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The *Xanthomonas campestris* type III effector XopJ proteolytically degrades proteasome subunit RPT6

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Keywords: Plant-pathogen interactions; bacterial type-III effectors; *Xanthomonas campestris\textit{ pv. vesicatoria} ; XopJ; protease; 26S proteasome; NPR1

One sentence summary

A type III effector from Xanthomonas acts as a protease to inhibit the proteasome and interferes with proteasomal turnover of defense signaling components.
Footnotes

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Abstract

Many animal and plant pathogenic bacteria inject type III effector (T3E) proteins into their eukaryotic host cells to suppress immunity. The 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 XopJ, a YopJ-family effector from the plant pathogen Xanthomonas campestris pv. vesicatoria, interacts with the proteasomal subunit RPT6 in planta to suppress proteasome activity resulting in the inhibition of salicylic acid (SA)-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 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 interaction of RPT6 with XopJ is dependent on ATP-binding activity of RPT6 but proteolytic cleavage additionally requires its ATPase activity. Inhibition of the proteasome impairs the proteasomal turnover of NPR1, the master regulator of SA responses, leading to the accumulation of ubiquitinated NPR1 which likely interferes with 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.
**Introduction**

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

NPR1 (Non-expressor of PR1) represents an essential component of SA-signalling that acts as a transcriptional co-activator to induce transcriptional re-reprogramming including the induction of PR-(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 TGA transcription factors and drives the activation of SA-responsive gene expression (Fu and Dong, 2013). Subsequent phosphorylation serves as a signal for ubiquitination of NPR1 mediating its degradation via the 26S proteasome and thereby returning the target gene promoter into 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 defence signalling highlights the important role of the proteasome in plant defence. Accordingly, Arabidopsis 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 (PCD) in response to avirulent bacterial strains (Hatsugai et al., 2009). Thus, the 26S proteasome seems to control defence 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, de-ubiquitinates 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 (Galan and Wolf-Watz, 2006). In accordance with its central role in a wide array of cellular processes, including defence, the proteasome is targeted or exploited by bacterial toxins or type III effector proteins (Dudler, 2013). Certain strains of *Pseudomonas syringae* pv. *syringae* secrete syringolin A (SylA), which is a small non-ribosomal peptide that inhibits proteasome activity through binding to the catalytic subunits of the 20S CP and is required for full virulence on host plants, *Phaseolus vulgaris* (Groll et al., 2008). The initial discovery of the interaction between the bacterial type III effector protein XopJ from *Xanthomonas campestris* pv. *vesicatoria* and RPT6, a subunit of the 19S RP, provided a 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 defence responses to attenuate onset of necrosis in infected tissue and to alter host transcription. The development of XopJ-associated phenotypes on susceptible pepper plants was shown to be dependent on NPR1 (Üstün et al., 2013). Another T3E effector targeting RPT6 to inhibit proteasome activity is HopZ4 from *Pseudomonas syringae* pv. *lachrymans* (Ustun et al., 2014). Both T3Es belong to the widespread YopJ-family of effector proteins that are present among plant and animal pathogenic bacteria and were originally classified as cysteine proteases (Lewis et al., 2011). However, recent studies indicate that these effectors, in particular the archetypal member YopJ from *Yersinia pestis*, HopZ1a from *Pseudomonas* and AvrBsT from *Xanthomonas*, act as acetyltransferases on their targets or decoys to suppress immunity in animals and plants, respectively (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 YopJ-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 is 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, respectively. Interaction of RPT6 with XopJ is dependent on 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 proteasomal
turnover of NPR1 and thereby suppresses SA-mediated defence signalling.

Results

XopJ-mediates 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 (Fig. S1). However, when XopJ-HA was transiently co-expressed with RPT6-
GFP in leaves of Nicotiana benthamiana, GFP fluorescence was dramatically decreased as
compared to N. benthamiana leaves co-expressing RPT6-GFP with a catalytically inactive
XopJ variant C235A or the non-myristoylated variant G2A, respectively (Fig. 1A-D). To
allow for the direct comparison of signal intensities, all confocal pictures shown in Figure 1
were recorded with the same microscope settings. However, Figure 1E illustrates that RPT6-
GFP fluorescence becomes readily detectable in XopJ co-expressing 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 is 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 phosphatase 2 (SPP2) were examined in plants expressing XopJ and its variants. The
data revealed that XopJ wild type protein did not result in decreased SPP2 protein levels (Fig.
1G), indicating that XopJ specifically reduces RPT6-GFP protein levels. RT-PCR analysis
revealed that co-expression 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 co-expressed in N. benthamiana
to exclude that the reduction of RPT6-GFP protein levels is a general characteristic of
effectors that share similarities to cysteine proteases (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 2 hybrid analysis and *in planta* bimolecular fluorescence complementation (BiFC) showed that XopJ is not able to interact with human RPT6 (Fig S3A and B). Thus, XopJ is not able to recruit *Hs*RPT6 to the plasma membrane and also does not lead to destabilization of *Hs*RPT6-GFP in *N. benthamiana* (Fig. S3C and D). These data support the notion that XopJ specifically triggers the degradation of RPT6 from plants.

**Degradation of RPT6 is protease-dependent.** To determine the mechanism underlying the XopJ-triggered reduction of RPT6 protein levels, co-expression 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 (serine-, cysteine-, aspartic-, and metallo-proteases 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.

