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An RNA exosome subunit mediates cell-to-cell trafficking of a homeobox mRNA via plasmodesmata

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Messenger RNAs (mRNAs) function as mobile signals for cell-to-cell communication in multicellular organisms. The KNOTTED1 (KN1) homeodomain family transcription factors act non–cell autonomously to control stem cell maintenance in plants through cell-to-cell movement of their proteins and mRNAs through plasmodesmata; however, the mechanism of mRNA movement is largely unknown. We show that cell-to-cell movement of a KN1 mRNA requires ribosomal RNA–processing protein 44A (AtRRP44A), a subunit of the RNA exosome that processes or degrades diverse RNAs in eukaryotes. AtRRP44A can interact with plasmodesmata and mediates the cell-to-cell trafficking of KN1 mRNA, and genetic analysis indicates that AtRRP44A is required for the developmental functions of SHOOT MERISTEMLESS, an *Arabidopsis* KN1 homolog. Our findings suggest that AtRRP44A promotes mRNA trafficking through plasmodesmata to control stem cell–dependent processes in plants.

Cell-to-cell communication in multicellular organisms promotes cell fate specification and coordination of development. As one way to transmit information between cells, plants selectively traffic transcription factors through plasmodesmata, cell wall–embedded channels that connect the cytoplasm of neighboring cells (*1*–*3*). The maize KNOTTED1 (KN1) homeodomain transcription factor was the first mobile protein found to use this trafficking pathway (*4*). Previous studies have identified regulators of KN1 protein trafficking (*5*–*7*); however, KN1 protein traffics with its mRNA (*4*). Selective trafficking of mRNAs in plants is prevalent (*8*–*12*); however, the mechanism by which this occurs through plasmodesmata has not been addressed. Here, we identify *Arabidopsis* ribosomal RNA–processing protein 44A (AtRRP44A) as an essential factor for the cell-to-cell trafficking of KN1 mRNA and show that AtRRP44A-dependent mRNA trafficking is required for cell-to-cell protein trafficking and stem cell functions in plants.

Isolation of KN1 trafficking mutants

We previously established a genetic screen in *Arabidopsis* to identify regulators of KN1 cell-to-cell trafficking by using a "trichome rescue system" (*7*, *13*). Trichomes are hairlike extensions of the leaf epidermis. Their development requires the cell-autonomous activity of GLABROUS1 (GL1), a MYB transcription factor (*14*). In our system, a fusion protein of green fluorescent protein (GFP), GL1, and the KN1 C-terminal trafficking domain ($KN1^C$) is expressed in the mesophyll cell layers of leaves of trichomeless *gl1* mutants by using the Rubisco small subunit 2b promoter (*pRbcS::GFP~GL1~KN1^C*) (*7*). Trafficking of GFP~GL1~$KN1^C$ to the epidermis rescues trichome formation in this line; thus, trichome number is an output for KN1 trafficking. In an ethyl methanesulfonate (EMS) mutagenesis screen of trichome rescue lines, we isolated two mutants that lacked trichomes, which were initially referred to as *rb31-7* and *mk5-140* (Fig. 1, A to C). Consistent with the loss of trichomes, epidermal GFP~GL1~$KN1^C$ accumulation decreased significantly in the mutants (Fig. 1, D to F) despite similar expression compared with that of the parental control trichome rescue lines in mesophyll cell layers (Fig. 1, G to I). These observations suggest that both mutants reduced the trafficking of GFP~GL1~$KN1^C$ from the mesophyll cell layers to the epidermis. To investigate whether trichome loss in the mutants was simply due to a reduction in transgene expression, we measured GFP~GL1~$KN1^C$ fluorescence in mesophyll cells of the mutants. We found that it was ~50 to 70% of the level in the parental trichome rescue lines (fig. S1A). However, this reduction was not the cause of trichome loss because plants hemizygous for the trichome rescue transgene also had ~50% expression, and this was sufficient for trichome rescue (fig. S1, A and B). Thus, we confirmed that KN1 trafficking was inhibited in *rb31-7* and *mk5-140* mutants.

