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HOST-PATHOGEN INTERACTIONS

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

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The SCF (Skp1–Cul1–F-box protein) ubiquitin ligase complex plays a pivotal role in various biological processes, including host-pathogen interactions. Many pathogens exploit the host SCF machinery to promote efficient infection by translocating pathogen-encoded F-box proteins into the host cell. How pathogens ensure sufficient amounts of the F-box effectors in the host cell despite the intrinsically unstable nature of F-box proteins, however, remains unclear. We found that the *Agrobacterium* F-box protein VirF, an important virulence factor, undergoes rapid degradation through the host proteasome pathway. This destabilization of VirF was counteracted by VirD5, another bacterial effector that physically associated with VirF. These observations reveal a previously unknown counterdefense strategy used by pathogens against potential host antimicrobial responses.

INTRODUCTION

The pathogen *Agrobacterium* genetically transforms plants, causing crown gall disease, and, under laboratory conditions, can transform virtually any eukaryotic species, from fungal to human cells (*1*). During transformation, a single-stranded copy of the bacterial transferred DNA (T-DNA) is exported into the host cell and ultimately integrated into the host genome (*2*). Within the host cell, the T-DNA is packaged into a nucleoprotein complex (T-complex) in which it is coated with the bacterial virulence (Vir) protein VirE2 (*3*). In addition, the plant factor VIP1 (VirE2-interacting protein 1) directly binds to VirE2, facilitating nuclear import of the T-complex and its subsequent targeting to the host chromatin (*4*–*6*). Upon *Agrobacterium* infection, host plants activate the mitogen-activated protein kinase (MAPK) defense signaling, leading to phosphorylation of VIP1 (*7*). This phosphorylation event enables the translocation of VIP1 into the nucleus, where it induces expression of several stress-responsive genes, including the pathogenesis-related *PR1* (*7*, *8*). This finding suggests that *Agrobacterium* co-opts the host defense response to efficiently transport the T-complex into the nucleus.

Once the T-complex reaches the host nucleus, it needs to be uncoated before T-DNA integration or its expression. This is mediated by the *Agrobacterium* F-box protein VirF, one of the bacterial effectors that are translocated into the host cell (*9*). As a subunit of the SCF (Skp1–Cul1–F-box protein) ubiquitin E3 ligase complex, VirF targets VIP1 as well as its associated protein VirE2 for ubiquitin-mediated, proteasome-dependent degradation (*9*). This VirF-mediated targeted proteolysis seems to be indispensable for *Agrobacterium* infection because deletion of the *virF* locus or mutations in the F-box domain of VirF substantially attenuate virulence (*10*–*12*).

An infection strategy that uses pathogen-encoded F-box proteins is employed not only by *Agrobacterium* but also by other viral (*13*–*15*) and bacterial pathogens (*16*), including the human pathogen *Legionella pneumophila* (*17*, *18*). The apparent paradox of this strategy is that F-box proteins are inherently short-lived because of their own proteolysis, which is mediated by autoubiquitination activity (*19*, *20*) or other E3 ubiquitin ligases (*21*–*23*). Assuming that only a limited amount of virulence proteins is translocated into the host cell, it would be critical for pathogens to protect their F-box effectors against degradation. Invading pathogens do not export the gene that encodes the F-box effector to the host cell and therefore must stabilize their exported F-box proteins. How this challenge is met by the pathogens remains unknown. Here, we demonstrate that *Agrobacterium* uses another exported virulence protein, VirD5, to stabilize VirF, which otherwise undergoes rapid degradation through the host ubiquitin-proteasome pathway. The role of VirD5 in the infection process was confirmed by the observations that an *Agrobacterium* strain lacking VirD5 exhibited significantly attenuated virulence on tomato plants. Thus, we propose that *Agrobacterium* has evolved VirD5 to counteract the host-induced degradation of VirF, thereby maximizing infection efficiency.

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RESULTS

VirD5 is localized to the cell nucleus

VirD5 is a bacterial host range factor (*24*) that shows no apparent homology to other proteins, making it difficult to determine its biological function. Although early genetic studies had indicated that VirD5 is not essential for *Agrobacterium* pathogenesis (*25*–*28*), additional work demonstrated that VirD5 is translocated into the host cell (*24*), suggesting that it may play a role within the host cell during *Agrobacterium* infection. Consistent with this notion, VirD5 (accession number, AAF77175) contains four potential eukaryotic nuclear localization signals (NLSs): ^{170}KRKR^{173}, ^{324}RRLGAPERTAYERWSKR^{340}, ^{766}KKDLEAKSVGVRQKKKE^{782}, and ^{817}RRVYDPRDRAQDKAFKR^{833}. Indeed, the cyan fluorescent protein (CFP)–tagged VirD5 (CFP-VirD5) protein was observed exclusively in the cell nucleus when transiently expressed in *Nicotiana benthamiana* leaves (Fig. 1A). Thus, our data further suggest that *Agrobacterium* VirD5 has evolved to function in plant cells and, more specifically, in the plant nucleus.