**XopJ possesses protease activity *in vitro* and *in vivo*.** To clarify whether XopJ acts as a protease itself, protease activity was monitored using a FRET-based protease detection kit containing a substrate peptide library of more than 2 x 10^6 peptide variants with proteinase K serving as a positive control (Kapprell et al., 2011). Recombinant MBP-XopJ, purified from *E. coli*, showed a significant increase in protease activity when compared to MBP alone (Figure 3A). This activity was dependent on an intact catalytic triad, because the MBP-XopJ C235A variant did not result in a significant rise in protease activity and remained at MBP background levels (Fig. 3A). Incubation of MBP-XopJ together with MBP-RPT6 diminished the protease activity, probably by out-competing binding of library peptides to XopJ (Fig. 3A). Application of a cocktail of protease inhibitors confirmed that XopJ indeed displays protease activity, as its activity was blocked similar to that of the positive control (Fig. S4A). To further dissect this effect, specific protease inhibitor substances were tested in an *in vitro* assay. The results showed that protease activity of XopJ is inhibited by cysteine protease inhibitor E-64 but not by inhibitors of proteases from other classes (Fig. S4B), further corroborating the notion that XopJ acts as a cysteine protease. Neither chymostatin, which is a
specific inhibitor of α-, β-, γ-, δ-chymotrypsin, nor serine protease inhibitor aprotinin, were able to diminish protease activity of recombinant XopJ (Fig. S4B). Serine and cysteine protease inhibitor leupeptin also did not reduce protease activity of XopJ, as it is highly specific towards the cysteine proteases papain and cathepsin B (Umezawa, 1976). The reduction of protease activity of XopJ by PMSF can be explained by the fact that PMSF also inhibits cysteine proteases at high concentrations (van der Hoorn et al., 2004). To test in vivo protease activity of XopJ, XopJ-HA, C235A-HA, and an empty vector control (EV) were transiently expressed in N. benthamiana and protease activity was measured in crude extracts 48 hpi. A significant increase in protease activity in plant extracts expressing XopJ-HA could be detected in comparison to EV or the enzymatic inactive C235-HA variant that remained at background protease activity levels (Fig. 3B). Treatment with a mix of protease inhibitors diminished XopJ-dependent protease activity to EV or C235A levels (Fig S4C).

XopJ degrades RPT6 in a protease-dependent manner. To further investigate the proteolytic activity of XopJ towards RPT6, same amounts of purified recombinant MBP-RPT6 (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 4A). Mutation of the Cys-235 residue within the catalytic triad of XopJ as well as the addition of protease inhibitors abolished degradation of MBP-RPT6 (Fig 4B), supporting 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 co-incubation with the C235A variant (Fig S6A). Incubation of GST-XopJ together with recombinant MBP-RPT6 purified from E. coli also resulted in a reduction of RPT6 protein abundance (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 to MBP or MBP-XopJ C235A treated extracts (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 regulatory particle (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 proteolytical 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 lysine residue at position 206 to an alanine (K206A) abrogated the interaction with XopJ in yeast, while a point mutation in the Walker B motif (E260A) did not affect binding of XopJ to RPT6 (Figure 5A).

To investigate in planta interactions, bimolecular fluorescence complementation (BiFC) assays were performed using agrobacterium-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 derivate Venus (Venus\(^{C155}\)) and transiently expressed in *N. benthamiana* leaves. Reconstitution of the fluorescence signal after transient co-expression with XopJ fused to the N-terminal 173 amino acids of Venus (Venus\(^{N173}\)) indicated that XopJ interacts with RPT6 Walker B mutant (E260A) at the plasma membrane, but not with RPT6 Walker A mutant (K206A) in planta (Fig 5B). The interaction of XopJ and RPT6, as well as the homodimerization of the cytosolic fructose-1,6-bisphosphatase (FBPase), served as a positive control (Fig 5B). Agroinfiltration of XopJ-Venus\(^{N173}\) together with FBPase-Venus\(^{C155}\) or FBPase-Venus\(^{N173}\) together with RPT6 (K206A) or (E260A)-Venus\(^{C155}\) served as negative controls and did not result in 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 XopJ-mediated degradation of RPT6 (E260A). Towards this end, co-expression studies in *N. benthamiana* of XopJ-HA together with 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 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
RPT6 Walker B mutant and also clearly recruits it to the plasma membrane upon co-
expression (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 proteasome-mediated turnover of NPR1. Previous results demonstrated that
XopJ-mediated inhibition of the proteasome interferes with SA-dependent defence responses
and that this effect is dependent on NPR1 (Üstün et al., 2013). The transcriptional co-activator
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 accumulation of ubiquitinated proteins and the
transcriptional co-activator NPR1. Thus degradation of RPT6 likely interferes with protein
turnover of ubiquitinated proteins (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 this whether XopJ triggers the accumulation of
ubiquitinated NPR1, NPR1-GFP was transiently co-expressed with XopJ. This also led to an
accumulation of NPR1-GFP fusion protein (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 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 accumulation of NPR1-GFP and hence did not
enhance NPR1 protein ubiquitination (Fig 6B).

In order to investigate whether XopJ can promote stabilization of NPR1 during bacterial
infection, susceptible pepper plants were infected with Xcv wild type bacteria or a XcvΔxopJ
deletion strain (Üstün et al., 2013). When NPR1 levels were monitored by immuno blotting at 3 dpi, Xcv wild type infected leaves showed a stronger NPR1 signal than those infected with XcvΔxopJ (Fig. 6C). This indicates that inhibition of the proteasome by XopJ impedes 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 (TE), nuclear depleted (ND) and enriched (NE) fractions revealed that XopJ-mediated inhibition of the proteasome occurred in each fraction (Fig 6D). To monitor proper separation of nuclear and cytosolic fractions, blots were additionally probed with antibodies against the cytosolic sucrose-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 type III effectors (T3E), translocated by the type III secretion system (T3SS), 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 is not well understood. We have previously shown that the Xcv T3E XopJ targets the proteasome subunit RPT6 to suppress salicylic-acid mediated defense signaling and hence promotes survival of bacteria during infection (Üstün et al., 2013). However, the underlying mechanism of XopJ-triggered proteasome inhibition remained unknown.

