Arabidopsis cyclophilins direct plasmodesmata-targeting of mobile mRNA via organelle hitchhiking

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Abstract

Plants selectively transport mobile mRNAs through intercellular pores, plasmodesmata (PD), to distribute spatial information for synchronizing meristematic differentiation with environmental dynamics. However, how plants recognize and deliver mobile mRNAs to PD remains unknown. Here, by using RNA-live cell imaging, we show that mobile mRNAs hitchhike on organelle trafficking to transport to PD. Perturbed cytoskeleton organization or organelle trafficking severely disrupts the subcellular distribution of mobile mRNAs. We further show that Arabidopsis rotamase cyclophilins (ROCs), which are organelle-localized RNA-binding proteins (RBPs), specifically bind mobile mRNAs on the surface of organelles to direct PD-targeting. Arabidopsis *roc* quadruple mutants showed reduced in PD-targeting of mobile mRNAs, along with phenotype alterations. ROCs can move intercellularly and form RNA-protein complexes in phloem, suggesting the roles of ROCs in delivery of mobile mRNAs through PD. Our results highlight that an RBP-mediated hitchhiking system is purposely recruited to orient plant-mobile mRNAs to PD for intercellular transport.

Introduction

Intercellular and inter-organ communication play a pivotal role for multicellular organisms to synchronize and coordinate diverse developmental and physiological programs. In addition to serving as templates for protein translation, many mRNAs can move intercellularly and function systemically to transmit and incorporate environmental cues into developmental programs. The use of mRNAs as information molecules provides an efficient remote-control system for cells to record and distribute environmental changes. The mobile mRNAs are usually transcribed in local tissues that perceive external stimuli, then undergo cell-to-cell or long-distance delivery through phloem. Upon being translocated to the destiny, the translocated mRNAs can exert their function to regulate growth and development. The functional importance of mobile mRNAs in regulating development has been revealed in many plant species, including the regulation of Arabidopsis floral initiation ^{1,2}, potato tuber formation ³, and tomato leaf development ^{4,5}. Mobile mRNAs can also move between plant species. Hetero-grafting with two plant species showed that mobile mRNAs can move between grafted plants or host-parasitic plants ⁶⁻¹⁰, which suggests that the machineries required for mRNA movement may be conserved in these plants.

Use of grafting experiments and biochemical analyses of phloem exudates, the RNA mobile motifs and protein components involved in long-distance movement and phloem translocation of mobile mRNAs have been revealed ¹¹⁻¹⁴. However, successful delivery of mobile mRNAs to remote tissues requires sequential intra- and intercellular translocation steps, including intracellular transport of mobile mRNAs to plasmodesmata (PD), cell-to-cell movement through PD, loading into vasculature for long-distance trafficking along phloem, and unloading to recipient tissues. Currently, our understanding of intracellular transport of mobile mRNAs to PD remains limited. We previously showed that the mobile but not non-mobile mRNAs are selectively transported to PD to initiate cell-to-cell movement, which suggests that intracellular PD-targeting is a critical step to determine the movement of mobile mRNAs ¹⁵. However, the

mechanisms involved in active delivery of mobile mRNAs and RNA-binding proteins (RBPs) responsible for bridging mobile mRNAs and trafficking machineries remained unidentified.

In this study, we addressed how endomembrane trafficking conveys PD-targeting of mobile mRNAs. Our mRNA live-cell imaging analysis showed that mobile mRNAs attach on organelles to deliver to PD. We identified Arabidopsis rotamase cyclophilins (ROCs) and showed that ROCs specifically bound mobile mRNAs on the surface of organelles to achieve PD-targeting. Arabidopsis ROCs belongs to a large gene family of cyclophilins, which are ubiquitous expressed proteins that possess peptidyl-prolyl *cis-trans* isomerase activity with some members localize to secretory vesicles^{16,17}. In addition, certain cyclophilins can bind viral RNA *in planta*^{18,19}, which suggests a potential role of cyclophilins in RNA metabolism. Of note, cyclophilins have been identified in phloem exudates of *Brassica napus*²⁰. However, whether cyclophilins are non-cell-autonomous RBPs that shuttle plant endogenous mobile mRNAs into phloem for long-distance transport remains to be investigated. Here, we showed that Arabidopsis ROCs specifically bound to mobile but not non-mobile mRNAs. In addition, live-cell imaging analysis showed that ROCs co-localized with mRNAs and organelle markers, suggesting that ROCs bind mobile mRNAs on organelles to direct PD-targeting of mobile mRNAs. Our results support a model of the ROC-mediated hitchhiking strategy recruited to deliver mobile mRNAs to PD for intercellular transport.

Results

Distinct modes of actin- and microtubule-mediated mobile mRNA trafficking

Cytoskeletons constitute a framework supporting dynamic transport of organelles and cargoes in eukaryotic cells²¹. To examine how the cytoskeleton directs intracellular transport of mobile mRNAs to PD, we exploited an MS2-based mRNA live-cell imaging system¹⁵ (Supplementary Fig. 1a) and probed the dependence of mobile *FLOWERING LOCUS T (FT)* mRNA transport on cytoskeletons. We first examined the association of mobile *FT* mRNA and actin during *FT* mRNA transport by transiently expressing the MS2 mRNA live-imaging system with fluorescence-conjugated Lifeact, a 17-amino-acid peptide that binds to actin filaments²². In cells expressing Lifeact-mCherry, we detected co-localization of *FT* mRNA with Lifeact-labeled actin filaments while the microfilaments extended from the nucleus to cell periphery (Fig. 1a). Time-lapse images showed *FT* mRNA transported along Lifeact-labeled actin filaments on transvacuolar strands (Supplementary Video 1), which supports the involvement of actin in transport of mobile mRNA. To examine the requirement of actin microfilaments in *FT* mRNA transport, we applied the actin polymerization inhibitor latrunculin B (LatB) to *N. benthamiana* leaves expressing the mRNA live-imaging system. After LatB application, Golgi transport showed reduced motility and resided at the cell periphery (Fig. 1b), which indicates interference with actin-driven intracellular transport. Meanwhile, the transport of *FT* mRNA foci was arrested in the cytoplasmic space and cell periphery (Fig. 1b and Supplementary Video 2), which supports the requirement of actin in *FT* mRNA movement.

