Supplementary Information for

Plant ESCRT Protein ALIX Coordinates with Retromer Complex in Regulating Receptor Mediated Sorting of Soluble Vacuolar Proteins

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Materials and Methods

Plasmid construction

Double restriction enzyme digestion (Takara) or recombination (Vazyme) methods were performed to clone corresponding genes to plasmids. For the constructs used for yeast two hybrid analysis, target genes were cloned into pGBK7 and pGADT7 vectors by double digestion or recombination. To generated constructs for transient expression in Arabidopsis protoplast, corresponding genes were amplified and cloned into pBI221 vectors modified to containing EYFP, Cerulean, mRFP, 3×Myc, 3×HA, or 5×Myc tags under 35S or UBQ10 promoter (1). For the constructs used in expression of recombinant proteins, PCR products were cloned into pGEX6P-1 (GE) vector via double restriction enzyme digestion. The constructs His-SUMO-BRO1D, His-SUMO-ALIXD, and His-SUMO-PRR were generated as described previously (2). For making Vps26Apro::gVps26A-GFP/mCherry, Vps29pro::gVps29-mCherry and AtVSR1pro::GFP-gAtVSR1 transgenic plants, the native promoter and genomic DNA of indicated genes were fused with fluorescent proteins and cloned into pBI121 or pCAMBIA1300 by recombination method. All constructs were verified by Sanger sequencing. Primers used for plasmid construction, genotyping, or RT-PCR are listed in Table S1.

Plant materials

The Arabidopsis T-DNA insertional mutants vps26a-1 (GABI_311E08), vps26b-1 (SALK_142592) and vps29-3 (SALK_010106) were obtained from TAIR and screened by genotyping PCR. For the ALIX mutant, the alix-1, alix-2 and DEXpro::ALIX-RNAi transgenic plant was generated as described previously (2, 3), while alix-4 (SALK_063124) was obtained from NASC. Double mutants were generated by crossing and screened by genotyping PCR. To generate the transgenic plants, all of the plasmids were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into WT Col-0 by floral dip. Arabidopsis WAVE lines stably expressing fluorescent protein-tagged organelle markers for protein subcellular localization were obtained from NASC. Double fluorescent tagged transgenic plants in WT indicated mutants were generated by crossing.

Plant growth and chemical treatments

Surface sterilized Arabidopsis seeds were grown on plates with half-strength Murashige and Skoog (MS) medium (pH 5.7) with 1% (w/v) sucrose and 0.8% (w/v) agar at 22 °C under a long day (16 h light/8 h dark) photoperiod. For phenotype analysis, 6 days old seedlings on plates or 24 days old plants on soil were observed and quantified. For DEX
induction, seeds were sown directly on half-strength MS agar plate with or without 10 μM DEX for indicated time before CLSM observation or protein extraction. FM4-64 dye uptake experiments and image collection were performed as previously described (1). Wortmannin (Wort) was dissolved in dimethyl sulfoxide (DMSO) and added in the liquid medium at 33 μM for 2h and 10 μg/mL for 1 h with seedlings or developing embryos before CLSM observation, respectively.

**Transient expression in Arabidopsis**

The Arabidopsis suspension cell, plant system biology dark type culture (PSB-D) was kept by subculture every 5 days. The detail procedures for protoplasts isolation and transient expression were described previously (1, 4). The protoplasts transferred with indicated plasmids were then incubated at 23°C for 12 h before confocal imaging analysis or protein extraction.

Agrobacterium mediated transient gene expression in Arabidopsis seedlings was described previously (5). Briefly, Agrobacterium strain PMP90 containing pCambia1300-UBQpro::aleurain-mRFP binary vector cultured at 28 ℃, 200 rpm for 20 h was harvested and the concentration was adjusted to OD₆₀₀=2 in 5% (w/v) sucrose liquid medium. The 4 days seedlings with indicated genotype were submerged in Agrobacterium culture and vacuum infiltrated two times for 1 minutes. Then the Agrobacterium solution was discarded and the seedlings were transferred to half strength MS medium for further 3 days incubation before confocal microscopy imaging.