KN1 trafficking mutants encode AtRRP44A

Both *rb31-7* and *mk5-140* mutants behaved as single recessive loci. We mapped them by sequencing M3 pools of mutants or nonmutant siblings to ~44× coverage. Using the MutMap+ pipeline (*15*), we identified potentially causal point mutations of *rb31-7* and *mk5-140* within the same gene, At2g17510 [guanine (G) to adenine (A) and cytosine (C) to thymine (T), which causes disruptive Cys^{551} to Tyr and Pro^{781} to Leu amino acid substitutions, respectively] (Fig. 1, J and K). At2g17510 encodes AtRRP44A, which is a subunit of the RNA exosome. We confirmed this as the causal gene by complementing trichome rescue defects in both mutants by using a TagRFP-T (hereafter RFP, red fluorescent protein) fusion protein that was expressed by its native regulatory sequences (*pAtRRP44A::AtRRP44A~RFP*) (Fig. 1, L and M), so we renamed our alleles as *atrrp44a-4* and *atrrp44a-5*, respectively. Null alleles of *AtRRP44A* in *Arabidopsis* are lethal (*16*, *17*), as in yeast, *Drosophila*, and human cells (*18*–*21*); thus, ours are likely to be weak alleles. The RNA exosome is a multiprotein complex that is involved in the processing and degradation of a wide range of RNAs in eukaryotes, and RRP44 is a catalytic subunit with 3′-to-5′ exoribonuclease and endoribonuclease activities (*22*–*24*). Two conserved domains, RNB (ribonuclease B) and PIN (pilT N-terminus), are responsible for these activities, respectively (Fig. 1K). The mutations in *atrrp44a-4* and *atrrp44a-5* were in highly conserved residues in the RNB domain (Fig. 1K and fig. S2A) (*25*–*27*) in the vicinity of the RRP44 catalytic center (fig. S2B) (*28*), which supports the notion that they are important for function.

atrrp44a mutants enhance *stm* and *cct8* phenotypes

KN1 can fully complement the shoot meristem defects of its *Arabidopsis* homolog SHOOT MERISTEMLESS (STM), whose mobility is essential for meristem maintenance (*13*, *29*, *30*). *atrrp44a-5* mutants also blocked trichome rescue by a *GFP~GL1~STM^C* fusion, suggesting that AtRRP44A also participates in STM trafficking (fig. S3). Thus, we next investigated whether AtRRP44A affects STM function. *atrrp44a* mutants had no obvious defects in shoot meristem size (Fig. 2, A, B, and E, and fig. S4A); however, they significantly enhanced the meristem phenotypes of *stm-10*, a weak allele of *stm* (*31*). Shoot meristems of *atrrp44a-4*; *stm-10* double mutants were significantly smaller than *stm-10* single mutants (Fig. 2, A to E) and terminated in many of the *atrrp44a-5; stm-10* double-mutant seedlings (22 out of 24) (fig. S4B). Similarly, meristems arrested in many of the *atrrp44a-4; stm-10* double mutants (fig. S4C), which resulted in seedlings with fewer leaves (fig. S4D). Consistent with these phenotypes, the expression of *STM* and of additional shoot meristem genes *CLAVATA3* (*CLV3*) and *WUSCHEL* appeared disorganized and/or reduced in the *atrrp44a; stm-10* double-mutant meristems, and *CLV3* expression was significantly lower in quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments (fig. S4, E and F). Collectively, our data suggest that AtRRP44A is required for STM-dependent shoot meristem maintenance, perhaps by controlling STM RNA trafficking.

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To further support a role for *atrrp44a* in meristem development, we attempted to enhance our weak alleles by combining in a hemizygous state with the *atrrp44a-1* null allele (Fig. 1J) (*17*). Indeed, these combinations significantly reduced the shoot meristem size (fig. S4G). We also assayed genetic interactions between *AtRRP44A* and *CCT8*, a regulator of KN1 trafficking (*7*) that is required for STM function in stem cell maintenance. The *atrrp44a; cct8-1* double mutants developed dwarf shoots with fasciated stems [n = 20 of 20 plants (*atrrp44a-4; cct8-1*); 18 of 18 plants (*atrrp44a-5; cct8-1*)] (Fig. 2, F and G, and fig. S5). The double mutants also occasionally made nonfasciated shoot branches, which terminated prematurely [n = 10 of 20 plants (*atrrp44a-4; cct8-1*); 11 of 18 plants (*atrrp44a-5; cct8-1*)] (Fig. 2H and fig. S5B). These phenotypes were not observed in the single mutants, suggesting that AtRRP44A regulates the balance of proliferation and differentiation in the shoot meristem through a chaperonin-dependent pathway. Collectively, our data support the idea that AtRRP44A is required for the function and trafficking of KN1 and STM, which are essential for meristem maintenance.

AtRRP44A can localize to plasmodesmata

Given our hypothesis that AtRRP44A promotes cell-to-cell trafficking of STM, we next examined whether its expression overlaps with STM in the shoot meristem. We transformed the native AtRRP44A~RFP fusion construct into plants that were heterozygous for the *atrrp44a-1* null allele. This fusion construct was functional, as it complemented the lethality of the null allele in the subsequent generation (fig. S6A). AtRRP44A~RFP expression was observed throughout the shoot meristem and flower primordia (Fig. 3A and fig. S6B), which overlapped with STM expression (*32*), as well as in epidermal and mesophyll layers of leaf primordia (Fig. 3B), where GFP~GL1~KN1^{C} traffics in the trichome rescue system. Thus, AtRRP44A was expressed broadly, including in the shoot meristem where STM traffics. The fusion protein accumulated in nuclei, as expected, mainly in the nucleolus in meristem cells and throughout the nucleoplasm in leaf primordia (Fig. 3, A and B) (*16*).

