**EDITOR’S CHOICE**

*Ralstonia solanacearum* novel E3 ubiquitin ligase (NEL) effectors RipAW and RipAR suppress pattern-triggered immunity in plants

Masahito Nakano, Kenji Oda and Takafumi Mukaihara*

**Abstract**

*Ralstonia solanacearum* is the causal agent of bacterial wilt in solanaceous crops. This pathogen injects more than 70 effector proteins into host plant cells via the Hrp type III secretion system to cause a successful infection. However, the function of these effectors in plant cells, especially in the suppression of plant immunity, remains largely unknown. In this study, we characterized two *Ralstonia solanacearum* effectors, RipAW and RipAR, which share homology with the IpaH family of effectors from animal and plant pathogenic bacteria, that have a novel E3 ubiquitin ligase (NEL) domain. Recombinant RipAW and RipAR show E3 ubiquitin ligase activity *in vitro*. RipAW and RipAR localized to the cytoplasm of plant cells and significantly suppressed pattern-triggered immunity (PTI) responses such as the production of reactive oxygen species and the expression of defence-related genes when expressed in leaves of *Nicotiana benthamiana*. Mutation in the conserved cysteine residue in the NEL domain of RipAW completely abolished the E3 ubiquitin ligase activity *in vitro* and the ability to suppress PTI responses in plant leaves. These results indicate that RipAW suppresses plant PTI responses through the E3 ubiquitin ligase activity. Unlike other members of the IpaH family of effectors, RipAW and RipAR had no leucine-rich repeat motifs in their amino acid sequences. A conserved C-terminal region of RipAW is indispensable for PTI suppression. Transgenic *Arabidopsis* plants expressing RipAW and RipAR showed increased disease susceptibility, suggesting that RipAW and RipAR contribute to bacterial virulence in plants.

**INTRODUCTION**

Plants have a sophisticated immune system, the so-called pattern-triggered immunity (PTI), to sense invading organisms by recognizing evolutionally conserved molecules called pathogen-microbe-associated molecular patterns (PAMPs/MAMPs), e.g. flagellin, elongation factor-Tu, peptidoglycan, lipopolysaccharides, cold shock protein and chitin, through pattern recognition receptors at the cell surface [1, 2]. Recognition of PAMPs/MAMPs induces a series of plant cellular responses including ion-flux changes across the plasma membrane, remodelling of the cytoskeleton, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), and expression of defence-related genes, inhibiting the colonization of pathogens [2–4].

To evade or suppress plant PTI responses, Gram-negative phytopathogenic bacteria use the Hrp type III secretion system to inject a large repertoire of virulence proteins, the so-called type III effectors, into plant cells [5, 6]. For example, strains of the bacterial wilt pathogen *Ralstonia solanacearum* have 60 to 75 effectors called Rips (*Ralstonia* injected proteins) [7–9]. After injection, effectors localize to subcellular compartments in plant cells and exert their virulence functions. Recent studies have revealed the enzyme activities of effectors, including protease, phosphatase, acetyltransferase, ribosyltransferase, uridylyltransferase, glutamyl cyclotransferase, nucleoside hydrolase, trehalose phosphate synthase and ubiquitin ligase [10–12].

Ubiquitination is a eukaryote-specific protein modification that involves the covalent attachment of an 8 kDa ubiquitin protein to a substrate protein, which regulates the degradation, localization and activity of the conjugated protein, and therefore plays an important role in various eukaryotic cellular processes [13, 14]. Protein ubiquitination is...
achieved by the stepwise action of three enzymes, namely a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) [15]. E1 mediates the thioester linkage between its own cysteine residue and the C-terminal glycine residue of ubiquitin in an ATP-dependent manner. The ubiquitin tag is then transferred to a cysteine residue of E2 and finally bound to a lysine residue of a substrate protein through the ubiquitin ligase activity of E3. On the basis of their structural and functional properties, eukaryotic E3 ubiquitin ligases are divided into two main types, the HECT (homologous to the E6-AP carboxyl terminus) and RING (really interesting new gene) types [14, 16].