**Immunofluorescence labeling in Arabidopsis roots**

The roots collected from 5-d-old seedlings were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) buffer plus 0.1% (v/v) Triton X-100. Then, the samples were digested, permeabilized and blocked by 2% (w/v) driselase, 3% (v/v) Nonidet P-40 supplement 10% (v/v) DMSO, and 3% (w/v) bovine serum albumin, respectively. The roots were then incubated with anti-VSR antibody at 4°C overnight. After washing by PBS three times, the roots were probed with Alexa 568 goat anti-rabbit IgG (Invitrogen) antibody for confocal observation (6).

**Confocal microscopy and FRET analysis**

The 5-d-old Arabidopsis seedlings or developing embryos in silique from 8 weeks plant with indicated genotypes were collected and imaged with Leica TCS SP8 confocal laser-scanning microscope. The ×63 (NA 1.20) water immersion objective with a sequential acquisition was used for imaging. For each experiment, 10 individual seedlings or embryos were observed and confocal imaging that represent >75% of the roots and embryos revealing similar expression levels and patterns. Images were
processed using Adobe Photoshop software (http://www.adobe.com). The Pearson coefficients plug-in (Coloc 2) in Image J (Wayne Rasband, NIH, https://imagej.nih.gov/) was used to determine the colocalization relationships between two fluorescent tagged proteins. The range of $r$ value is between -1 and +1, where 0 indicates no correlation, -1 and +1 indicate perfect anti-correlation or perfect correlations, respectively. To determine the colocalization percentage of the GFP-ALIX with Vps26A-mCherry or Vps29-mCherry in embryo or seedling, the total punctae number of Vps26A-mCherry or Vps29-mCherry and the punctae that were colocalized with GFP-ALIX vesicles were manually counted (2, 3, 7). For the quantification of the distribution percentage of Vps26A-GFP, Vps29-GFP or GFP-ALIX among different organelles, the total punctae number of organelle markers (mCherry-Rha1, VHA-a1-RFP and mCherry-SYP32) and the punctae that were colocalized with Vps26A-GFP, Vps29-GFP or GFP-ALIX were counted in embryo or seedling. The total number of quantified punctae was indicated in the corresponding figure legends or figures. False color code depicts relative fluorescent intensity. Colocalization and punctae number were quantified from at least five individual roots.

Fluorescent Resonance Energy Transfer (FRET) acceptor bleaching analysis was performed on the Leica SP8 confocal system according to the manufacturer’s instructions. The protoplasts isolated from PSBD cell that transiently expressed Cerulean and EYFP fusion proteins were used for photobleaching at 514 nm laser with 200Hz speed in full power intensity. The signal intensity of Cerulean donor fluorescence was imaged and documented before and after photobleaching a region of interest of EYFP fusions to <10% of initial intensity. FRET efficiency was calculated as $\text{FRET} = 100 \times \frac{D_{\text{post}} - D_{\text{pre}}}{D_{\text{post}}}$, where $D_{\text{pre}}$ and $D_{\text{post}}$ represent the fluorescence intensity of Cerulean and its fusions before and after bleaching, respectively. FRET efficiency quantification and statistical analysis were obtained from at least 10 protoplasts. The Cerulean-EYFP fusions linked by 18 amino acid (SSSELSGDEVGGTSGSEF) was used as positive control and the co-expressed free Cerulean and EYFP were used as negative control.

**Yeast two-hybrid (Y2H) analysis**

Y2H analysis was performed according to the manufacturer’s instructions using MatchMaker GAL4 Two-Hybrid System 3 (Clontech). For the pairs analysis of Y2H, the indicated cDNAs of genes were cloned into pGBK7T and pGADT7 vectors and co-transformed into yeast AH109 by heat shock method. Positive transformants were selected on synthetic drop-out agar medium without Trp and Leu (SD-2) at 30°C for three days. The interactions of positive colonies were screened on SD medium lacking His, Trp, and Leu (SD-3) and SD medium lacking Ade, His, Trp, and Leu (SD-4)
containing 0–10 mM 3-amino-1,2,4-triazole (3-AT) for 3 days. Each experiment was conducted at least two times independently and similar results were obtained.

**Protein preparation**

To extract protein from protoplasts, protoplasts transformed with indicated plasmids were first washed with 250 mM NaCl and followed by centrifugation at 100 g for 10 min to collect cells. Cells were resuspended in ice cold lysis buffer containing 50mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 5\% (v/v) glycerol, 0.5\% (v/v) Triton X-100, and 1×Complete Protease Inhibitor Cocktail (Roche Diagnostics, CA). The total cell extracts were then centrifuged at 16,000 g for 10 min at 4°C followed by adding SDS loading buffer.

