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Supplementary Materials for

Agrobacterium delivers VirE2 protein into host cells via clathrin-mediated endocytosis

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at advances.sciencemag.org/cgi/content/full/3/3/e1601528/DC1)

- movie S1 (.avi format). Comovement of VirE2 with FM4-64–labeled vesicles.
- movie S2 (.avi format). Restriction of VirE2 trafficking in ES1-induced aggregation of SYP61-containing endosomes.

Supplementary Materials

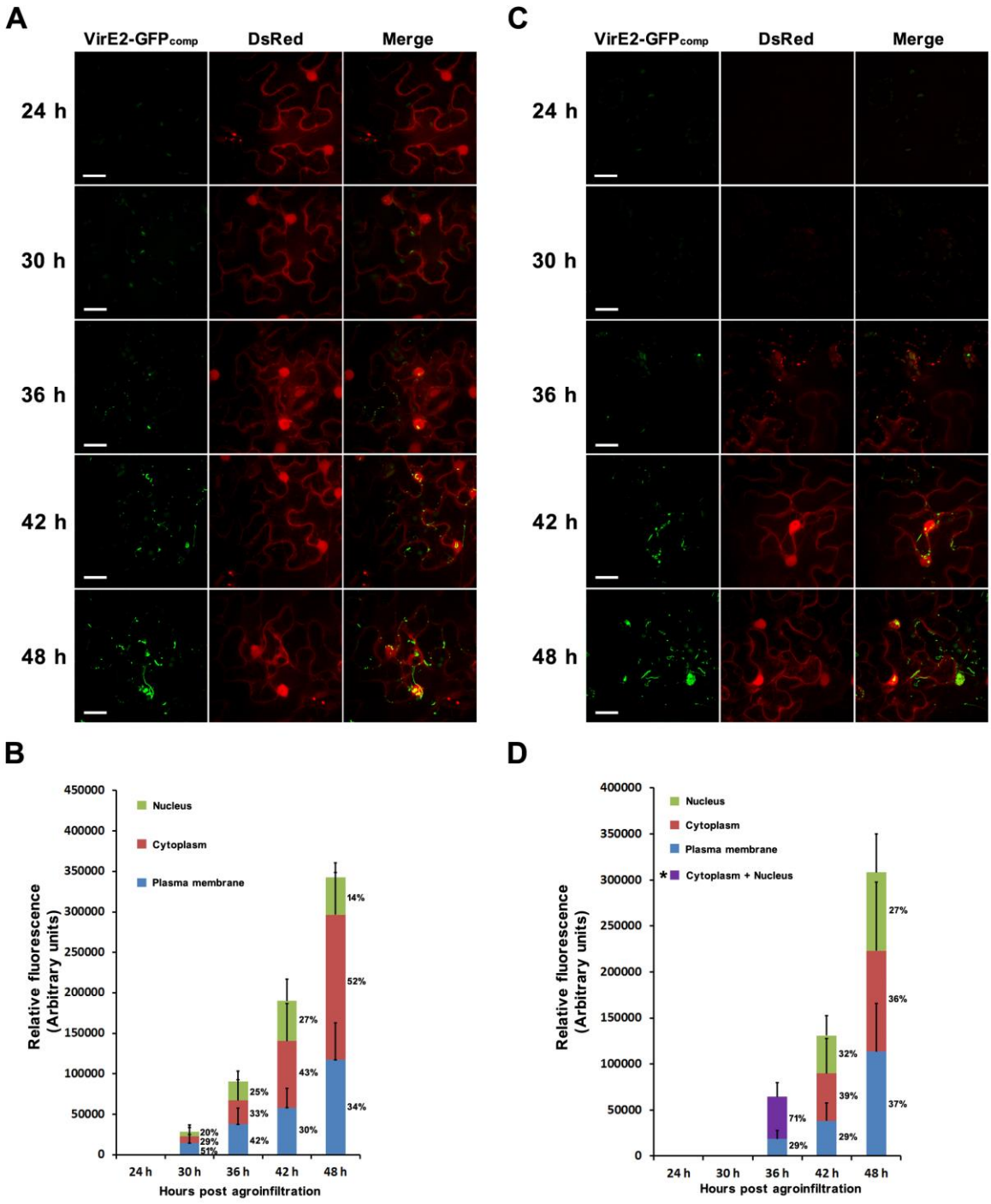

fig. S1. Time-course study of VirE2 subcellular localizations inside *N. benthamiana* cells.