To date, several type III effectors that mimic the HECT- and RING-type E3 ubiquitin ligases have been identified from animal- and plant-pathogenic bacteria. The Salmonella enterica effector SopA contains a HECT-like domain in its C-terminal region and regulates host inflammatory responses through E3 ubiquitin ligase activity [17]. The Pseudomonas syringae pv. tomato DC3000 effector AvrPtoB contains a C-terminal domain that is structurally similar to the RING domain and that degrades host pattern recognition receptors, such as Arabidopsis FLS2, EFR, CERK1 and BAK1, through E3 ubiquitin ligase activity to suppress PTI responses [18, 19].

IpaH family effectors have been identified from several animal- and plant-pathogenic bacteria, and several plant-symbiotic bacteria, such as Shigella spp., Salmonella enterica, Yersinia spp., P. syringae and Rhizobium spp. [20]. IpaH family effectors have a NEL (novel E3 ubiquitin ligase) domain, which is structurally distinct from the RING- and HECT-type E3 ubiquitin ligase domains [21]. To date, the E3 ubiquitin ligase activity of NEL-domain-containing effectors has been demonstrated for Shigella flexneri (IpaH9.8, IpaH7.8, IpaH4.5, IpaH3, IpaH1.4, IpaH0722, IpaH2.5) [20, 22–25], Salmonella enterica (SspH1, SspH2 and SrpR) [21, 26] and Sinorhizobium fredii (NopM) [27]. In all of the aforementioned functionally studied NEL effectors and almost all NEL effectors identified by genome sequencing of animal- and plant-pathogenic bacteria, a leucine-rich repeat (LRR) domain is paired with the NEL domain. Crystal structure analysis of Salmonella SspH2 has revealed the two-domain architecture of SspH2 consisting of the LRR and NEL domains [21]. The LRR domain of NEL effectors is required not only for substrate recognition by binding to target proteins [28, 29], but also for the autoinhibition of its E3 ubiquitin ligase activity by sequestering the catalytic cysteine residue of the NEL domain in the absence of target proteins [21, 30, 31]. Therefore, the LRR domain of NEL effectors is indispensable for the function of NEL effectors [16, 32].

NEL effectors from animal-pathogenic bacteria have been demonstrated to contribute to bacterial pathogenesis by facilitating bacterial vacuolar escape in macrophages, modulation of inflammatory responses, and colonization within host tissues [33–36]. However, it remains unclear whether and how NEL effectors from phytopathogenic bacteria contribute to pathogenesis in plants. In this study, we investigated the NEL effectors RipAW and RipAR from Ralstonia solanacearum. These two effectors had the NEL domain but not LRR motifs, and therefore can be considered unusual NEL effectors. Recombinant RipAW showed an E3 ubiquitin ligase activity in vitro, which depends on the catalytic cysteine residue conserved in the NEL domain. We also show that RipAW localizes to the cytoplasm of Nicotiana benthamiana cells and suppresses plant PTI responses in an E3 ubiquitin ligase activity-dependent manner.

**METHODS**

**Plant growth conditions**

*N. benthamiana*, *Capsicum annuum* and *Solanum lycopersicum* were grown in a controlled environment room at 25 °C with 16 h light (60 µmol m⁻² s⁻¹) and 50% relative humidity. *Arabidopsis thaliana* Col-0 was grown at 22 °C with 8 h light. Five- to six-week-old plants (time after seeding) were used for plant assays.

**Bacterial strains and growth conditions**

The bacterial strains used in this study are listed in Table S1 (available with the online Supplementary Material). The growth conditions, media and antibiotics used for the strains of Escherichia coli, Ralstonia solanacearum and Agrobacterium tumefaciens were described previously [37–39]. *P. syringae* pv. *tomato* strain DC3000 was grown at 28 °C in King’s B medium [40].

**Cloning of effector genes and site-directed mutagenesis**

The open reading frames (ORFs) of ripAW and ripAR were PCR-amplified from the genomic DNA of *Ralstonia solanacearum* strain RS1000 with the primer sets listed in Table S2 and cloned into the pCRII-TOPO vector (Invitrogen), yielding the plasmids pCRII-ripAW and pCRII-ripAR. The plasmid pCRII-ripAW C177A was produced by PCR-based site-directed mutagenesis using a mutagenic primer set (Table S2).

