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Supplementary Materials for

Agrobacterium Counteracts Host-Induced Degradation of Its Effector F-Box Protein

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MATERIALS AND METHODS

Plasmids

To analyze the subcellular localization of VirD5, the coding sequence of VirD5 (AAF77175) was PCR-amplified from the tumor-inducing (Ti) plasmid pTiA6 of the *Agrobacterium* A348 strain and cloned into the KpnI site of pSAT5-ECFP-C1 (*1*), resulting in pSAT5-ECFP-VirD5. Free DsRed2 was expressed from pSAT6-DsRed2-C1 (*1*).

To generate constructs for the BiFC assay (*2*), the coding sequence of VirD5 was cloned as PCR-amplified KpnI-SmaI fragments into pSAT6-nEYFP-C1 and pSAT6-cEYFP-C1 (*1*), resulting in pSAT6-nEYFP-VirD5 and pSAT6-cEYFP-VirD5, respectively. For nYFP- and cYFP-VirF fusions, the coding sequence of VirF was cloned as PCR-amplified SalI-BamHI fragments into pSAT6-nEYFP-C1 and pSAT6-cEYFP-C1, resulting in pSAT6-nEYFP-VirF and pSAT6-cEYFP-VirF, respectively. Plasmids for expression of nYFP-VirE3 and cYFP-VirE3 have been described (*3*).

For coimmunoprecipitation, the coding sequence of VirF was cloned as the SalI-BamHI fragment of pSAT6-nEYFP-VirF into the same sites of pSAT6-Myc-C1 (*1*), resulting in pSAT6-Myc-VirF. The expression cassette was then excised with PI-PspI from pSAT6-Myc-VirF and inserted into the same site of the pPZP-RCS1 binary vector (*4*), resulting in pRCS1-Myc-VirF. For the CFP-VirD5 fusion, the expression cassette was excised with I-CeuI from pSAT5-ECFP-VirD5 and inserted into either pPZP-RCS1 binary vector (*4*) or pRCS1-Myc-VirF.

To generate GST-VirD5, the coding sequence of VirD5 was cloned as PCR-amplified SmaI-NotI fragments into the GST fusion vector pGEX-5X-1 (GE

Healthcare).

For cell-free degradation assay, the coding sequence of VirD5 was cloned as the KpnI-SmaI fragment of pSAT6-nEYFP-VirD5 into the same sites of pSAT1-3xFLAG-C1 (*1*), resulting in pSAT1-3xFLAG-VirD5. To clone the mutant VirF (L26A, P27A), the mutant forward megaprimer was first PCR-amplified from pSAT6-Myc-VirF using a forward primer 5'-ACGGGTCGACATGAGAAATTCGAGTTTGCGTG-3' and a reverse primer containing the corresponding mutations 5'-CAGCACGTGGTCtgccgcATTTAGTAATTCTG-3'. Next, the full-length mutant VirF sequence was PCR-amplified using the same forward megaprimer and a reverse primer 5'-ATATGGATCCTCATAGACCGCGCGTTGATCGA-3' and cloned into the SalI-BamHI sites of pSAT6-Myc-C1. For Myc- and 3xFLAG-VirD2 fusions, the coding sequence of VirD2 was PCR-amplified from pSTT-VirD2 (*5*) and cloned into the SalI-BamHI sites of pSAT1-3xFLAG-C1 and pSAT6-Myc-C1. To generate the dominant-negative CULLIN1 ($CUL1^{DN}$), the coding sequence of the C-terminal deletion mutant of *Arabidopsis* CULLIN1 (amino acid residues 1 to 420) was PCR-amplified from an *Arabidopsis* cDNA library (*6*) and cloned into the XhoI-SalI sites of pSAT1-3xFLAG-C1. The resulting expression cassettes were excised with AscI or PI-PspI from pSAT1- or pSAT6-based constructs, respectively, and inserted into the corresponding site of the pPZP-RCS1 binary vector (*4*).

For ubiquitination assay, the coding sequence of *Arabidopsis* ubiquitin (At4g05050) was PCR-amplified from an *Arabidopsis* cDNA library (*6*) and cloned into the SalI-BamHI sites of pSAT1-3xFLAG-C1 (*1*). The resulting expression cassette was excised by AscI and

inserted into the same site of the pPZP-RCS1 binary vector (*4*).

To generate the LexA DNA binding domain (DBD)-CUL1 fusion construct, the coding sequence of the *Arabidopsis* CUL1 was PCR-amplified from an *Arabidopsis* cDNA library (*6*) and cloned into the EcoRI-SalI sites of pSTT91 (*7*). For the LexA DBD-CUL1^{DN} fusion construct, the coding sequence of CUL1^{DN} was PCR-amplified from an *Arabidopsis* cDNA library and cloned into the EcoRI-SalI sites of pSTT91. To generate the GAL4 activation domain (AD)-RBX1 fusion construct, the coding sequence of the *Arabidopsis* RBX1 was PCR-amplified from an *Arabidopsis* cDNA library and cloned into the EcoRI-SalI sites of pGAD424 (Clontech). The LexA DBD fusion of TOP1 (*Saccharomyces cerevisiae* DNA topoisomerase I) (*8*), and GAL4 AD fusions of ASK1 (*Arabidopsis* SKP-like 1) (*5*) and TMV MP (*Tobacco mosaic virus* movement protein) (*9*) have been described.

