A bacterial type III effector hijacks plant ubiquitin proteases to evade degradation

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Abstract

Gram-negative bacterial pathogens inject effector proteins inside plant cells using a type III secretion system. These effectors manipulate plant cellular functions and suppress the plant immune system in order to promote bacterial proliferation. Despite the fact that bacterial effectors are exogenous threatening proteins potentially exposed to the protein degradation systems inside plant cells, effectors are relative stable and able to perform their virulence functions. In this work, we found that RipE1, an effector protein secreted by the bacterial wilt pathogen, *Ralstonia solanacearum*, undergoes phosphorylation of specific residues inside plant cells, and this promotes its stability. Moreover, RipE1 associates with plant ubiquitin proteases, which contribute to RipE1 deubiquitination and stabilization. The absence of those specific phosphorylation sites or specific host ubiquitin proteases leads to a substantial decrease in RipE1 protein accumulation, indicating that RipE1 hijacks plant post-translational modification regulators in order to promote its own stability. These results suggest that effector stability or degradation in plant cells constitute another molecular event subject to co-evolution between plants and pathogens.
Introduction

Plants and microbial pathogens have undergone a complex co-evolution process. As potential hosts without an adaptive immune system, plants have developed detection mechanisms to perceive pathogenic threats, activating immune responses and developmental rearrangements in order to hinder the development of disease (Bentham et al., 2020). Pathogens correspond to this with the development of virulence activities aimed at suppressing the activation of immune responses and manipulating other plant cellular functions in order to promote pathogen proliferation and, subsequently, the development of disease (Wang et al., 2022). Studies on plant-pathogen interactions keep discovering new components of this co-evolution process, reflecting an unpredictable complexity.

One of the major virulence strategies of gram-negative bacterial pathogens is the injection of different effector proteins directly inside host cells using a type-III secretion system (T3SS). Those proteins, termed type-III effectors (T3Es) carry out numerous different virulence functions inside host plant cells, being essential for the development of disease (Cai et al., 2023; Macho, 2016; Toruño et al., 2016). However, as per the aforementioned co-evolution, plants have developed intracellular receptors carrying nucleotide-binding and leucine-rich repeat domains (NLRs) able to detect T3Es or their activities, by monitoring important plant proteins or cellular functions, perceiving perturbations in them, and subsequently activating immune responses (Kourelis and van der Hoorn, 2018). Therefore, although T3Es are collectively essential for the development of disease, each one of them carries the potential of being perceived by the plant immune system in resistant host plants containing the appropriate NLRs.

When a bacterial pathogen injects an effector that gets recognized by the plant immune system, pathogen evolution may drive the loss or modification of this effector (McCann and Guttmann, 2008), or the emergence of other effectors that may suppress this recognition or the subsequent downstream signalling (Rufián et al., 2023; Wu and Derevnina, 2023). In this situation, the recognized effector could exert its virulence
activity without triggering immunity.

It is generally assumed that T3Es travel through the T3SS needle in an unfolded state (Ghosh, 2004). Inside host cells, T3Es may associate with plant enzymes to undergo post-translational modifications that contribute to effector folding, subcellular localization, and/or function (Popa et al., 2016). Besides achieving appropriate folding and functional protein conformations, it is worth considering the possibility that T3E proteins may be recognized by plant cells as non-self-proteins, and therefore be exposed to protein regulatory processes of plant cells, including their potential degradation (Dikic, 2017; Su et al., 2020). Whether such regulatory processes constitute a threat for T3E proteins and how they escape from degradation is currently unknown.

*Ralstonia solanacearum* is one of the most destructive bacterial plant pathogens worldwide. This soil-borne bacterium invades plants through the roots, reaching the vascular system, and subsequently colonizes the whole plant using xylem vessels (Xue et al., 2020). *R. solanacearum* pathogenicity requires the activity of T3Es, and it is noteworthy that certain *R. solanacearum* strains secrete more than 70 different T3Es (Sabbagh et al., 2019). One of these T3Es, named RipE1, was originally validated as a protein injected into plant cells through the T3SS (Mukaihara et al., 2010); we subsequently found that RipE1 can be recognized by the plant immune system (Kim et al., 2023; Sang et al., 2020). Despite RipE1 recognition, we have also found that *R. solanacearum* strains are able to secrete additional effectors to inhibit RipE1-triggered immunity, including RipAY (Sang et al., 2018; Sang et al., 2020), RipAC (Yu et al., 2020), and RipD (Wang et al., 2023). In this work, we found that RipE1 undergoes phosphorylation and ubiquitination in plant cells. In the absence of phosphorylation, RipE1 ubiquitination is dramatically enhanced, and the subsequent stability of RipE1 protein is severely reduced. Moreover, we found that RipE1 associates with plant ubiquitin proteases (ubiquitin carboxyl-terminal hydrolases, UCHs), which contribute to RipE1 de-ubiquitination and protein stability. Finally, we show that RipE1...
phosphorylation and NbUCH-mediated deubiquitination are independent mechanisms that, together, lead to RipE1 stability in plant cells.
Results

RipE1 phosphorylation in plant cells contributes to protein stability

In order to understand better the biochemical properties of RipE1 in plant cells, we used Agrobacterium-mediated transient expression of *RipE1* fused to a green fluorescent protein (GFP) tag in leaves of the model Solanaceae plant *Nicotiana benthamiana*. We then performed affinity purification of RipE1-GFP followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Among the resulting RipE1 peptides, we were able to identify five phosphorylated Serine (S)/Threonine (T) residues (*Figures 1A, S1A and S1B*). Given the close proximity of T53, S58, and S59, we generated a mutant RipE1 where these residues were replaced by Alanines (A) (RipE1<sup>3A</sup>) and a mutant where all five phosphorylated residues were mutated (RipE1<sup>5A</sup>). Upon expression in *N. benthamiana* leaves, both mutants showed lower protein accumulation compared to wild-type RipE1, and this phenomenon was particularly significant for the RipE1<sup>5A</sup> mutant (*Figures 1B and 1C*). All RipE1 versions showed similar RNA accumulation (*Figure 1D*), indicating that the impaired protein accumulation is not caused by reduced gene expression, and suggesting that phosphorylation in these residues contributes to the stability of RipE1 protein in plant cells.

In eukaryotic cells, ubiquitination of proteins may lead to their degradation through the 26S proteasome (Dikic, 2017; Su et al., 2020). Interestingly, we found that the RipE1<sup>5A</sup> mutant is strongly ubiquitinated in plant cells (*Figures 1E and 1F*); the strong ubiquitination of both RipE1<sup>3A/5A</sup> mutants was particularly evident when protein loading was tuned to balance RipE1 accumulation (*Figures S1C-E*). Altogether, these results suggest that RipE1 phosphorylation in these residues is required to avoid RipE1 ubiquitination and maintain wild-type levels of RipE1 accumulation.
RipE1 interacts with UBIQUITIN CARBOXYL-TERMINAL HYDROLASES in plant cells

Upon transient expression in *N. benthamiana* cells, RipE1-GFP is localized at the cytoplasm and the cell periphery, showing also a weak accumulation in the nucleus (Figure S2A). To identify RipE1-interacting proteins in plant cells, we searched for peptides present in RipE1 affinity-purified samples in the LC-MS/MS analysis mentioned above. Among peptides present in RipE1-GFP samples and absent in the GFP control, we found a remarkable accumulation of peptides corresponding to UBIQUITIN CARBOXYL-TERMINAL HYDROLASES (UCHs) (Figure 2A). The UCH family is not well defined in *N. benthamiana*, but the current version of the genome includes 13 genes encoding proteins annotated as UCHs and showing a percentage of identity higher than 50% when compared to the NbUCHs identified by LC-MSMS and to their closest Arabidopsis homologs, AtUBP12 and AtUBP13 (Figure 2A, S2B and Table S1).