VirD5 interacts with VirF

To elucidate the role of VirD5 in *Agrobacterium* infection, we next examined possible interactions between VirD5 and other known exported Vir proteins (VirD2, VirE2, VirE3, and VirF) (*24*, *29*) in plants, using bimolecular fluorescence complementation (BiFC) (*30*). Only VirF interacted with VirD5 (Fig. 1B). When coexpressed in leaf epidermal cells of *N. benthamiana*, the N-terminal half of yellow fluorescent protein (YFP)

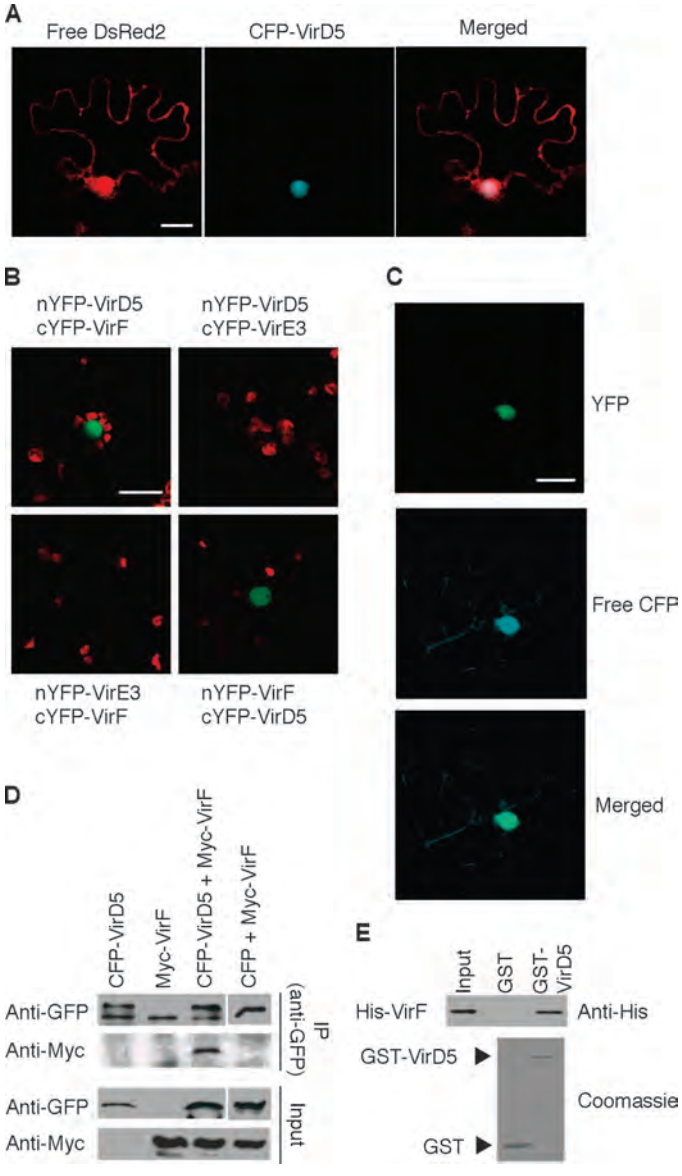

Fig. 1. VirD5 interacts with VirF in the nucleus. (A) CFP-tagged VirD5 was transiently coexpressed with free DsRed2 in *N. benthamiana* leaf epidermal cells by microbombardment. CFP-VirD5 colocalized with the nuclear DsRed2. Scale bar, 20 μm. Images are single confocal sections. (B) In vivo BiFC assay for the VirD5-VirF interaction in *N. benthamiana* leaves. VirE3 was used as negative control. Scale bar, 20 μm. YFP signal is in green, and plastid autofluorescence is in red. (C) Nuclear localization of the VirD5-VirF complexes. The YFP signal is derived from the interacting nYFP-VirD5 and cYFP-VirF. Free CFP is a reference protein that visualizes the cell cytoplasm and the nucleus. YFP and CFP signals are in green and blue, respectively; merged image represents overlay of the BiFC and CFP signals. Scale bar, 20 μm. All images are single confocal sections. (D) Coimmunoprecipitation assay for the VirD5-VirF interaction. CFP-VirD5 and Myc-VirF expressed in *N. benthamiana* leaves were immunoprecipitated (IP) with anti-GFP antibody, followed by immunoblotting with anti-GFP or anti-Myc antibody. (E) GST pull-down assay for the VirD5-VirF interaction. His-VirF was detected by anti-His antibody (top). GST and GST-VirD5 proteins were detected by Coomassie blue staining (bottom). Each experiment was performed at least twice with similar results.

