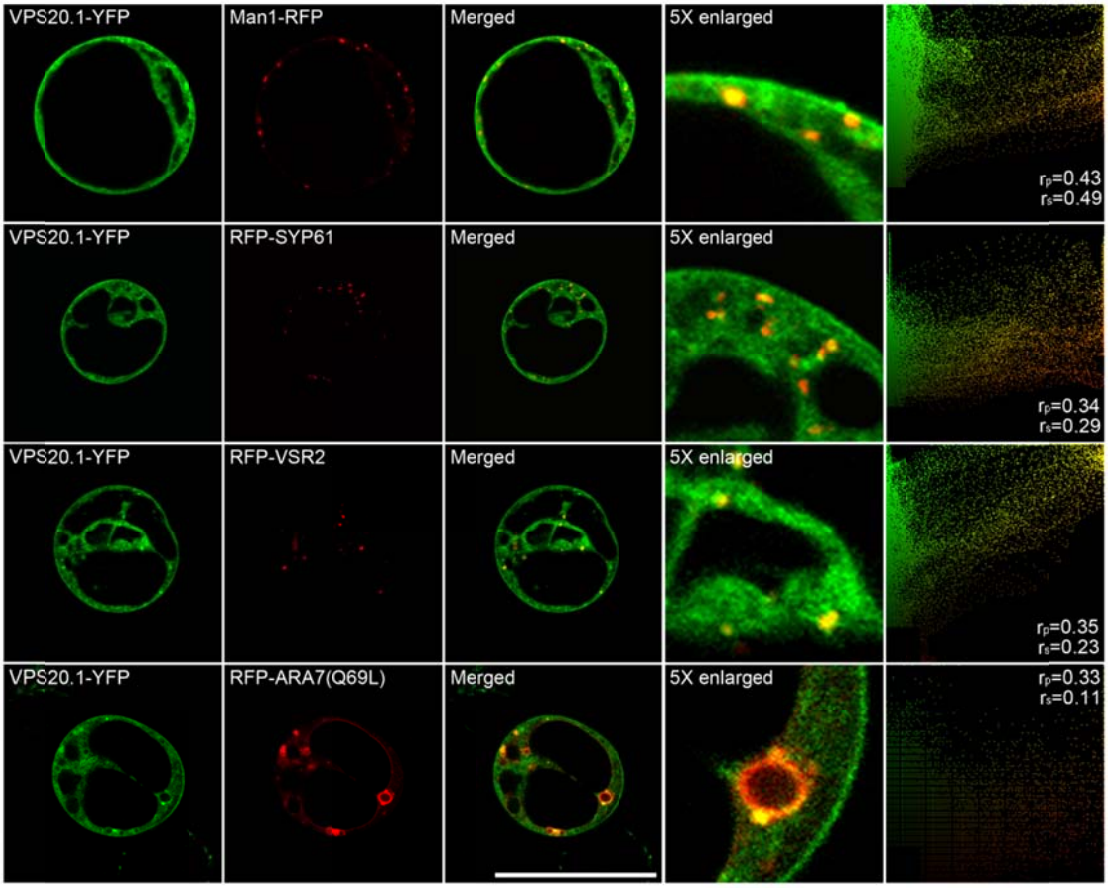
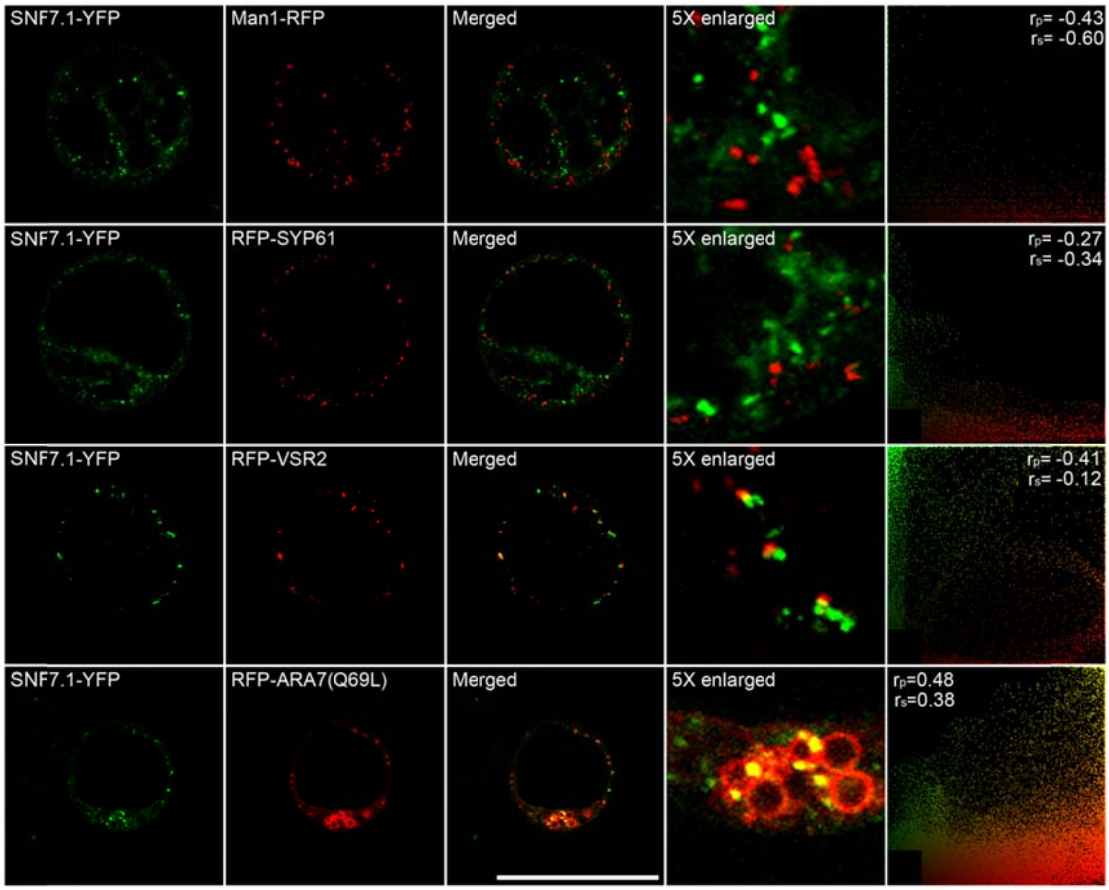


