Rho-of-plant activated root hair formation requires Arabidopsis YIP4a/b gene function

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ABSTRACT

Root hairs are protrusions from root epidermal cells with crucial roles in plant soil interactions. Although much is known about patterning, polarity and tip growth of root hairs, contributions of membrane trafficking to hair initiation remain poorly understood. Here, we demonstrate that the trans-Golgi network-localized YPT-INTERACTING PROTEIN 4a and YPT-INTERACTING PROTEIN 4b (YIP4a/b) contribute to activation and plasma membrane accumulation of Rho-of-plant (ROP) small GTPases during hair initiation, identifying YIP4a/b as central trafficking components in ROP-dependent root hair formation.

KEY WORDS: ROP, YIP, Root hair, Secretion, Trans-Golgi network

INTRODUCTION

Root hair formation in plants underlies strict spatial control and in Arabidopsis thaliana (Arabidopsis) root hairs emerge from the basal (root tip-oriented) ends of hair-forming epidermal cells. Members of the Rho-of-plant (ROP) small GTPase protein family provide early markers for the specific plasma membrane domain marking the incipient site of hair initiation (Molendijk et al., 2001; Jones et al., 2002). The mechanistic framework underlying polar ROP localization has been studied in the context of tip growth (Gu et al., 2003; Hwang et al., 2010; Chang et al., 2013; Huang et al., 2013) and polar positioning of the root hair initiation site (Fischer et al., 2006; Kiefer et al., 2015; Stanislas et al., 2015). Regulation of ROP cycling between a GTP-bound active and a GDP-bound inactive form sequestered in the cytosol represent a key factor.

However, little is known about trafficking to and regulation of ROP accumulation at the root hair initiation site. Here, we report that the trans-Golgi network (TGN)-localized YPT-INTERACTING PROTEIN 4a and YPT-INTERACTING PROTEIN 4b (YIP4a/b) contribute to activation and plasma membrane accumulation of ROPs, identifying YIP4a/b as central trafficking components in ROP-dependent root hair initiation.

RESULTS AND DISCUSSION

We have previously shown that the redundantly acting YIP4a and YIP4b proteins are required for cell elongation and act on secretory trafficking of some proteins and cell wall components via the TGN (Gendre et al., 2011, 2013). Strikingly, our analyses of yip4a yip4b double mutant roots indicated an almost complete absence of root hairs compared with wild type (WT) (Fig. 1A and Fig. S1A,B), with rare or no visible bulges, whereas the single yip4a and yip4b mutants have a similar or slightly higher hair density than wild type, respectively (Fig. S1A,B). Furthermore, expressing YIP4a under its own promoter is sufficient to restore hair formation (Fig. S1A,B). This suggests that both YIP4 proteins are required for hair initiation and act redundantly at an early stage.

In the Arabidopsis root, epidermal cell fate acquisition and subsequent differentiation into hair cells (trichoblast) or non-hair cells (atrichoblast) depends on their position relative to the underlying cortical cells. Mutations that result in a failure to specify trichoblast identity cause the formation of fewer or no hairs and ectopic hair cell specification results in additional hairs. Analyses of the expression pattern of a root hair file-specific marker, the promoter of EXPANSIN7 driving green-fluorescent protein (EXP7::GFP) (Cho and Cosgrove, 2002; Singh et al., 2008) revealed that EXP7::GFP expression starts immediately prior to the formation of the first hair bulges and continues during tip growth, but is absent from non-hair cell files (Cho and Cosgrove, 2002) (Fig. 1B). The pattern of EXP7::GFP expression was not affected by loss of YIP4a and YIP4b function but, unlike in wild type, expression ceased once cells had fully elongated (Fig. S1C). Moreover, the expression of YIP4b driven by the trichoblast-specific COBL9 promoter was observed in trichoblast cell files, as expected (Fig. 1C). These results indicate that it is unlikely that defects in root hair formation in yip4a yip4b are due to a failure of epidermal cell type specification.