We next investigated how AtRRP44A might facilitate trafficking. Previous studies that used microinjection or the trichome rescue system indicated that KN1 protein promotes the trafficking of its mRNA (*4*, *13*), suggesting that KN1 protein and mRNA traffic as an mRNA–protein (mRNP) complex. AtRRP44A binds to the exosome core complex, which is involved in the processing of rRNAs, mRNAs, and noncoding RNAs in *Arabidopsis* (*17*, *33*, *34*), but whether AtRRP44A participates in mRNA degradation or processing is unclear (*17*). Nonetheless, because AtRRP44A is a ribonuclease,

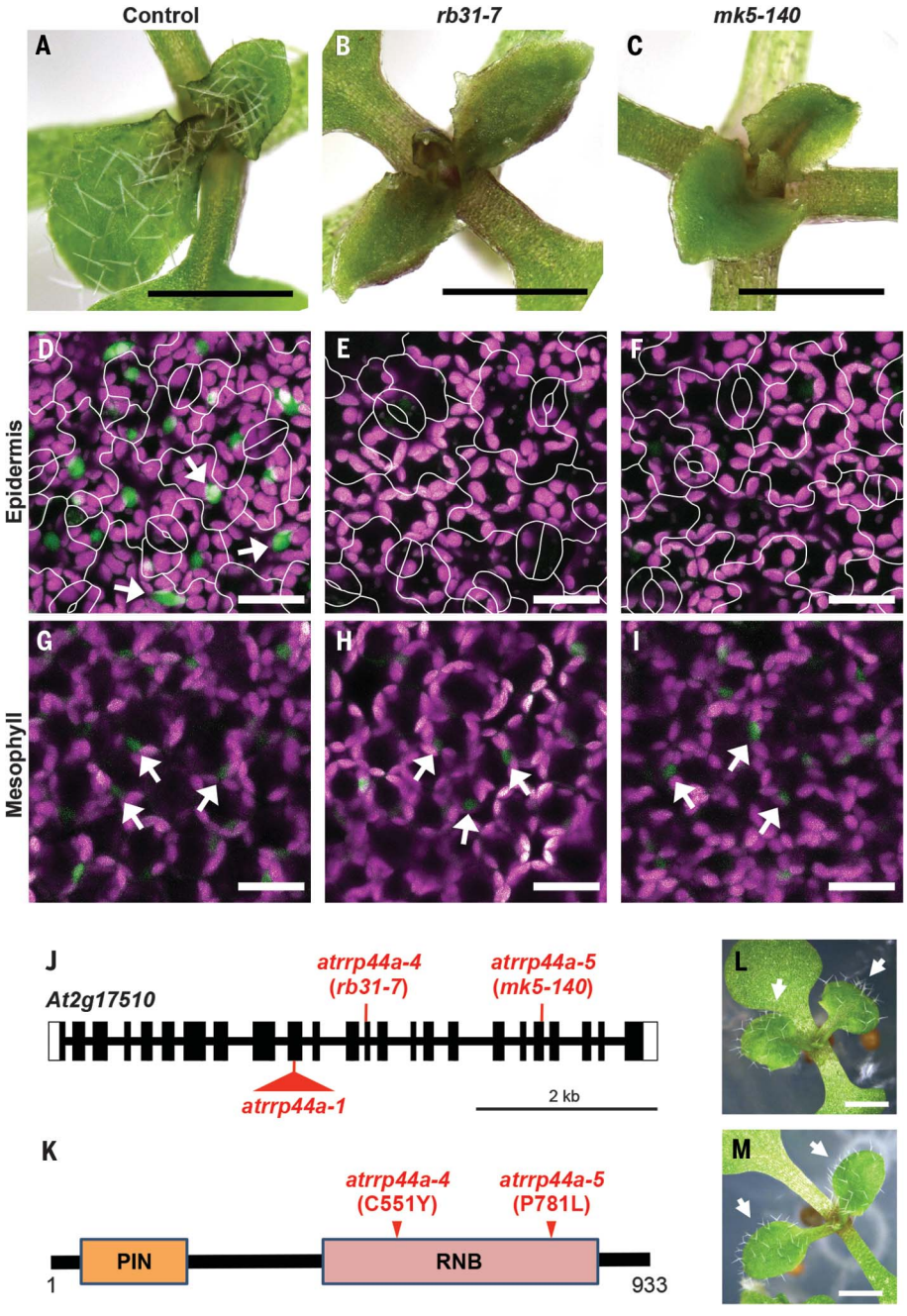

Fig. 1. AtRRP44A promotes KN1 trafficking. (**A** to **C**) Starting line for EMS mutagenesis (A) expresses GFP~GL1~KN1^{C} in mesophyll cells in a *gl1* mutant background and rescues trichome formation, whereas the *rb31-7* (B) and *mk5-140* (C) mutants lost trichome rescue. Scale bars, 1 mm. (**D** to **I**) Confocal images of a true leaf from the starting trichome rescue line show the accumulation of GFP~GL1~KN1^{C} in both the epidermis (D) and the mesophyll (G), whereas the *rb31-7* and *mk5-140* mutants have a severe reduction of GFP~GL1~KN1^{C} in the epidermis [(E) and (F), respectively] but normal accumulation in the mesophyll [(H) and (I), respectively]. The arrows show representative nuclei with GFP~GL1~KN1^{C} fusion protein. White lines show the outlines of epidermal cells. Chlorophyll autofluorescence is shown in magenta. Scale bars, 20 μm. (**J**) *AtRRP44A* (At2g17510) gene structure, showing different mutant alleles, *atrrp44a-1*, *atrrp44a-4* (*rb31-7*), and *atrrp44a-5* (*mk5-140*). Black bars indicate exons; lines and white boxes represent introns and untranslated regions, respectively. (**K**) AtRRP44A protein structure, showing different domains and the mutation sites in *atrrp44a-4* and *atrrp44a-5* in the RNB domain. (**L** and **M**) *atrrp44a-4* and *atrrp44a-5* mutants rescued by native expression of RFP-tagged AtRRP44A have normal trichome rescue (arrows). Scale bars, 1 mm.