fused to VirD5 (nYFP-VirD5) and the C-terminal half of YFP fused to VirF (cYFP-VirF) led to reconstitution of the YFP fluorescence (Fig. 1B), indicating interaction between the proteins. Similarly, the reciprocal combination (nYFP-VirF and cYFP-VirD5) also restored YFP fluorescence (Fig. 1B). The interacting complexes were observed exclusively in the cell nucleus and colocalized with the nuclear portion of coexpressed CFP (Fig. 1C), potentially reflecting the nuclear nature of both VirD5 (Fig. 1A) and VirF (*9*). No YFP signal was detected when cYFP-VirF was coexpressed with nYFP-VirE3, another *Agrobacterium* effector that is translocated into the host cell (*24*), or when nYFP-VirD5 was coexpressed with cYFP-VirE3 (Fig. 1B). In addition, nYFP-VirD5 did not interact with cYFP-VBF (VIP1-binding F-box protein) (fig. S1), a plant nuclear F-box protein that mimics the molecular function of VirF (*31*). We next confirmed the VirD5-VirF interaction in plant cells by an independent approach. CFP-VirD5 and Myc-tagged VirF (Myc-VirF) were transiently expressed in *N. benthamiana* leaves. Myc-VirF coimmunoprecipitated with CFP-VirD5 but not with free CFP (Fig. 1D). In an in vitro interaction assay, glutathione *S*-transferase (GST)–tagged VirD5 (GST-VirD5) specifically pulled down His-tagged VirF (His-VirF) (Fig. 1E). Together, these observations demonstrate that VirD5 interacts with VirF.

The F-box protein effector VirF is stabilized by VirD5

F-box proteins are intrinsically unstable (*19*, *20*). Therefore, the direct binding of VirD5 to VirF may function to stabilize the latter, ensuring that a sufficient amount of the bacterial F-box protein effector is available in the host cell to promote efficient infection. To test this hypothesis, we first examined whether coexpression of VirF and VirD5 in *N. benthamiana* alters the cellular amounts of VirF. Indeed, VirF accumulated at higher amounts in the presence of VirD5 in plant cells (fig. S2). Then, we analyzed the stability of VirF in the presence or absence of VirD5 by a cell-free degradation assay. Protein extracts were prepared from *N. benthamiana* plants transiently expressing Myc-VirF with or without FLAG-tagged VirD5 (FLAG-VirD5). In plant extracts containing Myc-VirF alone, VirF protein amounts rapidly declined over 30 min (Fig. 2, A and B). However, this degradation of VirF was delayed in the presence of FLAG-VirD5 (Fig. 2, A and B). In contrast, Myc-VirD2 was stable over 30 min, indicating that VirF destabilization in plant extracts is specific (Fig. 2, C and D). The stabilization effect of VirD5 on VirF was also specific because FLAG-VirD2 did not affect the degradation of VirF (Fig. 2, E and F).

VirF is targeted for degradation through the host ubiquitin-proteasome pathway

To shed light on the mechanism underlying the VirF degradation, we first tested the effect of the proteasome inhibitors MG132 and lactacystin on the stability of VirF. Pretreatment of *N. benthamiana* leaves with either proteasome inhibitor prolonged the half-life of VirF (Fig. 3, A and B, and fig. S3, A and B), suggesting that VirF is degraded through the host ubiquitin-proteasome pathway. Consistent with this notion, exogenous addition of the K0 ubiquitin mutant, which lacks all lysine residues required for ubiquitin polymerization, delayed the degradation of VirF (Fig. 3, C and D). These results prompted us to investigate whether VirF undergoes ubiquitination in vivo. For this purpose, Myc-VirF and FLAG-tagged ubiquitin (FLAG-Ub) were coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with MG132 to enrich the polyubiquitinated VirF species, total protein was extracted and immunoprecipitation was carried out with anti-Myc antibody. Subsequent immunoblot analysis using anti-FLAG or anti-Myc antibody revealed high–molecular weight protein species, an indication of polyubiquitination (Fig. 3E). The signal intensity of the high–molecular weight species was reduced when the leaves were pretreated with dimethyl sulfoxide (DMSO) instead of MG132

before harvesting (Fig. 3F). Together, these results indicate that VirF is polyubiquitinated and targeted for the proteasome-dependent degradation in the host cells.

The autocatalytic mechanism of F-box proteins is not involved in the degradation of VirF

Several F-box proteins promote their own degradation through auto-ubiquitination, which requires an intact F-box domain and its association with the core SCF (*19*, *20*). However, this is not likely for VirF because mutations in its F-box domain (Leu^{26}→Ala and Pro^{27}→Ala), which abolish the interaction of VirF with ASK1 (*Arabidopsis* SKP1-like 1) (*10*), did not substantially affect the degradation of VirF (Fig. 4, A and B). Moreover, VirD5 could still stabilize the mutant form of VirF (Fig. 4, A and B). These results suggest that VirF degradation may be mediated primarily by other E3 ubiquitin ligases rather than the auto-catalytic mechanism.