For the protein extracted from transgenic plants or seeds, 5-d-old Arabidopsis seedlings or dry seeds were grinded in liquid nitrogen followed by adding lysis buffer containing 1\% (w/v) SDS and then centrifuged at 16,000 g for 10 min at 4°C. Then the supernatant was extracted and boiled in sample loading buffer at 100°C for 10 min and performed immunoblotting on 12\% (w/v) SDS-PAGE gels. For the CS and CM fraction isolation, 7-d-old seedlings were ground in ice-cold extraction buffer (40 mM HEPES-KOH at pH 7.5, 1 mM EDTA, 10 mM KCl, 0.4 M sucrose, 0.5 mM phenylmethanesulfonyl fluoride, 25 μg/mL leupeptin, and 1× Complete Protease Inhibitor Cocktail) and centrifuged at 700 g for 3 min to remove the cellular debris. Then the supernatant was further ultracentrifuged at 100,000 g for 60 min at 4°C to separate the soluble fraction and membrane fraction.

**Immunoprecipitation and immunoblotting**

Transformed protoplasts were first diluted with 3 folds volume of 250mM NaCl and harvested by centrifugation at 100 g for 5 min. The protoplasts were then resuspended in cold IP buffer (50 mM of Tris-HCl, pH 7.4, 150 mM of NaCl, 0.5 mM of EDTA, 5\% (v/v) glycerol, 0.2\% (v/v) Triton X-100, and 1×Complete Protease Inhibitor Cocktail) and lysed by passing through a 1-mL syringe with needle. The total cell lysates were then centrifuged at 700 g for 5 min at 4°C to remove intact cells and large cellular debris followed by 14,000 rpm centrifugation for 30 min at 4°C. The supernatant was then mixed with IP buffer containing 0.08\% (v/v) Triton X-100 and incubated with GFP-Trap beads (ChromoTek)) at 4°C in a top to end rotator for 4 h followed by 5 times wishing with cold washing buffer containing 0.05\% (v/v) Triton X-100. Then, the samples were boiled in 1×SDS sample loading buffer.

For the immunoblotting, protein samples were separated in 12\% (w/v) SDS-PAGE gels and transferred to nitrocellulose membrane membranes (Bio-Rad) followed by blocking in PBST with 5\% (w/v) milk and followed by appropriate antibodies
incubation. The Clarity Western ECL substrate solutions (Bio-Rad) were used to detect the luminescence.

**Recombinant protein purification and in vitro binding assay**
The expression of GST-Vps26A, GST-Vps26B or GST-Vps29 recombinant proteins in *Escherichia coli* BL21 (DE3) pLysS strain were induced by 0.2 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) at 18 °C for 10 h, followed by purification using the GST Spin Trap Kit (GE). The expression and purification of His-SUMO-BRO1D, His-SUMO-ALIXD, and His-SUMO-PRR recombinant proteins were described in previously research (2). All the proteins eluted and dialyzed against pull-down buffer (50 mM Tris-HCl, 100 mM NaCl, and 10% glycerol, pH 7.5) followed by protein concentrations determination using Bio-Rad Protein Assay (cat. no. 5000006) and Coomassie Blue staining with using the bovine serum albumin as a standard.

For the in vitro binding assay, pre-cooled nickel-charged affinity resin was saturated with His-SUMO-BRO1D, His-SUMO-ALIXD, and His-SUMO-PRR. Then, the resin bound His-SUMO-BRO1D, His-SUMO-ALIXD, and His-SUMO-PRR were incubated with 30 pmol GST, GST-Vps26A or GST-Vps29 in 200 μL cold pull-down buffer containing 0.3% (v/v) Triton X-100 for 2 h at 4 °C in a top to end rotator, respectively. The resin was then washed 10 times with cold binding buffer containing 0.1% (v/v) Triton X-100 followed by boiling in SDS sample buffer, separated by SDS-PAGE for immunoblot using indicated antibodies.

**Transmission electron microscopy study**
The samples used in transmission electron microscopy study were prepared previously (2). Immunogold labeling was performed as previously described with anti-VSR (40 μg/mL) (2), anti-12S globulin (dilution 1:2000) and anti-2S albumin (dilution 1:2000) (8), and gold-coupled secondary antibody was used at a 1:50 dilutions. TEM observation was performed via Hitachi H-7650 transmission electron microscope with a charge-coupled devise camera operating at 80 kV (Hitachi High-Technologies Corporation, Japan).