Upon transient expression, a GFP-tagged version of NbUCH15 localizes at the cell periphery and the nucleus (Figure S2C and S2D), showing partial co-localization with RipE1 fused to a red fluorescent protein (RFP) tag (Figure S2C and S2D). We confirmed the physical association between RipE1 and NbUCH proteins by targeted Co-immunoprecipitation (CoIP) of RipE1 and two of the identified candidates, namely NbUCH12 and NbUCH15 (Figure 2B). A RipE1 mutant in the predicted catalytic cysteine (C172A), which does not trigger cell death (Sang et al., 2020) also associated with NbUCH12/15 (Figure 2B). We also measured Förster resonance energy transfer (FRET) between RipE1-RFP and NbUCH15-GFP using fluorescence lifetime imaging (FLIM), and the results confirmed a direct interaction between RipE1 and NbUCH15 (Figure 2C and Figure S2E).

Reduced expression of *NbUCH* genes leads to the activation of immunity against *R. solanacearum*
Ubiquitin proteases encoded by NbUCH orthologs in Arabidopsis and N. tabacum have been previously described as negative regulators of immunity, since mutation or reduced expression of their corresponding genes enhance immune responses (Ewan et al., 2011). To perform a loss-of-function analysis of NbUCH genes, we generated constructs to silence them by RNA-interference (RNAi). RNAi constructs targeting either NbUCH05, NbUCH12, or NbUCH15 led to a reciprocal reduced expression of all these three genes (Figure S3A), suggesting that either of these constructs causes a general silencing of NbUCH genes. The reduced expression of NbUCH genes led to the gradual emergence of tissue collapse (Figure 3A), which correlated with ion leakage indicative of cell death (Figure 3B). This also correlated with an enhanced expression of the defence-related marker gene NbPR1 (Figure 3C) and an enhanced resistance of these tissues to R. solanacearum Y45 (Figure 3D), indicating that silencing of NbUCH genes leads to cell death associated to an activation of plant immunity.

The suppressor of the G2 allele of skp1 (SGT1) is an essential component of the plant immune system, required for the induction of disease resistance mediated by many intracellular immune receptors containing nucleotide-binding and leucine-rich repeat domains (NLRs) (Azevedo et al., 2002; Kadota et al., 2010). Accordingly, virus-induced gene silencing (VIGS) of SGT1 in N. benthamiana is commonly used to test for SGT1-dependent NLR-mediated cell death (Yu et al., 2019). Interestingly, the cell death caused by the silencing of NbUCH genes was abolished by VIGS of SGT1 (Figure 3E). This suggested two potential scenarios: (i) the previously described activity of UCHs as negative regulators of immunity requires SGT1, and/or (ii) a compromised accumulation or integrity of NbUCHs is perceived by an SGT1-dependent NLR, leading to the activation of immunity. The latter scenario prompted us to consider the following hypothesis: RipE1 could be targeting NbUCHs, and this targeting would lead to the activation of an NLR, and the subsequent activation of immune responses.
The potential targeting of NbUCHs does not underlie RipE1-triggered immunity

We have recently found that RipE1 is recognized by the NLR NbPtr1 (Kim et al., 2023). Accordingly, VIGS of NbPtr1 abolishes the cell death and immune responses triggered by RipE1 expression in plant cells (Figure 4A; (Kim et al., 2023). To further analyze whether the observed activation of immunity upon NbUCH gene silencing could be related to RipE1-triggered immunity, we performed VIGS of NbPtr1. Interestingly, VIGS of NbPtr1 did not affect the activation of immunity triggered by silencing NbUCH genes, as indicated by the development of cell death (Figure 4B) and the induction of NbPR1 expression (Figure 4C), indicating that the activation of immunity triggered by RipE1 (NbPtr1-dependent) and that triggered by silencing NbUCH genes (Ptr1-independent) are based on different mechanisms. This suggests that the potential targeting of NbUCHs does not underlie RipE1-triggered immunity.

NbUCHs contribute to RipE1 deubiquitination and protein stability in plant cells

After considering unlikely that NbUCHs are virulence targets responsible for RipE1-triggered immunity, we considered other potential functional associations between RipE1 and NbUCHs. Interestingly, in tissues undergoing RNAi-mediated silencing of NbUCH genes, we observed a reduced accumulation of RipE1 protein upon Agrobacterium-mediated transient expression (Figure 5A). NbUCH silencing did not significantly reduce the accumulation of a different R. solanacearum T3E, RipAA, in the same conditions (Figure 5A and 5B) or the accumulation of RipE1 RNA (Figure S4). These results suggest that NbUCH silencing has a specific impact over the accumulation of RipE1 protein. To confirm this hypothesis, we overexpressed NbUCH15 and subsequently expressed RipE1. The overexpression of NbUCH15 led to a significantly enhanced accumulation of RipE1-GFP (Figures 5C and 5D), indicating that NbUCH15 promotes RipE1 accumulation.

Given the direct correlation between NbUCH expression and RipE1 protein
accumulation, and our previous observation that RipE1 undergoes ubiquitination in plant cells (Figure 1), we considered the possibility that NbUCHs mediate the de-ubiquitination of RipE1, therefore promoting its stability. Indeed, we observed much stronger RipE1 ubiquitination upon silencing of NbUCH15 (Figures 6A, 6B and S5) and a significant inhibition of RipE1 ubiquitination upon NbUCH15 overexpression (Figures 6C and 6D). Given that the alteration of NbUCH gene expression leads to different accumulation of RipE1 protein, we tuned the loading of the samples in order to compare the ubiquitination of similar levels of RipE1, as indicated in the figure panels (Figures 6A-D). These results suggest that NbUCHs contribute to RipE1 stability by reducing its ubiquitination.

RipE1 phosphorylation and NbUCH-mediated deubiquitination are independent mechanisms leading to RipE1 stability

Given that both RipE1 phosphorylation and its association with NbUCHs contribute to RipE1 stability, we tested whether RipE1 phosphorylation is somehow required for the association with NbUCHs and subsequent deubiquitination. In order to test the interaction between NbUCH15 and RipE1/RipE1\(^{5A}\), we expressed RipE1/RipE1\(^{5A}\) from an inducible promoter and collected protein samples at an early time-point, when RipE1\(^{5A}\) accumulation is still comparable to wild-type RipE1. In these conditions, the RipE1\(^{5A}\) mutant showed a similar association with NbUCH15 in CoIP assays (Figure 7A), indicating that RipE1 phosphorylation in these residues is not required for association with NbUCH15.