Microtubule-dependent transport of viral mRNAs to PD is crucial for successful virus spreading²³. To probe the requirement of microtubules for PD-targeting of mobile mRNA, we treated cells expressing the mRNA live-image system with the microtubule depolymerizer oryzalin. Instead of abolishing intracellular transport in transvacuolar strands seen in the actin depolymerization experiment, disruption of microtubules eliminated PD localization of *FT* mRNA, which resulted in the homogenized distribution of *FT* mRNA in cytosol (Fig. 1c). In contrast, long-range trafficking of mobile mRNAs to the cell periphery remained robust (Supplementary Video 3), suggesting that microtubules are critical for guidance and docking of *FT* mRNA to PD. Our results suggest that the long-range intracellular distribution of mobile mRNAs is driven by actin-based cytoplasmic stream, whereas PD-targeting of mobile mRNAs at the cell cortex is microtubule-dependent.

Mobile *FT* mRNA associated with RAB5-containing compartments for PD-targeting

Our mRNA live-cell imaging revealed that although intracellular transport of mobile mRNAs and Golgi followed the same stream while traveling along transvacuolar strands, mobile mRNAs did not associate with Golgi for intracellular transport¹⁵ (Supplementary Fig. 1b). To further explore the underlying mechanisms, we treated cells expressing the MS2 mRNA live-imaging system and fluorescence-conjugated organelle markers with different inhibitors blocking endomembrane trafficking. When we applied brefeldin A (BFA), an inhibitor that interferes with endoplasmic reticulum (ER)-Golgi protein delivery and induces relocation of Golgi markers to ER²⁴, to cells expressing the mRNA live-cell imaging system and mCherry-labeled Golgi marker MAN49-mCherry, transport of MAN49-mCherry from ER to Golgi was blocked and resulted in retention of red fluorescent foci in ER (Supplementary Fig. 1c). On the contrary, *FT* mRNA continued to display punctate foci on the cell periphery in BFA- or DMSO-treated control cells (Supplementary Fig. 1d), which suggests that PD-targeting of mobile *FT* mRNAs is BFA-insensitive.

In root cells, treatment with concanamycin A (CMA) blocks H⁺-ATPase activity on the membrane, which inhibits vesicular acidification and induces endosomal aggregation, whereas wortmannin (Wort) interferes with phosphoinositide 3-kinase (PI3K) activity and blocks the formation of phosphatidylinositol 3-phosphate (PI3P) on the vesicular surface²⁵. CMA treatment of cells co-expressing MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-labeled RAB5 (multivesicular body [MVB] marker) increased the volume of MVBs (Fig. 2a, 2b and Supplementary Video 4). Although intracellular transport of *FT* mRNA was severely disrupted, MS2_{FD}-GFP-labeled *FT* mRNA remained attached on enlarged RAB5-positive compartments (Fig. 2b and Supplementary Video 4). With CMA treatment, *FT* mRNA and RAB5-positive compartments remained associated in cytoplasm but failed to target to PD (Fig. 2b and Supplementary Video 4). The attachment of mobile mRNAs with RAB5-positive compartments also occurred with another mobile mRNA, *AGL24* (Supplementary Fig. 2), suggesting that the endomembrane trafficking system conveys PD

transport of mobile mRNAs. Similar to CMA, Wort treatment increased the volume and cytoplasmic abundance of RAB5-positive compartments (Fig. 2c). Notably, intracellular transport of *FT* mRNA was differentially affected by CMA and Wort as compared with the DMSO-treated control: with CMA treatment, the association of *FT* mRNA and RAB5-positive compartments remained detectable in cytoplasm (Fig. 2b); however, Wort treatment interfered with the interaction between *FT* mRNA and RAB5-positive compartments, thus resulting in a homogenous distribution of *FT* mRNA at the cell periphery (Fig. 2c).

To verify the association of *FT* mRNA with RAB5-positive compartments, we used RNA-immunoprecipitation (RIP). We isolated RAB5-positive compartments with immunoprecipitation followed by RT-PCR to examine whether *FT* mRNA remained associated with RAB5 compartments (Fig. 2d). For that purpose, AtUBQ10-mCherry-RAB5, 35S-*FT*_{SL24} or 35S-*RFP*_{SL24} was agro-infiltrated in different combinations in *N. benthamiana* leaves. Extracts from agro-infiltrated leaves were used for immunoprecipitation with anti-mCherry antibodies to pull down mCherry-RAB5-positive compartments. In controls, RT-PCR analysis of total extracts detected *FT* or *RFP* mRNA (Fig. 2d, input). However, in RIP fractions, RT-PCR detected *FT* but not *RFP* mRNA (Fig. 2d, RIP), indicating that *FT* mRNA was specifically associated with RAB5-positive compartments. When the samples from RIP were treated with RNase A, *FT* mRNA was no longer detected on RT-PCR, which suggests that *FT* mRNA attached on the surface of RAB5-positive compartments (Fig. 2d). The attachment of *FT* mRNA on the surface of RAB5-positive compartments was further visualized by fluorescent intensity analysis. In time-series images of the co-transport of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-RAB5-labeled compartments, green fluorescence was mostly detected on the boundary of mCherry-labeled compartments during co-transportation (Supplementary Fig. 3). Thus, our results show that mobile *FT* and *AGL24* but not non-mobile *RFP* mRNA attached on the surface of RAB5-positive compartments to transport to PD.