**Antibodies**
The primary antibodies, anti-cFBPase (Agrisera, AS04 043), anti-Myc (Santa Cruz, SC-789), and anti-HA (Abcam, ab18181), anti-GST (BoAo, B1024), anti-His (Abclonal, AE003), anti-Vps26A (Agrisera, AS13 2632) were purchased from commercial companies. The anti-12S globulin, anti-2S albumin, anti-GFP, and anti-VSR were described previously (8, 9).
RNA extraction and RT-PCR analysis
Total RNA was extracted from 5-d-old seedlings using the Eastep Super RNA Extraction Kit (Promega, LS1040) according to manufacturer’s instructions. The first strand for each complementary DNA was synthesized by Monscript RTIII All-in-One Mix Kit (Monad, MR05101).

Quantification and statistical analysis
No statistical methods were applied to predetermine samples or outcomes. When the negative or positive controls were not working, the data were excluded. Sample numbers and the number of biological replicates for each experiment are indicated in the figure legends or above. Data are presented as mean values ± S.D. Two-tailed Student’s \( t \) test was used when data met criteria for parametric analysis. Differences in means were considered statistically significant at \( P<0.05 \). Significance levels are: *\( P<0.05 \); **\( P<0.01 \); and ***\( P<0.001 \). One-way ANOVA test followed by Tukey’s multiple test was applied in multiple samples significant differences calculation with SPSS software. All the experiments were repeated at least three times, independently.

Accession numbers
The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are \textit{ALIX} (AT1G15130), \textit{Vps26A} (AT5G53530), \textit{Vps26B} (AT4G27690), \textit{Vps29} (AT3G47810), \textit{AtVSR1} (AT3G52850), \textit{Vps35A} (AT2G17790), \textit{Vps35B} (AT1G75850), \textit{Vps35C} (AT3G51310), \textit{SNX1} (AT5G06140), \textit{SNX2A} (AT5G58440), \textit{SNX2B} (AT5G07120), \textit{Vps2.1} (AT2G06530), \textit{Vps20A} (AT5G63880), \textit{Vps24} (AT5G22950), \textit{Vps28} (AT4G21560), \textit{SNF7A} (AT2G19830), \textit{SNF7B} (AT4G29160), \textit{FYVE4} (AT1G61690), \textit{FREE1} (AT1G20110) and \textit{AMSH1} (AT1G48790).
Fig. S1. ALIX mutants and their defectives in transport of vacuolar proteins.

(A) A diagram of ALIX genomic region showing the position of the EMS-induced mutation in alix-1 plants and that of T-DNA insertions in alix-2 and alix-4 mutants.

(B) Knock down of ALIX by DEX-inducible RNAi causes seedling lethality. Seven-day-old seedlings of WT and DEX::ALIX-RNAi plants germinated on the plates with (+) or without (-) DEX were photographed. Scale bar, 1cm.

(C) Genotyping showed that embryos from the paler seeds of the alix-2 (+/-) and alix-4 (+/-) progenies correspond to homozygous mutants. The genomic DNA from pools of 15 embryos were extracted for analysis. LP, left genomic primer. RP, right genomic primer. BP, T-DNA border primer.

(D) The GFP-CT24 in developing seeds of the progenies derived from the self-pollinated alix-2 (+/-) or alix-4 (+/-) mutant siliques. Note that the paler seeds showed green fluorescent signal under UV light. Scale bars, 10 μm

(E) Confocal images of GFP-CT24 in developing embryo cells of the WT and alix mutants. Note the signal of GFP-CT24 (green) was shown in the extracellular spaces in alix mutants. Scale bars, 10 μm

(F) Phenotypes of the F2 population of alix-1(+/−) VAC2(+/−). Scale bars, 1 cm
Fig. S2. Mapping interactions between Arabidopsis ESCRT components and retromer subunits.

(A) Analysis of proteins co-immunoprecipitated with GFP or GFP-ALIX. Seedlings of GFP-ALIX or GFP-expressing plants were grown on MS for 5 days. Co-immunoprecipitation experiments were performed with total protein extracts from seedlings. Immunoprecipitates were analyzed by performing SDS/PAGE followed by silver staining. Black arrow (band 1) indicates the purification of GFP or GFP-
ALIX. White arrow (bands No. 2 and 3) indicates the bands for following tandem mass spectrometry analysis.