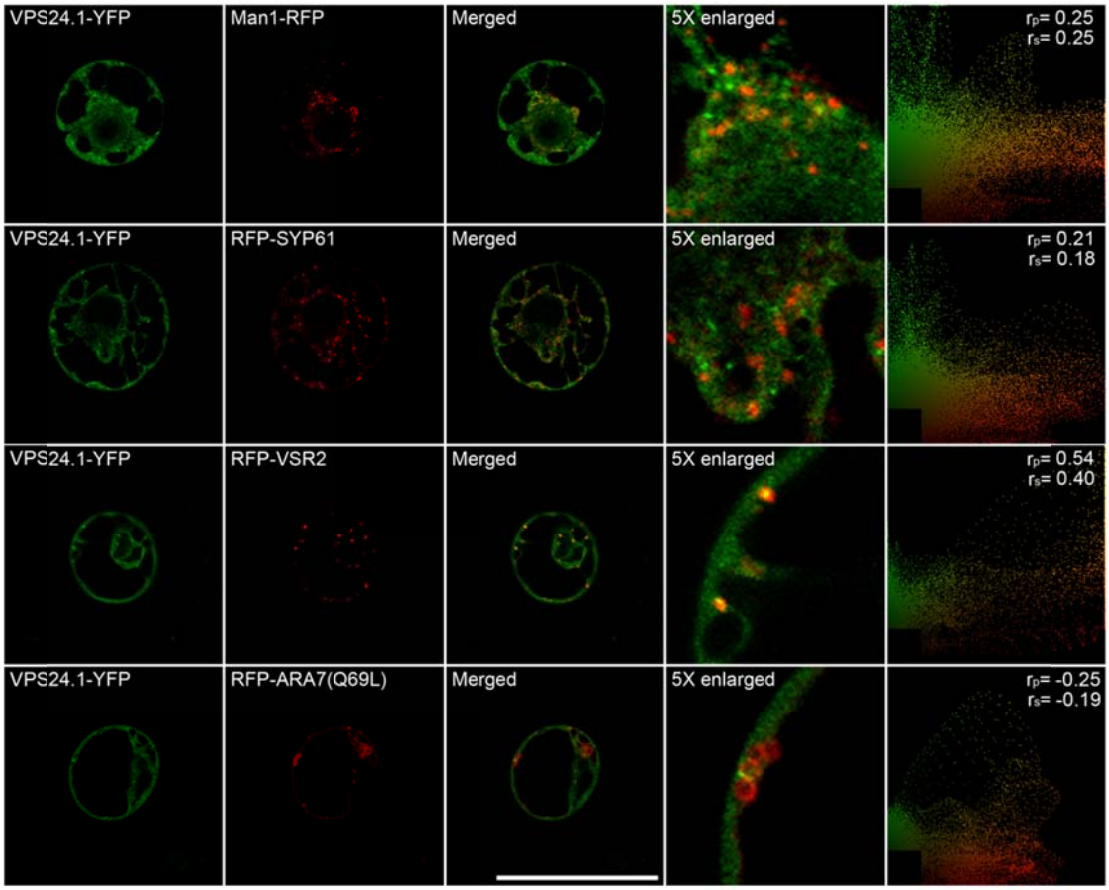
Supplemental Figure S1. Subcellular localizations of VPS20.1-YFP.

VPS20.1-YFP was coexpressed with different organelle markers including the Golgi marker Man1-RFP, the TGN marker RFP-SYP61, the PVC/MVB marker RFP-VSR2 and the enlarged PVC/MVB marker RFP-ARA7(Q69L) in *Arabidopsis* protoplasts. The scatterplots at the right-hand side of each row were obtained by using ImageJ program with the PSC colocalization plug-in. The corresponding Pearson or Spearman r values indicate the level of colocalization ranging from +1 for perfect colocalization to -1 for negative correlation. Scale bar = 50 μm.



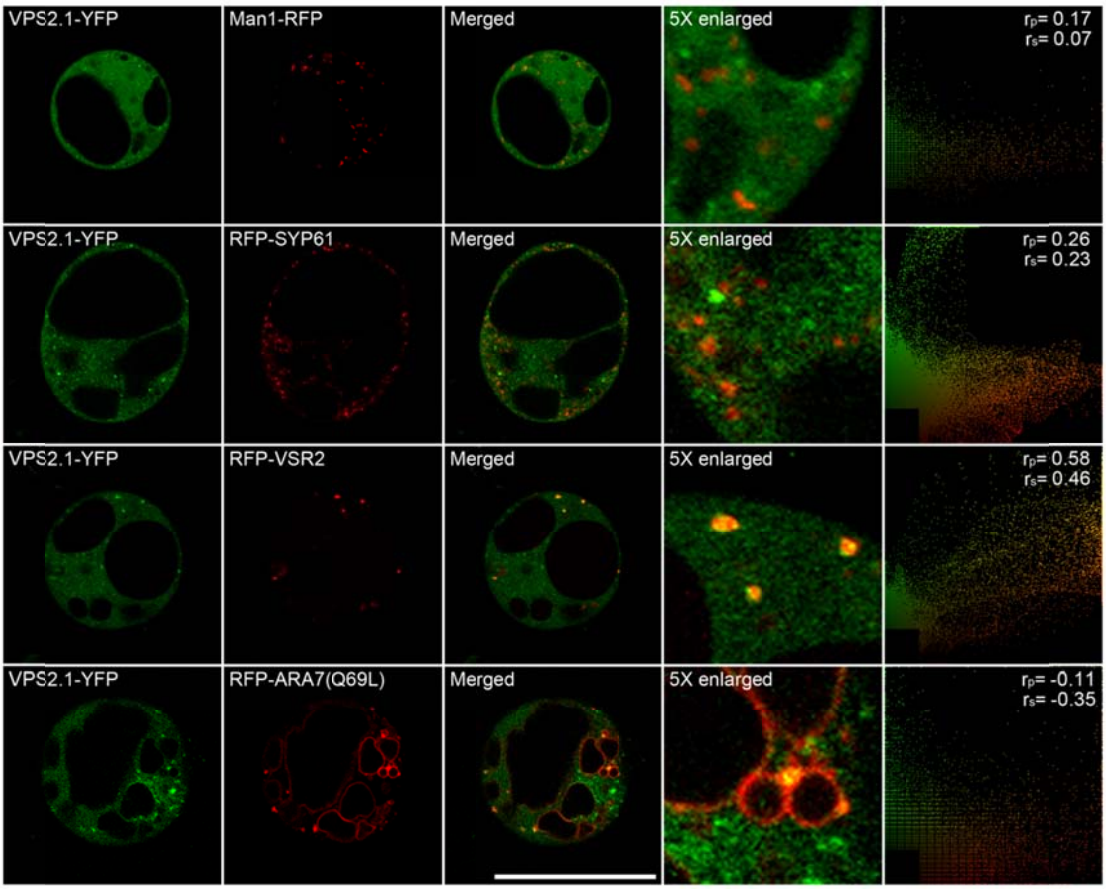
Supplemental Figure S2. Subcellular localizations of SNF7.1-YFP.