In the present 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 proteasomal turnover of NPR1. This provides a mechanistic link between XopJ and
its effect on SA-dependent defense responses during 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 a histidine,
glutamate and cysteine residue. Mutation of the cysteine residue interferes with the virulence
or avirulence activity 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 trans-acetyltransferase activity (Lee et al., 2012;
Jiang et al., 2013; Cheong et al., 2014). However, based on their secondary structure, it has
initially been hypothesized that these effectors are cysteine 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 JAZ1 in
soybean 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 co-expression with XopJ (Üstün et al., 2013).
However, in contrast to our previous approach we now recorded confocal images of
fluorescently labelled 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 co-expression of XopJ. Similarly, carefully controlling equal
protein loading and shorter exposition times robustly reveal differences in RPT6 protein
accumulation in transient co-expression experiments as compared to leaves expressing RPT6
and an empty vector 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 co-expression on the RPT6 protein level.

Several lines of evidence support the notion that XopJ acts as a cysteine protease to
specifically degrade RPT6: (1) degradation of RPT6 *in planta* is abolished in the presence of
a protease inhibitor cocktail; (2) recombinant *E. coli* produced MBP-XopJ cleaves peptides within a generic substrate library *in vitro*, which is inhibited by the cysteine protease inhibitor E-64 and (3) plant expressed XopJ is able to degrade recombinant RPT6 and vice versa. In addition, degradation of RPT6 was dependent on XopJ’s catalytic cysteine residue (Cys235), 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 T3SS of virulent Xcv (Üstün et al., 2013). Intriguingly, mutation in the myristoylation motif of XopJ by exchanging the glycine at position 2 to alanine affected XopJ’s ability to destabilize RPT6 *in planta*, indicating that 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 *in vitro* protease activity of *E. coli*-purified XopJ revealed that XopJ has detectable protease activity towards a peptide library as well as towards 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 plant.

Furthermore, XopJ-triggered degradation of RPT6 did not produce any detectable cleavage products as reported for other protease effectors such as AvrPphB or AvrRpt2 from *Pseudomonas* (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 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, respectively. RPT6 belongs to the AAA-ATPase family of proteins whose members are involved in 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 5, RPT6 forms a hexameric ring that has direct contact to the 20S catalytic core of the proteasome and which 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). As other AAA-ATPase family members, RPT6 possesses a so called Walker-A and a Walker-B motif, respectively, as integral parts of its ATP-binding site (Hanson and Whiteheart, 2005). The Walker-A motif directly interacts with the phosphates ATP 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; Weibezahn 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; Weibezahn et al., 2003; Dalal et al., 2004). Mutation of the Walker-A motif of RPT6 abolishes is ability to interact with XopJ and 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 full activation of XopJ’s protease activity or is 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 A. thaliana 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 defence.
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 holds also true during a compatible interaction of Xcv with pepper plants because NPR1 accumulates in wild type infected leaves but not in those inoculated with a Xcv *xopJ* knock-out 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 reaction 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 bacterial-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 *Pseudomonas* effector protein HopZ1a directly targets and acetylates JAZs, the negative regulators of jasmonic acid (JA) signaling, to antagonize SA dependent defence as a consequence of the activation of JA signaling (Jiang et al., 2013). Another effector from *Pseudomonas*, 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. Likewise other T3Es targeting hormonal signaling, Xcv effector XopJ also manipulates SA signaling indirectly through the inhibition of the proteasome via the degradation of proteasome subunit RPT6. As a consequence, proteasomal turnover of NPR1 is affected and leads to the accumulation of ubiquitinated NPR1. As turnover of NPR1 is also required for establishment of systemic acquired resistance (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 *Pseudomonas* 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 proteasomal turnover of NPR1 has to be analyzed in future studies. However, recent data demonstrates that SylA promotes the accumulation of ubiquitinated proteins in Arabidopsis, indicating SylA could also affect the turnover of regulators of SA signaling (Svozil et al., 2014).

Manipulation of the ubiquitin proteasome system (UPS) 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 UPS 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 how XopJ disables the proteasome function. The general interference of XopJ with the turnover of ubiquinated 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

Pepper (*Capsicum annuum* cv. Early Cal Wonder (ECW)) and tobacco plants (*Nicotiana benthamiana*) 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 µmol m⁻² s⁻¹ light and 75% relative humidity.

Infection of pepper plants

Xcv 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, Heidelberg, Germany) employing primers listed in Table S1 online. 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, Heidelberg, Germany). Point mutation variants of RPT6 were generated by site directed mutagenesis in the vector pGAD424 (Clontech). For the generation of the *HsRPT6* activation domain fusions, the coding region was amplified by PCR from cDNA derived from HeLa cells using the primers listed in Table S1, inserted into the vector pGAD424 (Clontech) and sequence verified. Direct interaction of two proteins was investigated by co-transformation 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 days and subsequent transfer to medium lacking Leu, Trp and His for growth selection and LacZ activity testing of interacting clones.
Plasmid construction for transient expression experiments

Construction of binary vectors expressing XopJ and its mutant variants XopJ G2A and C235A was described previously (Bartetzko et al., 2009; Ustun et al., 2014). The RPT6 K206A or E260A-GFP constructs were generated by site directed mutagenesis of the pENTR-D/TOPO (Invitrogen) clones. The HsRPT6-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 N. benthamiana cDNA, using the primers listed in Table S1. The resulting PCR fragments were inserted in the pENTR-D/TOPO (Invitrogen). Entry clones were subsequently recombined into pK7WGF2 (Karimi et al., 2002) using L/R-Clonase (Invitrogen).