**Protein expression and purification**

The coding region of each effector gene was PCR-amplified using the primer sets listed in Table S2 and cloned into the Ndel and HindIII double-digested PET28 plasmid (Novagen) using an In-Fusion HD cloning kit (Takara) to yield constructs expressing N-terminal His-tagged proteins. The resultant PET28-derived plasmids were transformed into E. coli BL21-Gold (DE3), and the transformants were grown in an Overnight Express Instant LB medium (Novagen) at 20 °C for 2 days. Cells were collected by centrifugation and disrupted in resuspension buffer (500 mM NaCl, 100 mM Tris-HCl pH 8.0) by sonication, and the insoluble debris was then removed by centrifugation. His-tagged proteins were purified from the supernatant using TALON metal affinity resin (Clontech) and dialysed using PD-10 desalting columns (GE Healthcare) with 25 mM Tris-HCl.
(pH 7.5) buffer. Protein concentrations were determined using a protein assay dye reagent (Bio-Rad) with BSA as a standard.

In vitro ubiquitination assays
Ubiquitination assays were performed in a 40 μl reaction mixture containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM ATP, 10 mM MgCl₂, 0.1 mM DTT, 0.5 μg UBE1 (Boston Biochem), 2 μg UbH5B (Boston Biochem), 2 μg HA-ubiquitin (Boston Biochem) and 1 μg purified RipAR-His or RipAW-His. The reaction mixtures were incubated at 37 °C for 1 h, and the reaction was stopped by adding Laemmli sample buffer (Bio-Rad) containing 100 mM DTT. Samples were separated by 10 % SDS-PAGE, transferred onto a PVDF membrane, and probed with an anti-HA (1:5000; Sigma) or anti-His (1:5000; GE Healthcare) antibody.

Agrobacterium-mediated transient expression (agroinfiltration)
The ORFs of ripAW and ripAR were PCR-amplified using the primer sets listed in Table S2 and cloned into SpeI-digested pEL2Ω-MCS vector [41] using an In-Fusion HD cloning kit to yield constructs expressing N-terminal HA-tagged proteins. The resultant plasmid was digested with Xbal and SpeI, and cloned into the SpeI site of pEL2Ω-MCS. The resultant pEL2Ω-derived plasmids were transformed into Agrobacterium tumefaciens strain GV3101. For agroinfiltration assay, cells of an overnight culture of Agrobacterium tumefaciens harbouring each plasmid were collected by centrifugation, washed with water and resuspended in infiltration buffer [10 mM MgCl₂, 10 mM MES (pH 5.6)] supplemented with 150 μM acetylsyringone at OD₆₀₀ 0.5. The bacterial cultures were incubated at 30 °C for 3 h with shaking before infiltration.

Subcellular localization ripAW and ripAW C177A genes were PCR-amplified using the primer sets listed in Table S2 and cloned into a BamHI-digested pEL2Ω-GFP vector [39] using an In-Fusion HD cloning kit to yield constructs expressing C-terminal GFP fusion proteins. The resultant pEL2Ω-GFP-derived plasmids were transiently expressed in N. benthamiana leaves by agroinfiltration. Leaf mesophyll protoplasts of N. benthamiana were prepared as described previously [38]. Briefly, leaves were harvested 2 days after agroinfiltration, rinsed with water, cut into strips and submerged in 0.5 M mannitol containing 5 mM MES-KOH (pH 5.7), 10 mM CaCl₂, 1 % cellulase Onozuka R-10 (Yakult) and 0.5 % Macerozyme R-10 (Yakult) with gentle shaking for 2 h in the dark. GFP fluorescence and the auto-fluorescence of chloroplasts were observed using a laser scanning microscope FV1200 (Olympus).