SNF7.1-YFP was coexpressed with different organelle markers including the Golgi marker Man1-RFP, the TGN marker RFP-SYP61, the PVC/MVB marker RFP-VSR2 and the enlarged PVC/MVB marker RFP-ARA7(Q69L) in *Arabidopsis* protoplasts. The scatterplots at the right-hand side of each row were obtained by using ImageJ program with the PSC colocalization plug-in. The corresponding Pearson or Spearman r values indicate the level of colocalization ranging from +1 for perfect colocalization to -1 for negative correlation. Scale bar = 50 μm.



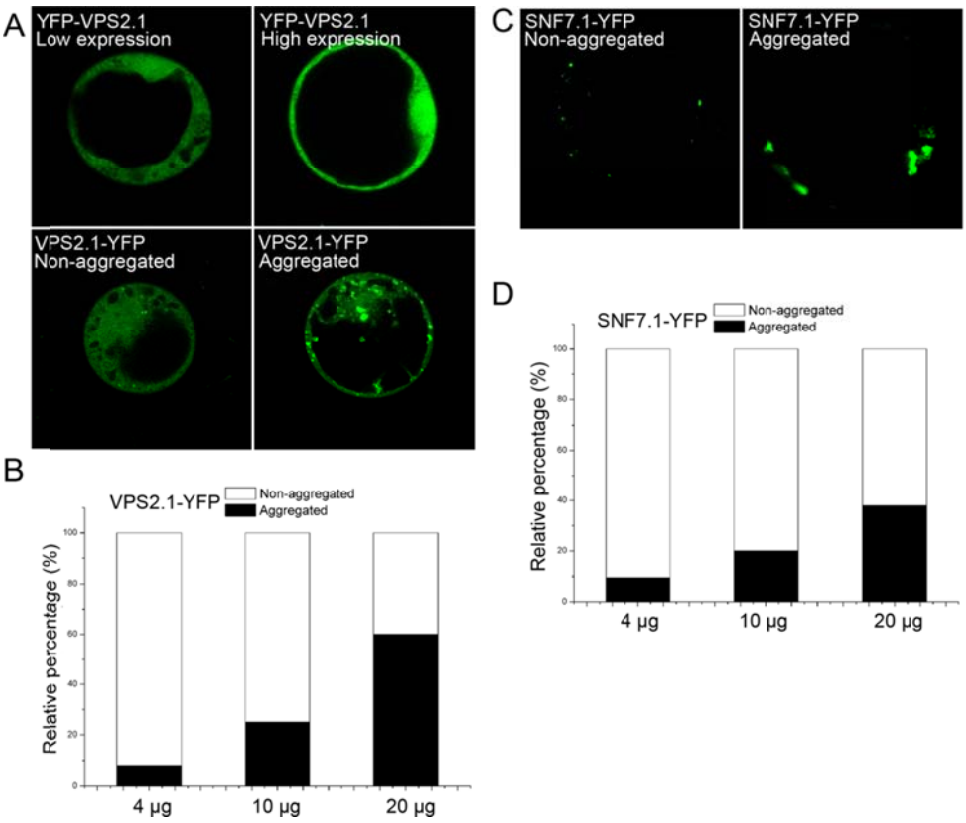
Supplemental Figure S3. Subcellular localizations of VPS24.1-YFP.

VPS24.1-YFP was coexpressed with different organelle markers including the Golgi marker Man1-RFP, the TGN marker RFP-SYP61, the PVC/MVB marker RFP-VSR2 and the enlarged PVC/MVB marker RFP-ARA7(Q69L) in *Arabidopsis* protoplasts. The scatterplots at the right-hand side of each row were obtained by using ImageJ program with the PSC colocalization plug-in. The corresponding Pearson or Spearman r values indicate the level of colocalization ranging from +1 for perfect colocalization to -1 for negative correlation. Scale bar = 50 μm.



