (GEFs), and GDP-dissociation inhibitor (GDI)-displacement factor (GDF) in a specific subcellular location.[2,3]

The Arabidopsis genome encodes 57 members of RabGTPases, which are well-known as an extensively expanded protein superfamily in plant lineages.[4] Among AtRabGTPases, the RabA1 group has the 9 largest members, RabA1a to RabA1i.[5–8] The RabA1 members appear to function in anterograde trafficking from trans-Golgi network/early endosomal compartments (TGN/EE) to the plasma membrane (PM) as well as in responses to salinity stress.[9,10] We have recently determined the crystal structure of RabA1a depending on its nucleotide binding state to GDP/GTP.[11] However, the location of RabA1a and its GDP- or GTP-bound states at subcellular level remains elusive.

To investigate the localization of RabA1a, we generated *Arabidopsis* transgenic plants expressing monomeric YFP (mYFP)-RabA1a under the 35S promoter. We also generated mYFP-RabA1a^{S27N} as the GDP-bound form and mYFP-RabA 1a^{Q72L} as the GTP bound form under the same promoter. The previous reports[6,12] and our crystal structure study[11] described that the conserved Ser27 in the p-loop is involved in capturing GDP; Gln72 in the switch II region is widely used to prepare a mutant with defective GTP hydrolysis ability. To exhibit the localization of RabA1a and mutant derivatives, we examined their subcellular distribution pattern when fractionated by ultracentrifugation into soluble and membrane fractions. All mYFP-fused RabA1a and its mutant proteins were expressed at the expected molecular size (50 kD) of the fusion proteins (Figure 1(a)). All fractionated RabA1a proteins were predominantly detected in the pellet fraction and weakly in the soluble fraction by anti-GFP antibodies (Figure 1(b)). As controls for the soluble and microsomal fractions, we included mYFP-PEN1 proteins from mYFP-PEN1 transgenic plants. As the intrinsic property of the PEN1 protein, mYFP-PEN1 was only detected in the pellet fraction. Additionally, we applied an anti-AALP antibody as a soluble fraction marker and an anti-VAMP722 antibody as a membrane-associated fraction marker. When we monitored the mYFP-tagged RabA1a and its mutant derivatives, the mYFP-RabA1a

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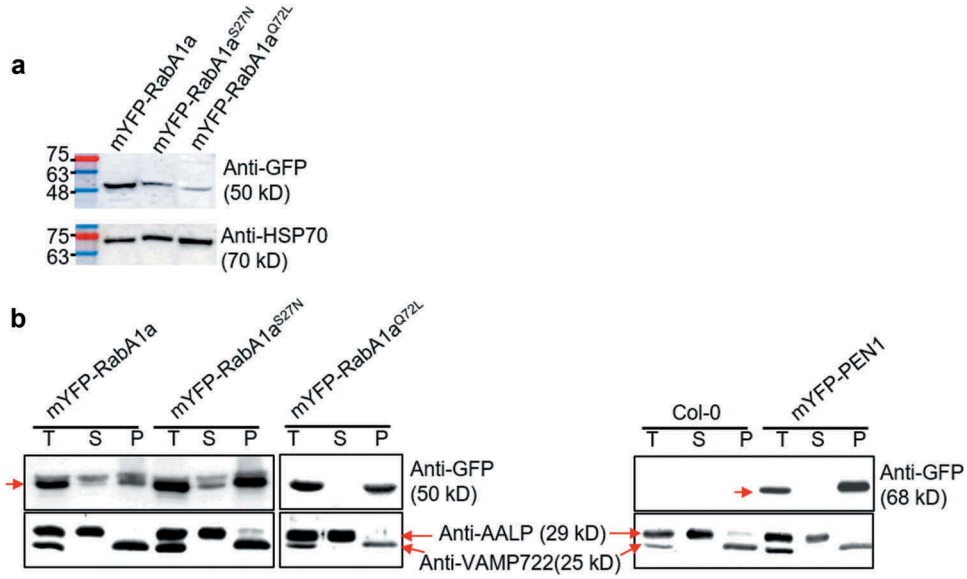