Co-localization of mobile *FT* mRNAs with organelles markers

To further examine whether mobile *FT* mRNA associates with endomembranous compartments *in vivo*, we conducted co-localization analyses. In the Golgi-bypassing unconventional protein secretion system, membrane transport is mediated by the trans-Golgi network (TGN) and MVBs for delivery of plasma membrane (PM)-destined or extracellular proteins²⁶. To determine whether these organelles are involved in PD-targeting of mobile mRNA, we co-expressed MS2_{FD}-GFP-labeled *FT* mRNA with mCherry-conjugated SYP41 (TGN marker), RAB5 (MVB marker) or RAB7 (tonoplast marker) in *N. benthamiana*²⁷. Live-cell imaging showed that *FT* mRNA foci co-localized with TGN (Fig. 3a) or MVBs (Fig. 3b) but did not significantly overlap with the tonoplast marker (Fig. 3c), which suggests that TGN and MVBs specifically mediate the transport of *FT* mRNA. TGN and MVBs are usually involved in targeting proteins to PM or vacuoles²⁶. To further examine whether TGN and MVBs are transported to PD, we co-expressed the TGN or MVB marker with a GFP-conjugated PD-localized protein, PLASMODESMATA-LOCATED PROTEIN 5²⁸

(PDLP5). Co-localization of PDLP5 with the TGN (Fig. 4a and 4b) or MVB marker (Fig. 4c and 4d) indicated that a subset of TGN and MVB was transported to PD, which is consistent with the notion that PD-targeting of *FT* mRNA mediated by TGN and MVB.

Arabidopsis cytoplasmic ROCs are organelle-localized RBPs with cell-to-cell mobility

In eukaryotic cells, RBPs are required to tether mRNAs for membrane-bound intracellular mRNA trafficking^{29,30}. To identify RBPs responsible for organelle attachment and phloem transport of mobile mRNAs, we sought to identify organelle-localized RBPs with the ability to move cell to cell. We examined the published Arabidopsis RBP databases^{31,32} and the proteomics databases of different endomembrane compartments³³. The combined analysis of these databases revealed at least 3 putative organelle-localized RBPs (Supplementary Table 1). Biolistic expression of GFP-fused putative endosomal RBPs in Arabidopsis leaves showed that the GFP-fused AT1G18080 and AT5G47210 proteins were retained in the bombarded cells (Supplementary Fig. 4), whereas GFP-fused At4G34870, which encodes ROC5/AtCYP18-4, and ROC3 (a ROC5 homolog) were mobile on biolistic assay (Fig. 5a and 5b). Cyclophilins are ubiquitous peptidyl-prolyl *cis-trans* isomerase with vesicular localization and RNA-binding ability found among some members^{17,19}. ROC5/AtCYP18-4 is a single-domain cyclophilin¹⁶. Arabidopsis contains 21 single-domain cyclophilins classified into 3 clades¹⁶. We examined the expression pattern of 5 closely related cytoplasmic cyclophilins, *ROC1*, 2, 3, 5 and 6³⁴. Real-time RT-PCR and histochemical analysis revealed that the promoter activity of these ROC genes mainly overlapped in vascular tissues, whereas *ROC1*, 3 and 5 showed a predominant expression level (Supplementary Fig. 5), which suggests the redundancy of cytoplasmic ROCs. Consistent with biolistic analysis, the 5 cytoplasmic ROCs proteins but not ER-localized ROC7 showed cell-to-cell movement in Arabidopsis transformants expressing GFP-fused cytoplasmic ROCs driven by a companion cell-specific promoter, *SUC2* (Supplementary Fig. 6). These data suggest that Arabidopsis cytoplasmic ROC proteins are mobile proteins.

ROCs specifically bind mobile *FT* mRNA on RAB5-containing organelles

To probe the roles of ROCs in coordinating PD-targeting of mobile mRNA and organelle trafficking, we examined the RNA-binding activities of ROCs. Co-incubation of ROCs with DIG-labeled, *in vitro*-transcribed *FT* mRNA resulted in a mobility shift of *FT* mRNA (Fig. 5c), which supports the *in vitro* RNA-binding activities of ROCs. This mobility shift of the RNA-ROC3 protein complex was further examined by competition assay with non-labeled mRNA (cold RNA): increasing amounts of cold *FT* or *GFP* mRNA gradually reduced the mobility shift of RNA-protein complexes, which suggests a non-specific and cooperative RNA binding of ROC3 under the *in vitro* conditions tested (Fig. 5c). To further verify the *in vivo* RNA-binding activities of ROC3, RIP was conducted. We co-expressed GFP-tagged ROC3 protein and *FT* or *RFP* mRNA in *N. benthamiana* leaves. RIP with antibodies against GFP showed that *FT* but not

RFP mRNA was co-immunoprecipitated with ROC3 protein (Fig. 5d), which indicates a selection machinery for exclusive transport of mobile mRNAs in living plants.