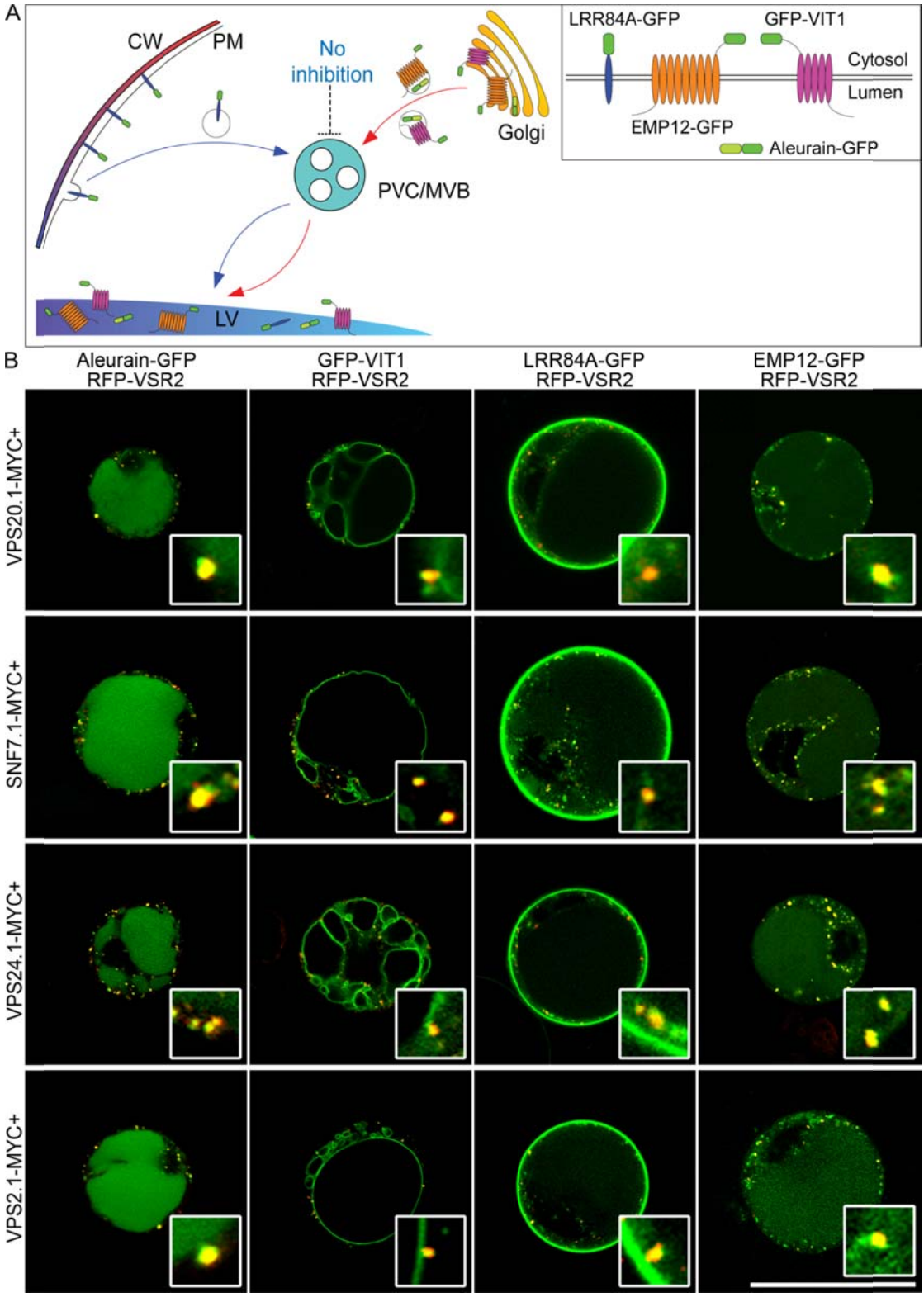
Supplemental Figure S4. Subcellular localizations of VPS2.1-YFP.

VPS2.1-YFP was coexpressed with different organelle markers including the Golgi marker Man1-RFP, the TGN marker RFP-SYP61, the PVC/MVB marker RFP-VSR2 and the enlarged PVC/MVB marker RFP-ARA7(Q69L) in *Arabidopsis* protoplasts. The scatterplots at the right-hand side of each row were obtained by using ImageJ program with the PSC colocalization plug-in. The corresponding Pearson or Spearman r values indicate the level of colocalization ranging from +1 for perfect colocalization to -1 for negative correlation. Scale bar = 50 μm.



Supplemental Figure S5. Overexpression of SNF7.1-YFP induced aggregates.