Immunostaining employing a YIP4b antibody revealed ubiquitous YIP4b expression in elongating hair and non-hair cells prior to hair formation (Fig. 1C) compared with the absence of signal in the yip4a yip4b double mutant at the same differentiation stage. However, expression of YIP4a or YIP4b from the trichoblast-specific COBL9 promoter in the yip4a yip4b background was sufficient to completely restore root hair development (Fig. 1C and Fig. S1A,B), suggesting that hair cell-specific expression of YIP4 is sufficient for YIP4 function in root hair development.
Following hair cell specification, comes hair initiation marked by bulging at the site of root hair formation, tip-growth and growth cessation (Grierson et al., 2014). As the absence of visible bulges in yip4a yip4b indicated that YIP4s may act at an early stage of root hair development, we investigated the recruitment of ROPs to the basal end of trichoblasts, preceding the formation of the bulge (Molendijk et al., 2001; Jones et al., 2002). We employed an anti-ROP antibody (Kiefer et al., 2015) directed against a conserved epitope in ROP2, ROP4 and ROP6. In cells exiting the meristematic zone, ROPs concentrate into patches at the basal end of the cell before a hair bulge is visible and remain concentrated at the tip of the bulge and in the growing hair (Molendijk et al., 2001; Jones et al., 2002) (Fig. S2A,B). Along the first 900 µm of the root tip, the length chosen to cover hair initiation until the first bulges become visible, yip4a yip4b had 2.9 times fewer ROP patches than wild type (14.3±11.1 and 37.5±5.5, respectively; n=35 seedlings; P<0.001) despite an identical number of cells (Fig. S2C-E). Noticeably, although ROP patches start to be visible at the same distance from the quiescent centre in both yip4a yip4b and wild type (424±71 µm and 403±51 µm, respectively; n=4 biological replicates with seven seedlings each; P=0.5), they disappear after 811±208 µm in yip4a yip4b compared with 1065±161 µm in wild type (beginning of root hair bulging; P=0.1). Furthermore, the ROP patches present in yip4a yip4b were significantly weaker (Fig. 2A,B) and also smaller compared with wild type, as reflected by a reduced patch area and a reduced patch length (Fig. 2C,D). Thus, lack of YIP4 resulted in a reduction in the number of cells displaying ROP patches, a reduction of patch intensity and size, as well as a failure to maintain ROP patches as cells elongate but without affecting the polar basal placement of ROP patches along the trichoblast (P=0.986; Fig. 2A insets; Fig. S2F).

Testing the requirement of ROPs for root hair development has proved difficult, as ROP single and double loss-of-function mutants have no effect on root hair number (Kang et al., 2017). Among the 11 Arabidopsis ROP genes, ROP2, ROP3, ROP4 and ROP5 have been detected in root trichoblasts by transcriptomic analysis (Brady et al., 2007), and ROP2, ROP4 and ROP6 localize at the site of initiation even before bulges occur, when expressed as fluorescent protein fusions under the control of their own promoter (Xu and Scheres, 2005; Stanislas et al., 2015) or when detected by immunolabelling (Molendijk et al., 2001). Moreover, manipulating either their quantity, by overexpression, or the activity of ROP2, ROP4 and ROP6, by blocking them in a constitutively active (CA) or inactive (dominant negative, DN) state, has profound effects on root hair development. For example, both CA-ROP4 and CA-ROP6 expression induces root hair swelling (Molendijk et al., 2001) and DN-ROP2 expression results in fewer and shorter hairs, whereas CA-ROP2 plants produce more and longer hairs than wild type (Jones et al., 2002). To observe the effects of loss of function of ROPs, we examined root hair length and root hair density in rop2, rop4 and rop6 single, double and triple mutants (Ren et al., 2016) (Fig. 3A-C). Root hair length, which provides a measure of ROP action on tip growth, was reduced by 20% in rop2 and rop4i-3 (an RNAi line displaying downregulation of ROP4, Fig. S3A) when compared with wild type. Hair length was reduced by about 40% in the rop2 rop4i-8 line and by about 70% in the rop2 rop4i-4 rop6 triple line (Fig. 3B). Hair density, scored as the number of hairs per mm root, reflects the action of ROP on hair initiation and was decreased by 22% in rop2 compared with wild type (Fig. 3C) but was not enhanced by additional downregulation of ROP4 or ROP6. However, rop2 rop4i-4 rop6 triple mutants displayed a 40% reduction in hair density compared with wild type. Thus, ROP2, ROP4 and ROP6 act redundantly on root hair length and root hair initiation. Interestingly, the redundancy in ROP signalling in root hair formation differs from that in leaf epidermal patterning, in which ROP2/4 and ROP6 act antagonistically (Fu et al., 2005), but is similar to that observed in petal development, in which they also act redundantly (Ren et al., 2016). ROP4 expression varied between rop4i lines (Fig. S3A), rendering a direct comparison difficult. Nevertheless, rop2 mutation had the strongest individual impact on hair density and length (Fig. 3A-C), while the overall strongest phenotype was observed in the rop2 rop4i-4 rop6 triple mutant, strongly suggesting a role for ROP4 in hair initiation and expansion.
The presence of root hairs in *rop2 rop4i-4 rop6* suggests that either additional ROPs are involved in root hair development or that residual ROP4 activity, owing to downregulation by RNAi, may account for the presence of the remaining hairs in the triple mutant. Indeed, size and number of the ROP patches were significantly decreased by 18 and 26%, respectively, in *rop2 rop4i-4 rop6* compared with wild type (Fig. 3D-F and Fig. S3B) but to a lesser extent than what was observed for *yip4a yip4b* (24 and 61%, respectively; Fig. 2C,D). These results indicate that the attenuation of ROP at the plasma membrane can significantly contribute to the lack of hairs in *yip4a yip4b*. Although ROP localization is not completely abolished, the *yip4a yip4b* mutant is almost hairless. Thus, additional YIP4-dependent factors may be required to proceed to tip growth. Alternatively, the quantity of ROP present in the patch is not sufficient or patches are not maintained long enough to trigger downstream processes in root hair development.