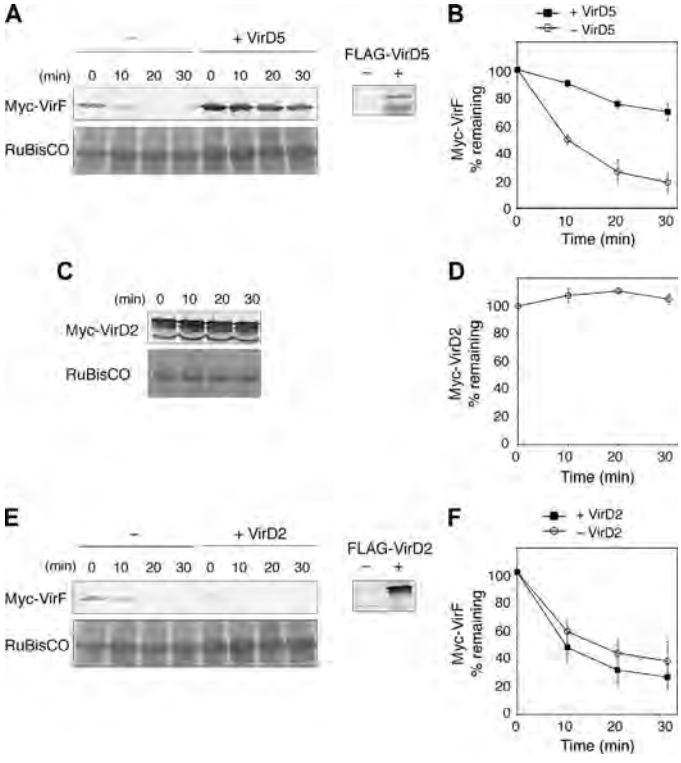

Fig. 2. VirF degradation and its stabilization by VirD5. (**A**) Myc-VirF is destabilized in a cell-free degradation assay. Myc-VirF was expressed alone (–) or coexpressed with FLAG-VirD5 (+VirD5) in *N. benthamiana* leaves. The resulting protein extracts were incubated for the indicated time periods. The putative RuBisCO large chain was used as loading control. The presence of FLAG-VirD5 in the plant extract was confirmed with anti-FLAG antibody (right). (**B**) Quantification of Myc-VirF degradation described in (A). (**C**) Myc-VirD2 is stable in the cell-free degradation assay. (**D**) Quantification of Myc-VirD2 degradation described in (C). (**E**) FLAG-VirD2 has no effect on VirF degradation. Myc-VirF was expressed alone (–) or coexpressed with FLAG-VirD2 (+VirD2). The presence of FLAG-VirD2 in the plant extract was confirmed with anti-FLAG antibody (right). (**F**) Quantification of Myc-VirF degradation described in (E). In all quantifications, relative protein amounts were normalized to the RuBisCO loading control. All quantified data are means ± SEM (n = 3 experiments).