BiFC Assay

Entry clones of RPT6 (K206A), (E260A) and HsRPT6 comprising the entire coding region of each cDNA were used in a L/R reaction with a Gateway System (Invitrogen)–compatible version of the BiFC vector pRB35S-GW-Venus \(^C\). Constructs were transformed into Agrobacterium tumefaciens C58C1 and transiently expressed by Agroinfiltration in N. benthamiana. The BiFC-induced YFP fluorescence was detected by confocal laser scanning microscopy (LSM510, Zeiss) after 48 hpi. The specimens were examined using the LD LCI Plan-Apochromat 25x/0.8 water-immersion objective for detailed pictures with excitation using the argon laser (458 or 488 nm line for BiFC and chlorophyll auto-fluorescence). The emitted light passed the primary beam-splitting mirrors 458/514 and was separated by a secondary beam splitter at 515. Fluorescence was detected with filter sets as follows: on channel 3, BP 530-560 and on channel 1, for red auto-fluorescence of chlorophyll.

Microscopic Analysis

Co-expression studies were performed as described previously (Bartetzko et al., 2009) using a Leica TCS SP5II or Zeiss LSM510 confocal microscope, respectively. For quantification, images were generated with identical cLSM settings (i.e. detector gain, optical slice, scanning time, magnification) using multiple infiltrated leaves with at least three independent
repetitions of Agrobacterium- infiltration. GFP quantification was determined by using the Leica software LAS_AF and Zeiss software ZEN.

**Transient expression assays and inhibitory studies**

For infiltration of *N. benthamiana* leaves, *A. tumefaciens* C58C1 was infiltrated into the abaxial air space 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 (OD$_{600}$) of 1.0. The cells were used for the infiltration directly after resuspension. Infiltrated plants were further cultivated in the greenhouse daily watering, and subjected to a 16 h light: 8 h dark cycle (25°C: 21°C) at 300 μmol m$^{-2}$ s$^{-1}$ light and 75% relative humidity.

For inhibitory studies, 100μM MG132 or 1% EtOH, 1x cOmplete ULTRA Protease Inhibitors (Roche Applied Science) or H$_2$O, were infiltrated into *A. tumefaciens*-inoculated *N. benthamiana* leaves at 42 hpi, and the leaves were collected at 48 hpi.

**Western blotting**

Leaf material was homogenized in sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris-HCl, pH 6.8; 9% β-mercapto-ethanol, 40% glycerol, 0.0005% bromophenol blue, 4% SDS) and, after heating for 10 min at 95 °C, subjected to gel electrophoresis. Separated proteins were transferred onto nitrocellulose membrane (Porablot, Machery und Nagel, Düren, Germany). Proteins were detected by either an anti-HA-Peroxidase high affinity antibody (Roche), anti-myc-Peroxidase antibody (Roche), anti-GFP antibody (Roche), anti-SPP2 (Chen et al., 2005), anti-Histone H3 antibody (Sigma), anti-NtNPR1 antibody or anti-ubiquitin antibody (Agrisera) via chemiluminescence (GE Healthcare). The anti-NtNPR1 serum (kindly provided by U. Pfitzner, University of Hohenheim) was generated by immunizing rabbits with a recombinant GST-fusion protein comprising the conserved salicylic acid-sensitive C-terminus with amino acids 386 to 588 of the *Nicotiana tabacum* NPR1 (Maier et al., 2011) (Genbank acc. no. AF480488_1). This portion of the NtNPR1 protein has 95% identity to the respective part of NPR1 from *Capsicum annuum* (Genbank acc. no. ABG38308.1).
Proteasome activity in crude plant extracts, or nuclear depleted and enriched fractions, was determined spectro-fluorometrically using the fluorogenic substrate suc-LLVY-NH-AMC (Sigma) according to Üstün et al. (Üstün et al., 2013). For semi-\textit{in vitro} proteasome inhibition assays 5 µg of \textit{E. coli} purified recombinant MBP-XopJ, MBP-C235A or MBP were mixed with 50 µg crude plant extracts from \textit{N. benthamiana}. The reaction was started after 1 h at 30°C by addition of 0.2 mM suc-LLVY-AMC. Released amino-methyl-coumarin (AMC) was measured every two minutes between \( t_0 \) and \( t_{120} \) 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 (RFU min\(^{-1}\)) or in percentage relative to controls, respectively.

**Protease Activity Assays**

For \textit{in vitro} protease assays, MBP, MBP-XopJ and MBP-XopJ C235A were expressed in \textit{E. coli} M15 cells. Bacteria were lysed by sonication. After centrifugation, the recombinant proteins were purified using amylose resin (New England Biolabs) according to the manufacturer’s instructions. The purity of the proteins was approximately 90% as analyzed by SDS-PAGE and commassie staining. For \textit{in vivo} protease activity EV, XopJ-HA or XopJ C235A-HA were expressed in \textit{N. benthamiana} by Agro-infiltration and harvested leaf material were homogenized in 200 µl extraction buffer [50 mM HEPES-KOH, pH 7.2, 2 mM ATP, 2 mM DTT, 250 mM sucrose]. After centrifugation the protein concentration of the supernatant was adjusted to 1 mg/ml with extraction buffer.