To elucidate whether ROC3 connects *FT* mRNA to organelle transport, we examined the subcellular localization of ROC3 in living plants. Co-expression of ROC3 with MS2_{FD}-GFP-labeled *FT* mRNA revealed co-localization of ROC3 and *FT* mRNA, which further supports an *in vivo* ROC3-*FT* mRNA interaction (Fig. 6a). In addition, ROC3 co-localized with RAB5-positive compartments (Fig. 6b and 6c), which supports that ROC3 bridges mobile mRNA and organelle trafficking. To further investigate the interaction between ROC3 and RAB5-positive compartments, we examined ROC3-RAB5 co-localization under pharmaceutical treatment. ROC3 and RAB5 subcellular distribution was not altered by BFA treatment (Fig. 6d). Under CMA treatment, ROC3 co-localized with enlarged RAB5 compartments accumulated in cytosol (Fig. 6e). Notably, ROC3-RAB5 and *FT* mRNA-RAB5 co-localization was concordantly affected by the chemicals tested (Fig. 2b and 6e), suggesting that ROC3 attached mobile mRNAs on RAB5-containing compartments for PD-targeting.

PD-targeting of *FT* mRNA is disturbed in Arabidopsis mutants defective in multiple cytoplasmic ROCs

To probe the requirement of ROCs in PD-targeting of mobile mRNAs *in vivo*, we examined PD-targeting of mobile *FT* mRNA in Arabidopsis mutants defective in cytoplasmic ROCs. We generated the Arabidopsis *roc1* mutant by using the CRISPR-Cas9 system (Supplementary Fig. 7a, details are provided in Methods), and obtained the T-DNA insertion mutants of *roc2* (Salk_067272), *roc3* (Salk_063724), and *roc5* (GABI_578C07) from the stock center (Supplementary Fig. 7b and 7c). Single, double and triple *roc* mutants showed no obvious phenotype, except flowering time was slightly delayed (Fig. 7a). However, the *roc1 roc2 roc3 roc5* quadruple mutant showed a developmental defective phenotype, with small plants, small rosette leaves and curved cauline leaves (Fig. 7b), which suggests the roles of ROCs in developmental regulation. To examine PD-targeting of mobile *FT* mRNA in *roc* mutants, the leaves of wild-type and quadruple mutants were excised and used for particle bombardment to transiently express the MS2 mRNA labeling system. Transient expression of MS2_{FD}-GFP labeled *FT* mRNA revealed impaired PD-targeting of *FT* mRNA in quadruple *roc* mutants (Fig. 7c and Table 1), which suggests that cytoplasmic ROCs act redundantly in mediating organelle-bound *FT* mRNA PD-targeting.

ROCs bind mobile mRNAs to form RNA-protein complexes in phloem

The intercellular mobility of ROCs (Fig. 5a and 5b; Supplementary Fig. 6) and the detection of cyclophilins in *B. napus* phloem exudates²⁰ suggest that ROCs may shuttle mobile mRNAs into phloem for long-distance translocation. To determine the involvement of ROCs in phloem translocation of mobile mRNAs,

we examined whether ROCs are detected in RNA-protein complexes of phloem exudates. We collected phloem exudates from broccoli (*Brassica oleracea*) as described ³⁵. Gel mobility-shift assay detected a high molecular-weight fragment on incubating *in vitro*-synthesized mobile mRNAs with broccoli phloem exudates (Fig. 8a). A similar gel-shift band was detected on incubating mobile mRNAs with proteins extracted from broccoli or Arabidopsis leaves (Fig. 8a), suggesting the formation of an RNP complex in Arabidopsis and broccoli. To examine whether ROC protein was detected in the RNP complex of phloem exudates, we performed size-exclusion chromatography to separate phloem proteins and identified the fractions containing protein complexes interacting with mobile mRNAs. Proteins from these fractions were separated on SDS-PAGE and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS). The peptides corresponding to broccoli ROC1 and ROC3 homologues were identified from mass spectrometry (Fig. 8c and 8d), suggesting that cytoplasmic ROCs form RNPs in phloem to confer long-distance movement of mobile mRNAs.

Discussion

Intra- and intercellular transport of RNAs is an efficient approach to incorporate spatial information to fine-tune cellular physiological processes. In multicellular organisms, the spatial distribution of mRNAs coupled with localized translation in distinct subcellular compartments is intimately associated with asymmetric development and viability of the cells ²¹. Up to 70% of examined mRNAs in *Drosophila* embryogenesis or one-third of investigated mRNAs in neurons exhibit specific subcellular localization ²¹. In metazoans, hitchhiking of mRNAs on the vesicular surface has been used to distribute mRNA intracellularly ^{21,30}. However, to transport RNAs across the plasma membrane, intercellular delivery requires RNAs to be incorporated into the lumen of secretory vesicles and released through extracellular vesicles (EVs) ^{36,37}. In plants, the cytoplasmic continuity provided by PD allows for cell-to-cell delivery of small RNAs and mobile mRNAs symplastically ^{12,15,38}. Thus, incorporating mRNAs into the lumen of secretory vesicles may not be essential to deliver mRNAs intercellularly in plant cells. In agreement with this notion, our mRNA live-imaging revealed that PD-targeting of mobile *FT* mRNA is mediated by an endomembranous trafficking pathway involving TGN and MVBs (Figs. 1-3). In addition, our *in vitro* gel-shift assay and *in vivo* mRNA live-cell imaging and RIP evidence supported that mobile *FT* mRNA is attached on the surface of organelles by organelle-localized RBPs, ROCs (Figs. 4-6). Thus, our results highlight that the hitchhiking strategy is used to target mobile mRNAs to PD.