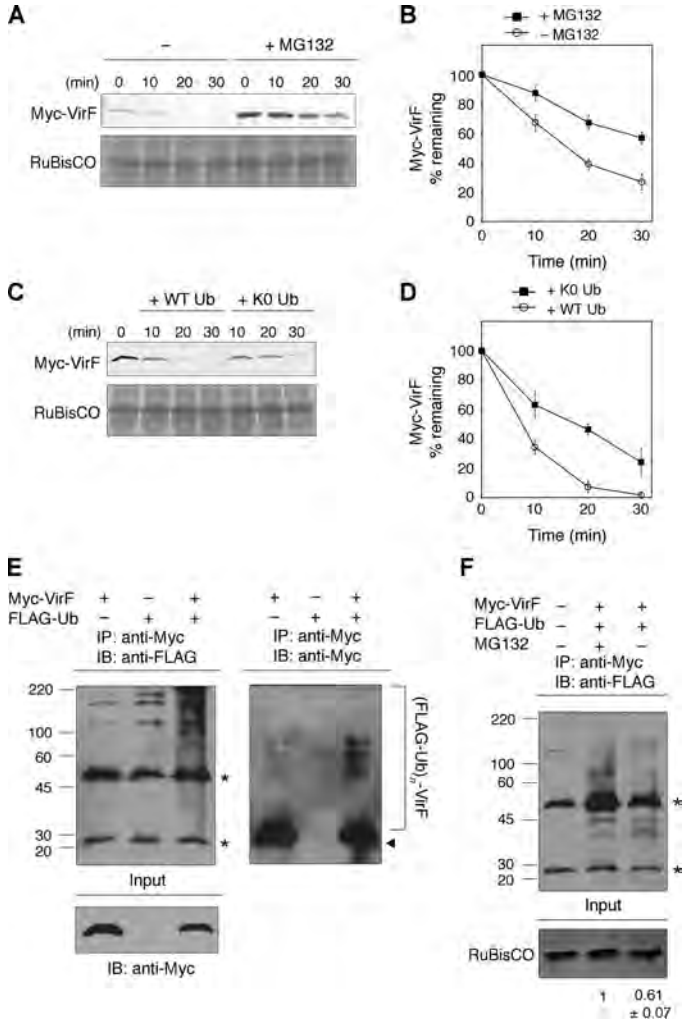

Fig. 3. VirF is degraded through the host ubiquitin-proteasome pathway. (**A**) Effect of the proteasome inhibitor MG132 on VirF degradation. *N. benthamiana* leaves transiently expressing Myc-VirF were treated with either DMSO (–) or 10 μM MG132 (+MG132) for 4 hours and analyzed by the cell-free degradation assay. (**B**) Quantification of Myc-VirF degradation described in (A). (**C**) VirF degradation is delayed by the K0 ubiquitin mutant. Recombinant wild-type (WT) or K0 ubiquitin (K0 Ub) (1 μg/μl) was added to protein extracts, and the stability of Myc-VirF was analyzed by the cell-free degradation assay. (**D**) Quantification of Myc-VirF degradation described in (C). (**E**) Ubiquitination of VirF. Myc-VirF and FLAG-ubiquitin were transiently coexpressed in *N. benthamiana* leaves. After pretreatment of the leaves with 10 μM MG132 for 4 hours, total proteins were immunoprecipitated (IP) with anti-Myc antibody, followed by immunoblotting (IB) with anti-FLAG (left) or anti-Myc antibody (right). Polyubiquitinated VirF species are indicated by a bracket [$(FLAG\text{-}Ub)_n$–VirF]. Arrowhead indicates nonubiquitinated VirF. (**F**) Omission of pretreatment of plants with MG132 reduced the amount of polyubiquitinated VirF. Asterisks indicate immunoglobulin G (IgG) heavy and light chains. Molecular mass markers (in kilodaltons) are indicated on the left. Relative polyubiquitinated VirF amounts are shown below the blot. All quantified data are means ± SEM (n = 3 experiments).

VirF degradation occurs through the host SCF pathway

Plants encode an unusually large number of F-box subunits (*32*), making the SCF complexes the largest class of E3 ubiquitin ligases for plants. Therefore, VirF degradation may be induced by a plant SCF complex. To explore this possibility, we generated a plant dominant-negative CULLIN1 ($CUL1^{DN}$). In a yeast two-hybrid assay, this C-terminal deletion mutant of *Arabidopsis* CULLIN1 (amino acid residues 1 to 420) interacted with ASK1, but not with RBX1, the catalytic core of the SCF complex (Fig. 4C). Similar dominant-negative forms of CUL1 have been used to identify substrates of mammalian SCF E3 ligases (*33*). We took advantage of this molecular tool to test potential involvement of a host SCF complex in VirF degradation. FLAG-tagged $CUL1^{DN}$ (FLAG-$CUL1^{DN}$) and Myc-VirF were coexpressed in *N. benthamiana* leaves, and the protein extracts were analyzed by a cell-free degradation assay. VirF proteins were stabilized in the presence of FLAG-$CUL1^{DN}$ (Fig. 4, D and E), suggesting that a plant SCF complex is at least partly responsible for the degradation of VirF in the host cell.