Measurement of chlorophyll content and ion leakage
The chlorophyll content of the leaves was spectrophotometrically measured as described by Markwell et al. [42]. Leaf greenness was determined using the chlorophyll meter SPAD-502 (Konica Minolta) according to the manufacturer’s instructions. The degree of cell death in plant leaves was quantified by the degree of electrolyte leakage from plant leaves. Leaf discs (8 mm in diameter) were collected from individual plants and floated on 1 ml water for 2 h at room temperature with gentle shaking. The ion conductivity of the water was measured using a conductivity meter LAQUAtwin (Horiba).

ROS measurement
ROS measurements were conducted using the chemiluminescence probe L-012 [43]. For PAMP treatment, leaf discs of N. benthamiana (4 mm in diameter) were floated on water overnight. The water was then replaced with 0.5 mM L-012 (Wako) solution (10 mM MOPS-KOH, pH 7.4) containing 100 mM flg22 (Funakoshi) or 100 μg ml⁻¹ chitin (Sigma). Chemiluminescence was continuously monitored using a microplate reader SH-8000Lab (Corona Electric).

qRT-PCR
Quantitative real-time PCR (qRT-PCR) was carried out as described by Nakano et al. [38] with slight modifications. Total RNA isolation and cDNA synthesis were conducted according to the manufacturer’s instructions. Total RNA was extracted from leaves using TRizol reagent (Invitrogen). RNA was purified using the RNeasy Plant Mini kit (Qiagen), and treated with RNase-Free DNase (Qiagen) to remove genomic DNA. cDNA was then synthesized with 1 μg RNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative PCR was carried out in a 10 μl reaction mixture containing 1 μl cDNA and 10 pM respective primers using a Power SYBR Green PCR master mix (Applied Biosystems) in triplicate on an Applied Biosystems 7900HT real-time PCR system. The cycling parameters were the same for all the primers: initial 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To check whether the unique products were amplified, dissociation curve analysis was performed at the end of the cycling. The expression level of NbEF1 or ACT2 was analysed as an internal control and used to normalize the values for the gene of interest.

MAPK assay
Phosphorylation of MAPKs was detected as described by Flury et al. [44]. Two days after agroinfiltration, N. benthamiana leaves were infiltrated with 1 μM flg22. Leaf discs (60 mg) were collected over the time-course, frozen in liquid nitrogen and ground to a fine powder. Proteins were extracted in 80 μl extraction buffer [0.35 M Tris-HCl (pH 6.8), 30 % glycerol, 10 % SDS, 0.6 M DTT, 0.012 % bromophenol blue], and leaf debris was removed by centrifugation at 11 000 g for 5 min. Total proteins (15 μl) were separated by 10 % SDS-PAGE and subjected to immunoblot analysis using a Phospho-p44/42 MAPK antibody (1:1000; Cell Signaling Technology). Immunodetection was performed using an ECL Prime Western blotting detection reagent (GE Healthcare).
Generation of transgenic plants

The plasmids pCRII-ripAW and pCRII-ripAR were double-digested with XbaI and SpeI, and the ORFs of ripAW and ripAR were cloned into the SpeI site of the pTA7002 vector [45]. The resultant pTA7002-derived plasmids were transformed into Agrobacterium tumefaciens strain GV3101, and transgenic Arabidopsis plants expressing ripAW and ripAR under the control of the dexamethasone (DEX)-inducible promoter were generated by the floral-dip method [46].

Bacterial inoculation and population assay

Transgenic Arabidopsis plants treated with 30 µM DEX with 0.01 % Silwet L-77 for 1 day were inoculated by spraying P. syringae pv. tomato DC3000 cells suspended in 10 mM MgCl₂ with 0.01 % Silwet L-77 (OD₆₀₀ 0.2). For measuring bacterial growth, a leaf disc (1 cm²) was taken from inoculated leaves 2 days after inoculation and homogenized in 1 ml water. Serial dilutions of the homogenate were spread on a King’s B plate containing 30 µg ml⁻¹ rifampicin.

