The Xanthomonas campestris Type III Effector XopJ Proteolytically Degrades Proteasome Subunit RPT6

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Many animal and plant pathogenic bacteria inject type III effector (T3E) proteins into their eukaryotic host cells to suppress immunity. The Yersinia outer protein J (YopJ) family of T3Es is a widely distributed family of effector proteins found in both animal and plant pathogens, and its members are highly diversified in virulence functions. Some members have been shown to possess acetyltransferase activity; however, whether this is a general feature of YopJ family T3Es is currently unknown. The T3E Xanthomonas outer protein J (XopJ), a YopJ family effector from the plant pathogen Xanthomonas campestris pv vesicatoria, interacts with the proteasomal subunit Regulatory Particle AAA-ATPase6 (RPT6) in planta to suppress proteasome activity, resulting in the inhibition of salicylic acid-related immune responses. Here, we show that XopJ has protease activity to specifically degrade RPT6, leading to reduced proteasome activity in the cytoplasm as well as in the nucleus. Proteolytic degradation of RPT6 was dependent on the localization of XopJ to the plasma membrane as well as on its catalytic triad. Mutation of the Walker B motif of RPT6 prevented XopJ-mediated degradation of the protein but not XopJ interaction. This indicates that the interaction of RPT6 with XopJ is dependent on the ATP-binding activity of RPT6, but proteolytic cleavage additionally requires its ATPase activity. Inhibition of the proteasome impairs the proteasomal turnover of Nonexpressor of Pathogenesis-Related1 (NPR1), the master regulator of salicylic acid responses, leading to the accumulation of ubiquitinated NPR1, which likely interferes with the full induction of NPR1 target genes. Our results show that YopJ family T3Es are not only highly diversified in virulence function but also appear to possess different biochemical activities.

Plants have been threatened by microbial infections throughout their phylogenetic history and thus evolved a sophisticated and multilayered immune system (Jones and Dangl, 2006). Phytohormonal signaling is instrumental to many aspects of plant defense, and salicylic acid (SA) has emerged as the central regulator of signaling pathways during induced immunity (Pieterse et al., 2012). SA signaling plays major roles during systemic acquired resistance (SAR), basal defense responses, and gene-for-gene resistance.

Nonexpressor of PR1 (NPR1) represents an essential component of SA signaling that acts as a transcriptional coactivator to induce transcriptional reprogramming, including the induction of PR (for pathogenesis-related) genes and genes associated with secretion (Wang et al., 2005; Pieterse et al., 2012). Upon pathogen attack, SA levels increase within the cytoplasm of the host cell, which then leads to the monomerization of NPR1 and translocation of the protein into the nucleus. Once in the nucleus, NPR1 can interact with different TGACG SEQUENCE-SPECIFIC BINDING PROTEIN transcription factors and drives the activation of SA-responsive gene expression (Fu and Dong, 2013). Subsequent phosphorylation serves as a signal for the ubiquitination of NPR1, mediating its degradation via the 26S proteasome and thereby returning the target gene promoter to the initial state (Spoel et al., 2009). It is assumed that the rate of NPR1 degradation by the proteasome determines the rate of mRNA production from SA-responsive genes (Spoel et al., 2009).

The significance of the proteasomal turnover of NPR1 during SA-mediated defense signaling highlights the important role of the proteasome in plant defense. Accordingly, Arabidopsis (Arabidopsis thaliana) mutant lines defective in proteasome function are impaired in certain immune responses (Yao et al., 2012), and particular proteasomal subunits have been proposed to act as a caspase-like enzyme during the induction of programmed cell death in response to avirulent bacterial strains (Hatsugi et al., 2009). Thus, the 26S proteasome seems to control defense mechanisms alongside its usual role as a multicatalytic protein complex that is essential for the degradation of ubiquitinated proteins. The 26S...
proteasome itself is composed of a 20S core particle (CP) that is capped on either end by a 19S regulatory particle (RP; Vierstra, 2009). The RP recognizes and binds ubiquitinated proteins, deubiquitinates and unfolds these substrates in an ATP-dependent manner, and controls the entry of unfolded target proteins to the proteolytic channel of the CP (Vierstra, 2009).

The injection of type III effector (T3E) proteins or bacterial toxins into the host cell is an efficient mechanism employed by many bacterial pathogens to suppress plant immunity and to promote disease development (Galán and Wolf-Watz, 2006). In accordance with its central role in a wide array of cellular processes, including defense, the proteasome is targeted or exploited by bacterial toxins or T3E proteins (Dudler, 2013). Certain strains of *Pseudomonas syringae* pv *syringae* secrete syringolin A (SylA), which is a small nonribosomal peptide that inhibits proteasome activity through binding to the catalytic subunits of the 20S CP and is required for full virulence on host plants, such as bean (*Phaseolus vulgaris*; Groll et al., 2008). The initial discovery of the interaction between the bacterial T3E protein *Xanthomonas* outer protein J (XopJ) from *Xanthomonas campestris* pv *vesicatoria* (Xcv) and Regulatory Particle AAA-ATPase6 (RPT6), a subunit of the 19S RP, provided, to our knowledge, the first example of a bacterial T3E directly targeting the proteasome (Üstün et al., 2013). The interaction of XopJ with RPT6 leads to the inhibition of the proteasome, which subsequently interferes with SA-dependent defense responses to attenuate the onset of necrosis in infected tissue and to alter host transcription. The development of XopJ-associated phenotypes on susceptible pepper (*Capsicum annuum*) plants was shown to be dependent on NPR1 (Üstün et al., 2013). Another T3E effector targeting RPT6 to inhibit proteasome activity is Hypersensitivity and pathogenesis-dependent outer protein Z4 (HopZ4) from *Pseudomonas syringae* pv *lachrymans* (Üstün et al., 2014). Both T3Es belong to the widespread *yersinia* outer protein J (YopJ) family of effector proteins that are present among plant and animal pathogenic bacteria and were originally classified as Cys proteases (Lewis et al., 2011). However, recent studies indicate that these effectors, in particular the archetypal member XopJ from *Yersinia pestis*, HopZ1a from *P. syringae* pv *syringae*, and Avirulence protein AvrBsT from *Xanthomonas euvesicatoria*, act as acetyltransferases on their targets or decoys to suppress immunity in animals and plants (Mukherjee et al., 2006; Lee et al., 2012; Jiang et al., 2013; Lewis et al., 2013; Cheong et al., 2014). In some cases, these effectors also display weak protease activity and lead to the degradation of potential target proteins, opening the possibility that XopJ family effectors can also act proteolytically (Ma et al., 2005; Szczesny et al., 2010; Zhou et al., 2011). However, the mechanism through which the interaction of XopJ with RPT6 leads to the inhibition of the proteasome and the role of NPR1 in this context are currently unknown. Here, we show that XopJ acts as a protease to directly degrade its target protein, RPT6, in a process that is dependent on the localization of XopJ at the plasma membrane and on an intact catalytic triad of the effector. Interaction of RPT6 with XopJ is dependent on the ATP-binding activity of RPT6, but proteolytic cleavage additionally requires its ATPase activity. The malfunction of the proteasome impairs the proteasomal turnover of NPR1 and thus results in the accumulation of ubiquitinated NPR1. We conclude that XopJ-mediated degradation of RPT6 connected with the inhibition of the proteasome prevents the proteasomal turnover of NPR1 and thereby suppresses SA-mediated defense signaling.