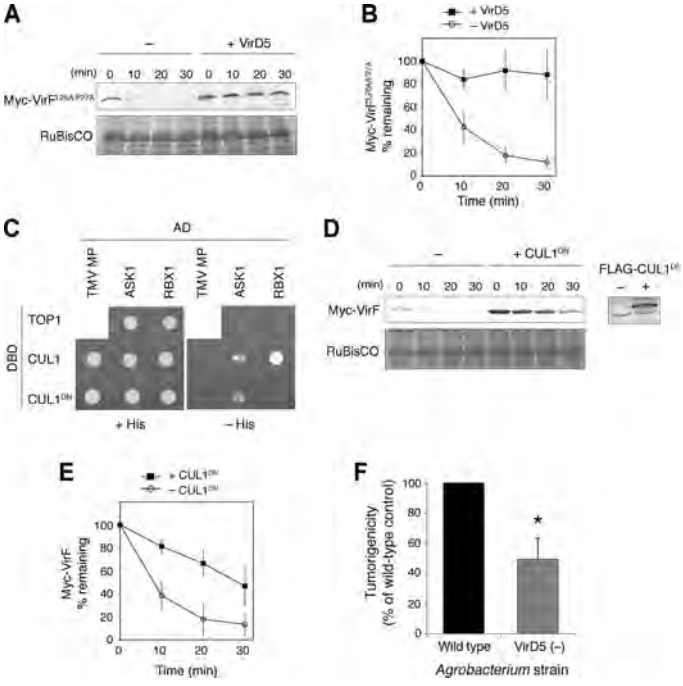

Fig. 4. Mechanism of VirF degradation and the requirement of VirD5 for *Agrobacterium* infection of tomato. (**A**) Cell-free degradation assay with the mutant VirF ($VirF^{L26A/P27A}$). Myc-$VirF^{L26A/P27A}$ was expressed alone (–) or coexpressed with FLAG-VirD5 (+VirD5) in *N. benthamiana* leaves. The resulting protein extracts were incubated for the indicated time periods. The putative RuBisCO large chain was used as loading control. (**B**) Quantification of Myc-$VirF^{L26A/P27A}$ degradation described in (A). (**C**) $CUL1^{DN}$ interacts with ASK1, but not RBX1, in a yeast two-hybrid system. Yeast cells expressing the indicated LexA DNA binding domain (DBD) fusions and GAL4 activation domain (AD) fusions were grown in the presence (+His) or absence of histidine (–His). *Tobacco mosaic virus* movement protein (TMV MP) and *Saccharomyces cerevisiae* DNA topoisomerase I (TOP1) were used as negative controls. (**D**) Effect of $CUL1^{DN}$ on VirF degradation. Myc-VirF was expressed alone (–) or coexpressed with FLAG-$CUL1^{DN}$ (+$CUL1^{DN}$) in *N. benthamiana* leaves. The resulting protein extracts were incubated for the indicated time periods. The presence of $CUL1^{DN}$ in the plant extract was confirmed with anti-FLAG antibody (right). (**E**) Quantification of Myc-VirF degradation described in (D). In all quantifications, relative protein amounts were normalized to the RuBisCO loading control. All quantified data are means ± SEM (n = 3 experiments). (**F**) VirD5 is necessary for efficient infection of tomato by *Agrobacterium*. Tomato seedlings were inoculated with either a wild-type *Agrobacterium* strain or a mutant strain that does not express VirD5 [VirD5 (–)]. Tumorigenicity was quantified as percentage of inoculation sites (50 to 70 per experiment) that developed a tumor and expressed as means ± SD (n = 3 experiments). The value of the wild-type control was set at 100%. *$P < 0.01$.

VirD5 is necessary for efficient infection of *Agrobacterium*

Although our data indicate an important role for VirD5 in the *Agrobacterium* infection, several previous genetic studies suggested that VirD5 is not essential for this process (*25*–*28*). However, most of these experiments were performed in plant species that do not require VirF for infection. If the main function of VirD5 is to stabilize VirF, its effect on infection should be tested in a host, such as tomato, in which VirF enhances infection (*12*). We thus inoculated the stems of tomato seedlings with either wild-type *Agrobacterium* or a mutant bacterial strain lacking *virD5* (*28*) and scored the resulting crown gall tumors. The number of the inoculation sites that developed a tumor was significantly decreased in the VirD5 (–) mutant compared to the wild-type bacterium (Fig. 4F), supporting the requirement of VirD5 for efficient *Agrobacterium* infection.

DISCUSSION

Host-pathogen interactions represent a never-ending arms race. Most organisms have evolved elaborate defense systems to fight against invading pathogens, whereas pathogens exploit or interfere with the host cellular functions to maximize their own infection efficiency. For example, RNA silencing serves as a well-conserved antiviral defense mechanism in both plants and animals (*34*). Conversely, many viral pathogens evade this defense by using their silencing suppressors that disrupt the host RNA silencing pathway (*34*). Such biotic interaction exemplifies a coevolution of defense and counterdefense between the host and the pathogens (*35*). Here, we reveal a previously unknown type of counterdefense strategy used by pathogens against the potential host antimicrobial response.

The *Agrobacterium* F-box protein VirF plays an essential role in uncoating of the T-complex in the host cells (*9*). Using a cell-free degradation assay, we demonstrated that VirF is unstable in protein extracts prepared from *N. benthamiana*, one of the natural host plants of *Agrobacterium*. This destabilization of VirF was reduced by the proteasome inhibitors MG132 and lactacystin, suggesting that the host ubiquitin-proteasome pathway is responsible for VirF degradation. Consistent with this notion, we observed that VirF undergoes polyubiquitination in the plant cells. Together, our data suggest the presence of a host defense mechanism that targets the *Agrobacterium* virulence factor for proteasome-dependent degradation. Because VirF effector function is critical for the *Agrobacterium* infection (*10*–*12*), a plant defense strategy that targets VirF makes biological sense. On the other hand, the destabilization of VirF might also represent the "unstable when active" phenomenon observed in other F-box proteins (*36*). Assembly of some SCF complexes leads to destabilization not only of their target proteins but also of the F-box adaptor proteins (*19*, *20*). However, this autocatalytic mechanism is not likely to be the case for VirF because the mutations in its F-box domain, which is required for the assembly of the SCF complex (*10*), did not stabilize VirF. Thus, VirF is likely destabilized through a host E3 ubiquitin ligase.

The fact that plants encode an unusually large number of F-box proteins (*32*) may reflect an evolutionary adaptation of the sessile organism

that necessitates broad protection against pathogens. Indeed, increasing evidence suggests that many plant F-box proteins play substantial roles in defense response (*31*, *37*, *38*). Thus, we suggest that during *Agrobacterium* infection, the host plant activates a cellular SCF complex containing an as-yet-unidentified F-box protein to destabilize VirF. This idea is further corroborated by the observation that disruption of the plant SCF function by a dominant-negative CUL1 led to the increased stability of VirF.