We next analysed the underlying cause for reduced ROP at the plasma membrane in *yip4a yip4b*. The amount of total ROP protein in wild-type and *yip4a yip4b* roots was not significantly altered (Fig. S4A,B). Importantly, the plasma membrane levels of EYFP-ROP2 were strongly reduced in *yip4a yip4b* compared with wild type (Fig. S5A-C), as observed for intrinsic ROP. Thus, reduction of ROP proteins at the plasma membrane in *yip4a yip4b* is not due to an overall reduction of ROP expression but is likely related to the secretory trafficking function of YIP4s. Whole-cell FRAP analyses blending the cell of interest and neighbouring cells expressing EYFP-ROP2 revealed that, after 180 min, fluorescence recovered to 26.8±4.9% in wild type compared with 12.7±1.7% in *yip4a yip4b* relative to the respective pre-bleach intensities. This substantial decrease in fluorescence recovery further supported the observation that plasma membrane delivery of newly synthesized ROP2 protein requires *YIP4a/b* function (Fig. 2E and Fig. S5D).

We then investigated whether the decrease of ROP at the plasma membrane could also be due to an attenuation of ROP activation in *yip4a yip4b*. ROP activity is positively regulated by guanine nucleotide exchange factors (ROP GEFs) that promote the exchange of GDP to GTP. Once active ROPs interact with effectors such as ROP-interactive CRIB-containing proteins (RICs), ROP inactivation relies on GTPase activation protein (ROP GAP) and inactivated ROPs are retrieved from the PM and sequestered in the cytosol by a guanine nucleotide dissociation inhibitor (ROP GDI) (Garcia-Mata et al., 2011). Constitutively activated ROP2 localizes to the PM in *Vicia faba* guard cells, whereas the dominant-negative variant is mostly cytoplasmic, indicating that the active form is predominantly membrane bound (Jeon et al., 2008). Moreover, reducing ROP activity by mutating *FERONIA (FER)*, a receptor-like kinase interacting with ROP-GEFs, severely affected the number of hairs produced (Duan et al., 2010; Huang et al., 2013). Pull-down experiments in which only activated ROPs are pulled down, employing their effector RIC1 as a bait, revealed 60% less active ROP2 in *yip4a yip4b* roots compared with the wild type (Fig. 4A; Fig. S6), very similar to the level observed in the *spk1-4* variant (Ren et al., 2016). However, introducing the ROP-GDI mutant *supercentipede1 (scn1-1)* or the constitutively active *CA-ROP2* form into the *yip4a yip4b* background did not restore hair growth. Instead, both the multiple bulge phenotype of *scn1-1* (Carol et al., 2005), as well as the multiple and ectopic hair phenotype of *CA-ROP2*, were suppressed in the *yip4a yip4b* background (Fig. 4B). Thus, genetic approaches that would enhance ROP levels by maintaining ROP at the plasma membrane via blocking its
transfer to the cytosol (scn1-1) or by increasing the active pool of ROP (CA-ROP2) did not restore hair initiation. This, combined with a reduced level of active ROP in the yip4a yip4b mutant, strongly suggests that YIP4 function is required for ROP activity and ROP localization to the plasma membrane.

YIP4a and YIP4b localize to the TGN and mediate secretory trafficking from the TGN in Arabidopsis roots. Thus, the hairless phenotype of the yip4a yip4b mutant suggests that YIP4-mediated secretory trafficking plays an important role at an early stage of hair formation. Interestingly, proteomic analysis of a hairless barley mutant revealed that the SECRETION-ASSOCIATED AND RAS-RELATED PROTEIN 1A (SAR1A) GTP-binding protein and a vacuolar ATP synthase subunit B (V-ATPase) are crucial for hair initiation (Janiak et al., 2012). Homologs of these two proteins in Arabidopsis are involved in secretory trafficking and YIP4 colocalizes with V-ATPase at the TGN, supporting a role for TGN-mediated

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**Fig. 3.** ROP2, ROP4 and ROP6 are required for root hair formation and elongation. (A) Representative images of roots of wild type Col-0 (WT) and the following ROP mutants: rop2, rop4i-3, rop6, rop2 rop4i-8, rop2 rop6, rop4i-5 rop6 and rop2 rop4i-4 rop6. Scale bar: 1 mm. (B) Measure of root hair length (in mm) in all genotypes mentioned above. More than 150 root hairs from at least ten growing roots for each genotype and each replicate (three replicates) were measured (n=450). (C) Hair density measurement (number of hairs per mm) for all genotypes mentioned above, n=30 roots per genotype (10 for each biological replicate). Data are means±d. analysed using Student’s t-Test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (D-F) Measure of the area (D), length (E) and number of existing patches (F) on a 480 µm region of wild-type and rop2 rop4 rop6 roots, just after the meristem. Data are average±s.d. (n=30 roots for each genotype) analysed using Kolmogorov–Smirnov test (**P<0.01, *P<0.05). See Fig. S3B for representative images.