we assessed whether it might affect trafficking indirectly by degradation or processing of KN1 or STM mRNA. However, when we examined mRNA levels and decay rates in dissected shoot apices or in seedlings by RT-qPCR, there were no differences in levels or stability of STM mRNA in *atrrp44a* mutants (fig. S7, A to D). We also assessed whether the trafficking function of AtRRP44A could be uncoupled from its RNA-processing activity by introducing a noncatalytic AtRRP44A mutant [AtRRP44A^{D489N}~RFP (D489N, Asp^{489}→Asn)] (*27*) driven by its native promoter into *atrrp44a* mutant trichome rescue plants. This construct fully rescued KN1 trafficking and trichome formation (fig. S7, E to G). These results suggest that AtRRP44A function in trafficking or trichome rescue is unrelated to its potential role in mRNA processing or degradation, although whether this noncanonical function promotes RNA trafficking in the meristem remains to be tested. We next hypothesized that AtRRP44A might directly participate in KN1 or STM mRNA trafficking, for example, by recruiting their mRNA to plasmodesmata or by transporting mRNA through plasmodesmata. In support of these ideas, AtRRP44A is found in the *Arabidopsis* cell wall proteome (*35*) and the plasmodesmal proteome (*36*) despite its predominant accumulation in nuclei (Fig. 3, A, B, and D) (*16*), suggesting that a fraction of the protein may associate with plasmodesmata. We could not detect plasmodesmata localization using our native AtRRP44A-tagged lines, so to test this possibility, we modified the AtRRP44A~RFP fusion by deleting two nuclear localization signals (NLSs) and adding a nuclear export signal (NES) (AtRRP44A$^{NLS\Delta}$~NES~RFP) to promote its export from the nucleus. Indeed, this modified protein accumulated outside of the nuclei (Fig. 3, C and D). We observed its localization in puncta that colocalized with aniline blue–stained plasmodesmata (75% of plasmodesmata showed AtRRP44A$^{NLS\Delta}$~NES~RFP signal, n = 227 of 302 plasmodesmata from 10 cells) (Fig. 3, E and F, and fig. S6C). Thus, our data support the idea that AtRRP44A interacts with plasmodesmata to promote trafficking.