As a counterdefense strategy, *Agrobacterium* has evolved another effector, VirD5, which is exported into the host cell and acts to bind and stabilize VirF. Thus, VirD5 functions as a positive regulator of the VirF protein and, by implication, *Agrobacterium* infection. Indeed, an *Agrobacterium* mutant that does not produce VirD5 significantly attenuated virulence in a host known to require VirF for efficient infection, further substantiating the role of VirD5 in the infection process. One limitation of our data, however, is that we were unable to identify mutations that specifically abolish the VirD5-VirF interaction and test the effects of such mutations on the bacterial virulence and VirF stability.

That VirD5, like VirF and VirE3, is a host range factor (*24*) not absolutely essential for infection in some hosts (*25*–*28*) suggests that in these hosts *Agrobacterium* may also exploit cellular factors that are functionally redundant with VirD5. Such functional redundancy between *Agrobacterium* effectors and host proteins is seen for VirE3 and VIP1 (*39*) and for VirF and VBF (*31*). Alternatively, in some hosts, the cellular F-box proteins, such as VBF (*31*), may substitute for VirF when the VirF protein amounts are reduced in the absence of VirD5.

Our observations reveal a previously unknown type of the host-pathogen molecular arms race that takes place over control of the stability of pathogen-encoded F-box protein effectors. Considering that diverse pathogens use their F-box proteins for infection, the host defense targeting such F-box proteins and the counterdefense strategy exploiting protective bacterial effectors could be potentially widespread.

MATERIALS AND METHODS

Sequence analysis

The WoLF PSORT (http://wolfpsort.org/) Web server was used to predict the NLSs of VirD5. The amino acid sequence of VirD5 (AAF77175) was submitted as the query.

Bimolecular fluorescence complementation

Microprojectiles were prepared with 200 ng of nYFP- and cYFP-fused constructs per shot and bombarded into *N. benthamiana* leaves with the Helios Gene Gun System (Bio-Rad). For studies of the subcellular localization of the VirD5-VirF interaction, free CFP was coexpressed from pSAT5-ECFP-C1 (*40*). Reconstitution of YFP fluorescence and free CFP fluorescence was detected with a Zeiss LSM 5 Pascal confocal microscope.

Transient expression by agroinfiltration

Agrobacterium EHA105 strain harboring an appropriate pPZP-RCS1 binary vector (*41*) was grown in LB medium supplemented with spectinomycin (100 μg/ml) overnight at 28°C. Cells were harvested by centrifugation and resuspended to OD_{600} (optical density at 600 nm) of 0.5 in infiltration buffer [10 mM $MgCl_2$, 10 mM MES (pH 5.5), 100 μM acetosyringone]. Bacterial suspension was incubated for 2 hours at room temperature and then infiltrated into the abaxial sides of 3- to 4-week-old *N. benthamiana* leaves with a 1-ml needleless syringe. Plants were grown for 48 hours under regular growth conditions before being harvested.

Coimmunoprecipitation assays from plant cells

Fusion proteins were transiently expressed in *N. benthamiana* leaves by agroinfiltration. Infiltrated leaves were harvested and ground into fine powder in liquid nitrogen. Total proteins were extracted from ground tissues in immunoprecipitation buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 3 mM dithiothreitol (DTT), 1× plant protease inhibitor cocktail (Sigma-Aldrich), and 50 μM MG132 (Calbiochem)]. Protein extracts were incubated with anti–green fluorescent protein (GFP) antibody (Clontech) for 3 hours at 4°C, followed by incubation with Protein G–Sepharose 4B (Invitrogen) for an additional 3 hours at 4°C to capture the immunocomplexes. After three washes with washing buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, and 1 mM EDTA], immunoprecipitates were eluted in SDS sample buffer. CFP-VirD5 and Myc-VirF proteins were detected by immunoblotting with anti-GFP antibody (Clontech) and anti–c-Myc antibody conjugated to horseradish peroxidase (Roche), respectively.

GST pull-down assays

GST and GST-VirD5 were expressed in *Escherichia coli* BL21(DE3) and immobilized on Glutathione Sepharose 4B (GE Healthcare). GST- and GST-VirD5–bound glutathione beads were incubated with purified His-VirF (*4*) in phosphate-buffered saline buffer containing 0.1% NP-40 for 2 hours at room temperature. After four washes with the same buffer, pulled-down proteins were resuspended in SDS sample buffer. His-VirF was detected by immunoblotting with anti-His antibody (Bethyl Laboratories) followed by anti-rabbit antibody conjugated to horseradish peroxidase (Pierce).