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**Fig. 4.** YIP4a and YIP4b contribute to ROP activation in vitro and to ROP activation of root hair development. (A) Total ROP2 expression in roots of 5-day-old seedlings of wild type, yip4a yip4b and spk1-4 (‘active’) compared with the active population of ROP pulled down by the effector RIC1 (‘total’). Five independent biological experiments were performed and quantifications are given in the graph below (n=5, data are average±s.d., ***P<0.001; **P<0.01, Student’s t-test, two-tailed). Examples of full blots can be seen in Fig. S6. (B) Root hair phenotype in 5-day-old seedlings of Col-0 (WT), yip4a yip4b, 35S:CA-ROP2 and scn1-1 mutants, and yip4a yip4b CA-ROP2 and yip4a yip4b scn1-1 triple mutants. Scale bar: 500 µm.
secretory trafficking in early hair development. Therefore, a reduced plasma membrane level of ROP or of its activator could be causing the root hair defects observed in yip4a yip4b.

To date, the mechanisms and factors underlying ROP localization at the plasma membrane remain unclear. For example, trafficking of ROP proteins from their site of synthesis on free polysomes in the cytosol to the plasma membrane was originally thought to be a direct process requiring post-translational lipid modification for membrane anchoring. The discovery that posttransylation modifying enzymes are intrinsic membrane proteins that are restricted to the endoplasmic reticulum (ER) called this view into question (Wright and Philips, 2006; Bracha-Drori et al., 2008). For animals, it has been proposed that Rho-type GTPases could reach the plasma membrane by two different means: at a slow rate through the vesicle trafficking machinery at the exit of the ER; and at a faster rate by a direct capture at the ER via GDI interaction (Garcia-Mata et al., 2011). In line with this, we detected ROP2/4/6 in SYP61-labelled secretory vesicles both by immunoprecipitation and by label-free quantitative proteomics (Fig. S7A-C). The low abundance of ROP2/4/6 in SYP61-labelled secretory vesicles when compared with YIP4a and YIP4b is consistent with the transient nature of ROP delivery through this compartment and the relatively small amount delivered to the plasma membrane. In contrast, YIP4s are resident proteins of the SYP61 compartment accumulating there at steady state. Our biochemical data together with the failure of scn1-1 mutations or overexpression of ROP2-CA to increase root hair formation in the yip4a yip4b mutant strongly suggest a role for post-Golgi, TGN-mediated trafficking in ROP localization. Consistent with this hypothesis, interference with TGN function by locking the ARF1 GTPase either in the GTP- or in the GDP-bound form reduced fluorescence of EYFP-ROP2 at the plasma membrane (Xu and Scheres, 2005). The recent discovery that RAC5/ROP meets its effector at the TGN before being potentially exocytosed to the plasma membrane (Stephan et al., 2014) further supports the view that ROPs enter post-Golgi, TGN-mediated trafficking in ROP localization.

Plasmid construction and plant transformation

1.3 kb of the COBL9 promoter were amplified, as well as the YIP4a and YIP4b ORF using the following primers: COBL9Pro-F-BamHI, 5′-atgattcc ceacaataatgaggctctcgagatctact-3′; COBL9pro-R-BamHI, 5′-tacctggttaggtc ttcctcaagggaggaattgg-3′; YIP4a-ATG-SpeI, 5′-atactagcttgccgcctagagatg-3′; YIP4a-ATG-norf, 5′-atacggttcggcttaggtcctagagatg-3′; YIP4b-ATG-SpeI, 5′-atactagcttgccgcctagagatg-3′; and yip4b-stop-norf, ataggccgcatgctctgctgccagaagatg-3′. The fragments were subsequently cloned into pGreen II 0229 (Basta resistance) by employing the following restriction sites: BamHI and SpeI-norf, respectively. The Agrobacterium tumefaciens strain C58C1 was transformed with pGreen COBL9::YIP4a or COBL9::YIP4b and used to transform the yip4a yip4b double mutant using the floral dip method (Clough and Bent, 1998). The construct and plant transformation to get yip4a yip4b expressing YIP4a::HA-YIP4a have been described previously (Gendre et al., 2013).

Confocal laser-scanning microscopy and immunolabelling

Fluorescence signals were viewed using a Zeiss LSM 780 confocal laser-scanning system mounted on a Zeiss Axio Observer Z1 inverted microscope, employing a water-corrected C-Apochromat 40× objective, numerical aperture 1.2 (Zeiss). GFP was detected using a 488 nm laser and 493-598 nm emission filter. Arabidopsis root whole-mount immunolabelling employed the protocol previously described (Gendre et al., 2011) with driselase treatment extended to 40 min for enhanced cell wall digestion in elongated root epidermal cells during ROP immunolocalization using rabbit anti-ROP at 1:250 (Kiefer et al., 2015), rabbit anti-YIP4b at 1:150 (Gendre et al., 2013) and anti-rabbit-DY633 at 1:400 (Agrisera). DY633 was excited using a 633 nm laser and detected using a 638-759 nm emission filter.
membrane intensity was calculated as a percentage of the mean intensity measured per individual cell relative to its pre-bleach intensity. A total of 20 cells from five individual plants (n=5) per genotype were quantified. The quantified cells had at least two fully bleached cells on either side to rule out recovery from lateral diffusion, etc. in neighbouring cells. Additionally, plasma membrane intensity of two non-bleached adjacent cells per root was determined and used to correct for loss of fluorescence caused by laser excitation during post-bleach image acquisition. Post-bleach intensity values were adjusted to set the intensity immediately after bleaching to zero.