AtRRP44A mediates KN1 mRNA trafficking

We next investigated whether AtRRP44A promotes mRNA trafficking by visualizing KN1 mRNA in the trichome rescue system (*37*). We inserted 24 repeats of the MS2 bacteriophage binding sequence (MBS) between the KN1 stop codon and 3′ untranslated region in our mesophyll-expressed trichome rescue construct to make *pRbcS::GFP~GL1~KN1^{C}~MBS*. This construct was introduced into plants that expressed an MS2 coat protein (MCP) ~RFP fusion in the epidermis (epiMCP) (Fig. 4A). MCP binds to MBS sequences, so if GFP~GL1~KN1^{C}~MBS mRNA traffics from the mesophyll to the epidermis, we expect to detect it through its binding by epiMCP~RFP in epidermal cells. Indeed, lines that were wild type (WT) for *AtRRP44A* showed many MCP~RFP puncta in the cytoplasm of epidermal cells (Fig. 4B), whereas plants that expressed MCP~RFP alone did not have puncta (fig. S8, A and B), indicating that GFP~GL1~KN1^{C}~MBS mRNA trafficked from mesophyll cell layers to the epidermis and formed fluorescent cytoplasmic puncta in association with MCP~RFP. By contrast, when we introduced the MBS–MCP system into *atrrp44a* mutants, their epidermal cells had approximately eightfold fewer fluorescent puncta (Fig. 4, C and D) despite similar levels of GFP~GL1~KN1^{C} protein in mesophyll cells (fig. S8, C and D). The puncta in *atrrp44a-5* were also smaller, suggesting that they contained fewer GFP~GL1~KN1^{C}~MBS mRNA molecules (fig. S8E). To confirm that this apparent loss of mRNA trafficking was not due to a reduction of GFP~GL1~KN1^{C}~MBS mRNA in *atrrp44a* mutants, we expressed MCP~RFP in mesophyll cell layers (mesoMCP, Fig. 4E). As expected, the mutants had similar numbers of KN1 mRNA puncta in mesophyll cells as the WT lines (Fig. 4, F to H). Thus, our data suggest that AtRRP44A is required for the cell-to-cell trafficking of KN1 mRNA in the trichome system, although its contribution to RNA trafficking in the meristem remains to be tested. To support this role, we examined whether AtRRP44A could bind to STM mRNA in vivo, using RNA immunoprecipitation–qPCR from extracts of shoot apices of *pAtRRP44A::AtRRP44A~RFP; atrrp44a-1* plants. Indeed, we found that AtRRP44A was associated with STM transcripts in vivo (fig. S8F).

KN1 mRNA trafficking promotes protein trafficking

We next investigated whether KN1 mRNA trafficking was important in the selective transport of a KN1 signal, by scoring trichome

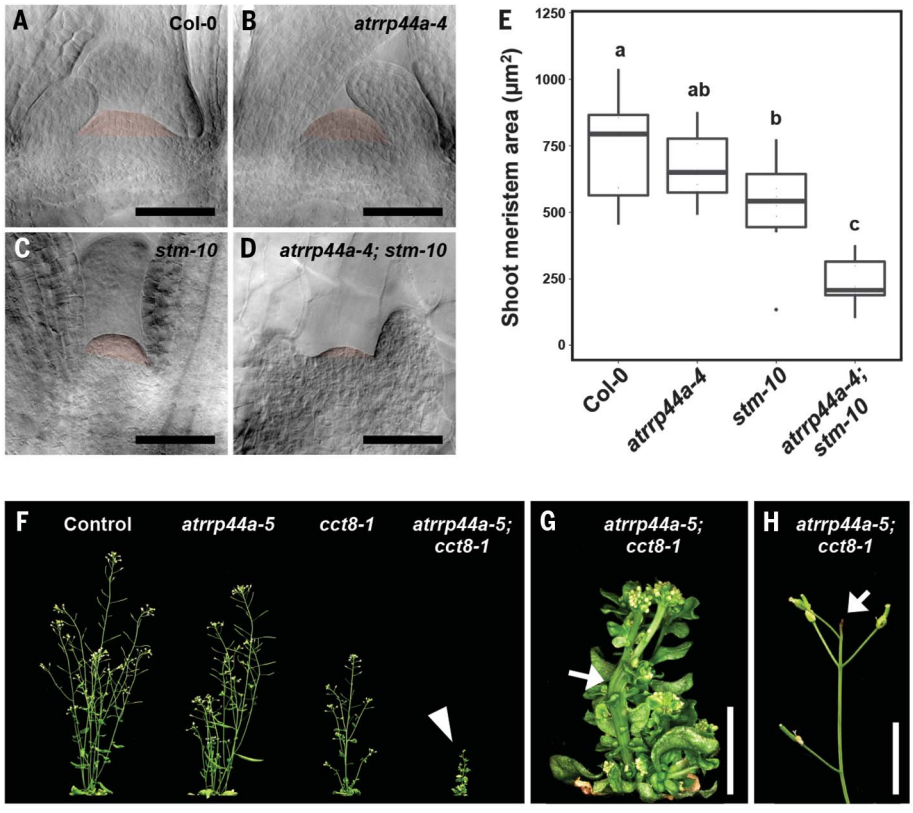

Fig. 2. *atrrp44a* mutants enhance *stm* and *cct8* phenotypes. (**A** to **E**) Meristem defects in *stm-10* were enhanced by *atrrp44a-4*. *stm-10* mutants had smaller meristems, and the meristem size was even smaller in the *atrrp44a-4; stm-10* double mutants; meristems were shaded in pink. Plants were grown for 14 days under short-day conditions. Bars topped by different letters (a, b, and c) are significantly different at $P < 0.05$ (Tukey's honest significant difference test). Scale bars, 50 μm. (**F** to **H**) *atrrp44a-5; cct8-1* double mutants developed enhanced shoot defects. *atrrp44a* and *cct8-1* single mutants had normal shoot development, although they were slightly shorter than the control lines, whereas the *atrrp44a-5; cct8-1* double mutants were severely dwarfed (arrowhead) (F), with fasciated stems (arrow) (G) and premature termination of the shoot meristem (arrow) (H). Scale bars, 1 cm.