Figure 1. Subcellular expression of RabA1a and its mutants in *Arabidopsis*. (a) The expressed mYFP-RabA1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} proteins were detected with an anti-GFP antibody. The loading amounts were detected with an anti-HSP70 antibody. (b) Subcellular fractionation of mYFP-tagged RabA1a proteins. Membrane association of mYFP-RabA1a and its derivatives were presented with fractionated soluble (s), and pellet (p, membrane) by ultra-centrifugation of total protein (t) extracts from the indicated transgenic plants. Anti-AALP and anti-VAMP722 antibodies were used as an internal control as a soluble and membrane fraction marker, respectively. Col-0 and mYFP-PEN1 transgenic plants were included for the control of fractionation.

protein was localized at the PM, the endosomes, and the cytosol, similar to the observation in the previous report (Figure 2(a)).[13,14] The mYFP-RabA1a^{S27N} protein was mainly localized both the PM and aggregates of endosomes and was minorly in the cytosol. However, mYFP-RabA1a^{Q72L} was mainly located at the PM and was drastically reduced in the endosomes and cytosol. Our results showed that mYFP-RabA 1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} proteins were membrane-associated and differentially positioned at the subcellular level.

To define the localization of mYFP-RabA1a, we used the amphiphilic styryl dye FM4-64 as a subcellular membrane marker, which sequentially labels the PM followed by a variety of endosomal, prevacuolar, and vacuolar compartments over time.[12] When FM4-64 was applied to the root tissue of 10-day-old transgenic *Arabidopsis* seedlings expressing mYFP-RabA1a, extensive co-localization at both the PM and endosomes was observed from the beginning of the staining until approximately 5 min later (Figure 2(a)). As shown in Figure 2(b), the respective possession ratio of RabA1a proteins was about 57.5% at the PM, 30.4% at the endosomes, and 12.1% at the cytosol. Using the same experimental approach used for mYFP-RabA1a, we applied FM4-64 to *Arabidopsis* root tissue expressing each of the predicted mutants. The dominant-negative GDP-bound form of YFP-RabA1a^{S27N} co-localized with FM4-64 at copious endosomes and the PM with about 43.5% and 40.7%, respectively (Figure 2(a, b)). Interestingly, endosomal localization of mYFP-RabA 1a^{S27N} was predominant compared to that of mYFP-RabA 1a. The dominant-positive form mYFP-RabA1a^{Q72L} mainly co-localized with FM4-64 at the PM by about 88.0%, but had drastically reduced co-labeling with endosome and cytosol signals at about 6.4% and 5.5%, respectively (Figure 2(a,b)).

To investigate the differential distribution of mYFP-RabA 1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} in a cell, we analyzed the intensity of mYFP-labeled subcellular membranous organelles and subsequently profiled the signals across the root cells of seedlings. The mYFP-RabA1a proteins localized both at the PM and endosomes. To determine how RabA1a proteins are correlated with FM4-64 at the PM or endosomes, we separately analyzed more than 30 images of root cells from FM4-64 stained mYFP-RabA1a seedlings, due to the subcellular localization of mYFP-RabA1a proteins either at the PM or endosomes. The mYFP-RabA1a proteins at the PM and endosomes showed Pearson's correlation coefficients of 0.71 and 0.83, respectively (Figure 2(c,d)). With the same approach, mYFP-RabA1a was applied to both mYFP-RabA1a^{S27N} and mYFP-RabA1a^{Q72L}, and mYFP-RabA1a^{S27N} apparently localized at endosomal aggregates (Pearson's r = 0.97) together with the PM (Pearson's r = 0.64) (Figure 2(e,f)), while mYFP-RabA1a^{Q72L} predominantly localized at the PM (Pearson's r = 0.83) and together with the endosome (Pearson's r = 0.59) (Figure 2(g,h)). These quantitative analyses of confocal images suggest that mYFP-RabA1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} proteins located distinctively and proportionally at the PM, endosomes, and the cytosol.