Measurement of \textit{in vitro} and \textit{in vivo} protease activity was performed according to manufacturer’s instructions using the P-CHECK protease detection kit (Jena Bioscience). Briefly, 5 µg of \textit{E. coli} purified recombinant protein or 50 µg of plant extract were mixed with Working Buffer I (pH=7.4) and 10 µl P-CHECK substrate solution. Proteinase K served as a positive control. In control experiments 1x cOmplete ULTRA Protease inhibitors or specific inhibitor substances (Roche Applied Science) were added to the reaction and incubated for 1 hour before the protease reaction was started by the addition of the P-CHECK substrate.
Measurement was carried out at 37°C for 2 hours 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 (RFU min⁻¹).

For semi-in vitro degradation assays, 1µg of *E. coli* purified recombinant proteins (MBP, MBP-RPT6, MBP-XopJ and MBP-XopJ C235A) were mixed with crude plant extracts expressing EV, XopJ-HA, XopJ C235A-HA or RPT6-GFP (+/- 1x cOmplete ULTRA Protease Inhibitors) and incubated overnight at 37°C with moderate shaking. Reactions were stopped by adding 4x 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 fine powder in liquid nitrogen and 5 ml extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% glycerol; 10 mM DTT; 10 mM EDTA; 1 mM NaF; 1 mM Na₂MoO₄.2H₂O; 1% (w/v) PVPP; 1% (v/v) 1x cOmplete ULTRA Protease inhibitor cocktail (Roche Applied Science); 1% (v/v) NP-40] added. Samples were cleared by centrifugation at 16,000×g for 15 min at 4°C and adjusted to 2 mg/ml total protein concentration. Immunoprecipitation was performed on 1.5 ml total protein by adding 20 µl GFP Trap-M beads (Chromotek) and incubation at 4°C for 2h. Beads were washed 4 times with TBS containing 0.5% (v/v) NP-40, immunoprecipitates eluted with 30 µl 2x 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 (CelLytic PN Isolation/Extraction Kit, 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, Frankfurt, Germany) and gene-specific primers (Table S1).

**Virus-induced gene silencing of N. benthamiana**

VIGS was performed as described previously (Ustun et al., 2012). Briefly, Agrobacterium strains with the pTRV1 vector and with pTRV2-GFPsil and PYL279-RPT6 (Üstün et al., 2013) (OD 600 = 1.0) were mixed in a 1:1 ratio, respectively, 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 analysed 14 d post infiltration.

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Figure 1: XopJ interferes with RPT6-GFP protein accumulation

(A) Empty vector, (B) XopJ-HA, (C) G2A-HA and (D) C235A-HA were transiently co-expressed together with RPT6 GFP in *N. benthamiana* using *Agrobacteria* infiltration. For confocal laser scanning microscopy (CLSM), samples were taken 48 h post inoculation, and images were generated with identical CLSM settings. (E) Microscope image of a RPT6-GFP and XopJ-HA co-expressing leave recorded with increased photomultiplier gain as compared to the images presented in Fig A to D. GFP fluorescence is shown in green and chlorophyll autofluorescence in red. The scale bar represents 20 μm. (F) Quantification of GFP intensity after co-expression of RPT6-GFP together with XopJ and its variants. GFP quantification was determined by using the Leica software LAS_AF. Co-expression with the empty vector (EV) control was set to 100 %. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *** P < 0.001. (G) Total proteins were extracted 48 h after infiltration with *Agrobacteria* harboring the respective XopJ and RPT6 expression constructs. 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 sucrose-phosphatase (anti-SPP2) served as a control and staining of the membrane with amido black showed equal loading. (H) XopJ does not affect RPT6 expression levels. RT-PCRs showing transgenic RPT6 mRNA in *N. benthamiana* leaves transiently co-expressing 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.

Figure 2: Destabilization of RPT6 is protease-dependent

(A) RPT6-GFP was transiently co-expressed together with EV, XopJ-myc and XopJ C235A-myc in *N. benthamiana* using Agroinfiltration. 42 hours post inoculation (hpi) 100μM MG132 or a protease inhibitor mix was infiltrated into Agrobacterium-inoculated leaves and leaf material was collected 48 hpi. Expression of RPT6-GFP was detected using an anti-GFP antibody. After stripping of the membrane, protein levels of XopJ and XopJ C235A were analyzed with an anti-myc 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. 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 empty vector (EV) control was set to 100%. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: **, P < 0.01. Experiments in (A) and (B) were repeated twice with similar results.

**Figure 3: XopJ displays protease activity in vitro and in vivo**

(A) 5 µg of MBP, MBP-XopJ, MBP-XopJ C235A, MBP-RPT6 and a mix of MBP-XopJ/MBP-RPT6 purified from *E. coli* were subjected for protease activity measurement using the P-CHECK protease detection kit. MBP and MBP-RPT6 served as a negative control. As a positive control, serine protease Proteinase K was included. A representative result of more than three repetitions with independent sets of purified proteins is shown. (B) Protease activity in *N. benthamiana* leaves transiently expressing XopJ-HA proteins. XopJ protein variants along with an empty vector (EV) control were transiently expressed in leaves of *N. benthamiana* using Agro-infiltration. After 48h, protease activity in total protein extracts was measured using the P-CHECK protease detection kit. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05; **, P < 0.01. The experiment was repeated twice with similar results.

