The C-Terminal Hypervariable Domain Targets *Arabidopsis* ROP9 to the Invaginated Pollen Tube Plasma Membrane

Dear Editor,

The small GTPase ROPs are molecular switches in diverse developmental and cellular processes in plants (Yang, 2002). Based on their genomic organization, ROPs have been classified into two subgroups (Winge et al., 2000). Type I ROPs regulate the dynamic organization of the actin cytoskeleton, a tip-focused Ca^{2+}-gradient, and the production of reactive oxygen species, thus playing essential roles in pollen tube growth presumably by integrating input signals into diverse output cellular activities (Nibau et al., 2006). The GTP-bound, active form of type I ROPs localize at the apical plasma membrane of pollen tubes to direct polar growth, while their GDP-bound, inactive forms are cytoplasmic (Yang, 2002). Localization of type I ROPs at the apical plasma membrane of pollen tubes is dynamically regulated by coordinated activities of their upstream regulators including guanine nucleotide exchange factors (RopGEFs), GTPase activating proteins (RopGAPs), and guanine nucleotide dissociation inhibitors (RhoGDIs) (Hwang et al., 2010).

Compared to the studies on type I ROPs, much less is known about the function and regulation of type II ROPs in pollen tubes. *Arabidopsis* ROP9/Rac7 was the only type II ROP whose expression in pollen tubes was detectable (Cheung et al., 2003). Overexpression of *ROP9* showed a dosage-dependent depolarization effect on pollen tubes (Cheung et al., 2003). However, its localization was excluded from the apical region (Cheung et al., 2003), distinct from that of type I ROPs. In addition, ROP9 localized at the invaginated pollen tube plasma membrane (IPTPM) that surrounds tobacco generative cells (Cheung et al., 2003). Indeed, an early study using non-discriminating anti-ROP antibody identified ROP signal at the IPTPM (Lin et al., 1996). However, it was unclear what determines the association of ROP9 at the IPTPM.

To determine the *cis*-factors mediating the association of ROP9 at the IPTPM, we first needed a fluorescent probe for this specific membrane structure. Lyn24GFP is a chimeric protein in which the N-terminal 24 amino acids of the Src family tyrosine kinase Lyn are fused to the N-terminus of GFP. Lyn24GFP associates with detergent-resistant membranes through myristoylation and palmitoylation in metazoan cells and is used as a fluorescent probe for lipid rafts (Gupta and DeFranco, 2003). We found that Lyn24GFP consistently labeled eyeglasses-shaped structures in *Arabidopsis* pollen tubes, beside its localization at the apical region (Figure 1A). A co-labeling experiment using the sperm-specific fluorescent reporter Pro_{HTR10}:HTR10–RFP (Ingouff et al., 2007) confirmed that the structure labeled by Lyn24GFP was indeed the IPTPM (Figure 1A), establishing Lyn24GFP as a fluorescent probe for the IPTPM.

To determine whether the association of ROP9 at the IPTPM depended on the bound guanine nucleotide, we compared the localization of ROP9 with its mutant versions. The constitutive active (CA) mutant remains GTP-bound whereas the dominant negative (DN) version does not undergo GDP–GTP exchange despite its interaction with RopGEFs (Yang, 2002). Co-labeling experiments with Lyn24GFP showed that wild-type ROP9 and both mutant variants remained at the IPTPM (Figure 1B), indicating that the localization of ROP9 at the IPTPM was independent of its activation status.

To test what determined the distinct localization of ROP9 at the IPTPM, we performed domain swapping experiments. Previous studies in leaf epidermal cells showed that different C-terminal hypervariable regions (HV) of ROPs were responsible for their membrane association (Ivanchenko et al., 2000; Lavy et al., 2002; Lavy and Yalovsky, 2006). We therefore swapped the N- and C-terminal domains between ROP9 and ROP3, one of the three type I ROPs highly expressed in pollen (Winge et al., 2000; Cheung et al., 2003). ROP3 has a typical CAAX motif at the C-terminus, while ROP9 has a 'GC–CG' box together with a CAAX motif (Figure 1C). Co-labeling with Lyn24GFP showed that no ROP3 was detected at the IPTPM (Figure 1C). However, the replacement of ROP3–HV with ROP9–HV resulted in an obvious signal at the IPTPM (Figure 1C), similar to what was observed for ROP9 (Figure 1B). On the contrary, ROP9–3HV was hardly detectable at the IPTPM (Figure 1C), suggesting that the C-terminal HV of ROP9 was critical for its association at the IPTPM. We also introduced either ROP9CA–3HV (ROP9CA with the HV of ROP3) or ROP3CA–9HV (ROP3CA with the HV of ROP9) transiently in tobacco pollen tubes. Similarly to what we observed in domain-swapped wild-type ROPs, the C-terminal HV determined the membrane distribution of ROPs at the IPTPM. That is, ROP9CA–3HV was not detected at