**RESULTS**

**XopJ Mediates the Destabilization of RPT6 Protein Levels**

Although various members of the YopJ-like effector family were already characterized as acetyltransferases (Lee et al., 2012; Cheong et al., 2014), recombinant XopJ does not possess measurable acetyltransferase activity in vitro (Supplemental Fig. S1). However, when XopJ-HA (for hemagglutinin) was transiently coexpressed with RPT6-GFP in leaves of *Nicotiana benthamiana*, GFP fluorescence was decreased dramatically as compared with *N. benthamiana* leaves coexpressing RPT6-GFP with the catalytically inactive XopJ variant C235A or the nonmyristoylated variant G2A (Fig. 1A–D). To allow for the direct comparison of signal intensities, all confocal images shown in Figure 1 were recorded with the same microscope settings. However, Figure 1E illustrates that RPT6-GFP fluorescence becomes readily detectable in XopJ-coexpressing leaves when the microscope settings are adjusted to increase the sensitivity of detection. Quantification of the GFP intensity signal revealed that XopJ-HA caused a significant reduction of RPT6-GFP fluorescence dependent on its localization (G2A) or enzymatic activity (C235A; Fig. 1F). Analysis of the protein levels of RPT6-GFP confirmed the microscopic data, as the RPT6-GFP signal was substantially decreased in the presence of XopJ-HA but not in the presence of its mutant variants (Fig. 1G). To rule out any toxicity effects that could result from the overexpression of XopJ-HA in *N. benthamiana*, endogenous protein levels of the cytosolic Sucrose Phosphatase2 (SP2) were examined in plants expressing XopJ and its variants. The data revealed that XopJ wild-type protein did not result in decreased SP2 protein levels (Fig. 1G), indicating that XopJ specifically reduces RPT6-GFP protein levels. Reverse transcription (RT)-PCR analysis revealed that coexpression with XopJ does not affect RPT6-GFP mRNA levels (Fig. 1H). This suggests that XopJ interferes with RPT6 protein stability.

In addition, RPT6-GFP and XopD from Xcv were transiently coexpressed in *N. benthamiana* to exclude that the reduction of RPT6-GFP protein levels is a general characteristic of effectors that share similarities to Cys proteases (Supplemental Fig. S2).

To investigate whether XopJ would also affect RPT6 from other kingdoms of the phylogenetic tree, we tested its interaction with RPT6 from humans, as both proteins share high similarity on amino acid level (80%). Yeast two-hybrid analysis and in planta bimolecular fluorescence complementation (BiFC) showed that XopJ
is not able to interact with human RPT6 (Supplemental Fig. S3, A and B). Thus, XopJ is not able to recruit HsRPT6 to the plasma membrane and also does not lead to the destabilization of HsRPT6-GFP in *N. benthamiana* (Supplemental Fig. S3, C and D). These data support the notion that XopJ specifically triggers the degradation of RPT6 from plants.

**The Degradation of RPT6 Is Protease Dependent**

To determine the mechanism underlying the XopJ-triggered reduction of RPT6 protein levels, coexpression of RPT6 and XopJ in the presence of different inhibitors was performed. The XopJ-dependent destabilization of RPT6-GFP in *N. benthamiana* was not affected by the proteasome inhibitor MG132 (Fig. 2A). In contrast, application of a commercially available mix of protease inhibitors (Ser, Cys, aspartic, and metalloproteases and aminopeptidases) abolished the XopJ-mediated decrease of RPT6 protein levels (Fig. 2A). Measurement of the proteasome activity in plants expressing XopJ in the presence or absence of protease inhibitors revealed that the mix of protease inhibitors abolished the proteasome-inhibiting ability of XopJ (Fig. 2B). These data indicate that XopJ might act as a protease with RPT6 as a substrate to inhibit proteasome activity.

Figure 1. XopJ interferes with RPT6-GFP protein accumulation. A to D, Empty vector (EV; A), XopJ-HA (B), G2A-HA (C), and C235A-HA (D) were transiently coexpressed together with RPT6-GFP in *N. benthamiana* using *A. tumefaciens* infiltration. For confocal laser scanning microscopy (CLSM), samples were taken 48 h post inoculation (hpi), and images were generated with identical CLSM settings. E, Microscope image of an RPT6-GFP- and XopJ-HA-coexpressing leaf recorded with increased photomultiplier gain as compared with the images presented in A to D. GFP fluorescence is shown in green, and chlorophyll autofluorescence is shown in red. Bar = 20 μm. F, Quantification of GFP intensity after coexpression of RPT6-GFP together with XopJ and its variants. GFP quantification was determined by using the Leica software LAS_AF. Coexpression with the EV control was set to 100%. Data represent means ± so (n = 3). Significant differences were calculated using Student's t test and are indicated by asterisks at P < 0.001. G, Total proteins were extracted 48 hpi with *A. tumefaciens* harboring the respective XopJ and RPT6 expression construct. RPT6-GFP protein levels were detected using an anti-GFP antibody. Expression of the XopJ variants was verified using an anti-HA antibody after stripping the same membrane. Analysis of the endogenous protein levels of cytosolic SPP2 (anti-NtSPP2) served as a control, and staining of the membrane with Amido Black showed equal loading. H, XopJ does not affect RPT6 expression levels. RT-PCR shows transgenic RPT6 mRNA in *N. enthamiana* leaves transiently coexpressing RPT6 with an EV control or XopJ-HA. Ubiquitin was used as an amplification control. All experiments were repeated at least three times with almost identical results.
XopJ Possesses Protease Activity in Vitro and in Vivo

To clarify whether XopJ acts as a protease itself, protease activity was monitored using a Förster resonance energy transfer-based protease detection kit containing a fluorogenic peptide Suc-LLVY-AMC at 30°C in a fluorescence spectrophotometer. The EV control was set to 100%. Expression of RPT6-GFP was detected using an anti-GFP antibody. After immunodetection of proteins, the membrane was stained with Amido Black to control for equal protein loading. B, N. benthamiana leaves transiently expressing XopJ-myc were treated with a cocktail of protease inhibitors or water 42 hpi. At 48 hpi, relative proteasome activity in total protein extracts was determined by monitoring the breakdown of the fluorogenic peptide Suc-LLVY-AMC at 30°C in a fluorescence spectrophotometer. The EV control was set to 100%. Data represent means ± SD (n = 3). Significant differences were calculated using Student’s t test and are indicated by asterisks at P < 0.01. The experiments in both A and B were repeated twice with similar results.