(B) Y2H analysis of the binary interactions of ALIX with SNXs (SNX1 and SNX2b). Transformed yeast cells were grown on either synthetic complete medium lacking leucine and tryptophan (with histidine, +His) as a transformation control, or synthetic complete medium lacking leucine, tryptophan, and histidine (without histidine, −His) for interaction assays.

(C) Y2H analysis of the binary interactions of Arabidopsis Vps26A, Vps26B, or Vps29 with ESCRT-III components (Vps2.1, Vps24, Vps20A, SNF7A, SNF7B, and FYVE4), FREE1, deubiquiting enzyme AMSH1, or ALIX. Transformed yeast cells were grown on either synthetic complete medium lacking leucine and tryptophan (with histidine, +His) as a transformation control, or synthetic complete medium lacking leucine, tryptophan, and histidine (without histidine, −His) for interaction assays.

(D) Immunoprecipitation (IP) assay shows association between ALIX and Vps26A, Vps26B, or Vps29. Arabidopsis protoplasts expressing EYFP, EYFP-Vps26A, EYFP-Vps26B, EYFP-Vps29, or EYFP-Vps28A with Myc-tagged ALIX were subjected to protein extraction and IP with GFP-trap followed by immunoblot with indicated antibodies.

(E) Purified recombinant proteins which were subjected to in vitro binding assay.

(F) In vitro binding assays of recombinant His-SUMO-BRO1D, His-SUMO-ALIXD, or His-SUMO-PRR with GST (lane 1), GST-Vps26A (lane 2), or GST-Vps29 (lane 3). Anti-His and anti-GST antibodies were used to detect bead-retained material. Arrows indicate GST-Vps26A and GST-Vps29 proteins pulled down by His-SUMO-BRO1D.
Fig. S3. Colocalization analysis of Vps29 with endosomal markers in embryo cells.

(A) Colocalization of Vps29-GFP with the MVB/PVC marker mCherry-Rha1 (left), TGN/EE marker VHA-a1-RFP (middle), and the Golgi marker mCherry-SYP32 (right) were analyzed with a confocal microscope in Arabidopsis embryo cells. Scale bars, 10 μm

(B) Percentage of Vps29-GFP positive punctae that colocalize with endosomal markers shown in A. The results were obtained from 7 individual embryos. Error bars represent the S.D. of percentages. n, total numbers of analyzed Vps29-GFP punctae.
Fig. S4. Colocalization analysis of Vps26 and Vps29 with endosomal markers in root epidermal cells.

(A) Colocalization of Vps26A-GFP with the MVB/PVC marker mCherry-Rha1 (top), TGN/EE marker VHA-a1-RFP (middle), and the Golgi marker mCherry-SYP32 (bottom) were analyzed with a confocal microscope in Arabidopsis root epidermal cells. Colocalization relationship was calculated by Pearson correlation. Scale bars, 20 μm.

(B) Colocalization of Vps26A-GFP with MVB/PVC marker mCherry-Rha1 after wortmannin treatment (+Wort). Scale bar, 20 μm.

(C) Colocalization of Vps29-GFP with the MVB/PVC marker mCherry-Rha1 (top), TGN/EE marker VHA-a1-RFP (middle), and the Golgi marker mCherry-SYP32 (bottom) were analyzed with a confocal microscope in Arabidopsis root epidermis cells. Colocalization relationship was calculated by Pearson correlation. Scale bars, 20 μm.

(D) Colocalization of Vps29-GFP with MVB/PVC marker mCherry-Rha1 after wortmannin treatment (+Wort). Scale bar, 20 μm.
Fig. S5. Subcellular localization analysis of GFP-ALIX in plants.

(A) Colocalization of GFP-ALIX with the MVB/PVC marker mCherry-Rha1 (left) and TGN/EE marker VHA-a1-RFP (right) were analyzed with a confocal microscope in Arabidopsis developing embryo cells. The percentage of GFP-ALIX with mCherry-Rha1 or VHA-a1-RFP colocalization is included in the bottom. The results were obtained from 5 individual embryos. n, total numbers of analyzed GFP-ALIX punctae. Scale bars, 10 μm.