The cell-to-cell movement of Arabidopsis ROC proteins (Fig. 5) and detection of ROC homologs in phloem exudates of *B. napus* ²⁰ suggest that ROCs may bind to mobile mRNAs and shuttle mobile RNAs into phloem for long-distance transport. Functional similarity between ROCs and viral movement proteins (MPs), which bind and guide viral RNAs to PD for viral cell-to-cell movement ²³, suggests the potential roles of ROCs in gating PD. The interaction between the ROC-RNA complex and PD components may gate PD to facilitate the cell-to-cell movement of mobile mRNAs into neighboring cells. However, unlike non-

specific RNA-binding of viral MPs, ROCs show RNA-binding specificity (Fig. 5d), which illuminates a delicate machinery for mobile mRNA movement to control plant development. Whether the function of ROCs is similar to that of viral MPs requires further investigation. In addition, how ROCs interact with PD components to gate PD mobility remains to be elucidated. Notably, recent findings indicate that during plant-pathogen interactions, plants small RNAs may be incorporated into EVs to transport into fungal cells ³⁷. Whether EVs participate in cell-to-cell movement of mobile mRNAs remains to be investigated.

The involvement of endomembrane transport in intracellularly localizing mRNAs has been extensively investigated in budding yeast, filamentous fungi, *Drosophila* oocytes, and neurons ²¹. In plants, recent study also revealed the involvement of endosomal trafficking in glutelin mRNA distribution during endosperm development ³⁰. These asymmetric-localized mRNAs can hitchhike on the organelles to transport to specific compartments. In the secretory pathway, TGN and MVBs are usually targeted to PM or vacuoles ²⁶; however, the proteins involving endomembranous trafficking have been localized at PD ³⁹, suggesting that endomembranous trafficking is a potential vehicle for cellular cargoes to approach PD. Indeed, in Arabidopsis, PD-mediated cell-to-cell movement of SHORT-ROOT (SHR) protein is concordant with localization of the TGN marker VTI12 and MVB marker Rha1; in addition, the movement of SHR is sensitive to CMA treatment ²⁵. Consistent with these results, our co-localization studies showed that a proportion of TGN and MVBs co-localized with the PD marker PDLP5 (Fig. 4). Thus, during the dynamic transport of TGN and MVBs at the cell cortex, a subset of TGN and MVBs may be transported to PD to release mobile mRNAs.

mRNAs docking on the surface of organelles for intracellular mRNA transport have been shown in fungal hyphae and animal cells. To bridge mRNA on the organelle surface, a protein or protein complex that recognizes both mRNAs and specific lipids on organelles is required. In *Ustilago maydis*, *septin* mRNA, which encodes a critical structural protein for hyphae polar growth, is transported on endosomes along microtubules during hyphae growth ²⁹. Tethering *septin* mRNA on endosomes requires protein complexes containing Rrm4, which encodes an RBP, and Upa1, which binds the phospholipid PI3P on vesicles ²⁹. In neurons, the RBP annexin A11 (ANXA11) recognizes phosphorylated phosphatidylinositol on the lysosomal surface, thus mediating the cotransport of RNA granules and lysosomes ⁴⁰. Notably, ROC1, 2, 3, and 5 belong to single-domain cyclophilins without a known lipid-binding domain ¹⁶; how ROCs specifically recognize mobile mRNAs and lipids remains unknown. The RNA mobile motifs that trigger mobile mRNA movement have been identified. In Arabidopsis, the tRNA-like sequence (TLS) or 5-methylcytosine (m⁵C) on a subset of mobile mRNAs is crucial for mRNA movement ^{13,14}. In pumpkin, a phloem polypyrimidine tract-binding protein, RBP50, interacts with a polypyrimidine tract-binding motif on some mobile mRNAs to form mobile RNP complexes for phloem translocation ¹¹. Whether ROCs can specifically bind with RNA mobile motifs to tether RNA on the organelles remains to be investigated.

PD is a symplastic channel containing a specialized membrane system, including PM lining the pore, and a centrally placed rod-like desmotubule derived from ER ⁴¹. Given that the size of TGN and MVBs (100~200 nm) is larger than the diameter of the PD sleeve (8-10 nm) ⁴², TGN and MVBs are not likely directly transported through the PD sleeve. Thus, the hitchhiking of mobile mRNAs on organelles may help RNA-protein complexes to access PD components by transporting the complexes to PD. However, how RNA-protein complexes transport through PD remains to be elucidated. The involvement of membrane trafficking in plant viral RNA movement is supported by the transport of viral replication vesicles derived from ER during Turnip mosaic virus infection. Notably, Turnip mosaic virus mRNA-containing vesicles move in an MVB-dependent manner that bypasses the Golgi apparatus ⁴³. Alternatively, in *Saccharomyces cerevisiae*, coordinated transport of *ASH1* mRNA and ER to daughter cells for local translation suggests a role for ER in intracellular mRNA targeting ²¹. Small molecules up to 10 kDa can traffic through the desmotubule lumen; however, large molecules such as ER lumen-targeted GFP do not move cell to cell ⁴⁴. Thus, the cell-to-cell movement of *FT* mRNA is not likely mediated through ER lumen. The current model for PD-mediated intercellular transport suggests that the wideness of the cytoplasmic sleeve, the space between the PM and desmotubule, determines PD conductivity ⁴¹. However, recent evidence shows that type I PD, which lacks a visible cytoplasmic sleeve, shows enhanced permeability to macromolecules, possibly contributed by specialized ER-PM membrane contact ⁴⁵. In addition, the mutation of multiple C2 domains and transmembrane region protein, a family of PD-localized ER-PM tethering proteins, could block PD conductivity ⁴⁶. Moreover, Arabidopsis with defective sphingolipid biogenesis exhibited exclusively type I (cytoplasmic sleeve-lacking) PD but enhanced PD permeability ⁴⁷. It is possible that the membranous architecture and ER-PM contact sites within PD may regulate mobile mRNA movement through PD. By recognizing the PD microdomain, MVBs may deliver internal contents by membrane fusion or interchange surface cargoes such as mobile mRNAs via a mechanism similar to that of lipid or protein transfer in membrane contact sites ⁴².