XopJ Degradates RPT6 in a Protease-Dependent Manner

To further investigate the proteolytic activity of XopJ toward RPT6, the same amounts of purified recombinant MBP-RPT6 (Supplemental Fig. S5) were incubated together with crude extracts from XopJ-expressing leaves. Subsequent western-blot analyses revealed that the amount of MBP-RPT6 was reduced in the presence of XopJ-expressing extracts (Fig. 3A). Mutation of the Cys-235 residue within the catalytic triad of XopJ as well as the addition of protease inhibitors abolished the degradation of MBP-RPT6 (Fig. 4B), suggesting the notion that XopJ acts as a protease to degrade RPT6. As recombinant MBP-XopJ also has protease activity in vitro, we tested whether purified XopJ is able to degrade plant-expressed RPT6-GFP. After incubation of MBP-XopJ together with RPT6-GFP, RPT6 protein levels decreased in the presence of wild-type XopJ but not upon coincubation with the C235A variant (Supplemental Fig. S6A). Incubation of glutathione S-transferase (GST)-XopJ together with recombinant MBP-RPT6 purified from E. coli also resulted in a reduction of RPT6 protein abundance (Supplemental Fig. S6B), further indicating that the proteolytic activity of recombinant XopJ is sufficient to degrade RPT6 in vitro. Moreover, N. benthamiana extracts incubated with MBP-XopJ have significantly
lower proteasome activities compared with MBP- or MBP-XopJ C235A-treated extracts (Supplemental Fig. S6C). Taken together, these data demonstrate that XopJ displays protease activity to degrade RPT6 and thereby inhibits proteasome activity in plant cells.

The Walker A Motif of RPT6 Is Required for XopJ Binding

RPT6 is one of six ATPases of the AAA protein family, being part of the RP of the 26S proteasome (Bar-Nun and Glickman, 2012). AAA proteins feature conserved Walker A and Walker B domains that bind and hydrolyze ATP, respectively, to generate a mechanical force that is used to unfold substrate proteins (Hanson and Whiteheart, 2005). Whereas ATP binding to the ATPase subunits of the proteasome is necessary for the association of the RP to the CP, substrate binding, and the opening of the proteolytic channel, ATP hydrolysis drives the unfolding of substrates that are destined to be degraded by the 26S proteolytic machinery (Bar-Nun and Glickman, 2012). In order to relate RPT6 functionality to its interaction with XopJ, a point mutation was generated in either of the two Walker motifs of RPT6 that rendered the protein defective in ATP binding (Walker A) or ATP hydrolysis (Walker B). Mutation of the Walker A motif in RPT6 by exchanging the Lys residue at position 206 to an Ala (K206A) abrogated the interaction with XopJ in yeast (Saccharomyces cerevisiae), while a point mutation in the Walker B motif (E260A) did not affect the binding of XopJ to RPT6 (Fig. 5A).

Figure 3. XopJ displays protease activity in vitro and in vivo. A, Five micrograms of MBP, MBP-XopJ, MBP-XopJ C235A, MBP-RPT6, and a mix of MBP-XopJ and MBP-RPT6 purified from E. coli was subjected to protease activity measurement using the P-CHECK protease detection kit. MBP and MBP-RPT6 served as negative controls. As a positive control, the Ser protease Proteinase K was included. A representative result of more than three repetitions with independent sets of purified proteins is shown. RFU, Relative fluorescence units. B, Protease activity in N. benthamiana leaves transiently expressing XopJ-HA proteins. XopJ protein variants along with an EV control were transiently expressed in leaves of N. benthamiana using agroinfiltration. After 48 h, protease activity in total protein extracts was measured using the P-CHECK protease detection kit. Data represent means ± so (n = 3). Significant differences were calculated using Student’s t test and are indicated by asterisks: *, P < 0.05; and **, P < 0.01. The experiment was repeated twice with similar results. RLU, Relative light units.

Figure 4. XopJ degrades RPT6 in a protease-dependent manner. XopJ has protease activity on recombinant MBP-RPT6 when transiently expressed in N. benthamiana leaves dependent on its catalytic activity. A, Extracts from N. benthamiana transiently expressing XopJ-HA or EV were incubated together with MBP-RPT6 purified from E. coli. RPT6 protein levels were monitored using an anti-MBP antibody, and XopJ protein accumulation was detected using an anti-HA antibody. Amido Black staining served as a loading control for the plant extracts used. B, Immunoblots using an anti-MBP antibody show MBP-RPT6 accumulation after incubation with crude extracts of N. benthamiana leaves expressing XopJ-HA or C235A-HA with (+) or without (−) protease inhibitors. Plants expressing the EV control served as a negative control. Anti-HA immunoblotting was performed to detect the proper expression of XopJ-HA proteins. Amido Black staining served as a loading control for the plant extracts used. The results in both A and B are representative of two independent experiments.
To investigate in planta interactions, BiFC assays were performed using *Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana*. To this end, RPT6 (K206A) and RPT6 (E260A) mutants were each fused to the C-terminal part of the yellow fluorescent protein (YFP) derivative Venus (Venus C155) and transiently expressed in *N. benthamiana* leaves. Reconstitution of the fluorescence signal after transient coexpression with the RPT6 Walker A motif is required for XopJ binding. A, XopJ interacts with RPT6 E260A (Walker B) but not with RPT6 K206A (Walker A) in a yeast two-hybrid assay. XopJ fused to the GAL4 DNA-binding domain (pGBT9) was expressed in combination with RPT6 E260A or K206A fused to the GAL4 activation domain. NRPT6, *Nicotiana tabacum* RPT6; –LT, yeast growth on medium without Leu and Trp; –HLT, yeast growth on medium lacking His, Leu, and Trp, indicating expression of the HIS3 reporter gene. B, BiFC in planta interaction studies of XopJ and RPT6 mutants. YFP confocal microscopy images show *N. benthamiana* leaf epidermal cells transiently expressing XopJ-Venus C in combination with RPT6 K206A or E260A-Venus C. A closeup of the same cells shows that the YFP fluorescence of XopJ-Venus C/RPT6 E260A-Venus C aligns with the plasma membrane. XopJ-Venus C and RPT6-Venus C and the dimerization of fructose-1,6-bisphosphatase (FBPase) within the cytosol served as positive controls. XopJ-Venus C with FBPase-Venus C or the RPT6 mutants together with FBPase-Venus C are included as negative controls. Bars = 20 μm, except for the closeup (5 μm). C, EV or XopJ-HA was transiently coexpressed together with RPT6-GFP, RPT6 K206A-GFP, or RPT6 E260A-GFP in *N. benthamiana* using agroinfiltration. Samples were taken 48 hpi, and images were generated with identical CLSM settings. GFP fluorescence is shown in green, and chlorophyll autofluorescence is shown in red. Arrows indicate cytosolic strands. Bars = 20 μm. D, Quantification of GFP intensity after coexpression of RPT6-GFP, RPT6 K206A-GFP, and RPT6 E260A-GFP together with XopJ. GFP quantification was determined using the ZEN software of Zeiss. Expression of XopJ was verified using an anti-HA antibody. All experiments were repeated three times with similar results.