RESULTS AND DISCUSSION

RipAW and RipAR are atypical IpaH family effectors with no LRR motifs

Using the type III effector repertoire of Ralstonia solanacearum RS1000 as query sequences for a homology search in the Pfam protein families database, we identified two effector proteins, namely, RipAW (Rip69) and RipAR (Rip61), that had a putative NEL domain (Fig. 1a). Although RipAR contained a long N-terminal region compared with RipAW, the two proteins shared high sequence similarity (Fig. S1). The NEL domains of RipAW and RipAR were located in their central region at amino acid positions 97–306 and 243–450, respectively, and shared 78.6 % similarity to each other. The putative catalytic cysteine residues and their surrounding amino acids that are conserved among the NEL...
effectors were also conserved in RipAW and RipAR (Fig. 1b). All of the previously characterized NEL effectors from animal-pathogenic bacteria and a plant-symbiotic bacterium have an LRR domain paired with the NEL domain [20]. Interestingly, unlike the already known NEL effectors, RipAW and RipAR had no LRR motifs in their N-terminal region; instead, they had a novel C-terminal region conserved between them (Figs 1a and S1).

In addition to RipAW and RipAR, the two putative NEL-domain-containing effectors RipV1 and RipV2 have been identified in the type III effector repertoire of the Ralstonia solanacearum species complex [9]. However, the ripV1 (hphx29) gene from Ralstonia solanacearum phylotype I strain RS1000 has an insertion sequence in its NEL-domain-coding region [37], and the ripV2 gene could not be detected in the genome of phylotype I strains [9]. Therefore, we focused on RipAW and RipAR effectors in this study.

**Functional characterization of RipAW and RipAR in vitro**

To clarify the E3 ubiquitin ligase activity of RipAW and RipAR in vitro, we expressed and purified the N-terminal histidine (His)-tagged recombinant proteins of RipAW and RipAR using an E. coli expression system. It has been reported that the catalytic cysteine residue in the NEL domain is essential for the E3 ubiquitin ligase activity of NEL effectors [20, 21, 27]. Therefore, we changed the putative catalytic cysteine at position 177 of RipAW to alanine and purified the putative catalytically inactive RipAW C177A protein. When the haemagglutinin (HA)-tagged ubiquitin substrate was incubated with E1, E2 and RipAW, and the reaction mixture was separated by SDS-PAGE, a polyubiquitin ladder was specifically detected in Western blot analysis using an anti-HA antibody (Fig. 1c). This ladder disappeared when one of the E1, E2 and RipAW proteins was excluded from the reaction mixture. On the other hand, the C177A mutation in RipAW completely abolished formation of the polyubiquitin ladder, suggesting that the cysteine residue at position 177 of RipAW is the essential catalytic cysteine residue required for ubiquitination of the target protein. A polyubiquitin ladder was also formed in the reaction mixture containing E1, E2 and RipAR. These results clearly show that RipAW and RipAR have an E3 ubiquitin ligase activity. We tried to purify the putatively catalytically inactive RipAR C322A protein, but obtained no soluble mutant proteins in our experiment system. Therefore, we mainly examined RipAW in the following plant assays.

**RipAW is localized to the cytoplasm of N. benthamiana cells**

To examine the subcellular localization of RipAW in plant cells, we examined the localization of transiently expressed GFP, RipAW-GFP and RipAW C177A-GFP proteins in N. benthamiana protoplasts using an Agrobacterium-mediated transient expression system (agroinfiltration). The fluorescence signals of RipAW-GFP and RipAW C177A-GFP localized to the cytoplasm of protoplasts, whereas that of GFP localized to both cytoplasm and nucleus (Fig. 2). These observations indicate that RipAW functions in the cytoplasm of N. benthamiana cells and that its localization is not affected by the C177A mutation.

**Prolonged expression of RipAW induces leaf chlorosis in N. benthamiana**

To examine the effect of RipAW in plant cells, we transiently expressed the N-terminal HA-tagged RipAW and RipAW C177A proteins in N. benthamiana leaves using agroinfiltration. Western blot analysis using the anti-HA antibody showed that both the RipAW and RipAW C177A proteins were produced in the leaves 2 days after agroinfiltration (Fig. 3a). Expression of RipAW and RipAW C177A induced no visible changes in the infiltrated leaves compared with the vector control at least 3 days after agroinfiltration, whereas the expression of RipAW, but not RipAW C177A, induced leaf chlorosis 4 days after agroinfiltration (Fig. 3b). The chlorotic phenotype is usually associated with a reduction in chlorophyll content and/or cell viability [47–49]. Therefore, we measured the chlorophyll content of the infiltrated leaves. As expected, expression of RipAW, but not RipAW C177A, decreased the chlorophyll content of the leaves 4 days after agroinfiltration (Fig. 3c). However, a similar degree of electrolyte leakage was observed in the leaves expressing RipAW and RipAW C177A compared with the vector control both 3 and 4 days after agroinfiltration (Fig. 3d), suggesting that RipAW neither induces cell