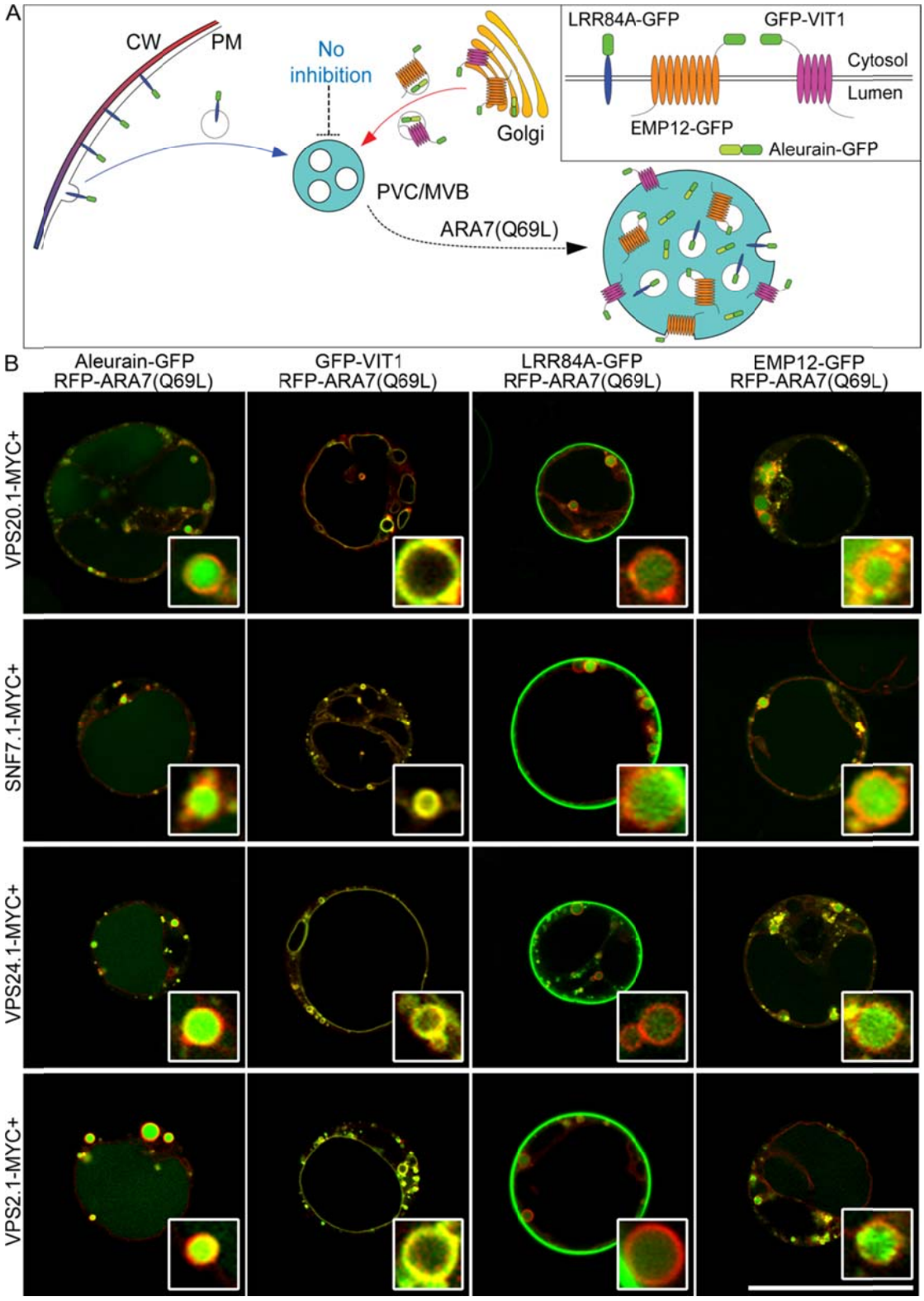
A, Representative images of protoplasts showing low expression or high expression of YFP-VPS2.1 signal and non-aggregated or aggregated VPS2.1-YFP signal. Scale bar = 50 μm. B, Percentage of protoplasts showing non-aggregated or aggregated YFP-VPS2.1 signal was correlated with the amount of plasmid DNA used in the transient expression. C, Representative images of protoplasts showing non-aggregated or aggregated SNF7.1-YFP signal. Scale bar = 50 μm. D, Percentage of protoplasts showing non-aggregated or aggregated SNF7.1-YFP signal was correlated with the amount of plasmid DNA used in the transient expression. Over 50 cells were calculated for each amount of plasmid DNA.



Supplemental Figure S6. Coexpression of ESCRT-III subunits did not change the expression pattern of the reporters on the LV.

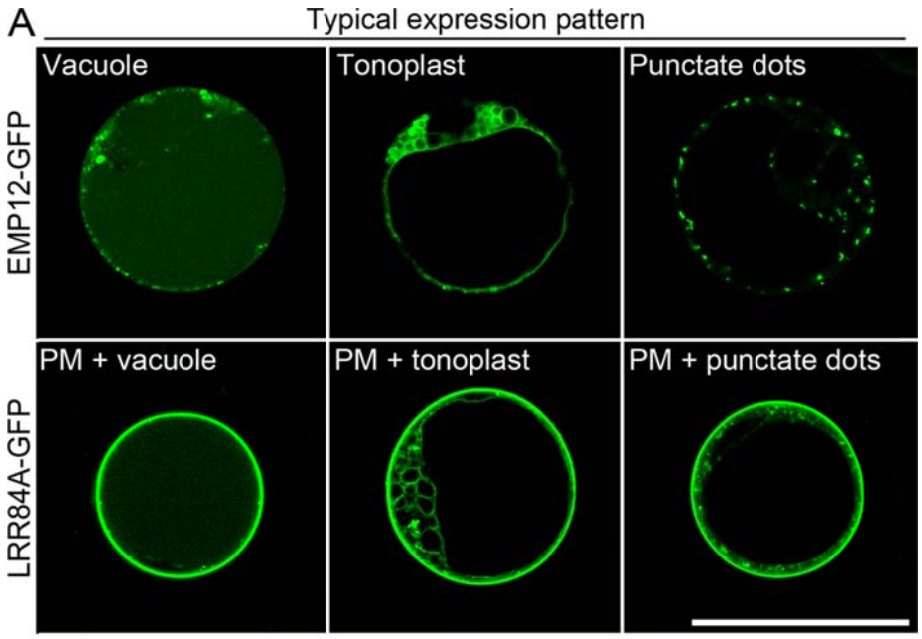
A, Schematic model showing the effect of ESCRT-III coexpression on the localization of the reporters on the LV. B, Coexpression of ESCRT-III subunits with four reporters

and RFP-VSR2 in *Arabidopsis* protoplasts. Scale bar = 50 μm.



Supplemental Figure S7. Coexpression of ESCRT-III subunits did not change the expression pattern of the reporters on the enlarged PVC/MVB. A, Schematic model showing the effect of ESCRT-III coexpression on the localization of the reporters on the enlarged PVC/MVB. B, Coexpression of ESCRT-III dominant negative mutants

with four reporters and RFP-ARA7(Q69L) in *Arabidopsis* protoplasts. Scale bar = 50 μm.