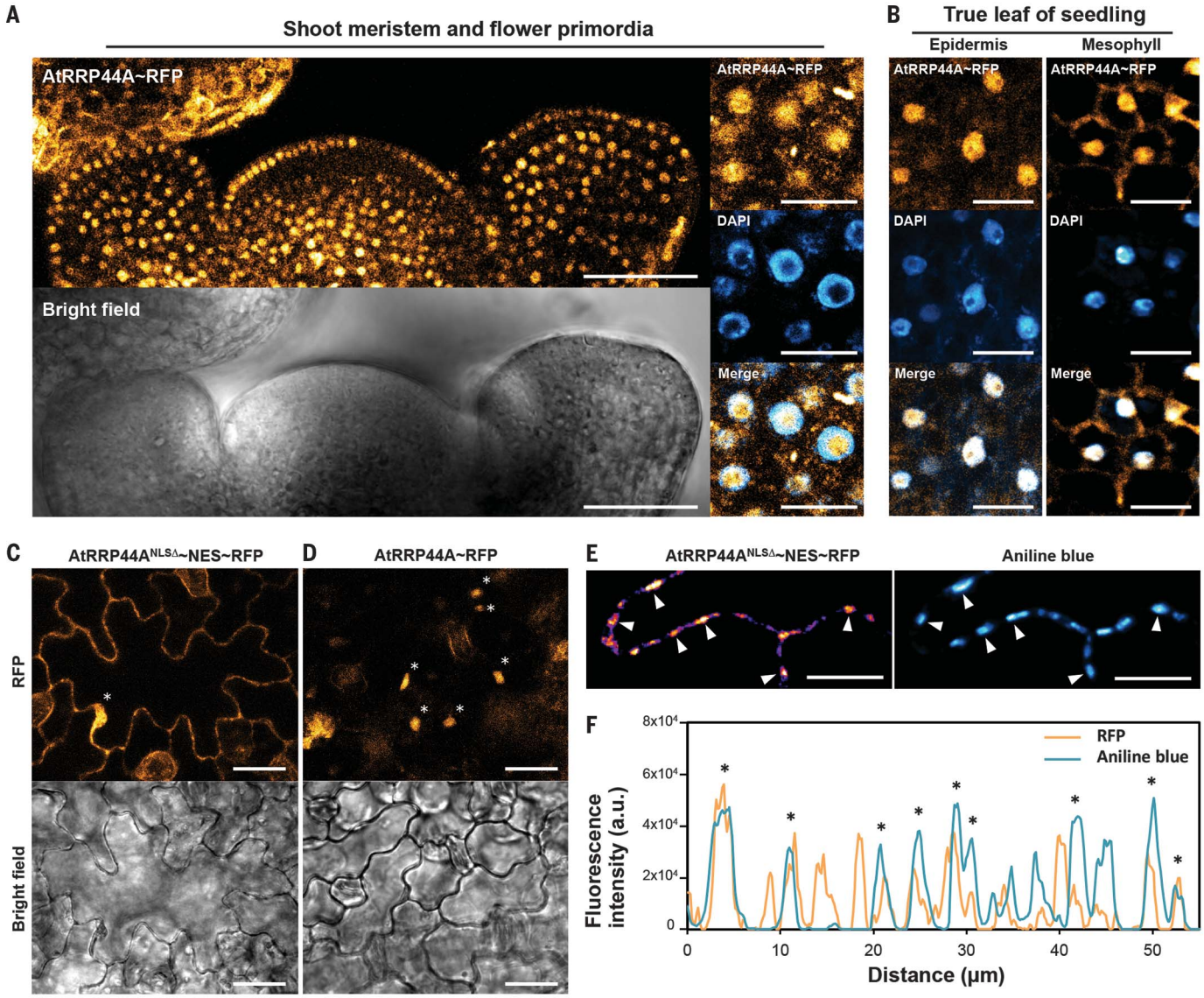

Fig. 3. Meristem expression and plasmodesmata localization of AtRRP44A. (**A** and **B**) AtRRP44A~RFP (orange) was expressed in the shoot meristem and flower primordia (A) and epidermal and mesophyll cells in leaf primordia (B). The fusion protein localized in nuclei in all tissues, as shown by colocalization with 4′,6-diamidino-2-phenylindole (DAPI) [blue in (A) and (B)]. Scale bars, 50 μm [for the left panels in (A)] and 10 μm [for the right panels in (A) and for (B)]. (**C** and **D**) Some of the AtRRP44A$^{NLS\Delta}$~NES~RFP protein (orange) was found outside of the nucleus and localized to the periphery of an epidermal cell (C), whereas native AtRRP44A~RFP protein (orange) was detected only in the nucleus (asterisks) (D). Scale bars, 20 μm. (**E** and **F**) AtRRP44A$^{NLS\Delta}$~NES~RFP (pink and orange) accumulated in puncta at the boundary between epidermal cells and colocalized with aniline blue staining of plasmodesmata (arrowheads) (E). Scale bars, 10 μm. Quantitative analysis indicated that AtRRP44A accumulated in ~75% of plasmodesmata (F). Orange and blue lines show the fluorescence intensities of RFP and aniline blue along cell-cell boundaries, respectively, and asterisks represent overlaps. a.u., arbitrary units.

rescue in the AtRRP44A WT lines expressing mesoMCP~RFP (Fig. 4E). We reasoned that binding of MCP~RFP to GFP~GL1~KN1^{C}~MBS mRNA in mesophyll cells might inhibit its trafficking because of the large size of the mRNP complex, which is similar to a previous study (*38*). Trichome rescue was reduced in mesoMCP lines compared to epiMCP lines or our original trichome rescue lines without the MBS–MCP system (no MCP) (Fig. 4I). GFP~GL1~KN1^{C} accumulation in the epidermis was also consistently decreased in mesoMCP lines compared with that of epiMCP lines (n = 16 plants from two independent transgenic lines) (Fig. 4, J and K). This result suggests that the cell-to-cell trafficking of KN1 mRNA promotes protein trafficking, for example, as an mRNP complex, and/or that the transported mRNA is translated de novo in the epidermis. Other homeodomain proteins also have the capacity to bind their mRNAs (*39*). Therefore, our data support the idea that AtRRP44A-mediated transport of a homeodomain mRNA plays a role in the cell-to-cell trafficking of a KN1 signal, possibly as an mRNP complex.

KN1 mRNA transiently associates with plasmodesmata

To support the idea that KN1 mRNA traffics cell to cell, we investigated whether KN1 mRNA associates with plasmodesmata, like other mobile mRNAs in plants (*40*, *41*). By imaging the