(B) Colocalization of GFP-ALIX with the MVB/PVC marker mRFP-AtVSR2 was analyzed with a confocal microscope in protoplasts of Arabidopsis suspension cells. The percentage of colocalization is included in the bottom. n, total numbers of analyzed GFP-ALIX punctae. Scale bars, 10 μm.

(C) Colocalization of GFP-ALIX with Vps26A-mCherry (left) or Vps29-mCherry (right) in root epidermis cells. The percentage of GFP-ALIX with Vps26A-mCherry or Vps29-mCherry colocalization is included in the bottom. The results were obtained from 5 individual embryos. n, total numbers of analyzed GFP-ALIX punctae. Scale bars, 10 μm.
Fig. S6. Phenotype analysis of indicated genotype of Vps26, Vps29 and ALIX plants.

(A) Phenotype of 7-day-old seedlings (left) and 24-day-old plants on soil (right) of vps26a-1 vps26b-1 double mutants, vps29-3, and complementation plants with indicated GFP fusions. Scale bars, 1 cm.

(B) Phenotype of 24-day-old plants of the indicated genotype. Scale bar, 1 cm.
Fig. S7. ALIX regulates the association of Vps26 and Vps29 to endosomes.

(A) RT-PCR analysis of Vps26A and Vps29 transcript levels. Total RNA from 7-day-old DEX treated WT or DEX::ALIX-RNAi plants was used for quantitative RT-PCR analysis using gene-specific primers. Actin was included as internal controls for quantitative RT-PCR.

(B) Fluorescence intensity of Vps26A-GFP and Vps29-GFP in the embryo cells of WT or alix-2. False color code was used for intensity visualization. Scale bars, 10 μm.

(C) Quantification (mean ± SD) of the number of Vps26A-GFP and Vps29-GFP punctuated structures per 25×25 μm² in embryo cells of WT and alix-2. n, total numbers of analyzed Vps26A-GFP or Vps29-GFP punctae. **P < 0.01, ***P < 0.001 in Student’s t-test. Note that reduction of the number of Vps26A-GFP and Vps29-GFP vesicles in alix-2.

(D) Localization of Vps26A-GFP or Vps29-GFP and TGN marker VHA-a1-RFP in root epidermis cells of DEX treated WT or DEX::ALIX-RNAi plant. Scale bars, 10 μm.

(E) Pearson colocalization coefficients of VHA-a1-RFP with Vps26A-GFP or Vps29-GFP signals in WT and ALIX-RNAi plant as shown in D. The results were obtained from 10 individual seedlings. ***P < 0.001 in Student’s t-test.

(F) Localization of Vps26A-GFP or Vps29-GFP and Golgi marker mCherry-SYP32 in root epidermis cells of DEX treated WT or DEX::ALIX-RNAi plant. Scale bars, 10 μm.
(G) Pearson colocalization coefficients of mCherry-SYP32 with Vps26A-GFP or Vps29-GFP signals in WT and ALIX-RNAi plant as shown in F. The results were obtained from 10 individual seedlings. n.s., P > 0.05 in Student’s t-test.
Fig. S8. Colocalization analysis of GFP-AtVSR1 with endosomal markers in root cells in ALIX-RNAi mutant.

(A) Colocalization analyzed of GFP-AtVSR1 with the MVB/PVC marker mCherry-Rha1 in the root epidermal cells of DEX::ALIX-RNAi seedlings without (-) or with (+) DEX induction, respectively. Scale bars, 10 μm.

(B) Colocalization analyzed of GFP-AtVSR1 with the TGN/EE marker VHA-a1-RFP in the root epidermal cells of DEX::ALIX-RNAi seedlings without (-) or with (+) DEX induction, respectively. Arrowheads indicate the mis-localization of GFP-AtVSR1 signals at PM. Scale bars, 10 μm.
Fig. S9. Immunoelectron micrograph of WT and *alix*-2 embryo cells with anti-2S albumin.

(A) Ultrathin sections prepared from high-pressure frozen/freeze-substituted developing embryo cells of *alix*-2 were labelled anti-2S albumin antibody. Note the accumulation of large clusters of vesicles labelled with anti-2S albumins. Scale bar, 500 nm.

(B) Enlarged image of the white outline area showing the gold particle labelling.
Fig. S10. Working model of ALIX function at the endosome membrane.

ALIXs are cytoplasmic proteins that can be recruited to membrane of MVB/PVCs and TGN via (A) the ESCRT components SNF7 or (B) retromer core complex in WT plant.