Figure 5. The RPT6 Walker A motif is required for XopJ binding. A, XopJ interacts with RPT6 E260A (Walker B) but not with RPT6 K206A (Walker A) in a yeast two-hybrid assay. XopJ fused to the GAL4 DNA-binding domain (pGBT9) was expressed in combination with RPT6 E260A or K206A fused to the GAL4 activation domain. NRPT6, *Nicotiana tabacum* RPT6; –LT, yeast growth on medium without Leu and Trp; –HLT, yeast growth on medium lacking His, Leu, and Trp, indicating expression of the HIS3 reporter gene. B, BiFC in planta interaction studies of XopJ and RPT6 mutants. YFP confocal microscopy images show *N. benthamiana* leaf epidermal cells transiently expressing XopJ-Venus C in combination with RPT6 K206A or E260A-Venus C. A closeup of the same cells shows that the YFP fluorescence of XopJ-Venus C/RPT6 E260A-Venus C aligns with the plasma membrane. XopJ-Venus C and RPT6-Venus C and the dimerization of fructose-1,6-bisphosphatase (FBPase) within the cytosol served as positive controls. XopJ-Venus C with FBPase-Venus C or the RPT6 mutants together with FBPase-Venus C are included as negative controls. Bars = 20 μm, except for the closeup (5 μm). C, EV or XopJ-HA was transiently coexpressed together with RPT6-GFP, RPT6 K206A-GFP, or RPT6 E260A-GFP in *N. benthamiana* using agroinfiltration. Samples were taken 48 hpi, and images were generated with identical CLSM settings. GFP fluorescence is shown in green, and chlorophyll autofluorescence is shown in red. Arrows indicate cytosolic strands. Bars = 20 μm. D, Quantification of GFP intensity after coexpression of RPT6-GFP, RPT6 K206A-GFP, and RPT6 E260A-GFP together with XopJ. GFP quantification was determined using the ZEN software of Zeiss. Expression of XopJ was verified using an anti-HA antibody. All experiments were repeated three times with similar results.

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XopJ fused to the N-terminal 173 amino acids of Venus (Venus\textsuperscript{N173}) indicated that XopJ interacts with the RPT6 Walker B mutant (E260A) at the plasma membrane but not with the RPT6 Walker A mutant (K206A) in planta (Fig. 5B). The interaction of XopJ and RPT6, as well as the homodimerization of the cytosolic FBPAse, served as a positive control (Fig. 5B). Agroinfiltration of XopJ-Venus\textsuperscript{N173} together with FBPAse-Venus\textsuperscript{C155} or FBPAse-Venus\textsuperscript{C155} together with RPT6 (K206A) or (E260A)-Venus\textsuperscript{C155} served as negative controls and did not result in the reconstitution of fluorescence (Fig. 5B).

Given the fact that the RPT6 Walker B mutant retained its ability to interact with XopJ, it was further investigated whether the interaction between both proteins leads to the XopJ-mediated degradation of RPT6 (E260A). Toward this end, coexpression studies in N. benthamiana of XopJ-HA together with the RPT6 wild type or its two mutant variants each fused to GFP were performed. A microscopic analysis revealed that XopJ was not able to recruit the RPT6 Walker A mutant to the plasma membrane and also did not lead to its degradation (Fig. 5C). This is likely owing to the lack of interaction between both proteins. In contrast, XopJ interacts with the RPT6 Walker B mutant and also clearly recruits it to the plasma membrane upon coexpression (Fig. 5C). Quantification of the GFP intensity showed that this variant is not destabilized by XopJ (Fig. 5D). Subsequent analysis of the protein levels by western blotting are consistent with the microscopic data (Fig. 5E) and indicate that, despite XopJ’s ability to interact with the RPT6 Walker B mutant, the effector is not able to trigger its degradation.

XopJ Inhibits the Proteasome-Mediated Turnover of NPR1

Previous results demonstrated that XopJ-mediated inhibition of the proteasome interferes with SA-dependent defense responses and that this effect is dependent on NPR1 (Üstün et al., 2013). The transcriptional coactivator NPR1 has been shown to be continuously cleared by the proteasome in order to perpetuate SA-responsive defense signaling (Spoel et al., 2009). Transient expression of XopJ leads to the accumulation of ubiquitinated proteins, probably triggered by the inhibition of the proteasome (Üstün et al., 2013). Likewise, virus-induced gene silencing of RPT6 also causes a strong inhibition of the proteasome and the accumulation of ubiquitinated proteins and the transcriptional coactivator NPR1. Thus, the degradation of RPT6 likely interferes with the turnover of ubiquitinated proteins (Supplemental Fig. S7). Examination of endogenous NPR1 protein levels using an anti-NPR1 specific antibody revealed that transient expression of XopJ resulted in the accumulation of NPR1 protein comparable to that observed after treatment with the proteasome inhibitor MG132 (Fig. 6A). In contrast, N. benthamiana leaves transiently expressing the G2A or C235A mutant variant of XopJ did not show an increase in NPR1 protein abundance (Fig. 6A). To test whether XopJ triggers the accumulation of ubiquitinated NPR1, NPR1-GFP was transiently coexpressed with XopJ. This also led to an accumulation of the NPR1-GFP fusion protein (Supplemental Fig. S8). Subsequently, NPR-GFP was pulled down using GFP-trap beads, and western-blot analysis of the precipitates using an anti-GFP antibody showed that transient expression of XopJ, but not of C235A, caused an accumulation of NPR1-GFP similar to the MG132 positive control (Fig. 6B). Analysis of the ubiquitination status of the different precipitates revealed that transiently expressed XopJ enhanced NPR1 ubiquitination, proven by the accumulation of the characteristic ubiquitin smear (Fig. 6B). The XopJ C235A variant was not able to induce an accumulation of NPR1-GFP and hence did not enhance NPR1 protein ubiquitination (Fig. 6B).

In order to investigate whether XopJ can promote the stabilization of NPR1 during bacterial infection, susceptible pepper plants were infected with Xcv wild-type bacteria or an Xcv\text Delta xopJ deletion strain (Üstün et al., 2013). When NPR1 levels were monitored by immunoblotting at 3 dpi, leaves infected with wild-type Xcv showed a stronger NPR1 signal than those infected with Xcv\text Delta xopJ (Fig. 6C). This indicates that inhibition of the proteasome by XopJ impedes the turnover of NPR1 during Xcv infection of pepper plants.

Degradation of NPR1 is supposed to take place in the nucleus (Spoel et al., 2009), while XopJ is localized at the plasma membrane (Bartetzko et al., 2009). A cellular fractionation experiment of N. benthamiana leaves transiently expressing XopJ was performed to assess the inhibition of the proteasome by XopJ at spatial resolution. Measurement of proteasome activity in total extracts and nucleus-depleted and nucleus-enriched fractions revealed that XopJ-mediated inhibition of the proteasome occurred in each fraction (Fig. 6D). To monitor the proper separation of nuclear and cytosolic fractions, blots were additionally probed with antibodies against the cytosolic Suc phosphatase (Chen et al., 2005) and the nucleus-specific histone H3 (Fig. 6E). These data demonstrate that XopJ affects the activity of the proteasome in the cytosol as well as in the nucleus.