**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 used plant extracts. (B) The immunoblot using an anti-MBP antibody shows MBP-RPT6 accumulation after incubation with crude extracts of *N. benthamiana* leaves expressing XopJ-HA or C235A-HA +/-protease inhibitors. Plants expressing the empty vector control served as a negative control. An anti-HA immunoblot was performed to detect proper expression of XopJ-HA proteins. Amido black staining served as a loading control for the used plant extracts. The results in both experiments (A, B) are representative of two independent experiments performed.
Figure 5: RPT6 Walker A motif is required for XopJ binding

(A) XopJ interacts with RPT6 E260A (Walker B) but not with RPT6 K206A (Walker A) mutant 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. \(N/rPT6 = Nicotiana tabacum\) 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.

Yellow fluorescent protein (YFP) confocal microscopy images show \(N. benthamiana\) leaf epidermal cells transiently expressing XopJ-Venus\(^N\) in combination with RPT6 K206A or E260A-Venus\(^C\). A close-up of the same cells shows that the YFP fluorescence of XopJ-Venus\(^N\)/RPT6 E260A-Venus\(^C\) aligns with the plasma membrane. XopJ-Venus\(^N\) and RPT6-Venus\(^C\) and the dimerization of fructose-1,6-bisphosphatase (FBPase) within the cytosol serve as positive controls. XopJ-Venus\(^N\) with FBPase-Venus\(^C\) or the RPT6 mutants together with FBPase-Venus\(^N\) are included as negative controls. Scale bar represents 20 \(\mu\)m, except for the close-up (5\(\mu\)m). (C) Empty vector (EV) or XopJ-HA were transiently co-expressed together with RPT6 GFP, RPT6 K206A- 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 in red. Arrows indicate cytosolic strands. Scale bar represents 20 \(\mu\)m. (D) Quantification of GFP intensity after co-expression of RPT6-GFP, RPT6 K206A and RPT6 E260A-GFP together with XopJ. GFP quantification was determined by using the ZEN software of ZEISS. Co-expression with the EV control was set to 100 %. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: ** * P < 0.001. (E) XopJ does not degrade RPT6 Walker B mutant. Total proteins were extracted 48 h after infiltration with Agrobacteria harboring the respective XopJ and RPT6 expression constructs. RPT6-, RPT6 K206- and RPT6 E260A-GFP protein levels were detected using an anti-GFP antibody. Expression of XopJ was verified using an anti HA-antibody. All experiments were repeated three times with similar results.
Figure 6: XopJ inhibits proteasome-mediated turnover of NPR1

(A) XopJ-triggered accumulation of NPR1 protein levels. XopJ protein variants along with an empty vector (EV) control were transiently expressed in leaves of *N. benthamiana* using Agro-infiltration. MG132 treatment for 6 hours was included into the analysis as a positive control. The western blot was probed 48 hpi with an anti-NPR1 antibody directed against the tobacco NPR1 protein. Intensities of NPR1 bands were quantified by ImageJ and shown at the bottom of each panel. An anti-myc antibody was used to show 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 immune-precipitation with GFP-Trap beads, followed by immunoblot analysis of the precipitates using either anti-GFP or anti-Ubiquitin antibodies. Proper expression of XopJ proteins in the input fraction was monitored by using an anti-HA antibody. 

(C) XopJ affects NPR1 protein levels during *Xanthomonas campestris* pv. *vesicatoria* (Xcv) infection in pepper. Xcv or Xcv ΔxopJ were inoculated at a bacterial density of 2 x 10^8 cfu ml^-1 into leaves of pepper ECW 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 extracts (TE) were separated into nuclear depleted (ND) and nuclear 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 the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05; **, 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.
Supporting information

**Figure S1:** XopJ does not display acetyltransferase activity. Auto-acetylation activity *in vitro*. Acetylation reactions using MBP, MBP-XopJ, GST and GST-HopZ1a proteins. For acetylation reactions, proteins were incubated with 0.4 mCi 14C-acetyl CoA and 100 nM inositol hexakisphosphate (IP6) for 30 min at 30°C. Proteins were then separated by SDS-PAGE. Gels were stained with Coomassie and then analyzed by autoradiography. GST and GST-HopZ1a were used as negative and positive acetyltransferase enzyme controls, respectively. Acetylated proteins (GST-HopZ1a-AC) are labeled in the autoradiograph. GST=28 kDa; GST-HopZ1a=70 kDa; MBP=50 kDa, MBP-XopJ=100 kDa. Similar results were obtained in three independent experiments.

**Figure S2:** Cysteine Protease XopD does not comprise RPT6 accumulation. XopD along with an empty vector (EV) control were transiently co-expressed together with RPT6-myc in leaves of *N. benthamiana* using Agro-infiltration. Western blot analysis was performed 48 hpi with an anti-GFP (XopD) and anti-myc (RPT6) antibody. This experiment was repeated twice with similar results.