Overexpression of NbUCH15 was also able to rescue the low protein accumulation observed for RipE1\(^{5A}\) (Figures 7B and 7C), likely as a consequence of a strong inhibition in RipE1\(^{5A}\) ubiquitination mediated by NbUCH15 (Figures 7D and 7E; protein loading was tuned to analyze similar amounts of RipE1\(^{5A}\)). Altogether, these results suggest that RipE1 phosphorylation and NbUCH-mediated deubiquitination are independent mechanisms that, together, lead to RipE1 stability in plant cells.
Discussion

T3Es travel unfolded through the T3SS and, as polypeptides, need to achieve active conformations and the appropriate subcellular localization for their respective functions (Ghosh, 2004; Popa et al., 2016). Given that eukaryotic cells have mechanisms to recognize and deal with unnatural proteins (Li et al., 2022), it is unclear how these T3E polypeptides, as non-plant proteins, survive the protein degradation machinery in plant cells. In this work, we found that RipE1 is ubiquitinated in plant cells, and this ubiquitination promotes effector instability or degradation. This may constitute a defence mechanism against potentially threatening non-self proteins in plant cells. Despite such ubiquitination, we found that RipE1 stability in plant cells is promoted by phosphorylation and deubiquitination mediated by plant deubiquitinating enzymes.

Several bacterial effectors have been found to undergo post-translational modifications (PTMs) and to associate with eukaryotic factors in host cells, which contribute to their virulence activity by mediating their biochemical activation and/or subcellular localization (Popa et al., 2016). Phosphorylation in plant cells has been reported for several T3Es (Popa et al., 2016); among R. solanacearum T3Es, RipAY associates with thioredoxins and undergoes phosphorylation in plant cells in order to activate its GGCT activity (Fujiwara et al., 2016; Sang et al., 2018; Wei et al., 2017). In the case of RipE1, phosphorylation seems to prevent RipE1 ubiquitination and promote RipE1 stability, which could be a way for the effector to adapt to the threat of degradation by the plant protein degradation machinery (Figure 8). Interestingly, the identified phosphorylation sites are conserved among RipE1 sequences from different R. solanacearum strains (Figure S6), suggesting that the phosphorylation of these residues may be an important mechanism contributing to RipE1 stability and subsequent function. We hypothesize that RipE1 phosphorylation may directly counteract its ubiquitination, but it is also possible that phosphorylation is required for the appropriate folding of RipE1, and that the mutation of the phosphorylated residues to the non-phosphorylatable Alanine causes a severe misfolding that exacerbates the
ubiquitination and degradation of RipE1. In addition to this phosphorylation-mediated mechanism to promote protein stability, we also found that RipE1 associates with specific plant ubiquitin proteases from the UCH family. Plant UCHs seem to deubiquitinate RipE1 and promote effector stability; this also constitutes a novel adaptation mechanism for the pathogen, hijacking plant ubiquitin proteases to counteract ubiquitination in plant cells and subsequent degradation in order to promote effector virulence activities (Figure 8).

RipE1 is conserved in most *R. pseudosolanacearum* strains (corresponding to the phylotype I of the *R. solanacearum* species complex) sequenced to date (Kim et al., 2023; Sabbagh et al., 2019). Once RipE1 is secreted inside plant cells and overcomes the threat of degradation, as characterized in this work, RipE1 activities could be detected in resistant host plants containing the NLR Ptr1 (Kim et al., 2023), which would lead to the activation of disease resistance (Kim et al., 2023; Sang et al., 2020). Instead of losing RipE1 as a mean of pathogenic adaptation to resistant host plants, these strains contain other effectors with the potential to mask or suppress RipE1-triggered immunity (Sang et al., 2020; Wang et al., 2023; Yu et al., 2020). The strong conservation of RipE1 among *R. pseudosolanacearum* strains and the complex evolution undergone to keep RipE1 stable and counteract its potential detection suggest that RipE1 may play an important role in pathogen virulence.

RipE1-triggered immunity in *N. benthamiana* requires its predicted cysteine-protease catalytic activity (Sang et al., 2020) and the presence of NbPtr1 (Kim et al., 2023). After finding that silencing NbUCH triggers immune-associated HR, we first hypothesized that NbUCHs may be targets of RipE1, and this activity could be monitored by NbPtr1 to activate immune responses. However, two observations suggest that NbUCHs are not virulence targets of RipE1 that are guarded by NbPtr1: first, a RipE1-C172A mutant, which loses its catalytic activity and is not recognized by NbPtr1, still associates with UCHs (Figure 2); second, the HR triggered by the lack of UCHs is not abolished by silencing NbPtr1 (Figure 4). This prompted us to explore a different scenario to explain
the association between RipE1 and NbUCHs. Our subsequent data suggests that NbUCHs associate with RipE1, promoting RipE1 deubiquitination and RipE1 stability; in this sense, NbUCHs could act as “helpers” for the effector to contribute to its stability and activity (Figure 8). UCHs are essential proteins for plant cells. In particular, the Arabidopsis homologs of the NbUCHs characterized in this work, AtUBP12/AtUBP13, constitute an important regulatory node in different signaling pathways (Zhang et al., 2024). Among other functions, AtUBP12/AtUBP13 have been shown to belong to the polycomb group protein system to regulate gene silencing (Derkacheva et al., 2016), and to contribute to the stability of MYC2 to activate jasmonate-dependent responses (Jeong et al., 2017). Besides their initial characterization as negative regulators of immunity (Ewan et al., 2011), it has been recently shown that AtUBP12/AtUBP13 mediate the deubiquitination of the salicylic acid receptor NPR3 to suppresses plant immunity, playing an important role in the negative regulation of immune activation (Zhou et al., 2023). Given the essential activities carried out by the UBP/UCH-family proteins in plant cells, RipE1 hijacks an important regulatory node, which cannot be “shut down” by plant cells, in order to prevent its own degradation.

Finally, we set out to determine whether RipE1 phosphorylation may directly counteract ubiquitination or may otherwise contribute to the interaction with NbUCHs. Our results were clear to show that RipE1 phosphorylation in the reported 5 residues is not required for interaction with NbUCH15 (Figure 7). Moreover, overexpression of NbUCH15 was also able to rescue the low protein accumulation observed for RipE1<sup>SA</sup>, likely as a consequence of a strong inhibition in RipE1<sup>SA</sup> ubiquitination mediated by NbUCH15, suggesting that phosphorylation and NbUCH15-mediated deubiquitination are independent processes and may have an additive effect contributing to RipE1 stability in plant cells. It is worth noting that, similar to the case of RipE1, we have recently found that another <i>R. solanacearum</i> T3E, RipBM, also undergoes phosphorylation and associates with plant 14-3-3 proteins to prevent its degradation (Wei et al, in preparation). These results suggest that phosphorylation and association with important plant proteins may be a common strategy among T3Es to counteract
degradation and promote their stability in plant cells.
Materials and Methods

Plant materials

*N. benthamiana* plants were grown at 23 °C in a growth room under 16-h light/8-h dark photoperiod with a light-intensity of 130 mE m⁻² s⁻¹. Each plant was grown in one pot on soil with 1:1 mix of potting soil and vermiculite. After *R. solanacearum* inoculation, plants were moved to a 27 °C growth chamber with 75% humidity under a 14-h light/10-h dark photoperiod.