Cell-free degradation assay

Myc or 3×FLAG fusion protein alone or together was transiently expressed in *N. benthamiana* leaves by agroinfiltration. Infiltrated leaves were harvested and ground into fine powder in liquid nitrogen. For studies of effects of proteasome inhibitor MG132, leaves were infiltrated with 10 μM MG132 (Calbiochem) or mock-treated with 0.1% DMSO and incubated for an additional 4 hours before harvesting. For studies of effects of the proteasome inhibitor lactacystin, leaves were infiltrated with 10 μM lactacystin (Sigma-Aldrich) or mock-treated with distilled water and incubated for an additional 4 hours before harvesting. Cell-free degradation assay was performed as described with slight modifications (*42*). Total proteins were extracted from ground tissues in degradation buffer [50 mM tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 5 mM adenosine 5′-triphosphate, and 1× plant protease inhibitor cocktail (Sigma-Aldrich)]. Equal volumes of extracts were transferred to microfuge tubes and incubated at room temperature for the indicated periods of time. For studies of effects of the lysineless ubiquitin mutant, purified wild-type ubiquitin or K0 ubiquitin (Boston Biochem) was added to protein extracts at 1 μg/μl. Reactions were terminated by addition of SDS sample buffer, followed by boiling for 3 min. Myc- and 3×FLAG-tagged proteins were detected by anti–c-Myc 9E10 antibody (Covance) and anti-FLAG M5 antibody (Sigma-Aldrich), respectively, followed by anti-mouse antibodies conjugated to alkaline phosphatase (Sigma-Aldrich). For loading controls, a major band at about 50 kD [putative RuBisCO (ribulose-1,5-bisphosphate carboxylase oxygenase) large chains] on Coomassie-stained gels was used. All immunoblots were quantified with ImageJ software (National Institutes of Health).

Ubiquitination assay

Myc-VirF or FLAG-Ub alone or together was transiently expressed in *N. benthamiana* leaves by agroinfiltration. After 48 hours, the infiltrated leaves were treated with 10 μM MG132 and incubated for an additional

4 hours unless otherwise noted. Leaves were harvested and ground into fine powder in liquid nitrogen. Total proteins were extracted from ground tissues in immunoprecipitation buffer (see above). Protein extracts were incubated with anti–c-Myc 9E10 antibody (Covance) for 3 hours at 4°C, followed by incubation with Protein G–Sepharose 4B (Invitrogen) for an additional 3 hours at 4°C to capture the immunocomplexes. After four washes with washing buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, and 3 mM DTT], immunoprecipitates were eluted in SDS sample buffer. The presence of $(FLAG\text{-}Ub)_n$–VirF was analyzed by immunoblotting with anti–c-Myc antibody conjugated to horseradish peroxidase (Roche) or anti-FLAG M5 antibody (Sigma-Aldrich) followed by anti-mouse antibody conjugated to horseradish peroxidase (Sigma-Aldrich).

Yeast two-hybrid analysis

Plasmids were transformed into the *S. cerevisiae* strain L40 by means of the lithium acetate method and selected on leucine- and tryptophan-deficient medium. Protein interactions were assayed by growing cells for 3 days at 30°C on leucine-, tryptophan-, and histidine-deficient medium.

Tumorigenesis in tomato

The assay was performed as described (*31*) with the wild-type R10 strain or the *virD5* mutant VIK36 strain (*28*). Briefly, 11-day-old *Solanum lycopersicum* cv. Micro-Tom seedlings grown on Murashige and Skoog (MS) medium were punctured by a 27-gauge needle, inoculated with *Agrobacterium* suspension (OD_{600} = 0.02), grown for an additional 2 days on MS, and then further grown on MS supplemented with timentin (100 μg/ml). Tumors were scored 28 days after inoculation. All quantitative data were analyzed by the Student's *t* test; *P* values <0.01, corresponding to the statistical probability of greater than 99%, were considered statistically significant.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/4/195/ra69/DC1
Materials and Methods
Fig. S1. VirD5 does not interact with VBF.
Fig. S2. Increased amounts of VirF in the presence of VirD5 in plant cells.
Fig. S3. Lactacystin, a specific inhibitor of the 26*S* proteasome, stabilizes VirF.
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Abstracts

One-sentence summary: A plant pathogen prevents degradation of its key virulence factor in infected host cells.

Editor's Summary:
Maintaining Virulence

Agrobacterium tumefaciens is a bacterial pathogen that genetically transforms its hosts, which include several commercially important plants such as grapevines and roses, and induces neoplastic growths termed crown galls (tumors). *Agrobacterium*-induced genetic transformation and tumorigenesis require the integration of a bacterial DNA molecule into the host genome, a process that involves the virulence factor VirF, an F-box protein that is incorporated into the host cell machinery that targets proteins for degradation. Many F-box proteins, including VirF, are themselves unstable and become degraded, leading Magori and Citovsky to investigate how *Agrobacterium* stabilizes VirF. They found that VirD5, another effector protein produced by *Agrobacterium*, associated with VirF and prevented its degradation. Indeed, a strain of *Agrobacterium* lacking VirD5 formed fewer tumors on tomato plants than did a wild-type strain with VirD5. These findings suggest a general strategy by which other pathogens may stabilize their F-box effector proteins in infected cells.

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