![Fig. 2. Subcellular localization of RipAW in N. benthamiana cells.](https://example.com/fig2)

- GFP
- Chlorophyll
- Merged

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collapse nor reduces cell viability. These results suggest that RipAW affects some physiological processes of plant cells through its E3 ubiquitin ligase activity.

**Expression of RipAW and RipAR suppress PTI in N. benthamiana**

To clarify the function of RipAW in plant cells, we examined the effect of RipAW expression on plant PTI responses. We treated *N. benthamiana* leaf discs transiently expressing RipAW and RipAW C177A with flg22. Interestingly, expression of RipAW, but not RipAW C177A, significantly suppressed the flg22-triggered ROS burst compared with the vector control 2 days after agroinfiltration (Fig. 4a, b). Transient expression of RipAW, but not RipAW C177A, also suppressed the chitin-triggered ROS burst in *N. benthamiana* leaves (Fig. S3a, b). PAMP recognition leads to the expression of defence-related genes in plants. Expressions of *NbAcre132*, *NbPti5* and *NbWryk22* are rapidly induced by flg22 treatment of *N. benthamiana* leaves and used as markers for PTI responses [50–52]. Expression of RipAW, but not RipAW C177A, significantly suppressed the flg22-triggered expression of PTI marker genes (Fig. 4c). These findings show that RipAW suppresses plant PTI responses through its E3 ubiquitin ligase activity. The transient phosphorylation and activation of MAPKs are well-defined events in plant PTI responses. In *Nicotiana* plants, both WIPK and SIPK (*Arabidopsis thaliana* MPK3 and MPK6 orthologues) are transiently phosphorylated upon elicitor treatment [52, 53]. flg22-induced phosphorylation of WIPK and SIPK was detected in *N. benthamiana* leaves expressing RipAW and the vector control (Fig. 4d), suggesting that RipAW has little effect on MAPK activation in plant cells.

RipAR showed an E3 ubiquitin ligase activity *in vitro* (Fig. 1c), suggesting that RipAR can suppress plant PTI responses like RipAW. Therefore, we transiently expressed the N-terminal HA-tagged RipAR in *N. benthamiana* leaves and treated the leaves with flg22. Expression of RipAR suppressed the flg22-triggered ROS burst (Fig. S4a, b) and the

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**Fig. 3.** Effect of RipAW expression in leaves of *N. benthamiana*. (a) Immunoblot analysis of RipAW-HA and RipAW C177A-HA. *N. benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* harbouring the binary vector carrying the effector genes or empty vector (EV). Total protein was extracted from the leaves 2 days after agroinfiltration, separated by SDS-PAGE and subjected to immunoblot analysis using an anti-HA antibody (top panel). A gel was stained with Coomassie brilliant blue (CBB) to confirm equal loading (bottom panel). The experiment was performed three times with similar results, and a representative result is shown. IB, Immunoblot. (b) Morphological feature of *N. benthamiana* leaves transiently expressing RipAW-HA or RipAW C177A-HA. Photographs were taken 3 and 4 days after agroinfiltration. The experiment was performed three times with similar results, and a representative result is shown. (c) Chlorophyll content of leaves expressing RipAW-HA or RipAW C177A-HA. Foliar chlorophyll content was measured 3 and 4 days after agroinfiltration using a chlorophyll meter. Values are presented as means±SD from six independent leaves. The asterisk indicates statistically significant differences compared with the EV control (t-test, *P*<0.05). (d) Cell death in leaves expressing RipAW-HA or RipAW C177A-HA. The degree of ion leakage from the leaf discs was measured 3 and 4 days after agroinfiltration using a conductivity meter. Values are presented as means±SD from six independent leaves.
induction defence-related genes (Fig. S4c), but not the phosphorylation of MAPKs (Fig. S4d). These results show that RipAR can also suppress plant PTI responses.