Bacterial strains

*Agrobacterium tumefaciens* GV3101 carrying different constructs was grown on solid Luria-Bertani (LB) medium plates with the appropriate antibiotics. The concentration of each antibiotic was 25 μg mL⁻¹ rifampicin, 50 μg mL⁻¹ gentamicin, 50 μg mL⁻¹ spectinomycin, and 50 μg mL⁻¹ kanamycin.

*R. solanacearum* Y45 was grown on solid Bacto-agar and Glucose (BG) medium for 2 days at 28 °C. Then bacteria were cultivated overnight after inoculation in liquid BG medium.

Generation of plasmid constructs

The generation of expression plasmids for *ripE1* from *R. solanacearum* GMI1000 (Rsc3369) was previously described (Sang et al., 2020). The *ripE1* fragment in pDONR207 was used as template to generate *ripE1* phosphosite mutants by site-directed mutagenesis using the QuickChange Lightning Site-Directed Mutagenesis Kit (Life technologies, USA) following the manufacturer’s instructions and the primers indicated in Table S2. The triple 3A mutant fragment was cloned into pENTR-D-TOPO (Thermo Fisher Scientific, MA, USA). The 3A mutant was used as a template to generate the 5A mutant. RipE1 and 5A with addition of an eGFP fragment from pGWB505 were inserted into pER8 vector using one-step cloning recombination (Vazyme, China).
NbUCH12 (Niben101Scf07463g01012.1) and NbUCH15 (Niben101Scf09610g00015.1) CDS fragments were cloned into pEASY-Blunt. NbUCH15 or NbUCH12 full length CDS fragments were cloned into pCAMBIA1300-FLAG vector via digestion and ligation or one-step cloning recombination (Vazyme, China).

NbUCH fragments to target specific genes using an RNAi approach was designed using the Solgenomics VIGS online tool (https://vigs.solgenomics.net/). The pK7GWIWG2_I-RedRoot expression vector (Morcillo et al., 2020) was used for transient silencing. Each NbUCH specific fragment was amplified using the primers described in Table S2 and cloned into pENTR-D-TOPO, and then sub cloned into pK7GWIWG2-II via LR recombination. All the primer sequences are shown in Table S2.

**Transient expression in N. benthamiana**

Agrobacteria carrying the indicated constructs were infiltrated into 4-5-week-old N. benthamiana. The bacterial density used was OD$_{600}$ 0.2-0.5 for confocal microscopy, protein accumulation, and phenotypic assays, OD$_{600}$ 1.0 was used for VIGS assays, and OD$_{600}$ 0.25 or 0.5 was used for RNAi-mediated local silencing. Bacteria were suspended in infiltration buffer (10 mM MgCl$_2$, 10 mM MES pH 5.6, and 150 μM acetosyringone), and then infiltrated into plant leaves using a 1 mL needless syringe.

**Confocal microscopy**

Confocal microscopy was performed as previously described (Wang et al., 2019). Leaf epidermal cells of N. benthamiana were examined using a Leica TCS SP8 confocal microscope (Leica, Germany). The settings for excitation and emission were 488 nm (ex) and 500-550 nm (em) for GFP, and 561 nm (ex) and 580-630 nm (em) for RFP.

**Conductivity measurements**
Cell death in plant leaves was quantified as previously described (Sang et al., 2020; Yu et al., 2020) by measuring the electrolyte leakage using a conductivity meter (ThermoFisher, USA) or observing the autofluorescence using the BioRad Gel Imager (Bio-Rad, USA). Briefly, one day after Agrobacterium infiltration in *N. benthamiana*, one 13 mm leaf disk was immersed in 4 mL of distilled water for 1 h with gentle shaking and then transferred to a 6-well culture plate containing 4 mL distilled water in each well. The ion conductivity was then measured at different time intervals. Autofluorescence in intact *N. benthamiana* leaves was measured at 2.5 dpi.

**Protein extraction and Western blots**

Protein extraction and western blots were performed as previously described (Sang et al., 2020; Yu et al., 2020). Briefly, plant tissues were frozen and ground in liquid nitrogen and ground using a Tissue Lyser (QIAGEN, Hilden, Nordrhein-Westfalen, Germany) with a frequency of 25 s⁻¹ for 1 min. Ground tissues were then homogenized in protein extraction buffer (100 mM Tris pH 8, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 2 mM DTT, 1% (v/v) protease inhibitor cocktail, 2 mM PMSF, 1% (v/v) NP40, 10 mM sodium molybdate, 10 mM sodium fluoride, 2 mM sodium orthovanadate). The resulting protein samples were incubated at 70°C for 10 minutes in SDS loading buffer and loaded in SDS-PAGE acrylamide gels for western blot analysis. Immunoblots were analyzed using the antibodies indicated in the figures: anti-GFP (Abicode, M0802-3a), anti-luciferase (Sigma, L0159), anti-actin (Agrisera, AS13 2640), anti-ubiquitination(P4D1) (Santa Cruz Biotechnology sc-8017 HRP), anti-FLAG (Abmart, M20008), anti-RFP (Chromotek 5F8), antibodies. The custom antibody against NbUCH15, was generated by Abclonal. Protein signals were quantified using the Image J software.

**Chemical treatments**

MG132 (Sangon Biotech, China) powder was dissolved in DMSO to a final concentration at 10 mM. 50 μM MG132 diluted in water was infiltrated into plants 4 hours before collecting samples. In all assays to detect ubiquitination, samples were
collected after 50 μM MG132 treatment for 4 hours.

**Co-immunoprecipitation**

Co-immunoprecipitation assays were performed as previously described (Yu et al., 2020). Briefly, 500 mg of ground *N. benthamiana* leaves were resuspended in 1 mL protein extraction buffer as indicated above. Supernatants were filtered through micro bio-spin chromatography columns. The filtered extracts were incubated with 15 μL GFP-trap agarose beads (ChromoTek, Germany) at 4°C for 1 hour, followed by 4 washes with wash buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 10% glycerol, 2 mM DTT, 1% (v/v) protease inhibitor cocktail, 0.5% (v/v) NP40, 10 mM sodium molybdate, 10 mM sodium fluoride, 2 mM sodium orthovanadate). To detect ubiquitination, 50 μM MG132 and 10 mM NEM were added to the protein extraction buffer and 20 μM MG132 and 2.5 mM NEM were added to the wash buffer. The resulting protein samples were incubated at 70°C for 10 minutes in SDS loading buffer and loaded in SDS-PAGE acrylamide gels for western blot analysis.