(A) In WT plants, ALIX can recruit de-ubiquitin enzymes AMSHs as canonical ESCRT proteins for sorting membrane proteins and regulating MVB/PVCs biogenesis.

(B) ALIX forms homodimer and interact with the Vps26 and Vps29 through the Bro1 domain, thus regulating the stability and membrane association of retromer core complex. The retromer subunit Vps35 interacts with the C-terminus of VSRs in the recycling process.

(C) In alix mutant plants, retromer core complex has lower stability and membrane association, compared to that in WT, thus leading to the mis-localization of the VSR to PM and co-secretion of soluble vacuolar cargos.

Hence the plant ALIX functions as a retromer regulator in regulating VSRs-mediated soluble vacuolar cargos sorting.
Table S1. Primers used for plasmid construction, genotyping or RT-PCR.

| Construct name | Primer name | Sequence (from 5' start to 3' end) |
|----------------|-------------|-----------------------------------|
| vps26a-1       | Q1243-vps26a-1-F | AGTTGATTCTCGCCGTCTTTATCTC |
| Genotyping     | Q1244-vps26a-1-R | TTTTGTTCTACAGTAGTCG |
| vps26b-1       | Q1247-vps26b-1-F | AGGTACGGAACATCGCTCCAC |
| Genotyping     | Q1248-vps26b-1-R | CGGAATCCAGAGAAAAAGGAAC |
| vps29-3        | JA524-vps29-3-LP | GCGGCTGATCTACCTCTCTAAG |
| Genotyping     | JA525-vps29-3-RP | CAAAATAACAAAGCGAGCCAAC |
| alix-2         | alix-2-LP     | AGAATAGAGAAAGACCTCCCG |
| Genotyping     | alix-2-RP     | TGTTTGGTTTCGCGTTTTTC |
| alix-4         | alix-4-LP     | ATCCAAACACACTACAACCGAC |
| Genotyping     | alix-4-RP     | GAAAGTCCCTCTCAAGGCCCT |
| Vps26a-1       | JB79-vps26a-F | ATGAATTATCTTTGAGGC |
| RT-PCR         | JB80-vps26a-R | TCAAGATGCTCTTCCTCGAG |
| Vps29-3        | JB82-vps29-F | ATGGTGCTGGTATGGCATT |
| RT-PCR         | JB83-vps29-R | CTACGGACCAGAGCTTGAG |
| pGBK7/pGADT7-ALIX | JA407-AtBro1-F-EcoRI | cgccgatccATGGCTCTCTCAGCATCCTCAAT |
| ALIX point mutation | JA408-AtBro1-R-BamHI | cgccgatccTATTGCCTGATGATCCTTCCAC |
|                | JA409-alix-G260A-F | TCATGTCAGCTGAAAAGCGACACTCTCTCTATG |
|                |               | ATGAAGCTTGGTTTAGATATGGAAAAAGA |
|                | JA410-aliX-G260A-R                                   | TCTTTTCCATATCTAAAGCAAGGCTTCTCATCAGAAGAGTGCTGCTTTTCAGCTGCACATGA |
|----------------|-----------------------------------------------------|-----------------------------------------------------------------|
| pDONR-Vps26A  | N278-VPS26a-F                                      | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N279-VPS26a-R                                      | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-Vps26B  | N280-VPS26b-F                                      | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N281-VPS26b-R                                      | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-Vps29   | N282-VPS29-F                                       | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N283-VPS29-R                                       | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-Vps35A  | N284-VPS35a-F                                      | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N285-VPS35a-R                                      | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-Vps35B  | N286-VPS35b-F                                      | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N287-VPS35b-R                                      | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-Vps35C  | N288-VPS35c-F                                      | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N289-VPS35c-R                                      | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-SNX1    | N290-SNX1-F                                        | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N291-SNX1-R                                        | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pDONR-SNX2A   | N292-SNX2a-F                                       | TACAAAAAACAGGCTTGTAGAATTATCTTTCTGAGGCTTTCAAGCC                  |
|                | N293-SNX2a-R                                       | CAAGAAAGCTGGGTAAAGATGATGTCTTCTTCTTGACACCATCAA                  |