It has been demonstrated that the Sinorhizobium fredii NEL effector NopM suppresses the flg22-triggered ROS burst, but neither blocks phosphorylation of WIPK or SIPK nor suppresses expression of defence-related genes in N. benthamiana leaves [27]. In contrast to NopM, RipAW and RipAR suppressed both the flg22-triggered ROS burst and the induction of defence-related genes (Figs 4 and S4).

RipAW also suppressed the chitin-triggered ROS burst in N. benthamiana leaves (Fig. S3). In Arabidopsis, the P. syringae effector AvrPtoB, a RING-type E3 ubiquitin ligase, has been reported to interfere with plant PTI responses triggered by multiple PAMPs [54]. Previous studies have demonstrated that AvrPtoB is present in the plant cytoplasm [55], whereubiquitnates the cytoplasmic kinase domain of PAMP receptors including FLS2, EFR, CERK1 and BAK1 [18, 19]. RipAW and RipAR are localized to the cytoplasm of plant cells (Figs 2 and S2), suggesting that RipAW and RipAR target a cytoplasmic protein or domain in an
important component of the flg22- and chitin-triggered signalling pathway. However, in contrast to AvrPtoB, RipAW and RipAR do not appear to affect MAPK signalling in plant cells (Figs 4d and S5d). Thus, these NEL effectors may interfere with plant PTI responses downstream of the MAPK cascade. At this time, the potential targets of RipAW and RipAR in PTI signalling remain unclear.

**C-terminal region of RipAW is indispensable for the ability to suppress PTI**

The LRR domain is conserved in almost all NEL effectors in databases [20, 27] and is indispensable for target ubiquitination in NEL effectors whose substrates have been identified [16, 32]. The fact that RipAW contained no LRR motif (Fig. 1a) suggests that RipAW contains an as-yet-unidentified protein domain that is required for substrate recognition and the regulation of E3 ubiquitin ligase activity. To identify such a protein domain in RipAW, we constructed RipAW mutants, namely, RipAW ΔN and RipAW ΔC, in which the N- and C-terminal regions of RipAW, respectively, were deleted (Fig. 5a). We transiently expressed the N-terminal HA-tagged RipAW ΔN and RipAW ΔC in N. benthamiana leaves and confirmed their expression by Western blot analysis (Fig. 5b). Expression of RipAW ΔN, as well as that of the wild-type, induced leaf chlorosis and decreased the chlorophyll content of the

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**Fig. 5.** Characterization of RipAW region required for suppression of PTI responses. (a) Schematic diagram of the N- or C-terminally truncated RipAW. NEL domains are indicated by black boxes. The conserved C-terminal regions of RipAW and RipAR are indicated by grey boxes. Positions of amino acid residues are indicated by numbers. (b) Immunoblot analysis of RipAW ΔN-HA and RipAW ΔC-HA. N. benthamiana leaves were infiltrated with Agrobacterium tumefaciens harbouring the binary vector carrying the truncated effector genes or empty vector (EV). Total protein was extracted from the leaves 2 days after agroinfiltration, separated by SDS-PAGE and subjected to immunoblot analysis using an anti-HA antibody (top panel). A gel was stained with Coomassie brilliant blue (CBB) to confirm equal loading (bottom panel). The experiment was performed three times with similar results, and a representative result is shown. IB, Immunoblot. (c) Morphological features of N. benthamiana leaves transiently expressing RipAW ΔN-HA or RipAW ΔC-HA. Photographs were taken 4 days after agroinfiltration. The experiment was performed three times with similar results, and a representative result is shown. (d) Chlorophyll content of leaves infiltrated with Agrobacterium tumefaciens expressing RipAW ΔN-HA or RipAW ΔC-HA. Foliar chlorophyll content was measured 4 days after infiltration using a chlorophyll meter. Values are presented as means±SD from six independent leaves. The asterisk indicates statistically significant difference compared with the EV control (t-test, P<0.05). (e) flg22-triggered ROS production in leaves expressing RipAW ΔN-HA or RipAW ΔC-HA. Leaf discs were collected 2 days after agroinfiltration and treated with L-012 solution containing flg22. Chemiluminescence was monitored for 60 min. Values are presented as means±SD from eight independent leaves. (f) Cumulative photon counts in (e). The asterisk indicates statistically significant difference compared with the EV control (t-test, P<0.05).
leaves 4 days after agroinfiltration compared with the vector control, whereas expression of RipAW ΔC showed no effect on either phenotype (Fig. 5c, d). Similarly, the fig22-triggered ROS burst was significantly suppressed by expression of RipAW ΔN, but not of RipAW ΔC, in N. benthamiana leaves (Fig. 5e, f). These results clearly show that the conserved C-terminal region of RipAW is indispensable for the suppression of PTI responses. It is considered that RipAW interacts with an essential component of plant PTI signalling in the C-terminal region, ubiquitinating and degrading it through the host ubiquitin-proteasome system.