**Figure S3:** XopJ does not interact with RPT6 from humans. (A) XopJ fused to the GAL4 DNA-binding domain (pGBT9) was expressed in combination with RPT6 from humans fused to the GAL4 activation domain (pGAD424) in yeast strain Y190. Cells were grown on selective media before a LacZ filter assay was performed. XopJ/RPT6 served as positive control, while the empty pGAD424 vector served as negative controls. –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. LacZ, activity of the lacZ reporter gene. (B) BiFC *in planta* interaction studies of XopJ and *Hs*RPT6 mutants. Yellow fluorescent protein (YFP) confocal microscopy images show *N. benthamiana* leaf epidermal cells transiently expressing XopJ-VenusN in combination with RPT6-VenusC. The combination of XopJ-VenusN and *Hs*RPT6-VenusC did not result in YFP reconstitution. The scale bar represents 20 μm. (C) Empty vector or XopJ-HA were transiently co-expressed together with *Hs*RPT6-GFP in *N. benthamiana* using Agro-infiltration. For confocal laser scanning microscopy (CLSM), samples were taken 48 h postinoculation. GFP fluorescence is shown in green and chlorophyll autofluorescence in red. The scale bar represents 20 μm, except for the close up (5μM). (D) XopJ does not degrade RPT6 from humans. Total proteins were extracted 48 h after
infiltration with *Agrobacteria* harbouring the respective XopJ and HsRPT6 expression constructs. RPT6-GFP protein levels were detected using an anti-GFP antibody. Expression of XopJ was verified using an anti HA-antibody. Staining of the membrane with amido black showed equal loading. The experiment was repeated twice with similar results.

**Figure S4: XopJ exhibits protease activity in a protease-specific manner.** (A) 5 µg of MBP and MBP-XopJ purified from *E. coli* were incubated with or without protease inhibitors for 1 hour at 37°C and subjected for protease activity measurement using the P-CHECK protease detection kit. The reaction was monitored at 37°C in a fluorescence spectrophotometer. As a positive control, serine protease Proteinase K was included. A representative result of more than three repetitions with independent sets of purified proteins is shown. (B) Specific proteinase inhibitor substances (33µM Chymostatin, 28µM E-64, 10µM Leupeptin, 0,15µM Aprotinin and 4mM PMSF) were added to 5µg of MBP or MBP-XopJ one hour before the protease reaction was started and subjected for protease activity measurement using the P-CHECK protease detection kit. MBP-XopJ protease activity without proteinase inhibitors (minus MBP background fluorescence) was set to 100% for data analysis. (C) XopJ protein variants along with an empty vector (EV) control were transiently expressed in leaves of *N. benthamiana* using Agrobacterium-infiltration. After 48h, proteins extracts were incubated with or without protease inhibitors for 1 hour at 37°C and protease activity in total protein extracts was measured using the P-CHECK protease detection kit. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05.

**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.** (A, B) Extracts from *N. benthamiana* transiently expressing EV, XopJ-HA or C235A-HA (+/- Protease Inhibitors) were mixed together with MBP-RPT6 purified from *E. coli* and an aliquot of each reaction was stopped immediately by the addition of SDS loading buffer. RPT6 protein levels were monitored using an anti-MBP antibody.

**Figure S6: MBP-XopJ degrades RPT6 and inhibits proteasome activity.** (A) MBP, MBP-XopJ and MBP-XopJ C235A were purified from *E. coli* and incubated with crude plant extracts of *N. benthamiana* leaves expressing RPT6-GFP at 37°C overnight. RPT6 protein levels were monitored using an anti-GFP antibody and recombinant proteins were analyzed.
on a SDS-PAGE. The band intensities of RPT6-GFP bands were quantified by ImageJ and are shown at the bottom of each panel. (B) Equal amounts (1µg) of MBP-RPT6 together with GST-XopJ or GST were incubated at 37°C overnight and protein amounts were analyzed by western blotting with anti-MBP and anti-GST antibodies. (C) Crude plant extracts of N. benthamiana leaves were incubated with 5µg MBP, MBP-XopJ and MBP-XopJ C235A for 1 hour at 30°C and proteasome activity was measured by monitoring the breakdown of the fluorogenic peptide Suc-LLVY-AMC at 30°C. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: **, P < 0.01. The experiments in (A, B) were repeated three times with similar results.

**Figure S7: Virus-induced gene silencing of RPT6 in N. benthamiana mimics XopJ-triggered effects. (A) Phenotype of RPT6 - VIGS plants in comparison to the pTRV2-GFPsil control. Picture was taken 14 dpi. (B) Proteasome activity was measured in GFPsil and RPT6 silenced plants by monitoring the breakdown of the fluorogenic peptide Suc-LLVY-AMC at 30°C. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05. (C) Accumulation of ubiquitinated proteins and NPR1 protein levels were monitored in RPT6 silenced plants in comparison to control plant GFPsil using anti-ubiquitin and anti-NPR1 antibodies.

**Figure S8: XopJ triggers accumulation of NPR1-GFP.** XopJ-myc together with NPR1-GFP were transiently expressed in N. benthamiana using Agro-infiltration. MG132 treatment for 3, 6 and 18 hours was performed to monitor NPR1 accumulation. Samples were subjected to western blot analysis 48 hpi. An anti-GFP antibody was used to detect NPR1-GFP and XopJ-myc expression was confirmed with an anti-myc antibody.
Figure 1: XopJ interferes with RPT6-GFP protein accumulation

(A) Empty vector, (B) XopJ-HA, (C) G2A-HA and (D) C235A-HA were transiently co-expressed together with RPT6-GFP in N. benthamiana using Agrobacteria infiltration. For confocal laser scanning microscopy (CLSM), samples were taken 48 h post inoculation, and images were generated with identical CLSM settings. (E) Microscope image of a RPT6-GFP and XopJ-HA co-expressing leave recorded with increased photomultiplier gain as compared to the images presented in Fig A to D. GFP fluorescence is shown in green and chlorophyll autofluorescence in red. The scale bar represents 20 μm. (F) Quantification of GFP intensity after co-expression of RPT6-GFP together with XopJ and its variants. GFP quantification was determined by using the Leica software LAS_AF. Co-expression with the empty vector (EV) control was set to 100 %. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *** P < 0.001. (G) Total proteins were extracted 48 h after infiltration with Agrobacteria harboring the respective XopJ and RPT6 expression constructs. 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 sucrose-phosphatase (anti-SPP2) served as a control and staining of the membrane with amido black showed equal loading. (H) XopJ does not affect RPT6 expression levels. RT-PCRs showing transgenic RPT6 mRNA in N. benthamiana leaves transiently co-expressing 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.
Figure 2: Destabilization of RPT6 is protease-dependent