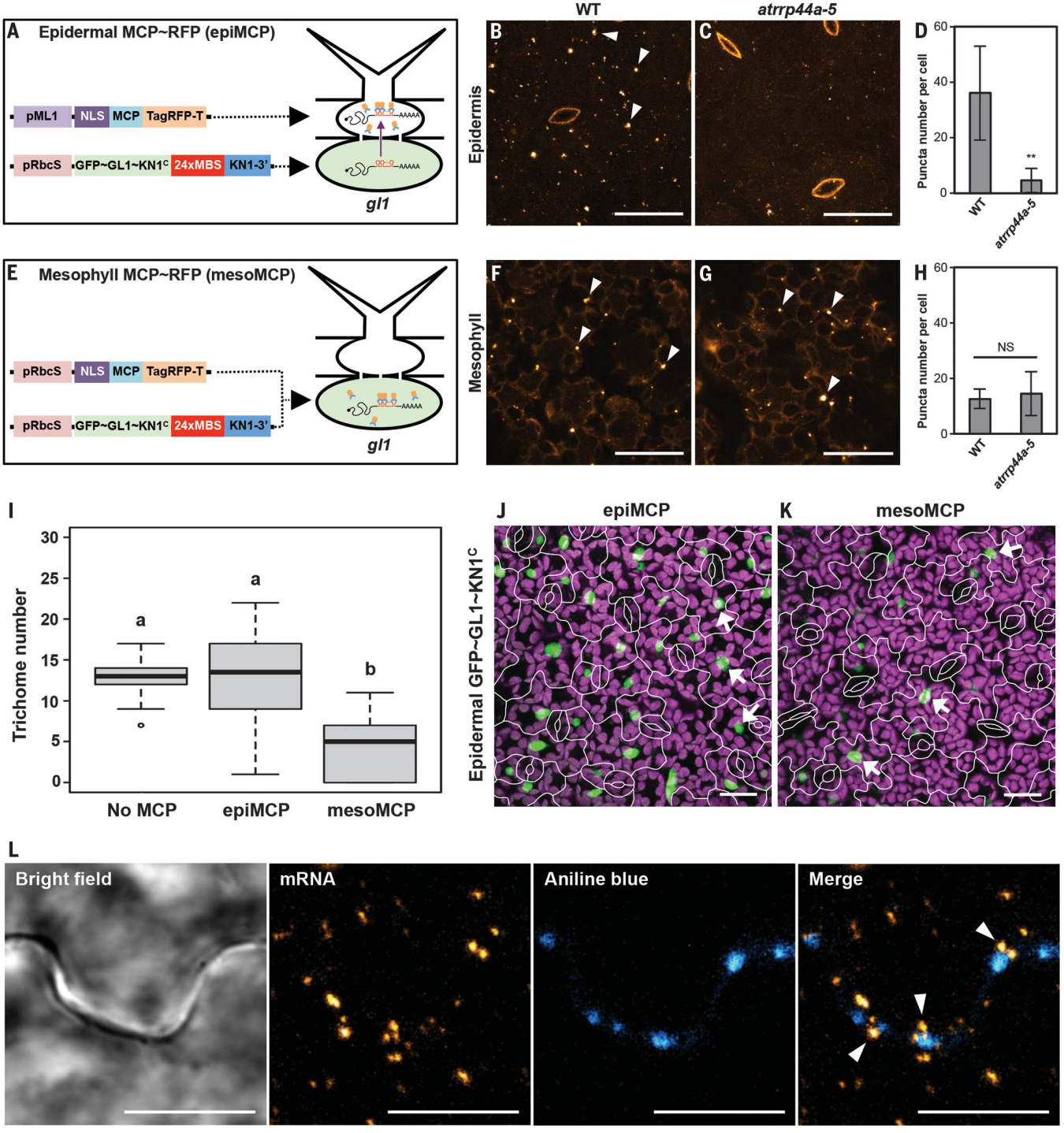

Fig. 4. AtRRP44A mediates KN1 mRNA trafficking. (**A** to **D**) To assay KN1 mRNA trafficking, GFP~GL1~KN1^{C}~MBS mRNA and MCP~RFP were expressed in the mesophyll layer and epidermis, respectively (epiMCP) (A). In lines WT for *AtRRP44A*, KN1 mRNA trafficked to the epidermis and formed many speckles in the cytoplasm of epidermal cells (orange, arrowheads) (B), whereas trafficking was strongly suppressed in *atrrp44a-5* mutants, which had approximately eightfold fewer epidermal fluorescent puncta compared with WT [(C) and (D)]. Scale bars, 20 μm. $^{**}P < 0.01$ (Welch's *t* test). (**E** to **H**) Coexpression of GFP~GL1~KN1^{C}~MBS mRNA and mesoMCP~RFP (E) indicated that *atrrp44a-5* mutants had similar numbers of KN1 mRNA puncta in mesophyll cells as in WT [orange, arrowheads in (F) to (H); $P = 0.145$, Welch's *t* test]. Scale bars, 20 μm. Error bars in (D) and (H) denote SD. NS, nonsignificance. (**I** to **K**) Expression of mesoMCP~RFP significantly reduced trichome rescue compared with epiMCP or lines without MBS–MCP expression (no MCP) (I) and also decreased GFP~GL1~KN1^{C} accumulation in the epidermis (K) in comparison with lines expressing epiMCP~RFP (J). Bars topped by different letters (a and b) are significantly different at $P < 0.01$ (Tukey's honest significant difference test). Arrows point to representative nuclei with GFP~GL1~KN1^{C} fusion protein. Chlorophyll autofluorescence is shown in magenta. White lines show the outlines of epidermal cells. Scale bars, 20 μm. (**L**) GFP~GL1~KN1^{C}~MBS mRNA (orange, arrowheads) associated with plasmodesmata, as shown by colocalization with aniline blue signals (blue). Scale bar, 10 μm.