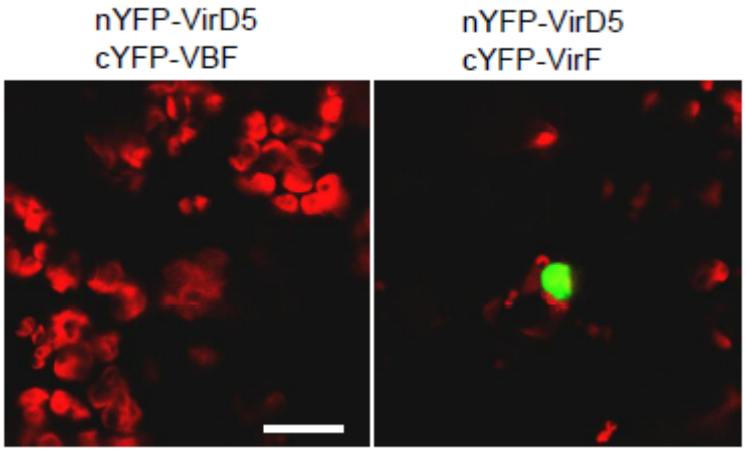

Fig. S1. VirD5 does not interact with VBF (VIP1-binding F-box protein)

BiFC assay for the VirD5-VBF interaction in *Nicotiana benthamiana* leaves. Reconstitution of YFP fluorescence was not detected when nYFP-VirD5 and cYFP-VBF were coexpressed in leaf epidermal cells of *N. benthamiana*. Experiments were peformed twice. The VirD5-VirF interaction was used as positive control. Bar = 20 μm. YFP signal is in green, and plastid autofluorescence is in red. All images are single confocal sections.

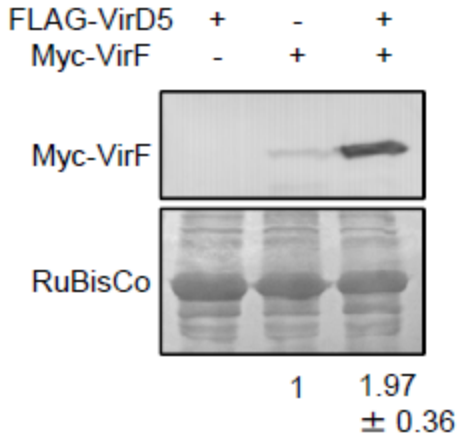

Fig. S2. Increased amounts of VirF in the presence of VirD5 in plant cells

Protein extracts were prepared from *N. benthamiana* leaves transiently expressing FLAG-VirD5 or Myc-VirF alone or together. Approximately 50 μg of total proteins were subject to immunoblotting with anti-Myc antibody. The putative RuBisCo large chain was used as loading control. Relative Myc-VirF amounts normalized to RuBisCo are shown below the blot as mean of three experiments ± SEM.

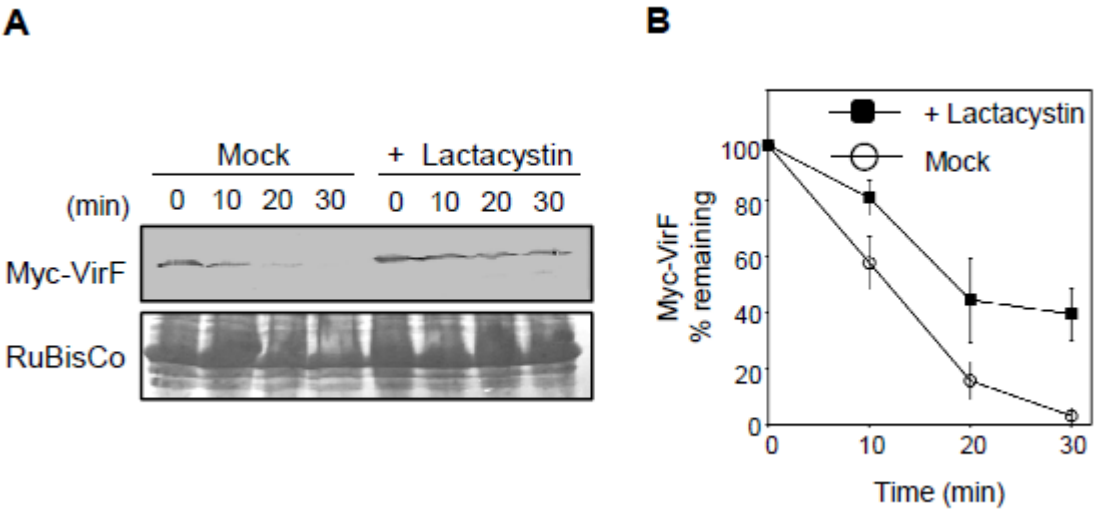

Fig. S3. Lactacystin, a specific inhibitor of the 26*S* proteasome, stabilizes VirF

(A) *N. benthamiana* leaves transiently expressing Myc-VirF were treated with either water (Mock) or 10 μM lactacystin (+Lactacystin) for 4 hours, and the resulting protein extracts were analyzed by the cell-free degradation assay. The putative RuBisCo large chain was used as loading control. **(B)** Quantification of Myc-VirF degradation described in (A). Relative protein amounts were normalized to the RuBisCo loading control. All quantified data are shown as mean of three experiments ± SEM.

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