Supplemental Information Titles and Legends

Extended Data Fig. 1 Images show the various steps in plant EVs isolation.

a, Leaves were harvested and rinsed; the petioles were removed. Next, leaves were placed in a syringe with infiltration buffer and vacuumed. **b**, The leaves were taped onto a 1ml syringe after infiltration buffer was vacuumed into leaves. **c**, Syringe with taped leaves was placed into a 50 ml conical tube. **d**, The apoplastic wash was collected by centrifuging the infiltrated leaves at 900 g at 4 °C. **e**, Scheme of EV isolation by differential ultracentrifugation from apoplastic wash of *Arabidopsis*. Sup, Supernatant. **f**, Confocal microscopy images of P40 and P100 fractions isolation by ultracentrifugation from apoplastic wash of *TET8pro:TET8-GFP* plants. Equivalent amounts of plants were inoculated with *B. cinerea* for 36 hours before P40 and P100 fraction isolation. Scale bars, 10 μm. **g**, GFP-labeled TET8 was detectable in both P40 and P100 EV fractions by western blot. The experiments were repeated three times independently with similar results. Source data are provided as a Source Data file.

Extended Data Fig. 2 Gene ontology (GO) enrichment analysis of proteins enriched in EVs.

a, **b**, The plant EV proteome from *B. cinerea* infected *Arabidopsis* plants was categorized based on GO terms related to biological process (**a**) and molecular function (**b**) through The *Arabidopsis* Information Resource Web site (www.arabidopsis.org).

Extended Data Fig. 3 TET8 and PEN1 localization.

a, Confocal microscopy images of TET8-YFP and ARA6-CFP at *B. cinerea* infection site on *N. benthamiana*. TET8-YFP was partially co-localized with ARA6-CFP signals. **b**, Fluorescent intensity was quantified for the images used in (**a**). Transections used for fluorescence intensity measurements are indicated by blue lines. Green and red lines represent histograms of ARA6-CFP and TET8-YFP fluorescent intensities, respectively. **c**, Confocal microscopy images of CFP-PEN1 and ARA6-YFP at the *B. cinerea* infection site on *N. benthamiana* . CFP-PEN1 and ARA6-YFP do not co-localize with ARA6-YFP signals. **d**, Fluorescence intensity was quantified for the images used in (**c**). Transections used for fluorescence intensity measurements are indicated by blue lines. Green and red lines represent histograms of CFP-PEN1 and ARA6-YFP fluorescent intensities, respectively. Scale bars, 10 μm.

Extended Data Fig. 4 Bottom loading EV separation by sucrose gradient centrifugation.

a, Pellets obtained from 100,000 g centrifugations (P100) were used to perform sucrose gradient centrifugation by both top and bottom loading. **b**, Six fractions were collected from bottom loading plant EV sucrose gradient centrifugation. TET8, AGO1, RH11, RH37, ANN1, ANN2 were detected by western blot. EV enriched (TAS1c-siR483, TAS2-siR453 and miR166) and non-EV-enriched (TAS1c-siR585 and TAS2-siR710) sRNAs were detected by RT-PCR. The experiments were repeated three times

independently with similar results. Source data are provided as a Source Data file.

Extended Data Fig. 5 Colocalization between EV associated RBPs with MVB marker ARA6 and EV markerTET8.

a, b, Fluorescent protein labeled RBPs were co-expressed transiently with MVB marker ARA6 (**a**) and EV marker TET8 (**b**) in *N. benthamiana*. Confocal microscopy was used to determine the localization of RBPs (AGO1, RH11, RH37, ANN1, ANN2) with ARA6 and TET8. AGO2 was used as a control. Scale bars, 10 μm. The experiments were repeated three times independently with similar results.

Extended Data Fig. 6 sRNAs specifically bound by AGO2, AGO4 and AGO5 were absent from plant EVs.

AGO2-associated miR393*, AGO4-associated siR1003, AGO5-associated miR390* and both AGO1 and AGO5-associated miR156 were detected in isolated plant EVs by sRNA RT-PCR. TAS1c-siR483, TAS2-siR453 and miR166 were used as positive control. TAS1c-siR585 and TAS2-siR710 were used as negative controls. The experiments were repeated three times independently with similar results. Source data are provided as a Source Data file.

Extended Data Fig. 7 AGO1 and RH37 selectively bind EV-enriched sRNAs in *N. benthamiana.*

YFP-AGO1 or RH37-CFP were co-expressed with EV-enriched (TAS1c-siR483, TAS2-siR453 and miR166) and non-EV-enriched sRNAs (TAS1c-siR585 and TAS2-siR710) in *N. benthamiana,* sRNAs were immunoprecipitated from plant total extraction using antibodies against GFP and detected by sRNA RT-PCR. IgG was used as a negative control. The experiments were repeated three times independently with similar results. Source data are provided as a Source Data file.

Extended Data Fig. 8 Verification of *rh11rh37* and *ann1ann2* double mutants.

a, The developmental phenotypes of the double mutant of *rh11rh37* and the real-time RT-PCR analysis of *RH11* and *RH37* expression in *rh11rh37* mutants. The data are presented as mean ± s.d., $n = 3$ biological replicates. The error bars indicate the standard deviation (s.d.). **b,** The real-time RT-PCR analysis of *RH11* and *RH37* expression in *rh11rh37* mutants. The data are presented as mean ± s.d., $n = 3$ biologically independent replicates. The error bars indicate the standard deviation (s.d.). **c**, The developmental phenotypes of the *rh11rh37#6* mutant that *RH37* expression was suppressed by artificial miRNA in *rh11* knockout mutant. **d,** RT-PCR analysis of *RH11* and real-time PCR analysis of *RH37* in *rh11rh37#6* mutant. The data are presented as mean ± s.d., $n = 3$ biologically independent replicates. The error bars indicate the standard deviation (s.d.). **e**, Phenotype of wild-type and *ann1ann2* mutant grown for 4 weeks in a growth chamber.

f, RT-PCR analysis of the expression levels of *ANN1* and *ANN2* in wild-type and *ann1ann2* double mutant. The statistical analysis in Extended Data Fig. 8b was performed using ANOVA Dunnett's multiple comparisons test. The statistical analysis in Extended Data Fig. 8d was performed using unpaired two-tailed Student's *t*-test. The small open circles represent the individual values. The asterisks indicate significant differences: $***P < 0.001$. The experiments were repeated three times independently with similar results. Source data are provided as a Source Data file.

Extended Data Fig. 9 EV-enriched sRNA amount was reduced in EVs isolated from EV-associated RBP mutants.

The relative level of both EV-enriched and non-EV-enriched sRNAs were examined by real-time RT-PCR in the total fraction and EV fraction from *ago1-27* (**a**), *rh11rh37* (**b**) and *ann1ann2* (**c**) mutants. The data are presented as mean ± s.d., $n = 3$ biological replicates. The error bars indicate the standard deviation (s.d.). The statistical analysis was performed using unpaired two-tailed Student's *t*-test. The small open circles represent the individual values. The asterisks indicate significant differences: $**P < 0.01$, $***P < 0.01$, NS, not significant.

Supplementary Table 1. EV-associated proteins detected in all three biological replicates with distinct peptide counts of five or more in each replicate.

Supplementary Table 2. EV associated RNA binding proteins detected in all three biological replicates with distinct peptide counts of five or more in each replicate.

Supplementary Table 3. Primers used in this study.