Pull-down assays
The ROP pull-down assays were performed as previously described (Ren et al., 2016). For quantification, data were analysed from five biological replicates using ImageJ software with the function of gel intensity analysis. The data are presented as the mean±s.d. (n=5) of the relative active ROP2 to the total amount of ROP. Statistical analyses were carried out using Student’s t-test.

Scoring of ROP patches
After immunolabelling, ROP patches along the first 900 µm region from the quiescent centre (QC) of the trichoblast length. Measurements were performed on 90 cells (n=90) pooled from the total root patch. The average of all patches were measured with a Zeiss LSM780 using the position setup within the Zen software. The number of ROP patches was manually counted within the 900 µm distance throughout the depth of the entire root tip. As the data were not normally distributed, the significance of differences between distributions was tested using the non-parametric, two-sample Kolmogorov–Smirnov (KS)-test (www.physics.csbsju.edu/stats/KS-test.n.plot_form.html) on 35 seedlings originating from five biological replicates (seven roots per replicate). Positions of the ROP patches between the closest to and the most distant from the QC followed a normal distribution. Thus, statistical analysis was performed using a Student’s t-test (two-tailed; sample with equal variance) on the average of the five biological replicates (n=5).

Image analysis of ROP patches
ImageJ (image.nih.gov/ij/) was used to characterize the shape of each patch (area and length) and its associated fluorescence intensity. Maximal projections of confocal sections (z=35) from two consecutive tiles of 212 µm were each processed equally using a median filter after 8-bit transformation. Particles were isolated using a fluorescence intensity-based threshold and a size exclusion filter to remove all particles that were not considered as a patch but as intracellular punctae or noise. For each patch, surface area and average fluorescence intensity were measured and their maximal length was characterized using the Feret’s diameter. The average and s.d. were calculated based on the pooled samples obtained from three independent replicates with 10 roots each (n=30). All ROP patches present in the root part analysed were measured and the average of all patches per root represents one sample (n). Significance was tested using the non-parametric, two-sample Kolmogorov–Smirnov (KS)-test (see above).

Quantification of polar ROP patch position
Quantification of polar ROP patch position was performed as previously described (Kiefer et al., 2015). In brief, the distance between the basal end of the trichoblast and the basal end of the ROP patch was divided by the total trichoblast length. Measurements were performed on 90 cells (n=90) pooled and obtained from three experiments, each employing 10 roots from which three cells with clearly distinguishable cell boundaries and ROP patches were measured. Significance of differences between distributions was tested using a non-parametric, two-sample Kolmogorov–Smirnov (KS)-test (P<0.05).

Western blot analyses
Western blots from gels loaded with 40 µg total protein extract per lane quantified by Bradford assay from roots of 7-day-old seedlings were washed and detected with mouse monoclonal anti-r-tubulin antibody clone B5-1-2 (Sigma-Aldrich, T5168) at 1:3500 dilution and horseradish peroxidase-coupled AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Europe, 115-035-003) at 1:10,000 dilution. Enhanced chemiluminescence (ECL) prime western blotting detection reagents (Amersham) were used according to the manufacturer’s instructions. Details on blot quantification can be found in the supplementary Materials and Methods.

Quantitative PCR
Details on DNase-treated total RNA extraction with an Omega Total RNA kit from five-day-old seedlings from three independent experiments, subsequent cDNA synthesis and quantitative PCR performed with a TaKaRa SYBR kit, as well as primer sequence information, can be found in the supplementary Materials and Methods.

ROP detection in SY61-positive immuno-purified vesicles
The immuno-purification procedure for SY61-positive vesicles, label-free proteomics and LC-MS/MS detection of ROP peptides are described in the supplementary Materials and Methods.

Accession numbers
Sequence data used in this research can be found in the TAIR database under the following accession numbers: YIP4a, AT2G18840; YIP4b, AT4G30260; ROP2, AT1G20090; ROP4, AT1G75840; ROP6, AT4G35020.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.G., M.G.; Software: T.V.; Validation: D.G., D.L.; Formal analysis: D.G., S.C., A.G.; Investigation: D.G., A.B., X.D., N.E., Y.B.; Resources: T.S., D.L., M.G.; Data curation: A.B.; Writing - original draft: D.G., M.G., R.P.B.; Writing - review & editing: D.G., A.B., X.D., Y.B.; Supervision: M.G., R.P.B.; Project administration: D.G.; Funding acquisition: M.G., R.P.B.