In this study, we found that the distinct proportion of subcellular localization of *Arabidopsis* RabA1a depends on its site-specific mutation as the GDP- or GTP- bound form. The proportion of RabA1a that localized to the PM, endosome, and cytosol were 57.5%, 30.4%, and 12.1%, respectively. This subcellular proportion of RabA1a was probably maintained and essential to

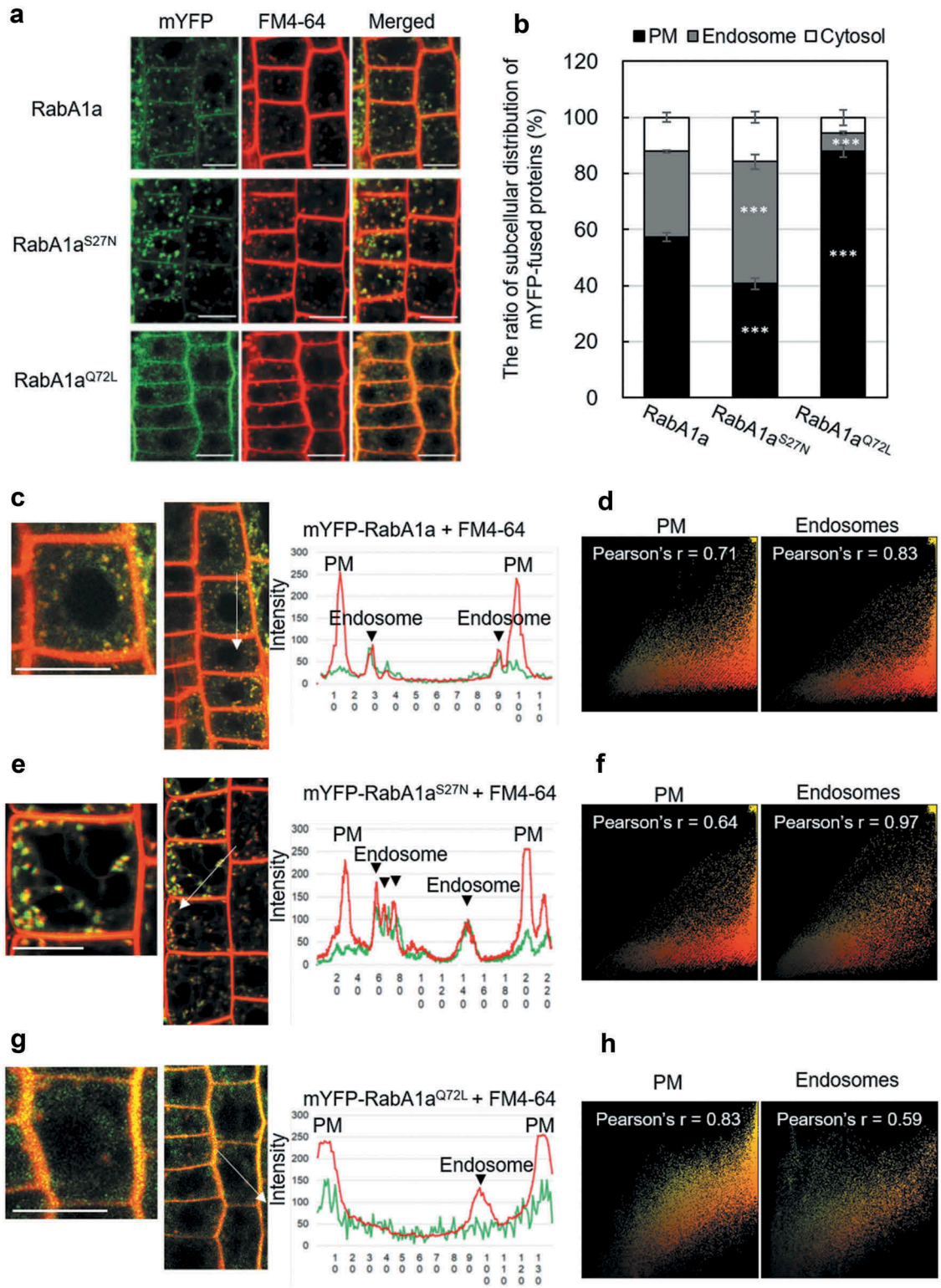

Figure 2. The distinct subcellular proportion of RabA1a, RabA1a^{S27N}, and RabA1a$^{Q72L.}$ (a) The mYFP-RabA1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} proteins co-localized with FM4-64-labeled plasma membrane (PM) and endosomes, and were weakly detectable in the cytosol of the *Arabidopsis* root. The respective possession rate of signals at the PM, endosomes, and cytosol were completely different depending on the form of RabA1a. Scale bar = 10 μm. (b) The quantitative analysis of the subcellular distribution of mYFP-RabA1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} at each of the PM, endosomes, and the cytosol. The ratio of mYFP signals at distinct subcellular organelles were measured using the FIJI 1.52i (National Institute of Health). For this analysis, more than 30 cells were used. Asterisks indicate significant differences; ***$P < 0.001$ Students' *t*-test, error bars show the SE (n = 30). (c, e, g) mYFP-RabA1a, mYFP-RabA1a^{S27N}, and mYFP-RabA1a^{Q72L} proteins were analyzed by intensity profiles at the PM or endosomes across the root cells. The left panels are the magnified images of white arrow area. The white arrow indicates the direction of the intensity profile as presented on the X-axis (nm). (d, f, h) The scattered plots with Pearson's coefficients (r-value) quantifying the degrees of colocalization at the PM or endosomes between FM4-64 and mYFP-RabA1a, mYFP-RabA1a^{S27N}, or mYFP-RabA1a^{Q72L}. The images of root cells (n = 30) were analyzed using PSC colocalization Plugin of the FIJI 1.52i, and the results are presented as scatter plots with Pearson's coefficients r-value.