**Large-scale immunoprecipitation and LC-MS/MS analysis**

Large-scale immunoprecipitation assays for LC-MS/MS analysis were performed as previously described (Yu et al., 2020). Briefly, 5 g of ground *N. benthamiana* leaves and 50 μL GFP-trap agarose beads were used following the co-immunoprecipitation procedure described above. Two more washes with wash buffer without NP40 were performed before LC-MS/MS analysis.

**RNA extraction and quantification RT-PCR**

Plant tissues were collected into 2 mL tubes with one metal bead, frozen in liquid nitrogen and then ground using a Tissue Lyser (QIAGEN, Hilden, Nordrhein-Westfalen, Germany) with a frequency of 25 s⁻¹ for 1 min. Total RNA was extracted using E.Z.N.A. Plant RNA kit (Biotek, China) following the manufacturer’s instructions without genomic DNA digestion. RNA samples were quantified using a Nanodrop spectrophotometer (ThermoFisher). 10 μL first-strand cDNA was synthesized using
500 ng RNA with iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer’s instructions. Quantitative RT-PCR solution was prepared using the Hieff® qPCR SYBR Green Master Mix (Yeason, China), and the reaction was performed using a CFX96 Real time system (Bio-Rad, USA). Primer sequences are shown in Table S2.

**RNA interference (RNAi) gene silencing**

Constructs for silencing indicated gene or carrying empty vector (EV) (as control) were expressed in 4-week-old *N. benthamiana* plants in same leaf, side by side, using Agrobacteria infiltration with an OD$_{600}$=0.5. Four discs were collected at 4 or 8 days post-infiltration to detect silencing efficiency using RT-PCR.

**Virus-induced gene silencing (VIGS)**

To silence *NbPtr1*, virus-induced gene silencing was performed as described before (Kim et al., 2023; Yu et al., 2019). In brief, Agrobacteria expressing pTRV1 and pTRV2-Ptr1 were mix in a ratio of 1:1 with a final dose of OD$_{600}$=1 and co infiltrated in 3-week-old *N. benthamiana* plants. pTRV2 empty vector was co expressed with pTRV1-EV as negative control. pTRV1-EV mixed with pTRV2-PDS was used as a control to indicate the silenced leaves. Plant tissues were collected at 9 days post-inoculation to detect silencing efficiency using RT-PCR.

**R. solanacearum growth in N. benthamiana**

The growth of *R. solanacearum* Y45 strain in *N. benthamiana* leaves has been previously described in detail (Wei et al., 2020; Yu and Macho, 2021). Briefly, *N. benthamiana* leaves expressing the indicated genes were infiltrated with a bacterial suspension containing $10^5$ CFU mL$^{-1}$. Plant tissues were collected 2 days after inoculation for bacterial quantification (Yu and Macho, 2021). Data analysis and representation were performed using Graphpad 7.0 software.

**FRET-FLIM assays**
Förster resonance energy transfer – fluorescence lifetime imaging (FRET-FLIM) experiments were performed as previously described (Rosas-Diaz et al., 2018; Yu et al., 2022). Briefly, donor proteins (fused to eGFP) were expressed in a pCAMBIA1300-GFP vector, and acceptor protein (fused to eRFP) were expressed from vector pGWB554 or pGWB2. FRET-FLIM experiments were performed on a Leica TCS SMD FLCS confocal microscope excitation with WLL (white light laser) and emission collected by a SMD SPAD (single photon-sensitive avalanche photodiodes) detector. Leaf discs of *N. benthamiana* plants transiently coexpressing donor and acceptor, as indicated in the figures, were visualized 30-36 hours after agroinfiltration. Accumulation of the GFP- and RFP-tagged proteins was estimated before measuring lifetime. The tuneable WLL set at 488 nm with a pulsed frequency of 40 MHz was used for excitation, and emission was detected using SMD GFP/RFP Filter Cube (with GFP: 500-550 nm). The fluorescence lifetime shown in the figures corresponding to the average fluorescence lifetime of the donor was collected and analyzed by PicoQuant SymphoTime software. Mean lifetimes are presented as mean ± SEM from at least three independent experiments.
Acknowledgements

We thank Rosa Lozano-Duran for critical reading of this manuscript, Xinyu Jian and Fangyuan Wu for technical and administrative assistance during this work, and all the members of the Macho and Lozano-Duran laboratories for helpful discussions. We thank the PSC Cell Biology and Proteomics core facilities for assistance with confocal microscopy and mass spectrometry. This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (grant XDB27040204), the Chinese 1000 Talents Program, and the Shanghai Center for Plant Stress Biology (Center for Excellence in Molecular Plant Sciences, Chinese Academy of Sciences). The authors have no conflict of interest to declare.

Author contributions

WY, ML, and APM planned and designed the research. WY and ML performed most of the experiments. WW, HZ, JL and YS performed additional experiments. CS contributed unpublished materials and ideas for the project. APM wrote the manuscript with input from all the authors.
References


Figure 1

**A**

Catalytic sites: C172 H203 D222

**B**

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**C**

![Relative protein accumulation graph]

**D**

![Graph showing relative gene expression]

**E**

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Figure 1. RipE1 undergoes phosphorylation in *N. benthamiana*, which contributes to protein stability.

(A) Schematic representation of RipE1 protein, indicating the position of phosphorylation sites, domain A, and catalytic sites.

(B) Western blot showing protein accumulation of GFP, RipE1-GFP, and RipE1 phosphodeficient mutants. *Agrobacterium* expressing GFP (as control), RipE1-GFP, 3A-GFP, or 5A-GFP (OD600 = 0.5) were infiltrated into the same leaf of *N. benthamiana*. Samples were taken at 30 hours post-infiltration (hpi), before the appearance of cell death. Blots were incubated with anti-Actin antibody to verify equal loading. This experiment was repeated 4 times, and the quantitation of the different repeats is shown in (C).

(C) Quantification of the relative protein accumulation of the different repeats of the assay shown in (B), measured using Image J. RipE1 values were normalized using the respective actin values and represented as relative to RipE1 (WT) for each repeat. Values indicate mean ± SE (n = 4 biological replicates). Different letters indicate significant differences (one-way ANOVA, Tukey’s test, p < 0.05).

(D) Quantitative RT–PCR (qRT–PCR) to determine the expression of *RipE1* in (B). Expression values are relative to the expression of the housekeeping gene *NbEF1a*. Values indicate mean ± SE (n = 9 biological replicates). Different letters indicate significant differences (one-way ANOVA, Tukey’s test, p < 0.05). Composite data from 3 independent biological replicates. Nd: not detected.

(E) Immunoprecipitation assays to determine the ubiquitination status of RipE1 and phosphodeficient mutants. Samples were collected 30 hpi, before the appearance of cell death. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. Protein marker sizes are shown for reference. This experiment was repeated 3 times, and the quantitation of the different repeats is shown in (F). A similar assay where the sample loading was adjusted to show similar accumulation of all RipE1 variants for comparison of their ubiquitination is shown in Figure S1C.