(**A**) *A. tumefaciens* EHA105*virE2::GFP11* was infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. Projected Z-series images were obtained at different time points post agroinfiltration. (**B**) Quantification of VirE2 subcellular localizations for panel A. The

intensity of VirE2-GFP$_{comp}$ signals associated with the plasma membrane, cytoplasm and nucleus was measured (n=30). (**C**) Wild type *N. benthamiana* leaves were infiltrated with evenly mixed *A. tumefaciens* strains EHA105*virE2::GFP11*(pGFP1-10), which is capable of delivering VirE2-GFP11 and the T-DNA expressing GFP1-10, and EHA105*virE2::GFP11*(pQH121-mC), which is capable of delivering VirE2-GFP11 and the T-DNA expressing free mCherry. Projected Z-series images were obtained at different time points after agroinfiltration. (**D**) Quantification of VirE2 subcellular localizations for panel C. The intensity of VirE2-GFP$_{comp}$ signals associated with the plasma membrane, cytoplasm and nucleus was measured (n=20). Scale bars, 20 μm. * Signals in the cytoplasm and nucleus were combined for calculation, as the transient expression of mCherry from the T-DNA delivered into the wild type *N. benthamiana* leaves at 36 hours after agroinfiltration was insufficient to show the nucleus.

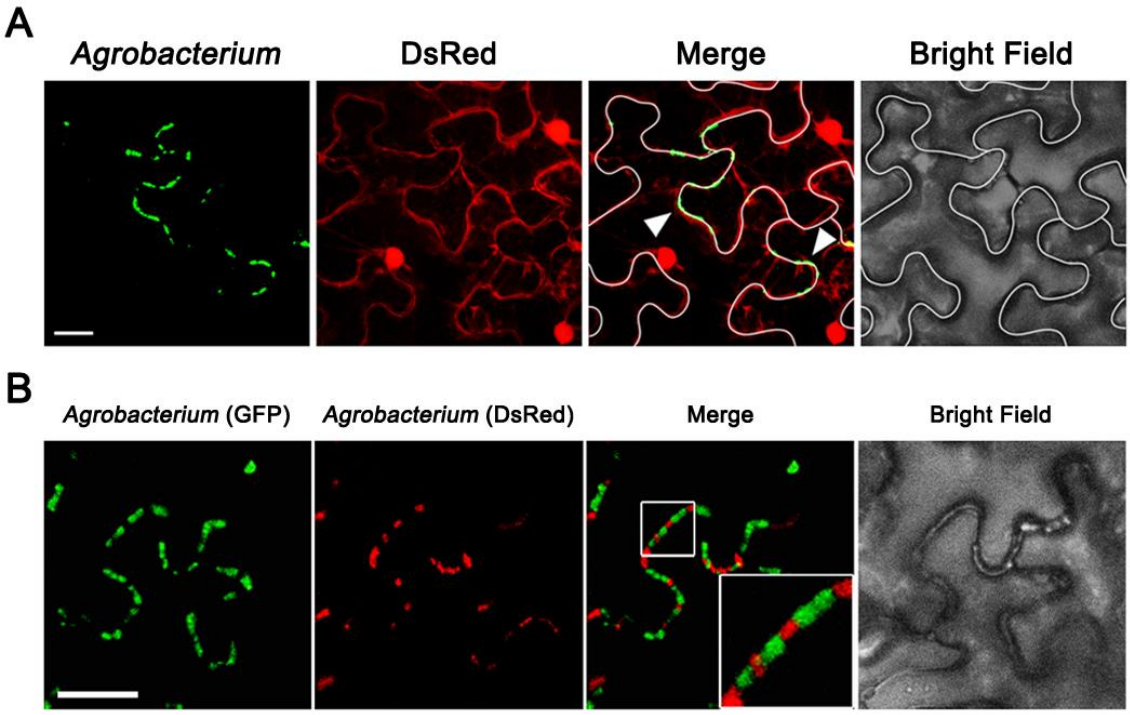

fig. S2. Accumulation of *Agrobacterium* at the intercellular space of infiltrated leaf epidermal cells. (**A**) Projected Z-series images of *N. benthamiana* (Nb308A) leaves infiltrated with GFP-labeled *A. tumefaciens* cells EHA105 (pVB-GFP). Images were obtained 2 days after agroinfiltration under confocal microscopy with an Olympus UAPO N 340 40× NA 1.15 water immersion objective. White lines were added to the images to indicate borders between leaf epidermal cells. (**B**) Projected Z-series image of wild type

N. benthamiana leaves infiltrated with evenly mixed GFP labeled *A. tumefaciens* cells EHA105 (pVB-GFP) and DsRed labeled *A. tumefaciens* cells EHA105 (pVB-RFP). Scale bars, 20 μm.