B

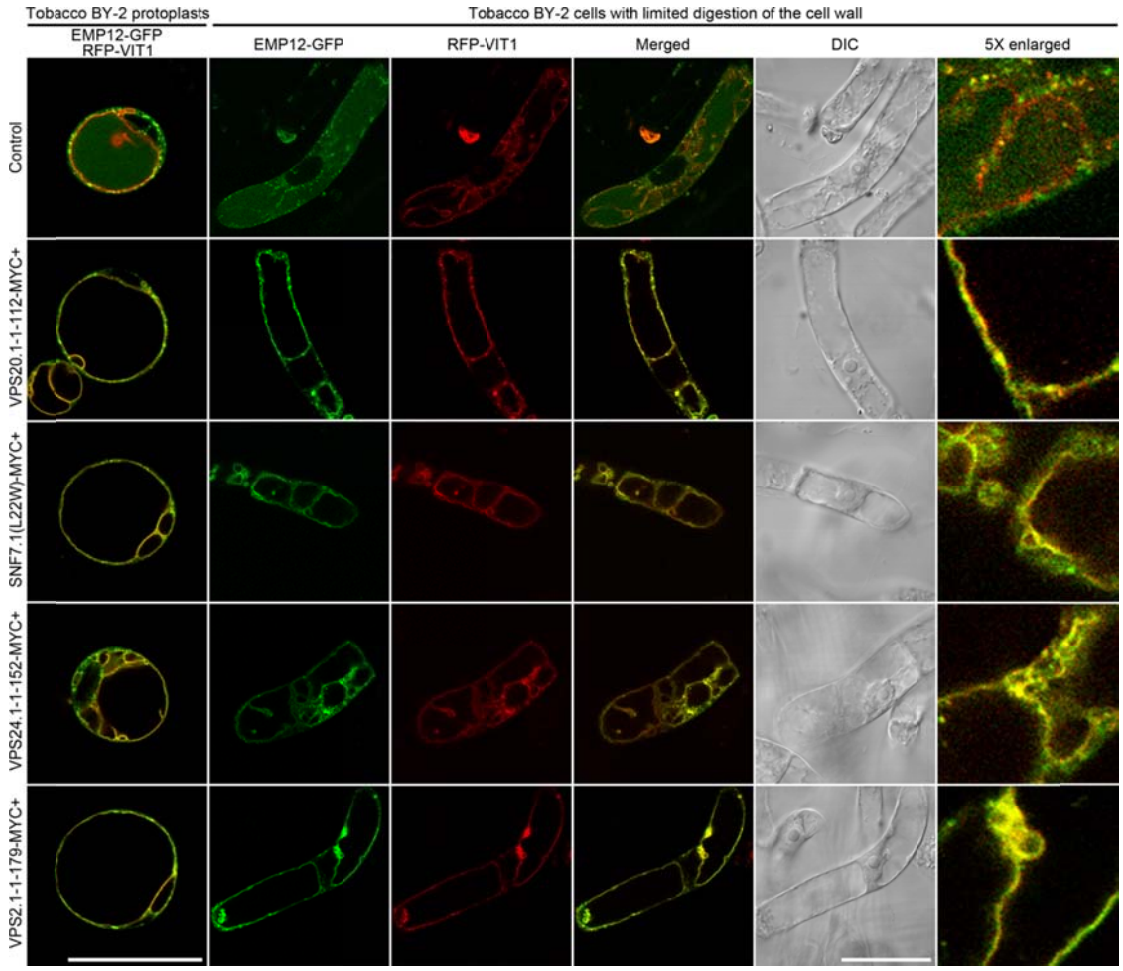
EMP12-GFP	Vacuole (%)	Tonoplast (%)	Punctate dots (%)
Control	91	0	9
+ SKD1(E232Q)	2	77	21
+ VPS20.1-1-112-MYC	5	75	20
+ SNF7.1(L22W)-MYC	6	66	28
+ VPS24.1-1-152-MYC	2	86	12
+ VPS2.1-1-179-MYC	6	70	24

LRR84A-GFP	PM+vacuole (%)	PM+tonoplast (%)	PM+punctate dots (%)
Control	88	0	12
+ SKD1(E232Q)	3	73	24
+ VPS20.1-1-112-MYC	9	65	26
+ SNF7.1(L22W)-MYC	31**	55	14
+ VPS24.1-1-152-MYC	6	75	19
+ VPS2.1-1-179-MYC	5	65	30

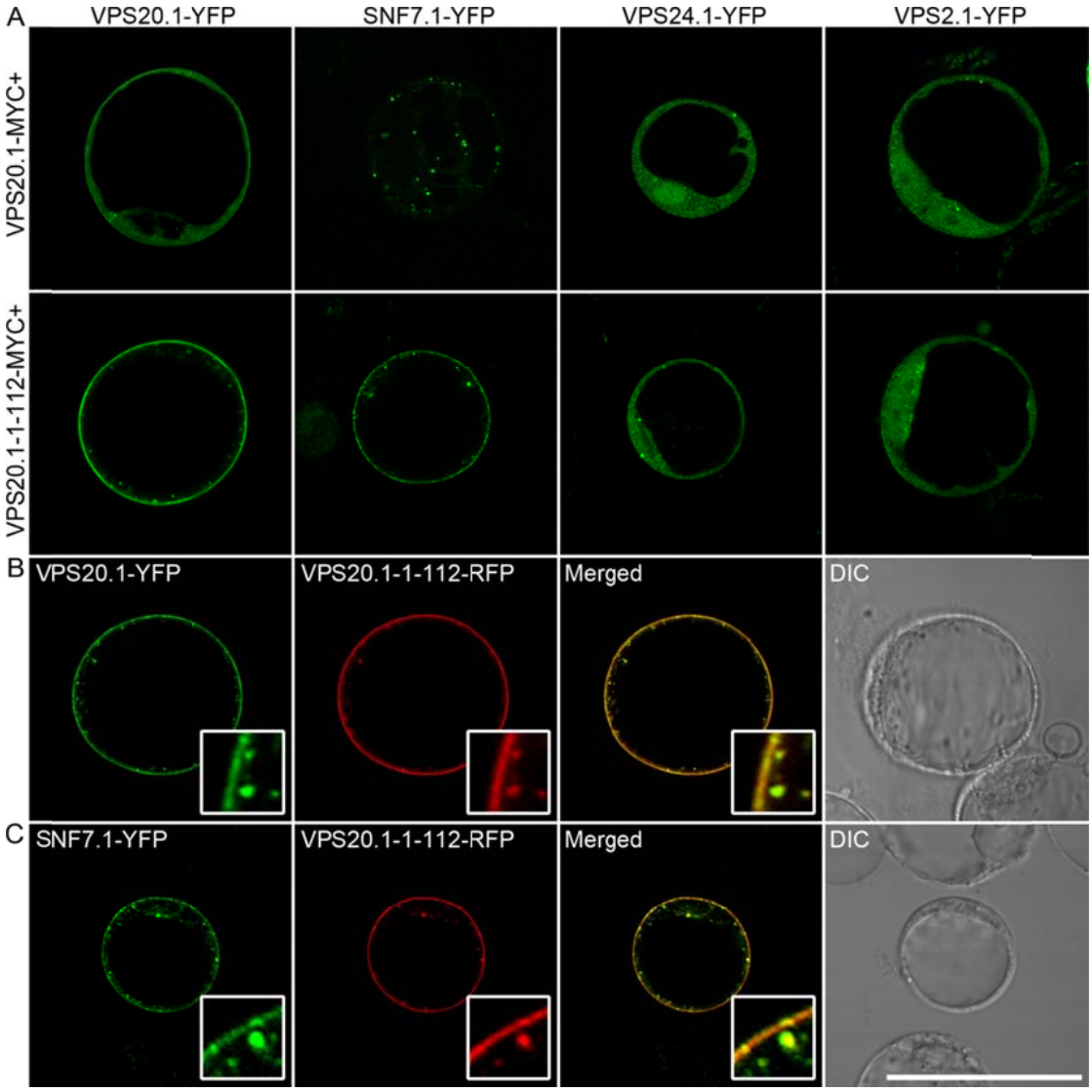
Supplemental Figure S8. Statistic analysis on the expression pattern of protoplasts coexpressing ESCRT-III dominant negative mutants with EMP12-GFP or LRR84A-GFP. ESCRT-III mutants were transiently coexpressed with EMP12-GFP or LRR84A-GFP in *Arabidopsis* PSBD cells.