(F) Quantification of the relative protein ubiquitination of the different repeats of the assay shown in (E), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation and represented as relative to the GFP control for each repeat. Values indicate mean ± SE (n = 3 biological replicates). Different letters indicate significant differences (one-way ANOVA, Tukey’s test, p < 0.05).
Figure 2

(A) Selected RipE1-GFP interactors identified by immunoprecipitation followed by LC-MS/MS. The table includes protein ID, peptide counts in GFP and RipE1-GFP samples, closest Arabidopsis orthologs, and the protein names used in this work.

(B) Co-immunoprecipitation assays to determine interactions between RipE1 (WT or C172A mutant) and NbUCH12/15. Agrobacterium containing the indicated constructs were infiltrated in N. benthamiana leaves and samples were taken 2 days post-infiltration (dpi). Immunoblots were analyzed using anti-GFP and anti-Flag antibodies, and protein marker sizes are provided for reference. These experiments were repeated 3 times with similar results.

(C) Interaction between RipE1-RFP and NbUCH15-GFP determined using FRET-FLIM upon transient co-expression in N. benthamiana leaves. Free RFP was used as a negative control. Fluorescence was visualized 30-36 hpi. Lines represent average values (n = 33), and error bars represent standard error. Asterisks indicate significant differences with the RFP control according to a Student’s t-test (**p < 0.01). Composite data from 3 independent biological replicates.
Figure 3

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B

Conductivity (μS/cm) vs. hours after sampling

C

Relative expression of NbPR1 at 8 dpi

D

Log10 (cfu/gFW)

E

TRV2-EV   TRV2-NbSGT1

NbUCH05

NbUCH12

NbUCH15
Figure 3. Silencing NbUCHs triggers SGT1-dependent immunity in *N. benthamiana*. NbUCH genes were silenced using RNAi in *N. benthamiana* leaves. An RNAi construct carrying the appropriate gene fragments (to silence each NbUCH gene) or an empty vector (as control) was expressed in the same leaf side-by-side using Agrobacterium (OD_{600}=0.5).

(A) Tissue collapse, indicative of cell death, triggered by silencing NbUCH genes. Photographs were taken at the indicated days post-infiltration (dpi) using a CCD camera. The infiltrated areas are delimited using dotted lines.

(B) Graph showing sample conductivity, indicating ion leakage from plant tissues caused by cell death. Leaf discs were collected 8 dpi, and ion leakage was measured at the indicated indicated times after sampling. Values indicate mean ± SE (n=3 biological replicates).

(C) Quantitative RT-PCR to determine the expression of the defense-related gene NbPR1. Samples were taken 8 dpi. Expression values are relative to the expression of the housekeeping gene NbEF1a. Values indicate mean ± SE (n=3 biological replicates).

(D) Growth of *Ralstonia solanacearum* Y45 in *N. benthamiana*. *R. solanacearum* was inoculated into *N. benthamiana* leaves after silencing NbUCH15 for 8 days, before the appearance of cell death. Leaf discs were collected 2 dpi for bacterial quantification. Values indicate mean ± SEM, (n=18 biological replicates from 3 independent repeats; each color corresponds to values from an independent replicate. Asterisks indicate significant differences compared to control according to a Student's t test (**** p < 0.0001).

(E) RNAi constructs for silencing NbUCHs or an empty vector (as control) were expressed in *N. benthamiana* undergoing VIGS of NbSGT1 or VIGS using an empty vector (EV) construct (as control). Tissue collapse, indicative of cell death, was recorded 16 dpi using a CCD camera. The infiltrated areas are delimited using dotted lines. Each experiment was repeat at least three times with similar results.
Figure 4. Immunity triggered by silencing \textit{NbUCH} does not require \textit{NbPtr1}.

Agrobacterium expressing an empty vector or a construct to induce virus-induced gene silencing (VIGS) of \textit{NbPtr1} were infiltrated in \textit{N. benthamiana} leaves.  
(A) Nine days after infiltration to induce VIGS of \textit{NbPtr1}, Agrobacterium expressing RipE1 or a GFP control were infiltrated into \textit{N. benthamiana} leaves. Tissue collapse, indicative of cell death, triggered by RipE1 expression was recorded 2 dpi. The infiltrated areas are delimited using dotted lines. Upper photographs were taken using a CCD camera from the adaxial side of the leaves, and lower pictures were captured using a UV camera from the abaxial side of the leaves and were flipped horizontally for representation.  
(B and C) Nine days after infiltration to induce VIGS of \textit{NbPtr1}, RNAi constructs for silencing \textit{NbUCH}s or an empty vector (as control) were expressed in \textit{N. benthamiana} leaves.  
(B) Tissue collapse, indicative of cell death, was recorded 12 dpi using a CCD camera. The infiltrated areas are delimited using dotted lines. Tissue collapse triggered by RipE1 (2 dpi) was used as control.  
(C) Quantitative RT-PCR to determine the expression of the defense-related gene \textit{NbPR1}. Samples were taken 8 dpi. Expression values are relative to the expression of the housekeeping gene \textit{NbEF1a}. Values indicate mean $\pm$ SE (n=3 biological replicates).
Figure 5

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- RipE1

- Actin

- Flag

- RIP1

B

Relative accumulation

RipE1

RipAA

C

RipE1-HA

Flag-RFP

NbUCH15-Flag

kDa

α-HA

α-Flag

α-Actin

D

Relative RipE1 accumulation

Flag-RFP

+RipE1

+RipAA

+RipE1

**
**Figure 5. NbUCH positive regulates RipE1 accumulation in *N. benthamiana*.**

(A and B) *NbUCH* genes were silenced using RNAi in *N. benthamiana* leaves. An RNAi construct carrying the appropriate gene fragments (to silence each *NbUCH* gene) or an empty vector (as control) was expressed in the same leaf side-by-side using Agrobacterium (OD$_{600}$=0.5). One day later, RipE1-GFP or RipAA-FLAG (as control) were expressed in same leaf using Agrobacterium (OD$_{600}$=0.1).

(A) Western blot showing the accumulation of RipE1-GFP and RipAA-FLAG protein accumulation. Blots were incubated with anti-Actin antibody to verify equal loading. Protein marker sizes are shown for reference. This experiment was repeated 3 times, and the quantitation of the different repeats is shown in (B).

(B) Quantification of the relative protein accumulation of the different repeats of the assay shown in (A), measured using Image J. RipE1 or RipAA values were normalized using the respective actin values and represented as relative to their respective empty vector control for each repeat. Values indicate mean ± SE (n = 3 biological replicates).

Asterisks indicate significant differences compared to each control according to a Student's t test (* p < 0.05, ** p < 0.01).

(C) Western blot to determine RipE1 protein accumulation after expression of NbUCH15 or Flag-RFP (as control). Agrobacterium expressing RipE1 was infiltrated 24 hours after expression of Flag-RFP (as control) or NbUCH15-Flag side-by-side in the same *N. benthamiana* leaf. Blots were incubated with anti-Actin antibody to verify equal loading. Protein marker sizes are shown for reference. This experiment was repeated 12 times, and the quantitation of the different repeats is shown in (D).