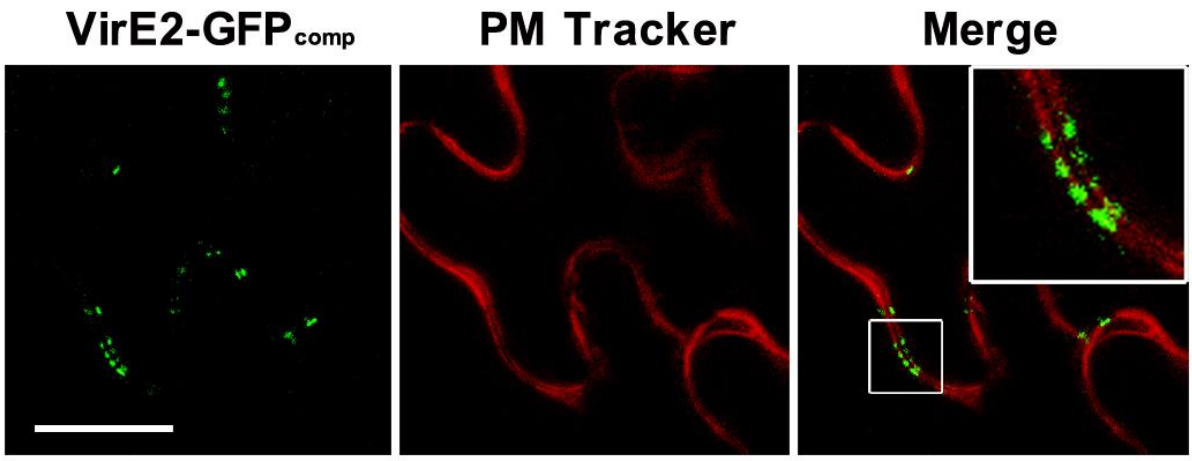

fig. S3. Accumulation of *Agrobacterium*-delivered VirE2 at the host plasma membrane.
Wild type *N. benthamiana* leaves were infiltrated with evenly mixed *A. tumefaciens* strains EHA105*virE2::GFP11*(pGFP1-10), which is capable of delivering VirE2-GFP11 and the T-DNA expressing GFP1-10, and EHA105*virE2::GFP11*(pm-rb), which is capable of delivering VirE2-GFP11 and the T-DNA expressing a plasma membrane (PM) tracker. Images were obtained 2 days after agroinfiltration under confocal microscopy with an Olympus UPlan SAPO 100× NA 1.4 oil immersion objective. Scale bars, 20 μm.