DISCUSSION

Adapted phytopathogenic bacteria are able to suppress plant innate immunity and to reprogram cellular pathways to promote bacterial multiplication and thus cause disease in host plants. During infection, bacterial T3Es, translocated by the type III secretion system, play a central role in the manipulation of the host cellular machinery. In general, these T3Es are essential for pathogen virulence by interfering with plant processes involved in defense responses (Jones and Dangl, 2006).

Although a range of studies have identified T3E target proteins, the enzymatic functions of many T3Es and their mode of action on their respective targets in plants are not well understood. We have previously shown...
Figure 6. XopJ inhibits the proteasome-mediated turnover of NPR1. A, XopJ-triggered accumulation of NPR1 protein levels. XopJ protein variants along with an EV control were transiently expressed in leaves of N. benthamiana using agroinfiltration. MG132 treatment for 6 h was included into the analysis as a positive control. The western blot was probed 48 hpi with an anti-NPR1 antibody directed against the N. tabacum NPR1 protein. The intensities of NPR1 bands were quantified by ImageJ and are shown at the bottom of the top gel. An anti-myc antibody was used to show the proper expression of XopJ proteins. Amido Black staining shows equal protein loading. B, XopJ leads to the accumulation of ubiquitinated NPR1. NPR1-GFP was transiently expressed together with EV, XopJ-HA, or XopJ C235A-HA in N. benthamiana. MG132 treatment served as a positive control and was performed as in A. Samples were taken 48 hpi, and total proteins (Input) were subjected to immunoprecipitation (IP) with GFP-Trap beads, followed by immunoblot analysis of the precipitates using either anti-GFP or anti-ubiquitin antibodies. The proper expression of XopJ proteins in the input fraction was monitored using an anti-HA antibody. C, XopJ affects NPR1 protein levels during Xcv infection in pepper. Xcv or Xcv ΔxopJ was inoculated at a bacterial density of $2 \times 10^8$ colony-forming units mL$^{-1}$ into leaves of cv Early Cal Wonder plants. MG132 treatment served as a positive control. NPR1 protein levels were detected by an anti-NPR1 antibody. D, XopJ also comprises proteasome activity in the nucleus. Nuclear fractionation was performed after transient expression of XopJ-HA in N. benthamiana. Crude plant total extracts (TE) were separated into nucleus-depleted (ND) and nucleus-enriched (NE) fractions. Proteasome activity was measured in each fraction by monitoring the breakdown of the fluorogenic peptide Suc-LLVY-AMC at 30˚C. The EV control was set to 100%. Data represent means ± SD (n = 3). Significant differences were calculated using Student’s t test and are indicated by asterisks: *, $P < 0.05$; and **, $P < 0.01$. E, Anti-SPP2 and anti-histone H3 antibodies were used as markers for cytoplasmic and nuclear proteins, respectively. Expression of XopJ was confirmed by an anti-HA antibody. All experiments were repeated three times with similar results.
that the Xcv T3E XopJ targets the proteasome subunit RPT6 to suppress SA-mediated defense signaling and hence promotes the survival of bacteria during infection (Üstün et al., 2013). However, the underlying mechanism of XopJ-triggered proteasome inhibition remained unknown.

In this study, we show that XopJ acts as a protease to degrade the proteasome subunit RPT6 in plant cells. Destabilization of RPT6 negatively impacts on proteasome function, affecting the proteasomal turnover of NPR1. This provides a mechanistic link between XopJ and its effect on SA-dependent defense responses during the Xcv infection of pepper plants.

XopJ from Xcv belongs to the YopJ superfamily of T3E proteins that are present in a wide range of animal and plant bacterial pathogens and symbionts, respectively (Lewis et al., 2011). YopJ-like effector proteins contain a conserved catalytic triad consisting of His, Glu, and Cys residues. Mutation of the Cys residue interferes with the virulence or avirulence function of these effectors, indicating that the enzymatic function is necessary for effector function within the host cell (Lewis et al., 2011). Recent studies revealed that several YopJ-like effector proteins possess auto- or transacetyltransferase activity (Lee et al., 2012; Jiang et al., 2013; Cheong et al., 2014). However, based on their secondary structure, it was initially hypothesized that these effectors are Cys proteases (Lewis et al., 2011), although potential substrate proteins have not yet been identified. HopZ1a from P. syringae pv syringae as well as AvrBsT from Xcv have been shown to possess weak protease activity in vitro when casein was used as a generic substrate (Ma et al., 2005; Szczesny et al., 2010). In addition, HopZ1a is able to destabilize 2-hydroxy isoflavone dehydratase or Jasmonate-Zim-Domain Protein1 (JAZ1) in soybean (Glycine max) host plants, although the biochemical mechanism of this destabilization is not clear (Zhou et al., 2011; Jiang et al., 2013). XopJ has no detectable acetyltransferase activity in vitro but leads to the degradation of its target protein, RPT6, inside plant cells. The findings presented here apparently contradict our previous reports, where we could not observe differences in RPT6 protein levels upon coexpression with XopJ (Üstün et al., 2013). However, in contrast to our previous approach, we now recorded confocal images of fluorescently labeled RPT6, expressed either alone or together with XopJ, using the same microscope settings. Using the same sensitivity allows direct comparison between images and clearly brings out the differences in fluorescence signal intensity that reflect the reduction in RPT6 protein levels upon the coexpression of XopJ. Similarly, carefully controlling equal protein loading and shorter exposition times robustly reveal differences in RPT6 protein accumulation in transient coexpression experiments as compared with leaves expressing RPT6 and an EV control. Thus, the novel results presented here are in no way contradictory to the previous findings of Üstün et al. (2013) but represent a more differentiated reassessment of the effect of XopJ coexpression on the RPT6 protein level.

Several lines of evidence support the notion that XopJ acts as a Cys protease to specifically degrade RPT6: (1) the degradation of RPT6 in planta is abolished in the presence of a protease inhibitor cocktail; (2) the recombinant E. coli-produced MBP-XopJ cleaves peptides within a generic substrate library in vitro, which is inhibited by the Cys protease inhibitor E-64; and (3) plant-expressed XopJ is able to degrade recombinant RPT6 and vice versa. In addition, the degradation of RPT6 was dependent on XopJ’s catalytic Cys residue (Cys-235), which is in line with previous results showing that the XopJ C235A mutant is not able to suppress proteasome activity and also does not delay the development of tissue necrosis in pepper plants when delivered by the type III secretion system of virulent Xcv (Üstün et al., 2013). Intriguingly, mutation in the myristoylation motif of XopJ by exchanging the Gly at position 2 to Ala affected XopJ’s ability to destabilize RPT6 in planta, indicating that the protease activity of in planta-expressed XopJ requires effector localization at the plant plasma membrane. This might indicate that a yet unidentified host cell factor or posttranslational modification of the effector is necessary for its activity. In contrast, measurement of the in vitro protease activity of E. coli-purified XopJ revealed that XopJ has detectable protease activity toward a peptide library as well as toward recombinantly produced RPT6 in the absence of a possible eukaryotic host factor. We currently have no clear explanation for this discrepancy; however, there could be quantitative differences in activity between XopJ in plant cells and recombinant protein in vitro that are associated with a modification of the effector by the host cell machinery. In order to elaborate this further, future experiments will aim to characterize the protease activity of XopJ purified from plants.