(D) Quantification of the relative protein accumulation of the different repeats of the assay shown in (C), measured using Image J. RipE1 values were normalized using the respective actin values and represented as relative to the control expressing Flag-RFP for each repeat. Values indicate mean ± SE (n = 12 biological replicates).

Asterisks indicate significant differences compared to each control according to a Student’s t test (** p < 0.01).
Figure 6

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Relative RipE1 ubiquitination

p=0.0699

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D

Relative RipE1 ubiquitination

p=0.0699
Figure 6. NbUCH15 promotes RipE1 stability by deubiquitynation.

(A and C) Immunoprecipitation assays to determine the ubiquitination status of RipE1. (A) Agrobacterium expressing RipE1-GFP was infiltrated 1 day after RNAi-mediated silencing of NbUCH15. An empty vector was used as RNAi negative control. Samples were collected 30 hpi, before the appearance of cell death. Given that NbUCH15 silencing compromises RipE1 protein accumulation, different volumes of the protein samples were loaded to show a comparable RipE1-GFP protein accumulation between different lanes after immunoprecipitation, allowing the detection of ubiquitination in the same amount of RipE1 protein. The relative loading volumes and protein abundance are indicated above lanes. A similar assay showing the loading of the same sample volumes (with different RipE1 accumulation) is shown in Figure S5A. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. The accumulation of native NbUCH proteins was detected using a custom anti-NbUCH antibody and the same volume of each sample. Protein marker sizes are shown for reference. This experiment was repeated 3 times, and the quantitation of the different repeats is shown in (B).

(B) Quantification of the relative RipE1 ubiquitination of the different repeats of the assay shown in (A), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation values and represented as relative to the empty vector control for each repeat. Values indicate mean ± SE (n = 3 biological replicates).

The p value compared to the control value using a Student’s t test is shown.

(C) Agrobacterium expressing RipE1-GFP was infiltrated 1 day after expression of NbUCH15-Flag or Flag-RFP (as control). Samples were collected 30 hpi, before the appearance of cell death. Given that NbUCH15 overexpression enhances RipE1 protein accumulation, different volumes of the protein samples were loaded to show a comparable RipE1-GFP protein accumulation between different lanes after immunoprecipitation, allowing the detection of ubiquitination in the same amount of RipE1 protein. The relative loading volumes and protein abundance are indicated above lanes. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. Protein marker sizes are shown for reference. This experiment was repeated 5 times, and the quantitation of the different repeats is shown in (D).

(D) Quantification of the relative RipE1 ubiquitination of the different repeats of the assay shown in (C), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation values and represented as relative to the control expressing Flag-RFP for each repeat. Values indicate mean ± SE (n = 5 biological replicates).

Asterisks indicate significant differences compared to the control according to a Student’s t test (* p < 0.05).
Figure 7. RipE1 phosphorylation and NbUCH-mediated deubiquitination are independent mechanisms leading to RipE1 stability.

(A) Co-immunoprecipitation assay to analyse the interaction between RipE1-5A and NbUCH15. Agrobacterium containing the indicated constructs were infiltrated in N. benthamiana leaves. In order to avoid stability issues with the RipE1-5A mutant, a estradiol (EST)-inducible vector was used, and samples were treated with 100 μM EST for 2.5 hours before being harvested at 2.5 dpi. Immunoblots were analyzed using anti-GFP and anti-Flag antibodies, and protein marker sizes are provided for reference. These experiments were repeated 3 times with similar results.

(B) Western blot to determine the accumulation of RipE1 or the RipE1-5A mutant after expression of NbUCH15 or Flag-RFP (as control). Agrobacterium expressing the RipE1 versions were infiltrated 24 hours after expression of Flag-RFP (as control) or NbUCH15-Flag side-by-side in the same N. benthamiana leaf. Blots were incubated with anti-Actin antibody to verify equal loading. Protein marker sizes are shown for reference. This experiment was repeated 9 times, and the quantitation of the different repeats is shown in (C).

(C) Quantification of the relative protein accumulation of the different repeats of the assay shown in (B), measured using Image J. RipE1 values were normalized using the respective actin values and represented as relative to the control expressing Flag-RFP for each repeat. Values indicate mean ± SE (n = 9 biological replicates).

Asterisks indicate significant differences compared to the control according to a Student’s t test (**** p < 0.0001).

(D) Agrobacterium expressing RipE1-5A-GFP was infiltrated 1 day after expression of NbUCH15-Flag or Flag-RFP (as control). Samples were collected 30 hpi, before the appearance of cell death. Given that NbUCH15 overexpression enhances RipE1 protein accumulation, different volumes of the protein samples were loaded to show a comparable RipE1-GFP protein accumulation between different lanes after immunoprecipitation, allowing the detection of ubiquitination in the same amount of RipE1 protein. The relative loading volumes and protein abundance are indicated above lanes. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. Protein marker sizes are shown for reference. This experiment was repeated 4 times, and the quantitation of the different repeats is shown in (E).

(E) Quantification of the relative RipE1 ubiquitination of the different repeats of the assay shown in (D), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation values and represented as relative to the control expressing Flag-RFP for each repeat. Values indicate mean ± SE (n = 4 biological replicates).

Asterisks indicate significant differences compared to the control according to a Student’s t test (**** p < 0.0001).
Figure 8. Simplified diagram showing a schematical model of RipE1 protein stability and activity in plant cells.

Upon injection inside plant cells, RipE1 is subjected to ubiquitination and subsequent degradation. RipE1 phosphorylation and UCH-mediated deubiquitination contribute to RipE1 stability. In susceptible hosts, phosphorylated stable RipE1 exerts its virulence activity through the association with virulence target(s). In resistant hosts, RipE1 activity is perceived by the presence of NbPtr1, leading to the activation of immune responses and disease resistance.
### Figure S1

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#### B

![Peptide fragmentation and mass spectrometry](image)

#### C

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#### D

- GFP: 1X
- RipE1: 4X
- 3A: 10X

![Ubiquitination analysis](image)

#### E

![Ubiquitination analysis](image)
Figure S1. RipE1 is phosphorylated in plant cells; RipE1 phosphorylation counteracts its ubiquitination.

(A) Phosphorylated peptides detected after immunoprecipitation of RipE1-GFP in *N. benthamiana* leaves followed by LC-MS/MS analysis. The number of the residues, peptide sequences, and mascot ion scores are shown. Phosphorylated residues are shown in red.

(B) Representative mass spectra of the phosphorylated peptides shown in (A).

(C) Immunoprecipitation assay to determine the ubiquitination status of RipE1 and phosphodeficient mutants. Agrobacterium carrying the indicated constructs were infiltrated as in Figure 1E. Samples were collected 30 hpi, before the appearance of cell death. Given the different RipE1 variants show different protein accumulation, different volumes of the protein samples were loaded to show a comparable RipE1-GFP protein accumulation between different lanes after immunoprecipitation, allowing the detection of ubiquitination in the same amount of RipE1 protein. The relative loading volumes and protein abundance are indicated above lanes. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. Protein marker sizes are shown for reference. This experiment was repeated 3 times, and the quantification of the different repeats is shown in (D).