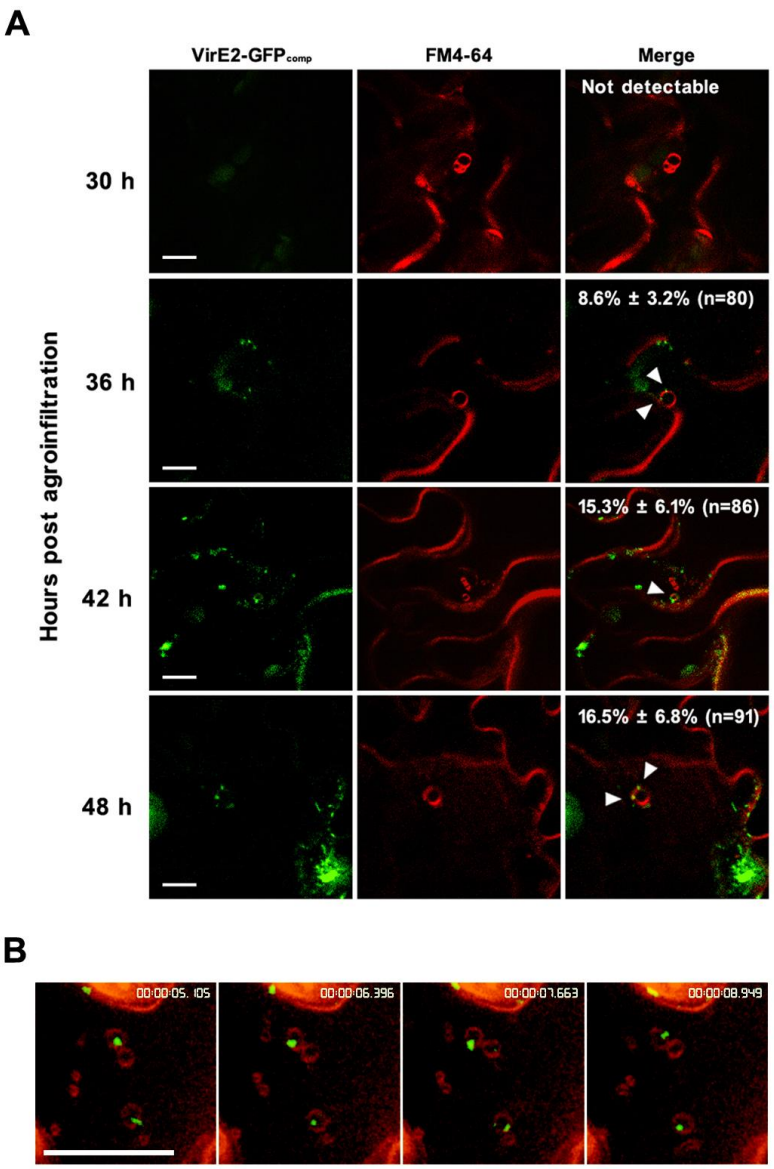

fig. S4. Colocalization of VirE2 with FM4-64–labeled endomembrane compartments in *N. benthamiana* epidermal cells. (**A**) Time course of VirE2 colocalization with endomembrane compartments. Wild type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells EHA105*virE2::GFP11(*pGFP1-10). Confocal microscopy with single optical sections was conducted at different time points post agroinfiltration. One hour before the confocal microscopy, the cells were stained with FM4-64. Percentages of detected FM4-64-stained endomembrane compartments colocalized with VirE2 are shown in the merge panels. Scale bar, 10 µm. (**B**) Time course of VirE2 comovement with endomembrane compartments. Wild type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells EHA105*virE2::GFP11*(pGFP1-10) and stained with FM4-64. The images were obtained 2 days after agroinfiltration (1 hour after FM4-64 staining). Scale bar, 20 µm.