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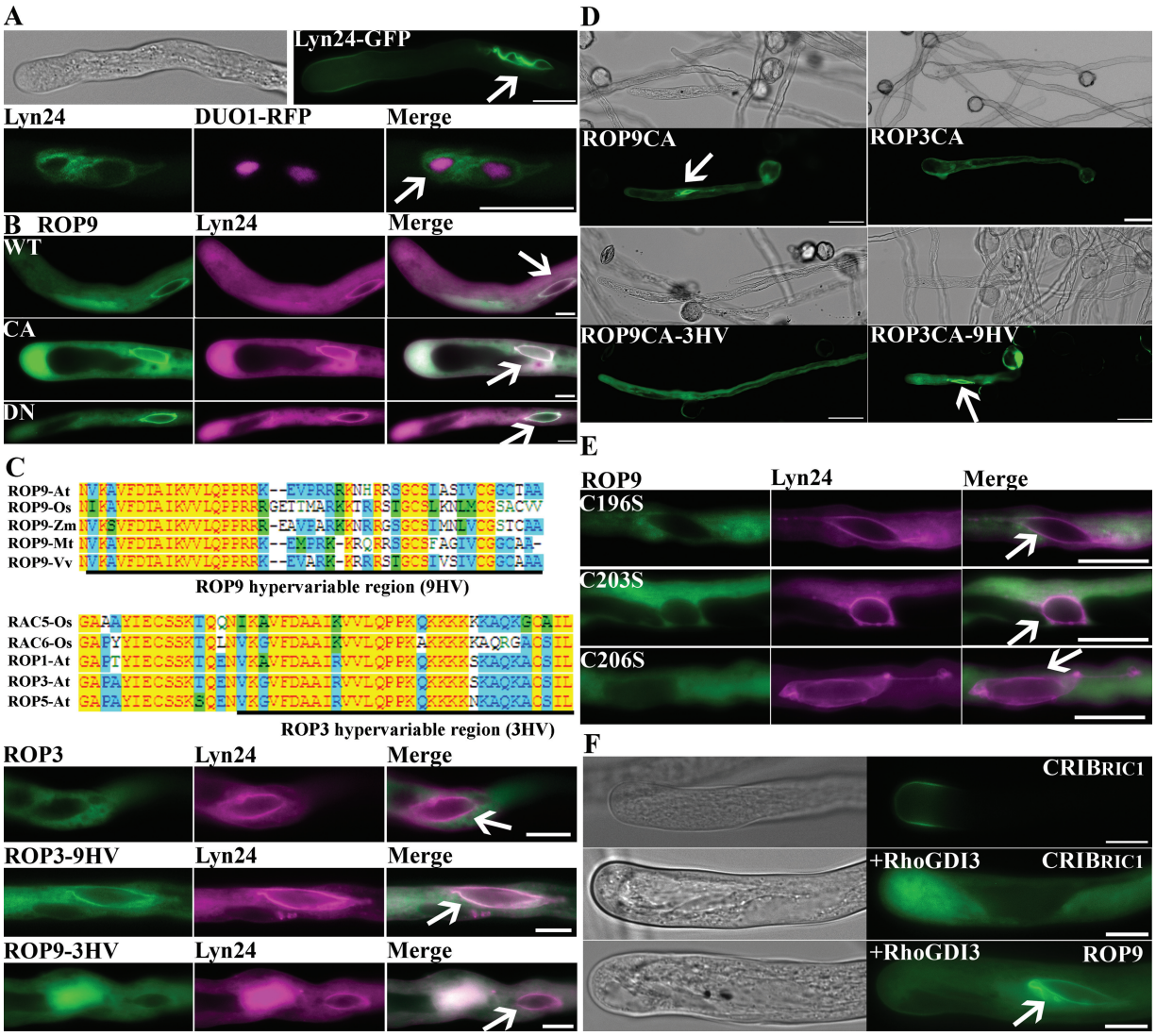

Figure 1. The C-Terminal Hypervariable Domain Targets *Arabidopsis* ROP9 to the Invaginated Pollen Tube Plasma Membrane.

(A) Lyn24 as a marker for the invaginated pollen tube plasma membrane (IPTPM). Top panel: a Pro_{LAT52}:Lyn24GFP transgenic *Arabidopsis* pollen tube (bright field and fluorescence images are side by side). Bottom panel: an *Arabidopsis* pollen tube transformed with both Pro_{LAT52}:Lyn24GFP and Pro_{HTR10}:DUO1–RFP. Images of GFP channel (green), RFP channel (magenta), and merge are shown from left to right.

(B) Regulated activity of ROP9 was not critical for its localization at the IPTPM. Tobacco pollen tubes transiently expressing Lyn24–GFP (Lyn24) together with RFP–ROP9 (WT), RFP–ROP9CA (CA), or RFP–ROP9DN (DN) are shown.