Furthermore, the XopJ-triggered degradation of RPT6 did not produce any detectable cleavage products, as reported for other protease effectors such as AvrPphB or AvrRpt2 from P. syringae (Shao et al., 2003; Chisholm et al., 2005). It is possible that RPT6 degradation products escape detection by the antibody directed against the respective fusion partner of RPT6 or that multiple XopJ cleavage sites are present in RPT6 and the resulting small peptide fragments that are not visible in our degradation assays.

Although the XopJ cleavage site within the RPT6 polypeptide chain is currently not known, a mutational analysis of RPT6 revealed some structural requirements for XopJ binding and degradation. RPT6 belongs to the AAA-ATPase family of proteins whose members are involved in a range of cellular processes and generally function by inducing conformational changes in substrate proteins during continuous cycles of nucleotide binding and hydrolysis (Hanson and Whiteheart, 2005). In concert with RPT1 to RPT5, RPT6 forms a hexameric ring that has direct contact with the 20S catalytic core of the proteasome and that is involved in substrate binding, opening of the gated channel in the 20S subunit, unfolding of proteins, and facilitating the translocation of the unfolded substrate through the AAA-ATPase ring.
into the 20S particle (Vierstra, 2009; Bar-Nun and Glickman, 2012). Substrate unfolding is the only process that requires the hydrolysis of ATP, while the other steps only depend on ATP binding (Benaroudj et al., 2003; Smith et al., 2004). Like other AAA-ATPase family members, RPT6 possesses so-called Walker A and Walker B motifs as integral parts of its ATP-binding site (Hanson and Whiteheart, 2005). The Walker A motif interacts directly with the ATP phosphates, and a mutation within this motif typically eliminates nucleotide binding and inactivates the AAA-ATPase protein (Hanson and Whiteheart, 2005). The Walker B motif is crucial for ATP hydrolysis, and a mutation within this motif blocks ATP hydrolysis but not binding (Babst et al., 1998; Weibezaehn et al., 2003; Dalal et al., 2004). As ATP binding but not hydrolysis is required for substrate binding by most, if not all, AAA-ATPases, mutations in the Walker B motif have been used to create substrate traps that bind but cannot release substrates (Babst et al., 1998; Weibezaehn et al., 2003; Dalal et al., 2004). Mutation of the Walker A motif of RPT6 abolishes ability to interact with XopJ; thus, the Walker A RPT6 mutant protein is not degraded by the effector. This indicates that ATP binding to RPT6 is required for its recognition by XopJ, because it has been shown that ATP-bound RPT6 represents the active form of the protein. The observation that a mutation of the Walker B motif does not affect XopJ binding but prevents RPT6 from being proteolytically degraded suggests that XopJ binds RPT6 similar to substrate proteins during proteasomal degradation and, hence, could act as a substrate mimic. The conformational change imposed during ATP hydrolysis might be necessary for the full activation of XopJ’s protease activity or may be required to expose a possible cleavage site within the RPT6 protein that is recognized by the effector.

Our experiments, including the destabilization of RPT6 by XopJ and virus-induced gene silencing of RPT6 in N. benthamiana, consistently suggest that XopJ triggers RPT6 degradation to reduce proteasome activity in plants, eventually leading to the accumulation of ubiquitinated proteins. Although most, if not all, RP subunits are essential for proteasome function in plants, analyses of weak mutant alleles for several RP subunits in Arabidopsis indicate that some have substrate-specific functions (Vierstra, 2009). Thus, it is conceivable that RPT6 has a specific function in proteasomal protein turnover during plant defense.

In addition, RPT6 plays important roles in 26S proteasome assembly in other eukaryotes (Ehlinger et al., 2013; Park et al., 2013). XopJ-triggered destabilization of RPT6 would prevent one of the earliest steps in 19S RP assembly (Tomko and Hochstrasser, 2013). As a consequence of a defective 19S RP assembly, ubiquitinated proteins would not be recognized and not directed to enter the proteolytic channel of the 20S CP, leading to an accumulation of ubiquitinated proteins, as observed during transient expression of XopJ in N. benthamiana (Üstün et al., 2013) or in RPT6-silenced N. benthamiana plants. Loss of other AAA-ATPase proteasome subunits, such as RPT2 in Arabidopsis, also results in a decreased 26S complex stability and, hence, inhibition of the proteasome activity (Lee et al., 2011), indicating the overall importance of RP AAA-ATPases during proteasome assembly.

Proteasome assembly itself is a complex process that is not fully understood in time and space (Tomko and Hochstrasser, 2013). Although it was shown that CP and RP components are imported separately into the nucleus, a recent study demonstrated that the 26S proteasome completes its assembly process in the cytoplasm and is able to enter the nucleus as a holocomplex (Pack et al., 2014). Thus, although XopJ itself is not a nuclear protein, its interference with proteasome assembly outside the nucleus eventually inhibits proteasome activity in the cytoplasm as well as in the nucleus, because it prevents the nuclear import of functional proteasome complexes.

Previously, it was shown that NPR1, the master regulator of SA signaling, must be constitutively cleared by the proteasome in the nucleus to perpetuate SA-responsive gene expression (Spoel et al., 2009). Indeed, XopJ prevents the proteasomal turnover of NPR1 in a myristoylation- and catalytic triad-dependent manner. This also holds true during a compatible interaction of Xcv with pepper plants, because NPR1 accumulates in wild-type infected leaves but not in those inoculated with an XcvΔxopJ knockout mutant. This provides a mechanistic link to previous findings that XopJ inhibits downstream SA responses (Üstün et al., 2013). Emerging data suggest that phytopathogenic bacteria developed T3Es to interfere with SA-dependent defense signaling. Based on its ability to induce programmed cell death in host plants, SA is considered as the central regulator of plant immunity against biotrophic and hemibiotrophic pathogens (Pieterse et al., 2012). Additionally, basal defense mechanisms such as bacteria-induced closing of stomata and callose deposition at the cell wall are partially dependent on SA (DebRoy et al., 2004; Melotto et al., 2006). Consequently, some T3Es are able to manipulate SA signaling, either directly or indirectly. For instance, XopD from Xcv acts as a sumo-protease to impair ethylene signaling and indirectly affects SA signaling (Kim et al., 2013). Moreover, the P. syringae effector protein HopZ1a directly targets and acetylates JAZs, the negative regulators of jasmonic acid (JA) signaling, to antagonize SA-dependent defense as a consequence of the activation of JA signaling (jiang et al., 2013). Another effector from P. syringae, HopX1, proteolytically degrades JAZ proteins to induce JA signaling and, hence, leads to the repression of SA signaling (Gimenez-Ibanez et al., 2014), providing further evidence that targeting hormonal signaling is an attractive strategy for plant pathogenic bacteria to inhibit plant immunity. Like other T3Es targeting hormonal signaling, the Xcv effector XopJ also manipulates SA signaling indirectly through the inhibition of the proteasome via the degradation of the proteasome subunit RPT6. As a consequence, proteasomal turnover of NPR1 is affected and leads to the accumulation of ubiquitinated NPR1. As the turnover of NPR1 is also required for the establishment of SAR, it is tempting to speculate that XopJ could...
play a role in the suppression of SAR during Xcv infection in pepper. A striking example for a bacterial proteasome-inhibiting toxin affecting SAR is the SylA peptide secreted by certain \textit{P. syringae} strains (Misas-Villamil et al., 2013). Similar to XopJ, SylA inhibits the proteasome activity in plant cells, although through a different mechanism (Groll et al., 2008). SylA blocks SA signaling to suppress SAR and thus creates a zone of SA-insensitive tissue to promote spreading of the bacteria from infection sites (Misas-Villamil et al., 2013). Whether SylA is also able to prevent the proteasomal turnover of NPR1 has to be analyzed in future studies. However, recent data demonstrate that SylA promotes the accumulation of ubiquitinated proteins in Arabidopsis, indicating that SylA could also affect the turnover of regulators of SA signaling (Svozil et al., 2014).