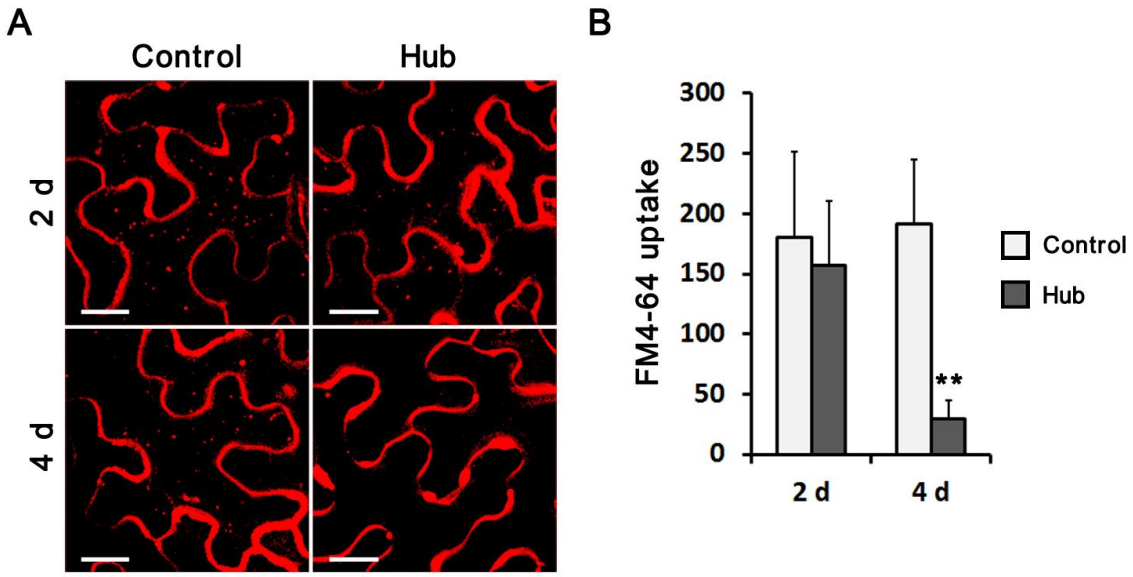

fig. S5. Expression of Hub impaired FM4-64 uptake in *N. benthamiana* epidermal cells. (**A**) Wild type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells EHA105(pXY01) or EHA105(pXY01-Hub) followed by FM4-64 staining at 2 days or 4 days after agroinfiltration. Projected Z-series images were obtained 5 hours post staining under confocal microscopy with an Olympus UAPO N 340 40× NA 1.15 water immersion objective. Scale bars, 20 μm. (**B**) Quantification of FM4-64 uptake. The number of endomembrane compartments stained by FM4-64 was calculated in each image (n=20). $^{**}p < 0.01$ (unpaired Student's *t* test).

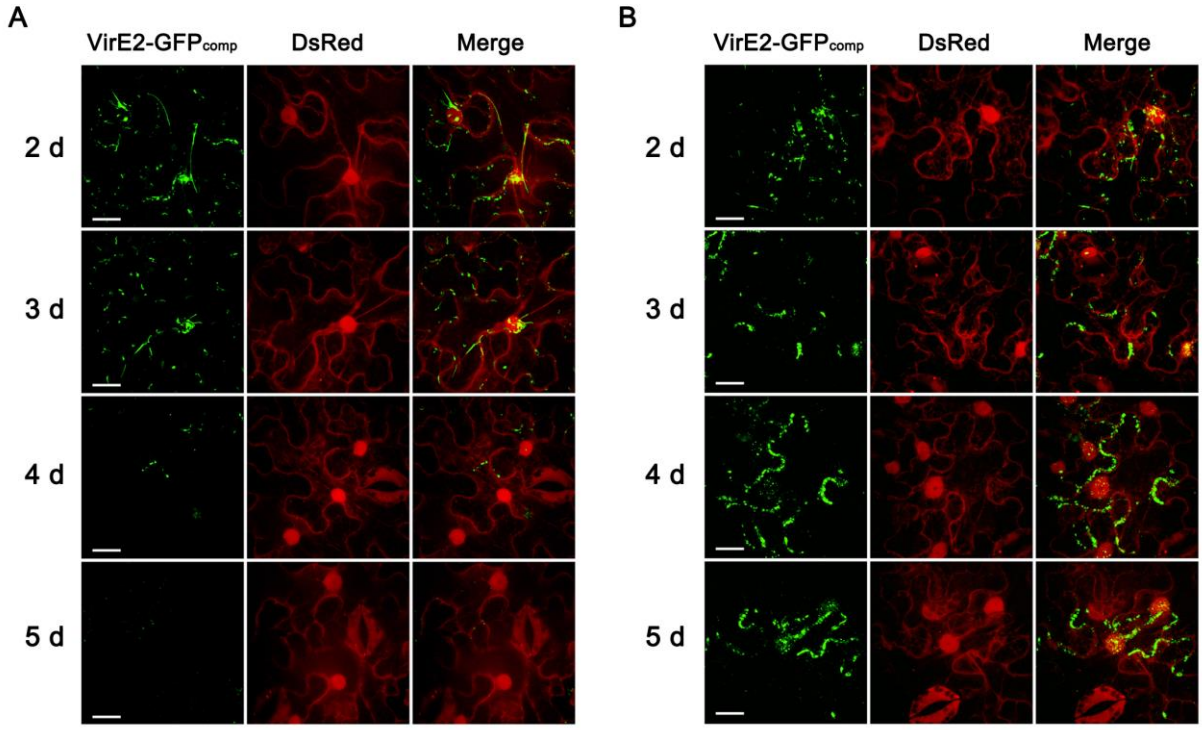

fig. S6. Expression of dominant-negative clathrin Hub impaired VirE2 departure from the plasma membrane in *N. benthamiana* epidermal cells. (**A**) Wild type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells EHA105*virE2::GFP11*(pXY01). (**B**) Wild type *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells EHA105*virE2::GFP11*(pXY01-Hub). Projected Z-series images are shown. Scale bars, 20 μm.