(C) The C-terminal hypervariable region (HV) of ROP9 was necessary for its localization at the IPTPM. The top panel shows alignment of ROP9 and its homologs from other plant species, as well as alignment of type I ROPs from *Arabidopsis* (At) and rice (Os). UNIPROT number (locus name) for ROP9–At is RAC7_ARATH (At4g28950); for ROP9–Os is RAC2_ORYSJ (Os05g0513800); for ROP9–Zm is Q9LEC5_MAIZE (ZEAMMB73_559671); for ROP9–Mt is A7UQU4_MEDTR (MTR_6g087980); for ROP9–Vv is A5ARL1_VITVI (VIT_11s0016g03640); for ROP1-At is RAC11_ARATH (At3g51300); for ROP3–At is RAC1_ARATH (At2g17800); for ROP5-At is RAC6_ARATH (At4g35950); for RAC5-Os is RAC5_ORYSJ (Os02g0834000); for RAC6-Os is RAC6_ORYSJ (Os02g0120800). The bottom panels show tobacco pollen tubes transiently expressing Lyn24–GFP (Lyn24) together with RFP–ROP3 (ROP3), RFP–ROP3–9HV (ROP3–9HV), or RFP–ROP9–3HV (ROP9–3HV).

(D) Association with the IPTPM was conferred by the C-HV of ROP9. Tobacco pollen tubes transiently expressing GFP–ROP9CA (ROP9CA), GFP–ROP3CA (ROP3CA), GFP–ROP9CA–3HV (ROP9CA–3HV), or GFP–ROP3CA–9HV (ROP3CA–9HV) are shown.

(E) Localization of ROP9 at the IPTPM relies on several cysteine residues at the C-terminal HV. Tobacco pollen tubes transiently expressing Lyn24-GFP (Lyn24) together with RFP–ROP9–C196S (C196S), RFP–ROP9–C203S (C203S), or RFP–ROP9–C206S (C206S) are shown.

(F) Localization of ROP9 at the IPTPM was independent of RhoGDI3. Tobacco pollen tubes transiently expressing $CRIB_{RIC1}$–YFP with CFP ($CRIB_{RIC4}$) or with RhoGDI3 (+ RhoGDI3) or expressing YFP–ROP9 with RhoGDI3 (+ RhoGDI3) are shown.

Arrows indicate the IPTPM. Bar = 10 μm for (A–C, E, F) and 50 μm for (D).

the IPTPM, similarly to the localization of ROP3CA, whereas ROP3CA–9HV was detected at the IPTPM, mimicking the localization of ROP9CA (Figure 1D).

Three conserved cysteine residues within ROP9–HV were previously shown to affect the plasma membrane association of type II ROPs (Ivanchenko et al., 2000; Lavy et al., 2002). Because the membrane association of ROP9 depended on a cell-specific mechanism (Lavy et al., 2002), we generated several point-mutated versions of ROP9 to determine which residues within ROP9–HV contributed to its localization to distinct membrane domains in pollen. Compared to wild-type ROP9, whose localization overlapped with Lyn24GFP at the IPTPM (Figure 1B), mutations at any of the three cysteine residues (C196S, C203S, C20S6) completely abolished its localization at the IPTPM (Figure 1E), indicating the essential roles of these cysteine residues in the association of ROP9 at the IPTPM.

The apical plasma membrane localization of type I ROPs in pollen tubes relies on the dynamic activity of RhoGDIs (Klahre et al., 2006; Hwang et al., 2010). To determine whether the localization of ROP9 at the IPTPM was also dynamically regulated by RhoGDIs, we transiently co-expressed RhoGDI3 with $CRIB_{RIC1N58}$ or ROP9 in tobacco pollen tubes. $CRIB_{RIC1N58}$ is the N-terminal 58 amino acids of RIC1, an effector that specifically interacts with a GTP-bound activated form of type I ROPs (Wu et al., 2001). When co-expressed with free CFP, $CRIB_{RIC1N58}$–YFP was localized at the plasma membrane of the apical flank in pollen tubes (Figure 1F), indicating where type I ROP–GTP is located. Co-expressed RhoGDI3 resulted in a substantial translocation of $CRIB_{RIC1N58}$–YFP from the apical flank to the cytoplasm (Figure 1E). Tubes expressing RhoGDI3 were extremely short in comparison to those of wild-type (data not shown), as previously reported for overexpression of Nt-RhoGDI2 (Klahre et al., 2006). By contrast, the localization of ROP9 on the IPTPM was not affected by co-expressed RhoGDI3 (Figure 1F). These results indicated that dynamic regulation of the asymmetric membrane localization of ROP9 was independent of RhoGDIs.

Taken together, we demonstrated that the dynamic localization of ROP9 at the IPTPM relies on its C-terminal HV, specifically three cysteine residues, but is independent of its bound guanine nucleotide or its dissociation inhibitors. It was postulated that ROPs localized at the IPTPM might be involved in driving the unidirectional migration of sperm cells against the bi-directional cytoplasmic streaming in pollen tubes (Lin et al., 1996). We found no defects in the *rop9-1* mutant for pollen tube growth *in vivo* or for male transmission (data not shown). However, the possibility that the IPTPM localization of ROP9 is involved in microtubule-driven sperm migration for competitive advantages under certain circumstances could not be excluded.

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