Manipulation of the ubiquitin proteasome system has emerged as a new virulence strategy of bacterial invaders to promote pathogenesis (Dudler, 2013). Recent advances in the plant immunity field revealed that several components of the ubiquitin proteasome system are required for plant immunity or exploited by pathogens (Marino et al., 2012). By showing that XopJ possesses protease activity resulting in the degradation of RPT6, we provide evidence for how XopJ disables the proteasome function. The general interference of XopJ with the turnover of ubiquitinated proteins also impedes the proteasomal turnover of NPR1, explaining our previous finding that XopJ interferes with SA-dependent defense responses (Üstün et al., 2013).

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

\textit{Pepper (Capsicum annuum 'Early Cal Wonder')} and \textit{Nicotiana benthamiana} plants were grown in soil in a greenhouse with daily watering and subjected to a 16-h-light/8-h-dark cycle (25°C/21°C) at 300 \textmu m \text{mol} m^{-2} s^{-1} light and 75% relative humidity.

**Infection of Pepper Plants**

\textit{Xanthomonas campestris pv. vesicatoria} infections for western blot analysis of endogenous NPR1 protein levels were performed as described previously (Üstün et al., 2013).

**Site-Directed Mutagenesis**

Site-directed mutagenesis of RPT6 constructs was carried out using the Quick-Change site-directed mutagenesis kit (Stratagene) employing the primers listed in Supplemental Table S1. All base changes were verified by sequencing.

**Yeast Two-Hybrid Analysis**

Yeast two-hybrid techniques were performed according to the Yeast Protocols Handbook and the Matchmaker GAL4 Two-Hybrid System 3 manual (both Clontech). Point mutation variants of RPT6 were generated by site-directed mutagenesis in the vector pGAD424 (Clontech). For the generation of the HisRPT6 activation domain fusion, the coding region was amplified by PCR from complementary DNA (cDNA) derived from HeLa cells using the primers listed in Supplemental Table S1, inserted into the vector pGAD424 (Clontech), and sequenced verified. Direct interaction of two proteins was investigated by cotransformation of the respective plasmids in the yeast strain AH109, followed by selection of transformants on medium lacking Leu and Trp at 30°C for 3 d and subsequent transfer to medium lacking Leu, Trp, and His for growth selection and \beta-galactosidase activity testing of interacting clones.

**Plasmid Construction for Transient Expression Experiments**

Construction of the binary vectors expressing XopJ and its mutant variants XopJ G2A and C255A was described previously (Bartetzko et al., 2009; Üstün et al., 2014). The RPT6 K206A and E260A-GFP constructs were generated by site-directed mutagenesis of the pENTR-D/TOPO (Invitrogen) clones. The HisRPT6-GFP construct was assembled by amplifying the entire coding region from cDNA from HeLa cells, while the NPR1-GFP construct was generated by amplifying the entire coding region from \textit{N. benthamiana} cDNA, using the primers listed in Supplemental Table S1. The resulting PCR fragments were inserted in the pENTR-D/TOPO (Invitrogen) clones. Entry clones were subsequently recombined into pK7WG2 (Karimi et al., 2002) using L/R Clonase (Invitrogen).

**BiFC Assay**

Entry clones of RPT6 (K206A), RPT6 (E260A), and HisRPT6 comprising the entire coding region of each cDNA were cloned in a Gateway system (Invitrogen)-compatible version of the BiFC vector pPB35S-GW-Venus-2. Constructs were transformed into \textit{Agrobacterium tumefaciens} C58C1 and transiently expressed by agroinfiltration in \textit{N. benthamiana}. The BiFC-induced YFP fluorescence was detected by CLSM (LSM510; Zeiss) after 48 hpi. The specimens were examined using the LD LCI Plan-Apochromat 25×/0.8 water-immersion objective for detailed images with excitation using the argon laser (488- or 498-nm line for BiFC and chlorophyll autofluorescence). The emitted light passed the primary beam-splitting mirrors at 458/514 nm and was separated by a secondary beam splitter at 515 nm. Fluorescence was detected with filter sets as follows: on channel 3, 530 to 560 band pass; and on channel 1, for red autofluorescence of chlorophyll.

**Microscopic Analysis**

Coexpression studies were performed as described previously (Bartetzko et al., 2009) using a Leica TCS SPSII or Zeiss LSM510 confocal microscope. For quantification, images were generated with identical CLSM settings (i.e. detector gain, optical slice, scanning time, and magnification) using multiple infiltrated leaves with at least three independent repetitions of \textit{A. tumefaciens} infiltration. GFP quantification was determined by using Leica software LAS AF and Zeiss software ZEN.

**Transient Expression Assays and Inhibitory Studies**

For infiltration of \textit{N. benthamiana} leaves, \textit{A. tumefaciens} C58C1 was infiltrated into the abaxial airspace of 4- to 6-week-old plants using a needleless 2-ml syringe. Agrobacteria were cultivated overnight at 28°C in the presence of appropriate antibiotics. The cultures were harvested by centrifugation, and the pellet was resuspended in sterile water to a final optical density at 600 nm of 1. The cells were used for the infiltration directly after resuspension. Infiltrated plants were further cultivated in the greenhouse with daily watering and subjected to a 16-h-light/8-h-dark cycle (25°C/21°C) at 300 \textmu m \text{mol} m^{-2} s^{-1} light and 75% relative humidity. For inhibitory studies, 100 \textmu M MG132 or 1% (v/v) ethanol, 1x Complete ULTRA Protease Inhibitors (Roche Applied Science), or water was infiltrated into \textit{A. tumefaciens}-inoculated \textit{N. benthamiana} leaves at 42 hpi, and the leaves were collected at 48 hpi.