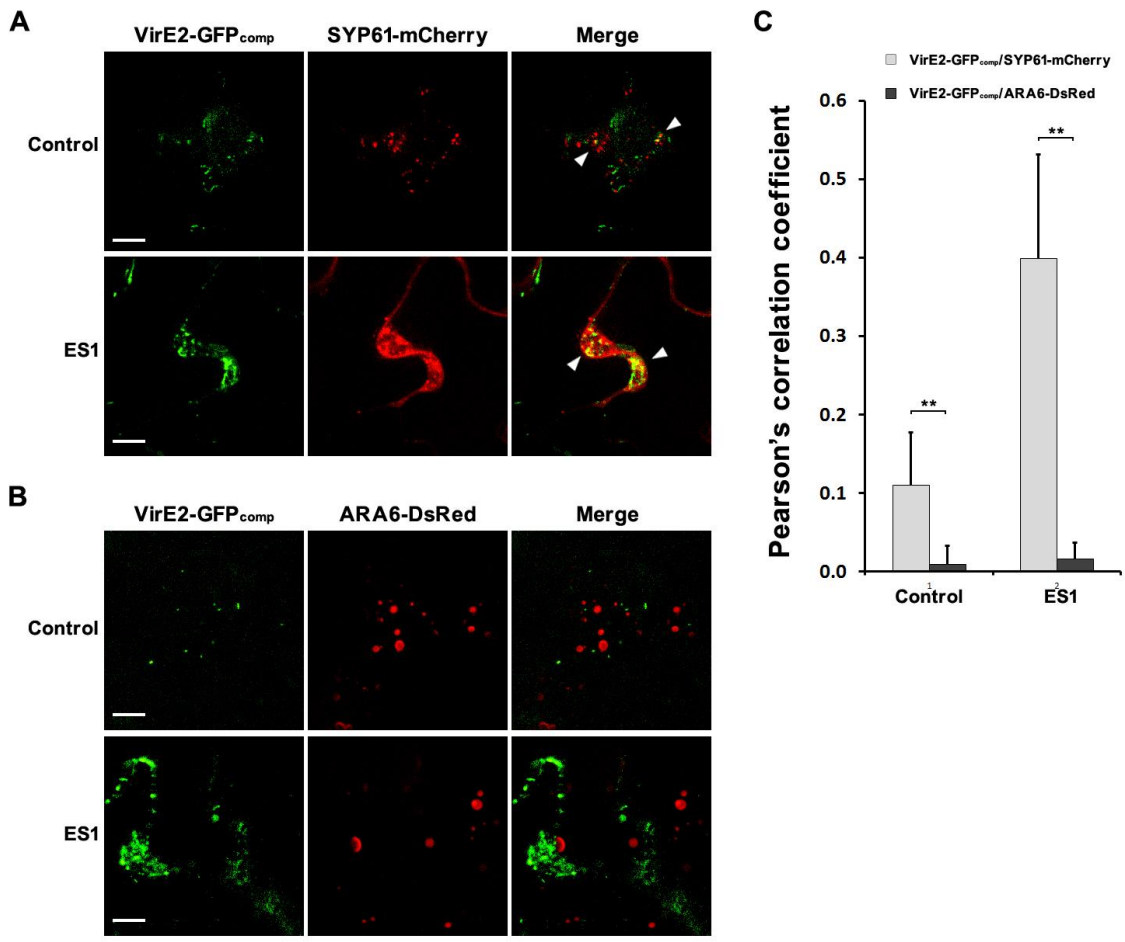

fig. S7. *Agrobacterium*-delivered VirE2 was associated with early endosomes rather than late endosomes. (**A**) *Agrobacterium*-delivered VirE2 was associated with early endosomes labeled with SYP61-mCherry. Wild type *N. benthamiana* leaves were infiltrated with evenly mixed *A. tumefaciens* cells EHA105*virE2::GFP11*(pGFP1-10) and EHA105*virE2::GFP11*(pXY01-SYP61-mC) together with (lower panel) or without (upper panel) chemical effector ES1. Single optical sections were obtained 2 days after agroinfiltration. (**B**) *Agrobacterium*-delivered VirE2 was not associated with late endosomes labeled with ARA6-DsRed. Wild type *N. benthamiana* leaves were infiltrated with evenly mixed *A. tumefaciens* cells EHA105*virE2::GFP11*(pGFP1-10) and EHA105*virE2::GFP11*(pXY01-ARA6-DsRed) together with (lower panel) or without (upper panel) chemical effector ES1. Single optical sections were obtained 2 days after agroinfiltration. (**C**) Estimation of the colocalization through Pearson's correlation coefficient using ImageJ plugin "Coloc 2" (n=30). $^{**}p < 0.01$ (unpaired Student's t test). Scale bars, 10μm .