**Contribution of ripAW and ripAR to bacterial virulence**

To elucidate the contribution of RipAW and RipAR to bacterial virulence, we constructed a *Ralstonia solanacearum* ΔripAW ΔripAR double mutant and examined its virulence in solanaceous host plants. The ΔripAW ΔripAR mutant showed no change in bacterial growth (Fig. S5a, c) nor a delay in symptom development (Fig. S5b, d) in tomato or pepper plants compared with the wild-type strain. This might be because *Ralstonia solanacearum* RS1000 has more than 70 effectors, and many functionally redundant effectors would exist in its effector repertoire.

*P. syringae* pv. *tomato* strain DC3000, the causal agent of bacterial speck disease in tomato, is one of the best characterized bacterial pathogens. Moreover, the *Arabidopsis*–*P. syringae* pathosystem has been widely used in recent studies to identify phytopathogen effectors that contribute to bacterial virulence in *Arabidopsis* plants from other pathosystems, such as *Pseudomonas* spp., *Xanthomonas* spp., *Hyaloperonospora* and *Phytophthora* [48, 56–60]. Therefore, we generated transgenic *Arabidopsis* plants expressing ripAW and ripAR under the control of the DEX-inducible promoter, and examined the aggressiveness of *P. syringae* in these plants. The ripAW and ripAR transgenic plants showed no morphological defects in their growth (Fig. 6a). Expression of ripAW and ripAR were rapidly induced by the DEX treatment (Fig. 6b). Bacterial growth of *P. syringae* pv. *tomato* DC3000 in plant leaves increased 10–100-fold in the transgenic plants expressing ripAW and ripAR compared with that in the wild-type plants 2 days after inoculation (Fig. 6c). This result clearly shows the contribution of ripAW and ripAR expression on bacterial virulence in the *Arabidopsis*–*P. syringae* pathosystem. Although the increased susceptibility of transgenic plants results from another pathosystem, we suggest that RipAW and RipAR may contribute to the virulence of *Ralstonia solanacearum* in certain plants.

**Concluding Remarks**

In this study, we performed the biochemical and *in planta* characterization of the *Ralstonia solanacearum* NEL-domain-containing effectors RipAW and RipAR. Unlike other members of the NEL effectors from animal- and plant-pathogenic bacteria and a plant-symbiotic bacterium, RipAW and RipAR contained no LRR domain, a typical protein–protein interaction domain that is indispensable for the function of NEL effectors. However, we demonstrated that the two effectors have an E3 ubiquitin ligase activity and significantly suppress plant PTI responses when expressed in *N. benthamiana* leaves. An essential cysteine residue in the NEL domain and a conserved C-terminal region of RipAW were indispensable for PTI suppression, suggesting that RipAW interacts with an essential component of plant immune signalling at the C-terminal region,
and ubiquitinates and degrades it through the host ubiquitin-proteasome system. Expression of RipAW and RipAR promoted growth of a susceptible P. syringae strain in Arabidopsis plants. Taken together, these results suggest that RipAW and RipAR contribute to bacterial virulence in plants.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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