Cooperation between actin and microtubules in subcellular trafficking is conserved among eukaryotes including plants. For example, in Arabidopsis, the overall distribution pattern of cellulose synthase is mediated by actin, but cellulose synthase is significantly targeted to PM subdomains coincident with cortical microtubules ⁴⁸. In tobacco pollen tubes, callose synthase is delivered along the actin cytoskeleton, but the positioning of callose synthase to callose plugs is fine-tuned by microtubules ⁴⁹. Consistently, our results revealed that the transport of *FT* mRNA in transvacuolar strands is mediated by actin, but PD-targeting of *FT* mRNA at the cell cortex depends on microtubules (Fig. 1). During intracellular movement of SHR protein in Arabidopsis root cells, pausing at actin-microtubule intersections is critical for further PD-targeting and cell-to-cell movement. This pausing event requires kinesin G, a motor protein associated with microtubules ⁵⁰. Thus, the interplay between actin and

microtubules and the involvement of motor proteins for intercellular delivery of mobile mRNAs deserves further investigation.

Methods

Plant materials and growth conditions

Nicotiana benthamiana or Arabidopsis plants were grown under a 16-h/8-h day/night cycle, with white fluorescent light at $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$; temperature of the growth chamber was set to 27°C for *N. benthamiana* and 22°C for Arabidopsis. Arabidopsis T-DNA insertion mutants were obtained from ABRC stock center.

Agro-infiltration in *N. benthamiana* leaves

The construction of pCAMBIA1390-CaMV35S-MS2_{FD}-GFP and pCAMBIA1390-CaMV35S-FT_{SL24} was as described¹⁵. The plasmids were introduced into *Agrobacterium tumefaciens* strain AGL1. The successful transformants were selected on LB media containing $50 \mu\text{g}\cdot\text{ml}^{-1}$ kanamycin at 28°C for 2 days.

A. tumefaciens strain AGL1 carrying individual constructs was cultured in LB media containing $50 \mu\text{g}\cdot\text{ml}^{-1}$ kanamycin, 10 mM MES, pH 5.7, and 20 μM acetosyringone at 28°C overnight. *Agrobacterium* cells were pelleted and resuspended in infiltration solution (10 mM MgCl₂, 10 mM MES, pH 5.7, and 200 μM acetosyringone) to OD₆₀₀ 1.0 and incubated at room temperature for 1 hr. Co-infiltration of *Agrobacterium* was conducted with bacterial solutions prepared as equivalent volume mixtures and infiltrated into the abaxial side of 3-week-old *N. benthamiana* leaves by using a needle-removed syringe.

Chemical treatment

Aniline blue fluorophore (ABF) (Biosupplies, Bundoora, Australia) was stocked as a 0.1-mg/ml solution in distilled water and stored at 4°C. Before detecting callose deposits, the stock solution was diluted 1:3 with infiltration buffer and infiltrated into *N. benthamiana* leaves.

Brefeldin A (BFA), concanamycin A (CMA), latrunculin B (LatB), and oryzalin stock solutions were prepared with DMSO in concentration at 10 mM, 1 mM, 4 mM, and 20 mM, respectively. Stock solutions were stored at -20°C. Before use, working solutions were prepared by diluting stock solutions with Milli-Q

water (final concentration: 50 μ M for BFA, 1 μ M for CMA, 20 μ M for LatB, and 20 μ M for oryzalin). Freshly-prepared working solutions were infiltrated into *N. benthamiana* leaves with a needle-free syringe before microscopy examination.

Confocal laser scanning microscopy and fluorescent intensity analysis

At 1 or 2 days after agro-infiltration or 10 min after ABF infiltration in tobacco leaves, a $\sim 0.5\text{-cm}^2$ sample was removed from infiltrated or the leading front region of infiltrated leaves¹⁵, covered with glass slides and observed under the confocal laser scanning microscope LSM880 (Carl Zeiss, Oberkochen, Germany). The settings for excitation laser/detection filters (in nm) were argon488/BP510-550 (for GFP) and DPSS561/BP590-650 (for mCherry and DsRED). High-resolution images were acquired with the Airyscan module mounted on the LSM880 system. Fluorescent intensity analysis was conducted with the Profile assay included in the ZEN software accompanying the LSM880 microscope.

RNA immunoprecipitation (RIP) analysis

N. benthamiana leaves with transiently expressed 35S-mCherry-RAB5 and 35S-*FT_{SL24}* or 35S-*RFP_{SL24}* were sampled 2 days after agro-infiltration. An amount of 5 g leaves was ground in liquid nitrogen and homogenized with 10 ml extraction buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 2.5 mM MgCl₂, 0.5% Triton X-100, 10% glycerol, 1X Complete protease inhibitor cocktail, 20U/ml RNase inhibitor) and incubated at 4°C for 15 min. The samples were centrifuged at 20000g for 10 min at 4°C to remove cell debris. Supernatant of total extract was diluted with 3X volumes of dilution buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 2.5 mM MgCl₂, 10% glycerol, 1X complete protease inhibitor cocktail, 20 U/ml RNase inhibitor). For RNA immunoprecipitation, 30 ml RFP-Trap-MA beads (Chromotek) was equilibrated and resuspended in 50 ml wash buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl, 2.5 mM MgCl₂, 0.2% Triton X-100, 10% glycerol, 0.5 mM DTT, 1X complete protease inhibitor cocktail, 20 U/ml RNase inhibitor). The beads and diluted supernatant of total extract were mixed and incubated at 4°C for 1 hr with gentle rotation. To recover the beads, the beads were magnetically separated, washed and resuspended in 1 ml wash buffer.