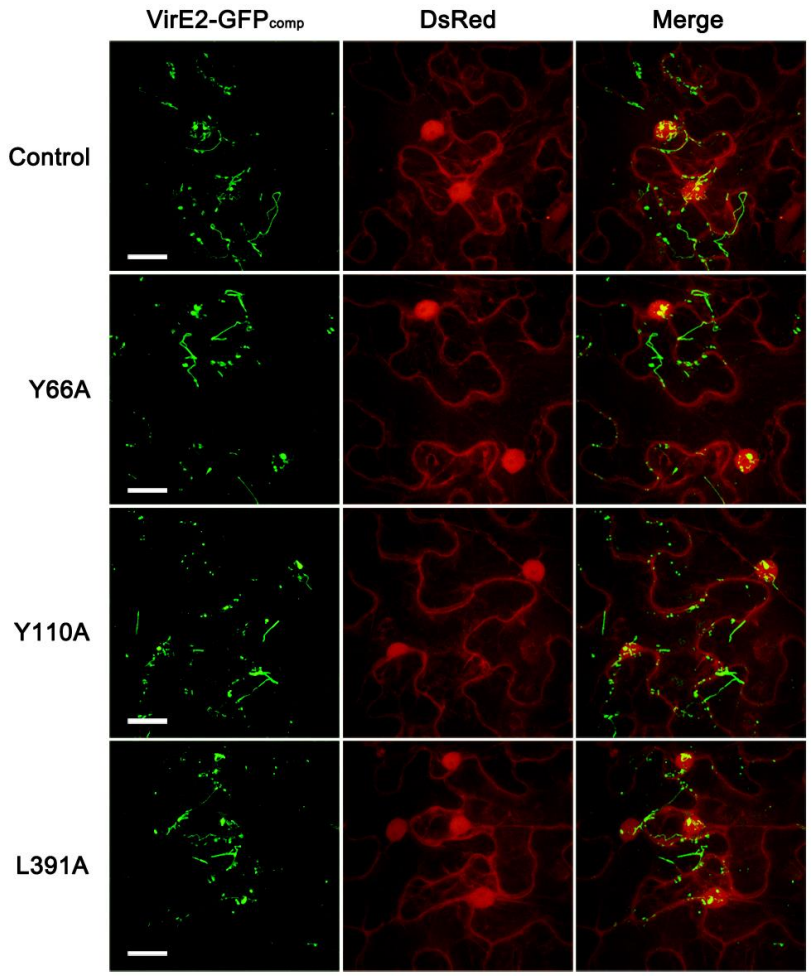

fig. S8. Mutations at other putative VirE2 endocytic sorting motifs did not affect VirE2 internalization in host cells. *N. benthamiana* (Nb308A) leaves were infiltrated with *A. tumefaciens* EHA105*virE2::GFP11* or VirE2 mutants, in which the corresponding tyrosine residues or leucine residues were substituted with alanine. Projected Z-series images were obtained at 2 days after agroinfiltration. Scale bars, 20 μm.

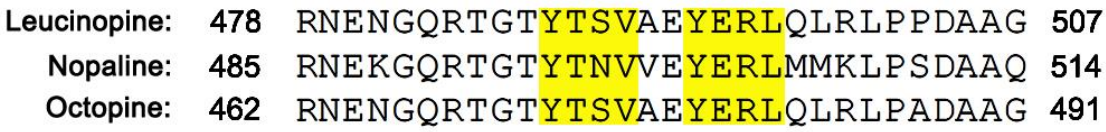

fig. S9. Sequence alignment analysis of VirE2 from different types of Ti plasmids. Sequence alignment revealed that the dual tyrosine-based endocytic motifs at the C-terminus were conserved on VirE2 proteins from different types of Ti plasmids.

table S1. Strains and plasmids used in the studies.