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Data availability
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Supplementary information
Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.168559.supplemental

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Rho-of-plant-activated root hair formation requires Arabidopsis YIP4a/b gene function

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Supplementary Materials and Methods

Western blot quantification. Western blots from gels loaded with 40 µg total protein extract per lane quantified by Bradford assay from roots of 7-day-old seedlings were incubated with rabbit anti-ROP (Stanislas et al., 2015) at 1:200 dilution followed by anti-rabbit HRP (GE healthcare) at 1:10000 dilution. The blot was rinsed and then detected with mouse anti-tubulin antibody (Sigma) at 1:3500 dilution and goat anti-mouse (Jackson Immunoresearch) at 1:10000 dilution. Enhanced chemiluminescence (ECL) prime western blotting detection reagents (Amersham) was used according to the manufacturer's instruction. Images of the blots were acquired using a LAS-3000 imaging system (Fujifilm) and bands were quantified with ImageJ according to the following recommendations http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. The quantification is based on the average ± s.d. of n=6 biological replicates. Statistical difference was assessed with a Student’s t-test (two-tailed distribution, two samples of equal variance).

ROP4 Quantitative PCR. DNAse-treated total RNA was extracted according to the manufacturer’s instruction (OMEGA Total RNA kit) from five-day-old seedlings grown on 1/2x MS agar medium. RNA integrity was verified on an agarose gel and by the RNA 260 nm/280 nm OD ratio, while presence of residual gDNA was verified by PCR. The experiment was repeated three times. Quantitative PCR performed with TaKaRa SYBR kit was done on subsequent cDNA using the following primers for ROP4 and ACTIN2 (AT3G18780): ROP4-QRT-F 5’-ACCATCCTGGTCAGTGCTAT-3’, ROP4-QRT-R 5’-ACACGCAGCGTTCTTCTTCTT-3’, Actin2-QRT-F 5’-TCCCTCAGCACATTCCAGAGAT-3’, Actin2-QRT-R 5’-AAGATTCTGACCTGCTCATC-3’. Relative expression of ROP4 to the ACT2 level was set as 1. A Student t-test was performed (n=3) to assess statistical differences with ***P<0.001 and **P<0.01.

The semi-quantitative PCR was realized once on one of the biological replicates described above. The primers were as follows: ROP4-RT-F 5’-TGAGTGCTTCCAGGGTATA-3’, ROP4-RT-R 5’-CAAGAACACGAGCGTTCTTCTTCTT-3’, Actin2-RT-F 5’-CCGCTATGTATGTCGCCATCAGAGAT-3’, Actin2-RT-R 5’-CCAGCAGCTTCCATCCCACAAA-3’.
Detection of ROP in SYP61-positive vesicles. SYP61 vesicles were isolated employing the immuno-purification procedure described previously (Wattelet-Boyer et al., 2016). In brief, *Arabidopsis thaliana* seedlings were grown in liquid culture for eight days and then ground in vesicle extraction buffer (HEPES 50 mM pH 7.5, 0.45 M sucrose, 5 mM MgCl₂, 1 mM DTT, 0.5% (w/v) PVP (Sigma), 1 mM PMSF). Intact pools of light vesicles were collected at the 33/8% interface of a 38/33/8% sucrose step gradient after overnight centrifugation at 150 000 g at 4°C. This membrane fraction was then resuspended in the resuspension buffer (50 mM HEPES pH 7.4, 0.25 M sucrose, 1.5 mM MgCl₂, 150 mM NaCl, 1 mM PMSF, protease inhibitor cocktail) and the total membrane fraction was used as input for immuno-precipitation (IP). IP was performed with magnetic Dynabeads coupled to protein A (Invitrogen) according to the manufacturer’s instructions. For each IP, 75 µl of beads were incubated with 7 µl of rabbit anti-GFP antibodies (Invitrogen) for 1 h with shaking at 4°C, washed with PBS-Tween (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.02 % Tween-20), equilibrated in the resuspension buffer for 10 min on ice and incubated with 1 ml of purified total membrane extract for 1 h with shaking at 4°C. Several washes were performed with 1 ml of resuspension buffer for 5 min with shaking at 4°C. Step gradient-purified total membrane fractions (IP input) and bead-immuno-purified fractions (IP output) were loaded at equal quantity on an SDS-PAGE gel and subjected to western-blotting. Purified rabbit anti-ROP antibody at 1:200 dilution was used to detect ROPs as described, previously (Kiefer et al., 2015). Label-free proteomics was used to identify ROP2, ROP4 and ROP6 protein peptides in the SYP61 IP fraction. In brief, IP samples were treated with 25 µl 1% (w/v) SDS for 30 min at 37°C, 0.3 µl DTT 2 M was then added with subsequent incubation for 30 min at 37°C, 2.3 µl iodoacetamide 1 M was added followed by 30 min incubation at 37°C and finally 7 µl 5x Laemmli loading buffer was added followed by incubation for 30 min at 37°C. Samples were loaded and subjected to SDS-PAGE electrophoresis, extracted from the gel and injected into an LC-MS/MS Q-Exactive with a gradient time of 120 min. Label-free quantitative data analysis was performed on raw LC-MS/MS data imported in Progenesis QI for Proteomics 2.0 (Nonlinear Dynamics Ltd). Data was processed by volume integration for 2-6 charge-state ions and calculation of protein abundance (sum of the volume of corresponding peptides). Quantitative data was considered for proteins quantified by a minimum of two peptides.
Fig. S1. Complementation of root hair density in yip4a yip4b expressing YIP4a and ceasing of EXP7 promoter activity in differentiated yip4a yip4 root hair cells.