RNA co-purified with the beads was extracted with TRIzol reagent (Ambion). The RNA pellet was dissolved in 13 ml of DEPC-treated water and subjected to RT-PCR. The reverse transcription reaction was performed with SuperScript III reverse transcriptase (Invitrogen) at 50°C for 1 hr. An amount of 2 ml cDNA was used for PCR reactions with the following conditions: 94°C 1 min for 1 cycle, 94°C 30 sec, 60°C 30 sec, 68°C 80 sec for 35 cycles, and 68°C 7 min for 1 cycle. In total, 5 ml PCR products was separated in 0.8% agarose gel. The primers used to detect transgenic *FT* RNA were forward:

5'GGAAGTTCATTTTCATTTGGAGAGA, reverse: 5'GACACGATGAATTCCTGCAGTGGGACTTGG; and transgenic *RFP* RNA: forward: 5'ATGCGCTCCTCCAAGAACGTCATC; reverse: 5'TCCACTAGTCAGGAACAGGTGGTGGCGGCCCTC.

CRISPR

To generate Arabidopsis *roc1* mutant, guide RNAs (gRNAs) were designed by using the web-tool CRISPR-P (<http://crispr.hzau.edu.cn/CRISPR2/>). The primer sequences for gRNAs were ROC1-gRNA1: 5'GGTCGTGGAAGGCTTAGACG; and ROC1-gRNA2: 5'TCATGTCTGAAGTATACCTTA. The DNA fragment containing gRNA was inserted into vectors. The resulting vector containing two-target gRNAs was introduced to Arabidopsis. Successful transformants were selected under antibiotics-containing medium. The transformants was subjected to sequencing analysis to identify *roc1* mutants (Supplementary Fig. 7a).

Particle bombardment assay

For transient expression in Arabidopsis, 5 µl (1 µg/µl) plasmid DNA combinations were coated on 1.25 mg gold particles with 1-µm diameter (Bio-Rad, Hercules, CA) and delivered onto the abaxial side of leaves from 3-week-old Arabidopsis plants. For co-expression of the MS2 mRNA-labeling system or putative endosomal RBPs, different combinations of plasmid DNA were mixed at a 1:1 (in volume) ratio. Particle bombardment was performed with Biolistic PDS-1000/He (Bio-Rad) under the following conditions: bombardment chamber vacuum degree = 27 mm-Hg; helium pressure = 650 psi; distance from stopping screen to the target tissues = 6 cm. At 48 hr after bombardment, images were obtained under the confocal laser scanning microscope LSM880 (Carl Zeiss).

Declarations

Acknowledgements

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Author contributions

KRL, NCH and TSY designed the experiments. KRL performed live-imaging experiments. NCH performed the RIP assay. NCH and YHC characterized *roc* mutants. KRL and TSY wrote the manuscript with inputs from NCH.

Competing interests

The authors declare no competing interests.

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Table

Table 1. Disruption of *FT* mRNA PD-targeting in Arabidopsis *roc1,2,3,5* quadruple mutant

	Number of cells with punctate <i>FT</i> distribution	Number of cells with homogenous <i>FT</i> distribution
Col	37 (88.10%)	5
<i>roc1,2,3,5</i>	8 (23.53%)****	26

**** $p < 0.0001$, one-sided Fisher’s exact test.

Figures

Figure 1. Cytoskeleton is required for intracellular transport of mobile *FT* mRNA (a) Co-localization of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-labeled actin (Lifeact-mCherry). N, nucleus. Scale bar =10 μm. (b) Arrest of transport of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-labeled Golgi (MAN49-mCherry) in cells with latrunculin B (LatB) treatment. Scale bar = 20 μm. (c) Distribution of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-labeled Golgi (MAN49-mCherry) in cells with oryzalin (Ory) treatment. N, nucleus. Scale bar = 10 μm.

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Figure 2. *FT* mRNA associated with RAB5-containing compartments for PD-targeting (a-c) Distribution of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-RAB5-labeled compartments in cells with (a) DMSO treatment for negative controls, (b) concanamycin A (CMA), or (c) wortmannin (Wort) treatment. Note that Wort treatment dissociated *FT* mRNA from RAB5-containing compartments and resulted in homogenous distribution of *FT* mRNA at the cell cortex. Scale bar = 20 μ m. (d) RNA-immunoprecipitation (RIP) analysis of mRNA associated with RAB5-containing compartments. Left panel, the illustration of RIP experiments. Antibodies against mCherry were used to immunoprecipitate mCherry-RAB5 containing compartments. Right panel, RT-PCR analysis of mRNA extracted before (input) or after RIP. For RNase treatment, the beads isolated after RIP were treated with RNase A for 30 min.