Strain and plasmid	Relevant characteristics	Source or reference
A. tumefaciens		
EHA105	C58 strain containing pTiBo542 without T-DNA	(*69*)
EHA105*virE2::GFP 11*	EHA105 derivative, with the GFP11 coding sequence inserted into *virE2* on pTiBo542	(*37*)
EHA105*virE2(Y488 A)::GFP11*	EHA105*virE2::GFP11* derivative, with a point mutation Y488A at *virE2*	This study
EHA105*virE2(Y494 A)::GFP11*	EHA105*virE2::GFP11* derivative, with a point mutation Y494A at *virE2*	This study
EHA105*virE2(Y488 A Y494A)::GFP11*	EHA105*virE2::GFP11* derivative, with point mutations Y488A and Y494A at *virE2*	This study
A348	A136 (pTiA6NC) (Octopine-type)	(*70*)
A348-*virE2(Y472A)*	A348 derivative, with a point mutation Y472A at *virE2*	This study
A348-*virE2(Y478A)*	A348 derivative, with a point mutation Y478A at *virE2*	This study
A348-*virE2(Y472A Y478A)*	A348 derivative, with point mutations Y472A and Y478A at *virE2*	This study
Plasmids		
pm-rb	A binary vector containing a plant plasma membrane marker; Km^r	(*39*)
er-gb	A binary vector containing a plant ER marker; Km^r	(*39*)
pXY01	A binary vector for target gene expression under the control of CaMV 35S promoter; Km^r	This study
pGFP1-10	pXY01 derivative, with GFP1-10 coding sequence under the control of CaMV 35S promoter; Km^r	This study
pXY01-Hub	pXY01 derivative, with Hub coding sequence under the control of CaMV 35S promoter; Km^r	This study

pXY01-SYP61-mC	pXY01 derivative, with SYP61-mCherry coding sequence under the control of CaMV 35S promoter; Km^r	This study
pXY01-ARA6-DsRed	pXY01 derivative, with ARA6-DsRed coding sequence under the control of CaMV 35S promoter; Km^r	This study
pCB301	A mini binary vector; Km^r	(*68*)
pXY301	pCB301 derivative, with T-DNA right border sequence deleted; Km^r	This study
pVB	pXY301 derivative, with a VirB promoter region; Km^r	This study
pVB-RFP	pVB derivative, with DsRed coding sequence under the control of VirB promoter; Km^r	This study
pVBA-RFP	pVB-RFP derivative, with kanamycin resistance cassette replaced by ampicillin resistance cassette; Amp^r	This study
pVB-GFP	pVB derivative, with GFP coding sequence under the control of VirB promoter; Km^r	This study
pBI121	A binary vector; Km^r	(*71*)
pQH121	pBI121 derivative, with *gusA* deleted; Km^r	This study
pQH121-mC	pQH121 derivative, with mCherry coding sequence under the control of CaMV 35S promoter; Km^r	This study
pMAL-c2x	MBP tag expression vector; Amp^r	New England Biolabs
pMBP-AP2MC	pMAL-c2x derivative, expressing MBP-AP2MC fusion protein; Amp^r	This study
pGEX-4T-1	GST tag expression vector; Amp^r	GE Healthcare
pGST-VirE2C	pGEX-4T-1 derivative, expressing MBP-VirE2C fusion protein; Amp^r	This study
pGST-VirE2C(Y488A)	pGEX-4T-1 derivative, expressing MBP-VirE2C(Y488A) fusion protein; Amp^r	This study
pGST-VirE2C(Y494A)	pGEX-4T-1 derivative, expressing MBP-VirE2C(Y494A) fusion protein; Amp^r	This study

pGST-VirE2C(Y488A /Y494A)	pGEX-4T-1 derivative, expressing MBP-VirE2C(Y488A /Y494A) fusion protein; Amp^r	This study

movie S1. Comovement of VirE2 with FM4-64–labeled vesicles.

A. tumefaciens strain EHA105*virE2::GFP11*(pGFP1-10) was infiltrated into wild type *N. benthamiana* leaves. FM4-64 staining was carried out 2 days after agroinfiltration. The epidermal cells were examined at 1 hour after FM4-64 staining. Scale bar, 20 μm. Time (h:min:s) is shown at the top right.

movie S2. Restriction of VirE2 trafficking in ES1-induced aggregation of SYP61-containing endosomes.

A. tumefaciens cells EHA105*virE2::GFP11*(pGFP1-10) and EHA105*virE2::GFP11*(pXY01-SYP61-mC) were mixed with ES1 (25 μM) and infiltrated into wild type *N. benthamiana* leaves. The epidermal cells were examined at 2 days after agroinfiltration. Scale bar, 20 μm. Time (h:min:s) is shown at the top right.