Representative images of different expression patterns were shown (A). Over 100 protoplasts were quantified for each coexpression experiment and statstic analysis data was presented as a percentage of protoplasts displaying each pattern (B). We noted that around 31% protoplasts displayed vacuole pattern in LRR84A-GFP and SNF7.1(L22W)-MYC coexpression experiments as indicated by double asterisk.

However, among these 31% protoplasts, half of them displayed dual localization on the tonoplast. Scale bar = 50 μm.

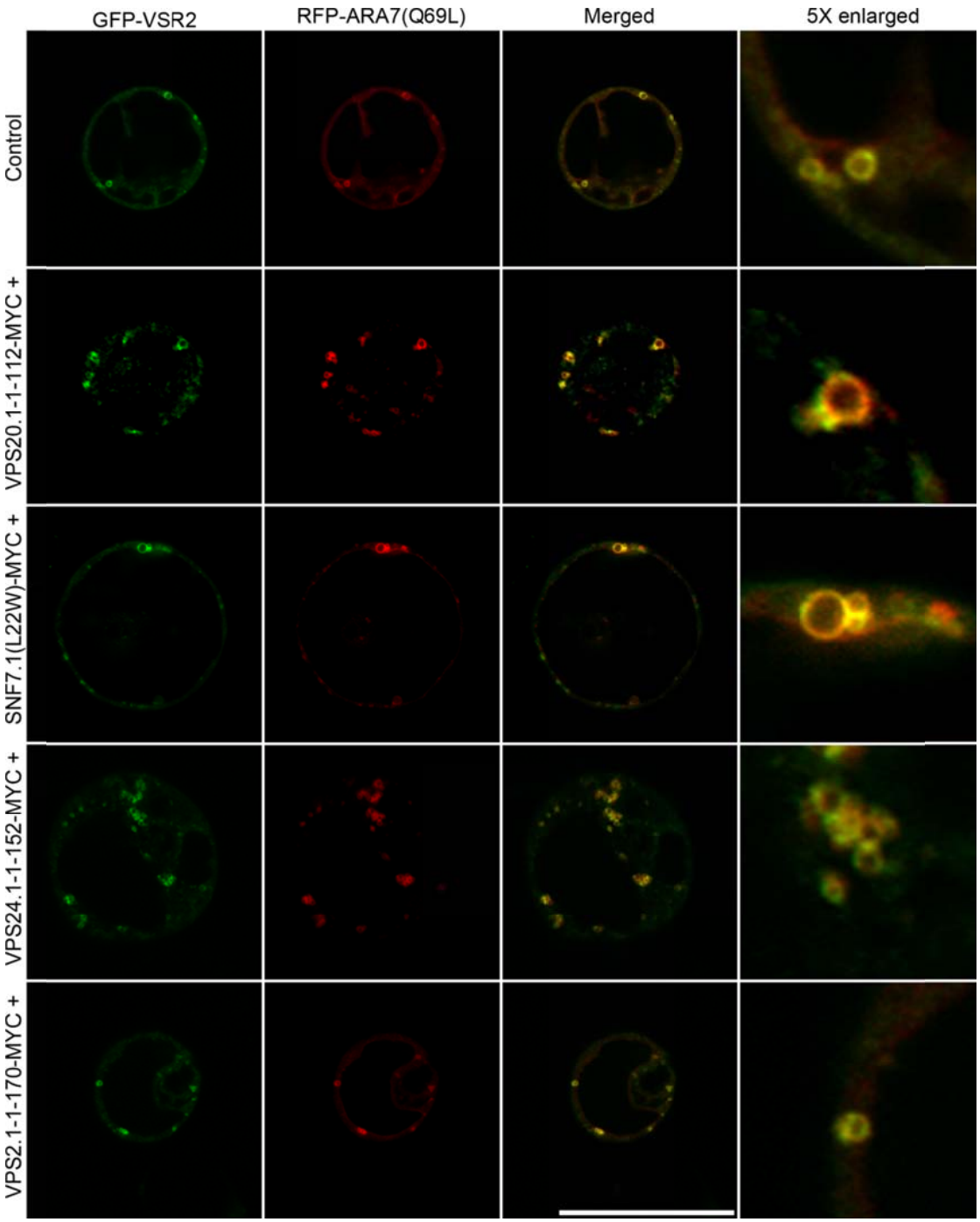


Supplemental Figure S9. Coexpression of *Arabidopsis* ESCRT-III dominant negative mutants altered the expression pattern of EMP12-GFP in tobacco BY-2 cells. *Arabidopsis* ESCRT-III mutants were transiently coexpressed with EMP12-GFP and RFP-VIT1 in tobacco BY-2 protoplasts or tobacco BY-2 cells with limited digestion of the cell wall by cellulicine for 45 minutes to loosen the cell wall. Scale bar = 50 μm.