**Western Blotting**

Leaf material was homogenized in SDS-PAGE loading buffer (100 \textmu M Tris-HCl, pH 6.8, 9% [v/v] \textbeta-mercaptoethanol, 40% [v/v] glycerol, 0.0005% [w/v] Bromphenol Blue, and 4% [v/v] SDS) and, after heating for 10 min at 95°C, subjected to gel electrophoresis. Separated proteins were transferred onto nitrocellulose membranes (Porablot; Macherey-Nagel). Proteins were detected by an anti-HA-peroxidase high-affinity antibody (Roche), anti-myc-peroxidase antibody (Roche), anti-GFP antibody (Roche), anti-SP2 antibodies (Chen et al., 2005), anti-histone H3 antibody (Sigma), anti-NPR1 antibody, or an anti-ubiquitin antibody (Agrisera) via chemiluminescence (GE Healthcare). The anti-NINPR1 serum (kindly provided by Ursula Pütz, University of Hohenheim) was generated by immunizing rabbits with a recombinant GST fusion protein comprising the conserved SA-sensitive C terminus with amino acids 386 to 588 of \textit{Nicotiana tabacum} NPR1 (Maier et al., 2011). GenBank accession no. AF480488.1). This portion of the NINPR1 protein has 95% identity to the respective part of NPR1 from pepper (GenBank accession no. ACG83081.1).
Measurement of Proteasome Activity

Proteasome activity in crude plant extracts, or nucleus-depleted and enriched fractions, was determined spectrofluorometrically using the fluorogenic substrate Suc-LYV-AMC (Sigma) according to Üstün et al. (2013). For semi-in vitro proteasome inhibition assays, 5 μg of Escherichia coli-purified recombinant MBP-XopJ, MBP-C235A, or MBP was mixed with 50 μg of crude plant extract from N. benthamiana. The reaction was started after 1 h at 30°C by the addition of 0.2 μM Suc-LYV-AMC. Released amino-methyl-coumarin was measured every 2 min between 0 and 120 min using a fluorescence spectrophotometer (Synergy HT; BioTek), with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Proteasome activity was calculated from the linear slope of the emission curve and is expressed as fluorescence units per minute or in percentage relative to controls.

Protease Activity Assays

For in vitro protease assays, MBP, MBP-XopJ, and MBP-XopJ C235A were expressed in E. coli M15 cells. Bacteria were lysed by sonication. After centrifugation, proteins were purified using amylase resin (New England Biolabs) according to the manufacturer’s instructions. The purity of the proteins was approximately 90% as analyzed by SDS-PAGE and Coomassie Blue staining. For in vivo protease activity, EV, XopJ-HA, and Xop C235A-HA were expressed in N. benthamiana by agroinfiltration, and harvested leaf material was homogenized in 200 μL of extraction buffer (50 mM HEPES-KOH, pH 7.2, 2 mM ATP, 2 mM dithiothreitol, and 250 mM Suc). After centrifugation, the protein concentration of the supernatant was adjusted to 1 mg mL\(^{-1}\) with extraction buffer.

Measurement of in vitro and in vivo protease activity was performed according to the manufacturer’s instructions using the P-CHECK protease detection kit (Jena Bioscience). Briefly, 5 μg of E. coli purified recombinant protein or 50 μg of plant extract was mixed with working buffer 1 (pH 7.4) and 10 μL of P-CHECK substrate solution. Proteinase K served as a positive control. In control experiments, 1× Complete ULTRA Protease Inhibitors or specific inhibitor substances (Roche Applied Science) were added to the reaction and incubated for 1 h before the protease reaction was started by the addition of the P-CHECK substrate. Measurement was carried out at 37°C for 2 h using a fluorescence spectrophotometer (Synergy HT; BioTek), with an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Protease activity was calculated from the linear slope of the emission curve and is expressed as fluorescence units per minute. For semi-in vitro degradation assays, 1 μg of E. coli purified recombinant proteins (MBP, MBP-RPT6, MBP-XopJ, and MBP-XopJ C235A) was mixed with crude plant extracts expressing EV, XopJ-HA, Xop C235A-HA, or RPT6-GFP (with or without 1× Complete ULTRA Protease Inhibitors) and incubated overnight at 37°C with moderate shaking. Reactions were stopped by adding 4× SDS loading buffer and examined via western blotting.

Protein Extraction and GFP Pull Down in N. benthamiana

Approximately 1 g of leaf material was ground to a fine powder in liquid nitrogen, and 5 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 10 mM dithiothreitol, 10 mM EDTA, 1 mM NaF, 1 mM NaMoO\(_4\)·2H\(_2\)O, 1% [w/v] polyvinylpyrroldione, 1% [v/v] 1× Complete ULTRA Protease Inhibitor cocktail [Roche Applied Science], and 1% [w/v] Nonidet P-40) was added. Samples were cleared by centrifugation at 16,000 g for 15 min at 4°C and adjusted to 2 mg mL\(^{-1}\) total protein concentration. Immunoprecipitation was performed on 1.5 mL of total protein by adding 20 μL of GFP Trap-M beads (Chromotivek) and incubation at 4°C for 2 h. Beads were washed four times with Tris-buffered saline containing 0.5% (v/v) Nonidet P-40, and immunoprecipitates were eluted with 30 μL of 2× SDS loading buffer and heating at 70°C for 10 min.

Nuclear Fractionation

Plant cell fractionation was performed by using the Cellytic PN isolation/extraction kit for plant leaves (Sigma) according to the manufacturer’s instructions.

RNA Extraction and Expression Analysis

Total RNA was isolated from leaf material and then treated with RNase-free DNase (Fermentas) to degrade any remaining DNA. First strand cDNA synthesis was performed from 2 μg of total RNA using Revert-Aid reverse transcriptase (Fermentas). For RT-PCR, cDNAs were amplified using Taq polymerase (New England Biolabs) and gene-specific primers (Supplemental Table S1).

Virus-Induced Gene Silencing of N. benthamiana

Virus-induced gene silencing was performed as described previously (Üstün et al., 2012). Briefly, A. tumefaciens strains with the pTRV1 vector and with pTRV2-GFPb and PYL29-RPT6 (Üstün et al., 2013; optical density at 600 nm = 1) were mixed in a 1:1 ratio, and the mixture was infiltrated into a lower leaf of a 4-week-old N. benthamiana plant using a 1-mL sterile syringe without a needle. Silenced plants were analyzed 14 d post infiltration.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. XopJ does not display acetyltransferase activity.

Supplemental Figure S2. Cys protease XopD does not comprise RPT6 accumulation.

Supplemental Figure S3. XopJ does not interact with RPT6 from humans.

Supplemental Figure S4. XopJ exhibits protease activity in a protease-specific manner.

Supplemental Figure S5. Protein inputs used for semi-in vitro degradation assays to demonstrate comparable MBP-RPT6 protein amounts prior to the incubation with extracts from XopJ-HA expressing leaves.

Supplemental Figure S6. MBP-XopJ degrades RPT6 and inhibits proteasome activity.

Supplemental Figure S7. Virus-induced gene silencing of RPT6 in N. benthamiana mimics XopJ-triggered effects.

Supplemental Figure S8. XopJ triggers accumulation of NPI-R-GFP.

Supplemental Table S1. Oligonucleotides used in this study.

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LITERATURE CITED


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