interface between epidermal cells, we found colocalization of GFP~GL1~KN1^{C}~MBS mRNA with aniline blue–stained plasmodesmata (Fig. 4L). By using time-lapse imaging, we found that KN1 mRNA puncta moved freely through the cytoplasm until they "met" a plasmodesma, where they paused for ~1 to 6 min before leaving (movies S1 and S2). We also observed KN1 mRNA–plasmodesmata interactions at the interface of epidermal and mesophyll cells (movie S3) and between leaf epidermal cells when expressed in a heterologous tobacco system (movie S4). Thus, our data support the idea that KN1 mRNA associates with plasmodesmata to traffic from cell to cell.

We demonstrate a role of KN1 mRNA trafficking in the selective transport of a KN1 signal and the role of AtRRP44A in this process (fig. S9). This role of AtRRP44A appears to be independent of its function in RNA metabolism and may involve additional factors that prevent its RNA-processing activity, despite its binding to the mobile homeodomain mRNA. Our finding that trafficking of maize KN1 mRNA is mediated by AtRRP44A in *Arabidopsis* suggests that this mechanism is required for plant stem cell function in diverse plants. Whether RRP44A functions in transport of other plant mobile RNAs remains to be seen.

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SUPPLEMENTARY MATERIALS

science.org/doi/10.1126/science.abm0840
Materials and Methods
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View/request a protocol for this paper from *Bio-protocol*.

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