(A-B) Representative images (A) and root hair density measurement (B) in WT, yip4a yip4b, yip4a and yip4b single mutants as well as yip4a yip4b double mutant complemented with YIP4a::HA-YIP4a and COBL9::YIP4a. Hairs were counted on a portion of 5 mm (from 5 to 10 mm away from the tip) on 20 plants per genotype (average ± s.d., n=20). Asterisks indicate statistical differences (**P<0.05, ***P<0.001 with Student t-test). (C) Representative images of WT and yip4a yip4b roots expressing EXP7::GFP (green). Propidium iodide counterstaining of cells walls (magenta).
Fig. S2. Number of ROP patches in the wild type and in the yip4a yip4b mutant.

(A) Representative images of Col-0 (WT) and yip4a yip4b roots immunostained with anti-ROP antibody (red) and nuclei stained with DAPI (blue). Below the overlay of the anti-ROP and DAPI images, the inverted ROP greyscale signal corresponding to the image above with the patches marked with an
asterisk and the ROP signal at the tip of growing root hair is indicated with an arrow. (B) Close-up images with the transmission image merged to better visualize ROP patch positioning with the root tip pointing to the bottom. Arrows indicate basal ends of cells. (C) Scheme representing a typical WT root starting from the quiescent centre, indicating the region where the number of ROP (d) and the number of cells (e) were counted. (D) Average ± s.d. of the number of ROP patches counted on the first 900 μm of the root starting from the quiescent centre. n=35 seedlings (7 per biological replicate, 5 replicates). Kolmogorov-Smirnov test with *** P<0.001. (E) Number of post-mitotic cells in WT and yip4a yip4b along the first 900 μm of the root (n=15 roots, average ± s.d.). (F) Relative ROP patch positioning in WT and yip4a yip4b roots (n=90 cells; 3 cells per root, 10 roots in each of the three replicates). Non-significative difference P=0.986 with Kolmogorov-Smirnov test.)
Fig. S3. ROP4 downregulation in rop4i-3, rop2 rop4i-8 and rop4i-5 rop6 and rop2 rop4i-4 rop6. (A) Quantitative real-time PCR analysis of the expression level of ROP4 in Col-0 (WT) and the above-mentioned mutants. The expression level of ACTIN2 is used as reference (n=3 biological replicates, ***P<0.001 with Student t-test). In inset, is a semi-quantitative PCR done on one of the biological replicates as visual reference. (B) Representative images of maximum projections (flattened image of the whole root depth) of WT and rop2 rop4 rop6 roots immunolabeled with anti-ROP antibody. The region spanning 480 µm from the beginning of the elongation zone (prior to root hair emergence). Arrows indicate the direction towards the root tip. Scale bar, 100 µm.
Fig. S4. The total amount of ROP remains unaffected in yip4a yip4b. (A) Representative Western blot showing anti-ROP and anti-tubulin (TUB) signals from roots of 7-day-old wild type (WT) and yip4a yip4b seedlings. (B) Quantification was performed with Image J based on 6 biological replicates (n=6, average ± s.d.) is displayed. A Student’s t-test, two-tailed with equal variance, indicated no statistical difference (P=0.3).
**Fig. S5.** *yip4a yip4b* mutants display less EYFP-ROP2 at the plasma membrane than wild type. (A) Representative image of *ROP2:EYFP-ROP2* line expressing EYFP-ROP2 in WT or in *yip4a yip4b*. Scale bar, 100 µm. (B) Fluorescence intensity quantification of EYFP-ROP2 in *ROP2:EYFP-ROP2* expressing lines at transversal plasma membrane around 400 µm from the root tip. n = 18 roots, 6 roots per replicate in 3 biological replicates. ***P<0.001 calculated with Mann-Whitney U-test as the data were not normally distributed. (C) Distinction between cytosolic (cytosolic YFP) and EYFP-ROP2 PM signal in the cell-type used for quantification in (B), indicating that it is mostly PM signal that is quantified. (D) Representative images of a 10-11 cells long strip in the transition zone of WT and *yip4a yip4b* expressing EYFP-ROP2 before and after bleaching (the entire width of the root was bleached with 10-15 iterations at full power of the 458, 488 and 514 nm laser lines). Fluorescence recovery after 30, 60, 90, 120, 150 and 180 min is monitored. Note that the cells are growing during the course of the experiment, indicating that they are still alive and functional.
**Fig. S6. Representative Western blots of the activated ROP2 pull down.**

From left to right: Coomassie staining (arrowhead indicates Rubisco protein, 53 kDa), Western blot with ROP antibody on total protein extract (arrowhead indicates the band chosen for quantification in Fig. 4A), Western blot with ROP antibody on the RIC1-pulled fraction (arrowhead indicates the band chosen for quantification in Fig. 4A).