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Figure 3. *FT* mRNA co-localizes with trans-Golgi network (TGN) and multivesicular body (MVB) (a) Co-localization of MS2_{FD}-GFP labeled *FT* mRNA with mCherry-labeled TGN marker SYP41. The co-localized spots are indicated by arrowheads. Scale bar = 20 μ m. (b) Co-localization of MS2_{FD}-GFP-labeled *FT* mRNA with mCherry-labeled MVB marker RAB5. The co-localized spots are indicated by arrowheads. Scale bar = 10 μ m. (c) Distribution of MS2_{FD}-GFP-labeled *FT* mRNA and mCherry-labeled tonoplast marker RAB7. Scale bar = 10 μ m.

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Figure 4. Co-localization of TGN and MVB with PD (a) Co-localization of GFP-labeled PD-localized protein PLASMODESMATA-LOCATED PROTEIN 5 (PDL5) and mCherry-labeled TGN marker SYP41. Scale bar = 20 μm . (b) Enlarged image of the area marked by yellow rectangle in (A). The co-localized spots are indicated by arrowheads. Scale bar = 5 μm . (c) Co-localization of GFP-labeled PDL5 and mCherry-labeled MVB marker RAB5. Scale bar = 20 μm . (d) Enlarged image of the area marked by yellow rectangle in (c). The co-localized spots are indicated by arrowheads. Scale bar = 5 μm .

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Figure 5. Arabidopsis cytoplasmic rotamase cyclophilins (ROCs) are organelle-localized RNA-binding proteins with cell-to-cell mobility (a-b) Cell-to-cell movement of ROC3 and 5 proteins in Arabidopsis leaf epidermal cells. 35Spro-DsRed and 35Spro-ROC3-GFP (a) or 35Spro-ROC5-GFP (b) were transiently co-expressed in Arabidopsis leaves by particle bombardment. Scale bar = 50 μ m. (c) Left panel, gel-mobility shift assay of ROC proteins co-incubated with *in vitro*-transcribed DIG-labeled *FT* mRNA. Middle panel, competition assay with increasing amounts of non-labeled cold *FT* mRNA showing the mobility shift of *FT* mRNA-ROC3 complexes. Right panel, non-specific competition assay with increasing amounts of non-labeled cold *GFP* mRNA showing the mobility shift of *FT* mRNA-ROC3 complexes. (d) Left panel, illustration of RNA-immunoprecipitation (RIP) analysis of mRNA associated with ROC-GFP. Right panel, RT-PCR analysis of mRNA extracted before (input) or after RIP with antibodies against GFP and *FT* (left panel) or *RFP* (right panel) primers.

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Figure 6. Co-localization of ROC3 with MVB marker RAB5 and mobile *FT* mRNA. (a) Co-localization of MS2_{FD}-GFP-labeled *FT* mRNA and ROC3-DsRed. White arrowheads indicate the co-localization spots. Scale bar = 5 μm. (b-c) Co-localization of ROC3-GFP and mCherry-RAB5 (b) at cell cortex (Scale bar = 10 μm) and (c) in transvacuolar strand (Scale bar = 5 μm). White arrowheads indicate the co-localization spots. (d) Brefeldin A (BFA) treatment has no effect on ROC3-RAB5 co-localization. White arrowheads indicate the co-localization spots. Scale bar = 20 μm. (e) Co-localization of ROC3-GFP and mCherry-RAB5 labeled MVBs in cells with concanamycin A (CMA) treatment. White arrowheads indicate the co-localization spots of ROC3 with enlargement and aggregation of RAB5-labeled compartments. Scale bar = 10 μm.

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Figure 7. PD-targeting of *FT* mRNA is disturbed in Arabidopsis mutants defective in multiple cytoplasmic ROCs. (a) Flowering time of different Arabidopsis ROC mutants indicated as total rosette leaf number. N=9 for all sets. *Student's t*-test was used to compare data between individual mutant lines and Col. * $p < 0.05$; *** $p < 0.001$. ns, not significant. Data are mean $\pm$ SD. (b) Left panel, 4-week old plants of Arabidopsis wild-type (Col) and *roc1,2,3,5* quadruple mutant. Right panel, rosette leaves (upper) and cauline leaves (lower) of wild-type (Col) and *roc1,2,3,5* quadruple mutant. (c) Transient expression of MS2_{FD}-GFP-labeled *FT* mRNA in leaf epidermal cells from wild-type (left) and *roc1,2,3,5* quadruple mutant (right). Scale bar = 20 μ m.

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Figure 8. ROCs are phloem RNA-binding proteins. (a) The ³²P-labeled mobile mRNA was synthesized and incubated with dialyzed broccoli phloem sap or leaf protein extract from broccoli or *Arabidopsis*. T, Tris buffer; P, phosphate buffer. (b) Biotin-labeled mobile mRNA or non-biotin-labeled mobile mRNA was incubated with fractionated phloem sap proteins. Mobile mRNA-associated proteins were co-purified using biotin-avidin purification system. Peptides identified with subsequent LC-/MS/MS analysis to sample (i) and sample (ii) are summarized in (c) and (d), respectively. The peptides hit to putative broccoli homologs to *Arabidopsis* ROC proteins.

Figure 8

ROCs are phloem RNA-binding proteins. (a) The ^{32}P -labeled mobile mRNA was synthesized and incubated with dialyzed broccoli phloem sap or leaf protein extract from broccoli or Arabidopsis. T, Tris buffer; P, phosphate buffer. (b) Biotin-labeled mobile mRNA or non-biotin-labeled mobile mRNA was incubated with fractionated phloem sap proteins. Mobile mRNA-associated proteins were co-purified using biotin-avidin purification system. Peptides identified with subsequent LC-/MS/MS analysis to sample (i) and sample (ii) are summarized in (c) and (d), respectively. The peptides hit to putative broccoli homologs to Arabidopsis ROC proteins.

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