function in the multiple interactions with effector molecules to sense salinity stress[14] and auxin-mediated response.[13] As a dominant-negative mutant, 43.5% of RabA1a^{S27N} localized mainly at endosomes, which appeared to be stabilized as enriched aggregates. In contrast, as the dominant-positive mutant, 88% of the RabA1a^{Q72L} positioned at the PM. This differential subcellular distribution may be occurring to stabilize or shift interactions with unknown effectors of RabA1a, depending on its specific mutant forms. RabGTPase employs and plays with many effector molecules to transport cognate vesicles from a donor station to a target station.[3] Therefore, the proportional distributions of RabA1a^{S27N} (GDP bound) in endosomes or RabA1a^{Q72L} (GTP bound) in the PM could help the stable interaction of specific effectors among varied interacting molecules to ensure the partitioned trafficking.

Although we have extensively studied the subcellular localization of Rab proteins, RabA1a to Raba1i among the RabA1 subfamily are still uncharacterized in details. Intrinsic properties of Rab proteins are most likely involved in its interactions with various effector molecules depending on GDP- or GTP-bound status. Therefore, it is important to know the localization of GDP-/GTP- bound form of Rab protein each. To avoid unintended artifacts of interactions between RabA1a and an organelle marker protein, we chose the amphiphilic styryl dye FM4-64 for the localization study. FM4-64 stains provided an excellent benefit to identify and monitor the intrinsic subcellular-proportional distribution of RabA1a, RabA1a^{S27N}, and RabA1a^{Q72L}.

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No potential conflicts of interest were disclosed

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