(D) Quantification of the relative protein ubiquitination of the different repeats of the assay shown in (C), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation and represented as relative to the GFP control for each repeat. Values indicate mean ± SE (n = 3 biological replicates). Different letters indicate significant differences (one-way ANOVA, Tukey’s test, p < 0.05). P values are shown for reference.

(E) Composite data representation of all the replicates shown in Figure 1E and S1C. Values indicate mean ± SE (n = 6 biological replicates). Different letters indicate significant differences (one-way ANOVA, Tukey’s test, p < 0.05).
Figure S2

A

GFP

RipE1-GFP

GFP + -
RipE1-GFP - +

70-
25-

kDa

B

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Figure S2
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Figure S2. RipE1 and NbUCH15 interact and partially co-localize in the cell periphery and nucleus.  
(A) Confocal microscopy images showing the subcellular localization of free GFP and RipE1-GFP. RipE1-GFP or GFP (as control) were expressed in 4-week-old *N. benthamiana* using Agrobacterium (*OD*$_{600}$=0.5). Microscopy images were captured 48 hours post-inoculation. A size bar (25 μM) is shown for reference. The right panel shows a western blot to verify the accumulation of these proteins, ruling out significant GFP cleavage in the RipE1-GFP samples. Blots were analyzed using an anti-GFP antibody, and protein marker sizes are shown for reference.  
(B) Phylogenetic tree showing the NbUCH proteins identified in this work, together with all the other proteins annotated as UCH in the *N. benthamiana* proteome, showing at least 50% identity when compared with NbUCH05, NbUCH12, and NbUCH15. The tree includes also the proteins encoded by the closest Arabidopsis orthologs, namely AtUBP12 and AtUBP13.  
(C) Confocal microscopy images showing the subcellular localization of RipE1 and NbUCH15 in *N. benthamiana*. RipE1-RFP or RFP (as control) were co-expressed with NbUCH15-GFP or GFP (as control) using Agrobacterium (final *OD*$_{600}$=0.5). Microscopy images were captured 46 hours post-inoculation. A size bar (10 μM) is shown for reference. Each experiment was repeated 3 with similar results. The right panels show the quantification of the fluorescent signals in the boxed areas.  
(D) Western blot to verify the accumulation of the proteins in (C). Blots were analyzed using an anti-GFP and anti-RFP antibodies, and protein marker sizes are shown for reference.  
(E) Western blot to verify the accumulation of the proteins in the FRET-FLIM experiments shown in Figure 2C. Blots were analyzed using an anti-GFP and anti-RFP antibodies, and protein marker sizes are shown for reference. An anti-actin antibody was used to verify equal loading.
Figure S3. Validation of the silencing efficiency in RNAi and VIGS assays.

(A) Quantitative RT-PCR to determine the expression of *NbUCH05*, *NbUCH12*, and *NbUCH15* in the experiments shown in Figure 3. Samples were taken 4 or 8 dpi. Expression values are relative to the expression of the housekeeping gene NbEF1a. Values indicate mean ± SE (n=3 biological replicates).

(B) Quantitative RT-PCR to determine the expression (silencing efficiency) of *NbPtr1*. Samples were taken 8 dpi. Expression values are relative to the expression of the housekeeping gene *NbEF1a*. Values indicate mean ± SE (n=3 biological replicates).

Each experiment was repeated at least three times with similar results.
Figure S4. The expression of RipE1 or RipAA is not significantly altered by silencing of NbUCH genes.

(A) Quantitative RT-PCR to determine the expression of RipE1 and RipAA in N. benthamiana tissues in the experiment shown in Figure 5A. Expression values are relative to the expression of the housekeeping gene NbEF1a. Values indicate mean ± SE (n =9 biological replicates). Composite data from 3 independent biological replicates.
Figure S5. RipE1 ubiquitination is enhanced upon silencing of *NbUCH15*.

(A) Immunoprecipitation assay to determine the ubiquitination status of RipE1 upon silencing of *NbUCH15*. Agrobacterium carrying the indicated constructs were infiltrated as in Figure 6A. Samples were collected 30 hpi, before the appearance of cell death. In this case, the same sample volumes were loaded into each lane, showing a reduced accumulation of RipE1, but nevertheless a stronger ubiquitination. Anti-GFP beads were used for immunoprecipitation. An anti-ubiquitin (P4D1) antibody was used to detect ubiquitinated proteins. The accumulation of native NbUCH proteins was detected using a custom anti-NbUCH antibody. This experiment was repeated 3 times, and the quantification of the different repeats is shown in (B).

(B) Quantification of the relative protein ubiquitination of the different repeats of the assay shown in (A), measured using Image J. Ubiquitination values were normalized using the respective protein accumulation and represented as relative to the empty vector control for each repeat. Values indicate mean ± SE (n = 3 biological replicates). P values are shown for reference according to a Student’s t test.

(C) Composite data representation of all the replicates shown in Figure 6A and S5A. Values indicate mean ± SE (n = 6 biological replicates). Asterisk indicates significant differences compared to the control according to a Student’s t test (* p < 0.05).
Figure S6. The phosphorylated residues in RipE1 are conserved among strains belonging to different phytypes within the *R. solanacearum* species complex.

Protein sequence alignment showing RipE1 versions in different *R. solanacearum* strains. The phosphorylated residues analyzed in this study are indicated in red, together with the conserved domain A, and the catalytic sites.
| Protein 1         | Protein 2         | Score 1 | Score 2 | Score 3 | Score 4 | Score 5 | Score 6 | Score 7 | Score 8 | Score 9 | Score 10 | Score 11 | Score 12 | Score 13 | Score 14 | Score 15 | Score 16 | Score 17 | Score 18 | Score 19 | Score 20 | Score 21 |
|------------------|-------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| Niben101Scf      | Niben101Scf       | 64.41   | 77.05   | 72.6    | 64.2    | 65.91   | 77.68   | 71.07   | 58.56   | 64.12   | 64.41   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   |
| Niben101Scf      | Niben101Scf       | 64.41   | 77.05   | 72.6    | 64.2    | 65.91   | 77.68   | 71.07   | 58.56   | 64.12   | 64.41   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   |
| Niben101Scf      | Niben101Scf       | 64.41   | 77.05   | 72.6    | 64.2    | 65.91   | 77.68   | 71.07   | 58.56   | 64.12   | 64.41   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   | 100     | 83.45   | 96.15   |

Table S1. Matrix showing the percentage of identity between UCH proteins in N. benthamiana.

Matrix showing the percentage of identity between NbUCH proteins identified in this work, together with all the other proteins annotated as UCH in the N. benthamiana proteome, showing at least 50% identity when compared with NbUCH05, NbUCH12, and NbUCH15. The matrix includes also the proteins encoded by the closest Arabidopsis orthologs, namely AtUBP12 and AtUBP13. Empty rows correspond to comparisons without a significant identity value.
### Table S2. Primers used in this study

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