| pGBKT7/pGADT7- | JA608-SNX2b-F-NdeI                                 | gggCATATGATGATGGGCTCAGAGAATGAC |
| SNX2B         |                                                     |                                                                  |
| JA609- SNX2b-R-BamHI | gggGGATCCCTTAAGAGCTCTCTCTATCGTATTG |
|----------------------|-----------------------------------|
| pGEX-6P-1-Vps26A     | JA452- VPS26a/b-F-BamHI-Spel     |
| JA455- VPS26a-R-XhoI  | cgcctgagTCAAGATGTCTCTCTTGGAG    |
| pGEX-6P-1-Vps26B     | JA456- VPS26b-R-XhoI             |
| JA453- VPS29-F-BamHI  | cgcctgagCTACGGACCAGAGCTGGTAG    |
| pGEX-6P-1-Vps29      | JA454- VPS29-R-XhoI              |
| JA407- AtBro1-F-EcoRI | cgcctgagATGGCTTTCTTCTGCTCTCTAAT  |
| pMAL-C2X-MBP-ALIX     | JA408- AtBro1-R-BamHI            |
| JA407- AtBro1-F-EcoRI | cgcctgagTCATTGCCTGTAGATCTCCAC   |
| pBI121-Vps26Apro: :gVps26A-GFP | R95-pBI121-VPS26A |
| R97- VPS26A- GFP      | ctatgaccatgatcgccaagcttAGCTCGTTTATGTGAGGACTAT |
| R96-GFP- VPS26A       | CAGGCTCAAGAGAAGACATCTATGAGTAAA GGAGAAGAICTTTTCAC |
| R94-pBI121-GFP/mcherr y | GTGAAAAAGTTTTCTCCTTCTCTATGAAGTGCCTCTGAGCCCTG |
| pBI121-Vps26Apro: :gVps26A-GFP | R95-pBI121-VPS26A |
| R98-                  | cgcctgagATGGCTTTCTTCTATGAGGACTAT |
|                       | GGCTCAAGAGAAGACATCTATGGTGAGCAA  |
| Construct                      | Description                                             | Sequence                                                                 |
|-------------------------------|---------------------------------------------------------|--------------------------------------------------------------------------|
| mCherry                       | Vps26A-mcherry                                         | GGGCGA                                                                   |
|                               | R99-mcherry-Vps26A                                     | TCGCCCTTGCTCACCATAGATGTCTCTTCCCTT GAGCC                                  |
|                               | R94-pBI121-GFP/mcherry                                 | cgatcggggaatctgctgctTTACTTTGACAGCTCGTCGTCATG                             |
| pBI121-Vps29pro::g Vps29-mCherry | R91-pBI121-VPS29                                      | ctatgaccatgattacgcaagctgATTCcatctcctctctctgattgctgatcactaacgt          |
|                               | R92-VPS29-mcherry                                      | CTACCAGCTCTTGTGCTAGATGGTGAAGCAA GGGCGA                                  |
|                               | R93-mcherry-VPS29                                      | TCGCCCTTGCTCACCATCTACGGACCAGAGCT GGTAG                                  |
|                               | R94-pBI121-GFP/mcherry                                 | cgatcggggaatctgctgctTTACTTTGACAGCTCGTCGTCATG                             |
| pCAMBIA1300-AtVSR1pro::GFP-gAtVSR1 | pVSR1_SP_GFP_gDNA F                                    | agctatgaccatgattacgAATTCCatctcctctctctctctgattgctgatcactaacgt          |
|                               | pVSR1_SP_GFP_gDNA R                                    | GCTCAACCATACCCATTGGAGATTCAAGATCA GAGAAACGAGAGAGT                         |
|                               | VSR1_GFP_gDNA F                                        | GCAATGGGTATGGTGAGCAAGGGCGAGGAGC                                         |
|                               | VSR1_GFP_gDNA R                                        | CAACGAATCTCTTTGTACAGCTCGTCATGCGCAAGGAGAGAGT                              |
|                               | GFP_VSR1_gDNA F                                        | CTGTACAAGAGATTCGGTTGGAGAAGAACA ATCTAAGGTTACATACCT                        |
|                               | GFP_VSR1_gDNA R                                        | taaaacgagccagctgcaAGCTTaatctctaatcaagtttattg                              |
| pCAMBIA1300-UBQ10-aleurain-mRFP | JB229-Aleu-BamHI-F                                     | gggGGATCCCGCTGGTTACAAATGT                                               |
|                               | JB238-Aleu-KpnI-R                                      | gggGTACCTCGCGTTACGGTTTTCCAGTTC                                          |
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