For ROP2 activity assays in WT, *yip4a yip4b* and *spk1-4*, total proteins were extracted from five-day-old seedlings grown on 1/2x MS agar medium. Twenty micrograms of MBP-RIC1-conjugated agarose beads were added to the protein extracts and incubated at 4°C for 2 h on a rocking table. The beads were washed four times in wash buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, and 0.5% (v/v) Triton X-100) at 4°C. GTP-bound ROP proteins that were associated with the MBP-RIC1 beads were boiled and used for analysis by western blotting with a ROP2-specific antibody. ROP2 polyclonal antibodies was generated against the peptide QFFIDHPGAVPTTNQG (Abicode). Prior to the pull-down assay, a fraction of total proteins was analyzed by immunoblot assay to determine total ROP2 (GDP bound and GTP bound, blot on the left). The amount of the GTP-bound active form of ROP2 (blot on the right) was normalized to that of total ROP2.
Fig. S7. ROP and YIP4 proteins are found in SYP61-positive vesicles.

(A) Stain-free loading control (Biorad) of SYP61 secretory vesicle (SVs) immunoprecipitation (IP) input from a sucrose-gradient with total membrane fraction and IP output from the bead fraction displaying equal loading. (B) Western blot of same loading as in (A) using first the goat anti-rabbit HRP coupled secondary antibody, only, without any primary antibody (blot on the left). On this blot we could clearly see the GFP antibodies around 50 kDa but could not detect any band around 20 kDa. The same membrane was then incubated with anti-ROP antibodies followed by incubation with the secondary antibody, which revealed a band around 21 kDa in the SYP61 IP fraction. Moreover, blotting anti-ECH antibody on another membrane (membrane 2) loaded exactly the same way as membrane 1 revealed a very strong enrichment of the targeted compartment SYP61 where ECH resides. (C) Label-free proteomics revealed the presence of ROP2, ROP4 and ROP6 common peptides (3 common peptides detected, hence it is impossible to differentiate the different ROPs), YIP4a and YIP4b (10 peptides detected), ECHIDNA (8 peptides detected) but not PM-H+-ATPase (AT2G24520) a marker for the plasma membrane, indicating no contamination from the PM fraction-in the SVs.
Fig.S8. Schematic model of the putative role of the secretory pathway in the plasma membrane-localization of type-I ROPs.

(A) Wild-type roots displaying a functional secretory system contain numerous ROP patches (red) at incipient hair initiation sites which persist at the tip of the growing hair. (B) When YIP4s (and their complex partner ECHIDNA) are lacking, the secretion of some cargos en route to the PM is impaired (Bouitte et al., 2013; Gendre et al., 2013), likely due to a defect in secretory vesicle formation (Bouitte et al., 2013). Mutants then lack hairs and show a drastic reduction in number and intensity of ROP patches. (C) Type-I ROP (ROP1-8) proteins synthesized at free polysomes in the cytosol require prenylation and the posttranslational modification of the C-termini at the surface of the endoplasmic reticulum (ER) for their membrane anchoring (Wright and Philips, 2006; Bracha-Drori et al., 2008; Feiguelman et al., 2018). Furthermore, the presence of ROP-GEF SPK1 both at the ER exit site (Zhang et al., 2010) and on punctae associated with the PM (Yanagisawa et al., 2018) reinforces the importance of the ER. Studies in yeast and mammals suggest that that Rho/Rac/Cdc42 could exit directly from the ER via a complex with RhoGDI after having been extracted from the organelle membrane (route 2, fast) or via vesicles (route 1, slow)(Slaughter et al., 2009; Garcia-Mata et al., 2011; Watson et al., 2014). By analogy, ROP may reach the PM by two different means in plants. ROPs are found in SYP61-vesicles, EYFP-ROP2 recovery after photobleaching is impaired at the plasma membrane and intensity of ROP patches is decreased in the secretion-defective yip4a yip4b mutant, suggesting that a part of ROP is taking the secretory pathway on its way to the PM. It remains open whether secretion of other factors crucial to
activate ROP at the PM, like ROP-GEF, are also impaired in yip4a yip4b resulting in the decrease in ROP patches. In addition, tight regulation of the ROP activation/inactivation cycle keeps ROP polarized. Data from yeast indicates that the initial polar aggregation of Cdc42 seems highly dependent on the GTPase cycles and the cytosolic pool of GTPase and only partially dependent of secretion via actin filaments (Wedlich-Soldner et al., 2004). Hence, a contribution by both the secretory pathway and the cytosolic ROP pool is feasible.

Supplemental references

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