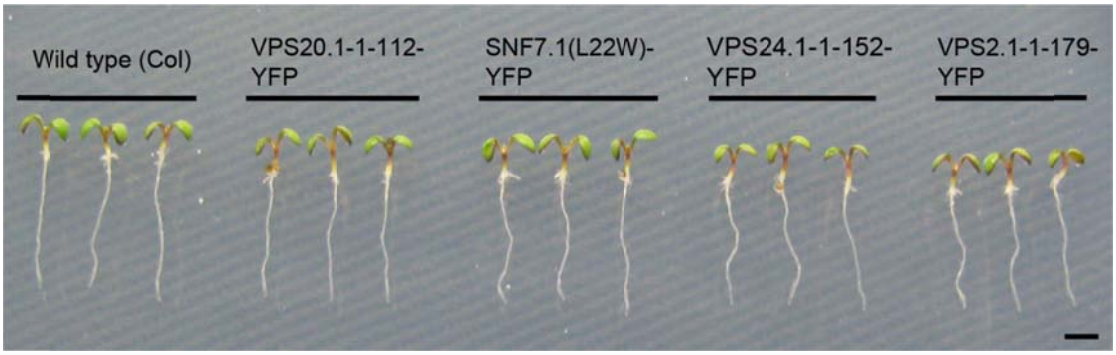


Supplemental Figure S10. Effect of VPS20.1-1-112 overexpression on the localization of the ESCRT-III subunits.

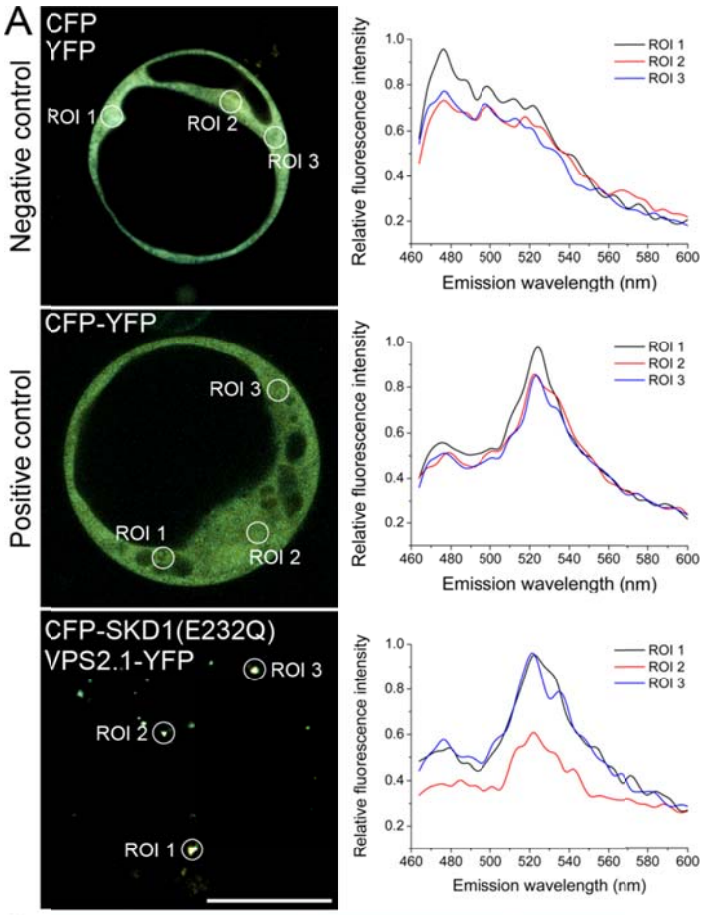
A, ESCRT-III YFP fusions were coexpressed with VPS20.1-MYC or VPS20.1-1-112-MYC in Arabidopsis protoplasts. B and C, VPS20.1-YFP or SNF7.1-YFP was coexpressed with VPS20.1-1-112-RFP in Arabidopsis protoplasts. Scale bar = 50 μm.



Supplemental Figure S11. Coexpression of ESCRT-III mutants with GFP-VSR2 and RFP-ARA7(Q69L). Scale bar = 50 μm.



Supplemental Figure S12. Wild type or transgenic *Arabidopsis* plants grown on MS plates without inducer. Scale bar = 2 mm.



B

	Signal ratio (522nm/476nm)	Interaction?
CFP + YFP (Negative control)	0.67±0.04	No
CFP-YFP (Positive control)	1.83±0.13	Yes
CFP-SKD1(E232Q) + VPS20.1-YFP	1.52±0.16	Yes
CFP-SKD1(E232Q) + SNF7.1-YFP	1.56±0.26	Yes
CFP-SKD1(E232Q) + VPS24.1-YFP	0.89±0.08	No
CFP-SKD1(E232Q) + VPS2.1-YFP	1.65±0.29	Yes

Supplemental Figure S13. SKD1(E232Q) directly interacts with ESCRT-III subunits. A, Representative images of the negative (CFP coexpressed with YFP) and positive (CFP-YFP tandem fusion expression) controls, and coexpression of CFP-SKD1(E232Q) and VPS2.1-YFP were shown in the left panel. The emission spectrum of three different regions is shown in the right panel. ROI, region of interest. Scale bar = 50 μm. B, Quantitative analysis of signal ratios (522nm/476nm) in protoplasts coexpressing CFP-SKD1(E232Q) and different ESCRT-III YFP fusions.