(A) RPT6-GFP was transiently co-expressed together with EV, XopJ-myc and XopJ C235A-myc in N. benthamiana using Agroinfiltration. 42 hours post inoculation (hpi) 100μM MG132 or a protease inhibitor mix were infiltrated into Agrobacterium-inoculated leaves and leaf material was collected 48 hpi. Expression of RPT6-GFP was detected using an anti-GFP antibody. After stripping of the membrane, protein levels of XopJ and XopJ C235A were analyzed with an anti-myc 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. 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 empty vector (EV) control was set to 100 %. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: **, P < 0.01. Experiments in (A) and (B) were repeated twice with similar results.
Figure 3: XopJ displays protease activity in vitro and in vivo

(A) 5 µg of MBP, MBP-XopJ, MBP-XopJ C235A, MBP-RPT6 and a mix of MBP-XopJ/MBP-RPT6 purified from E. coli were subjected for protease activity measurement using the P-CHECK protease detection kit. MBP and MBP-RPT6 served as a negative control. As a positive control, serine protease Proteinase K was included. A representative result of more than three repetitions with independent sets of purified proteins is shown. (B) Protease activity in N. benthamiana leaves transiently expressing XopJ-HA proteins. XopJ protein variants along with an empty vector (EV) control were transiently expressed in leaves of N. benthamiana using Agro-infiltration. After 48h, protease activity in total protein extracts was measured using the P-CHECK protease detection kit. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05; **, P < 0.01. The experiment was repeated twice with similar results.
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 used plant extracts. (B) The immunoblot using an anti-MBP antibody shows MBP-RPT6 accumulation after incubation with crude extracts of *N. benthamiana* leaves expressing XopJ-HA or C235A-HA +/-protease inhibitors. Plants expressing the empty vector control served as a negative control. An anti-HA immunoblot was performed to detect proper expression of XopJ-HA proteins. Amido black staining served as a loading control for the used plant extracts. The results in both experiments (A, B) are representative of two independent experiments performed.
Figure 5: RPT6 Walker A motif is required for XopJ binding

(A) XopJ interacts with RPT6 E260A (Walker B) but not with RPT6 K206A (Walker A) mutant 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. NtRPT6 = 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. Yellow fluorescent protein (YFP) confocal microscopy images show N. benthamiana leaf epidermal cells transiently expressing XopJ-VenusN in combination with RPT6 K206A or E260A-VenusC. A close-up of the same cells shows that the YFP fluorescence of XopJ-VenusN/RPT6 E260A-VenusC aligns with the plasma membrane. XopJ-VenusN and RPT6-VenusC and the dimerization of fructose-1,6-bisphosphatase (FBPase) within the cytosol serve as positive controls. XopJ-VenusN with FBPase-VenusC or the RPT6 mutants together with FBPase-VenusN are included as negative controls. Scale bar represents 20 μm, except for the close-up (5μm).

(C) Empty vector (EV) or XopJ-HA were transiently co-expressed together with RPT6 GFP, RPT6 K206A- 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 in red. Arrows indicate cytosolic strands. Scale bar represents 20 μm.

(D) Quantification of GFP intensity after co-expression of RPT6-GFP, RPT6 K206A and RPT6 E260A-GFP together with XopJ. GFP quantification was determined by using the ZEN software of ZEISS. Co-expression with the EV control was set to 100 %. Data represent the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *** P < 0.001.

(E) XopJ does not degrade RPT6 Walker B mutant. Total proteins were extracted 48 h after infiltration with Agrobacteria harboring the respective XopJ and RPT6 expression constructs. RPT6-, RPT6 K206- and RPT6 E260A-GFP protein levels were detected using an anti-GFP antibody. Expression of XopJ was verified using an anti HA-antibody. All experiments were repeated three times with similar results.
(A) XopJ-triggered accumulation of NPR1 protein levels. XopJ protein variants along with an empty vector (EV) control were transiently expressed in leaves of *N. benthamiana* using Agro-infiltration. MG132 treatment for 6 hours was included into the analysis as a positive control. The western blot was probed 48 hpi with an anti-NPR1 antibody directed against the tobacco NPR1 protein. Intensities of NPR1 bands were quantified by ImageJ and shown at the bottom of each panel. An anti-myc antibody was used to show 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 with GFP-Trap beads, followed by immunoblot analysis of the precipitates using either anti-GFP or anti-Ubiquitin antibodies. Proper expression of XopJ proteins in the input fraction was monitored by using an anti-HA antibody. (C) XopJ affects NPR1 protein levels during *Xanthomonas campestris* pv. *vesicatoria* (Xcv) infection in pepper. Xcv or Xcv ΔxopJ were inoculated at a bacterial density of 2 x 10⁸ cfu ml⁻¹ into leaves of pepper ECW 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 extracts (TE) were separated into nuclear depleted (ND) and nuclear 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 the mean SD (n = 3). Significant differences were calculated using Student’s t-test and are indicated by: *, P < 0.05; **, 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.
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