

Xanthomonas type III effector XopD targets SUMO-conjugated proteins *in planta*

Andrew Hotson,^{1†} Renee Chosed,^{2†} Hongjun Shu,³ Kim Orth² and Mary Beth Mudgett^{1*}

¹Department of Biological Sciences, Stanford University, Stanford, CA 94305-5020, USA.

²Department of Molecular Biology and ³AFCS Protein Chemistry Laboratory, University of Texas Southwestern Medical Center, Dallas, TX 75390-9148, USA.

Summary

Xanthomonas campestris pathovar *vesicatoria* (*Xcv*) uses the type III secretion system (TTSS) to inject effector proteins into cells of Solanaceous plants during pathogenesis. A number of *Xcv* TTSS effectors have been identified; however, their function *in planta* remains elusive. Here, we provide direct evidence for a functional role for a phytopathogenic bacterial TTSS effector *in planta* by demonstrating that the *Xcv* effector XopD encodes an active cysteine protease with plant-specific SUMO substrate specificity. XopD is injected into plant cells by the TTSS during *Xcv* pathogenesis, translocated to subnuclear foci and hydrolyses SUMO-conjugated proteins *in vivo*. Our studies suggest that XopD mimics endogenous plant SUMO isopeptidases to interfere with the regulation of host proteins during *Xcv* infection.

Introduction

Xanthomonas campestris pathovar *vesicatoria* (*Xcv*) is the causal agent of bacterial spot disease on tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*) plants (Jones *et al.*, 1998). When environmental conditions are warm and humid, this bacterium causes severe damage to foliage, stems and fruit, resulting in reduced fruit yield and significant economic losses worldwide (Pohronezny and Volin, 1983). Chemicals are used in the field to control *Xcv* growth; however, their efficacy is short-lived because of the evolution of chemically resistant *Xcv* strains (Jones *et al.*, 1995; Sahin and Miller, 1996). Thus, management of bacterial spot disease in agriculture is currently dependent on sanitation and the

use of pathogen-free seedlings and pathogen-resistant cultivars. Understanding the molecular events controlling *Xcv* pathogenesis in plants may lead to alternative methods of disease control.

The genetic and biochemical dissection of *Xcv*-plant interactions has revealed that the *hrp*-encoded type III protein secretion system (TTSS) is required for plant pathogenesis and persistence (Minsavage *et al.*, 1990; Buttner and Bonas, 2002). *Xcv* mutants lacking a functional TTSS are non-pathogenic when associated with host plants because they are not able to grow within the spaces between plant cells (apoplast). Bacterial growth in leaves is probably dependent on resources within host cells such as nutrients and water. Thus, TTSS is viewed as the essential conduit for translocating specific proteins (i.e. effector proteins) into the plant cell interior and liberating its abundant reserves.

It is intriguing that phytopathogenic bacteria use numerous and diverse effectors to infect plant cells. Computational and experimental approaches have been used to predict and/or confirm TTSS effectors in plant pathogens. A cDNA-AFLP analysis performed in *Xcv* identified a number of candidate effectors that are co-regulated with the *Xcv* TTSS pathogenicity island (Noel *et al.*, 2001). A clever TTSS effector screen performed in *Pseudomonas syringae* pv. *maculicola* functionally identified 13 effectors translocated into *Arabidopsis* cells (Guttman *et al.*, 2002). Concurrently, the draft genome sequence for *Pseudomonas syringae* pv. *tomato* was exploited to identify 36 TTSS-secreted proteins (Boch *et al.*, 2002; Fouts *et al.*, 2002; Guttman *et al.*, 2002; Petnicki-Ocwieja *et al.*, 2002; Zwiesler-Vollick *et al.*, 2002). The comparison of these effectors with the annotated genome sequences from other plant pathogens has aided in the identification of conserved and strain-specific effectors (Salanoubat *et al.*, 2002; da Silva *et al.*, 2002). These studies clearly reveal that phytopathogenic bacteria use a large army of proteins to alter plant physiology. The central question remaining is: How?

The most characterized TTSS effectors in phytopathogenic bacteria are avirulence (Avr) proteins (White *et al.*, 2000). Pathogen Avr effector proteins translocated by the TTSS into plant cells have two general fates (Mudgett and Staskawicz, 1999; Casper-Lindley *et al.*, 2002; Szurek *et al.*, 2002). If the host is resistant, it recognizes the Avr effector and immediately activates a resistance

Accepted 18 July, 2003. *For correspondence. E-mail mudgett@stanford.edu; Tel. (+1) 650 723 3252; Fax (+1) 650 723 6132. †These authors contributed equally to this work.

protein-dependent plant defence response that will limit the growth and spread of the invading bacterial pathogen. Conversely, if the host is susceptible and does not recognize the Avr effector, the invading bacterial pathogen will go unnoticed, multiply within the plant apoplast and eventually elicit plant disease symptoms. Thus, the outcome of these plant–pathogen interactions is defined at the molecular level by the presence or absence of functional alleles of bacterial Avr TTSS effectors and plant disease resistance proteins (Dangl and Jones, 2001).

Owing to selective pressures, one might predict that pathogens would lose Avr effectors to avoid plant host surveillance mechanisms. On the contrary, Avr effectors are maintained in strains and can mutate to avoid host recognition (Gassmann *et al.*, 2000; Vera Cruz *et al.*, 2000), implying that these proteins may function *in planta* as virulence factors. In fact, Avr proteins have been shown to elicit host-specific disease symptoms, to promote pathogen growth and to interfere with, evade and/or suppress host defences, clearly demonstrating that they contribute to pathogenesis (Ritter and Dangl, 1995; Reuber and Ausubel, 1996; Duan *et al.*, 1999; Jackson *et al.*, 1999; Chang *et al.*, 2000; Chen *et al.*, 2000; Tsiamis *et al.*, 2000; Abramovitch *et al.*, 2003). However, the molecular basis for these Avr-induced phenotypes remains elusive.

Hints as to the mechanism of Avr action have come from the study of two effector families conserved in both plant and animal bacterial pathogens. The YopT and YopJ effectors define two families of cysteine proteases functioning in bacterial pathogenesis. Both families mimic proteolytic activities of eukaryotic proteins that are essential for the normal maintenance of host signalling. The *Yersinia* YopT cysteine protease is cytotoxic in animal cells because it cleaves lipid-modified Rho GTPases near their C-termini causing them to be released from the host membrane (Shao *et al.*, 2002). Proteolytic inactivation of Rho GTPases by YopT is predicted to impair the ability of host cells to rearrange the actin cytoskeleton normally during macrophage phagocytosis and bacteria internalization in non-phagocytic cells. In plant cells, protease activity of the *Pseudomonas* YopT homologue AvrPphB is required for the induction of the hypersensitive response (HR) and for autoproteolytic cleavage of the mature AvrPphB polypeptide. Interestingly, processing of AvrPphB exposes a potential N-terminal myristoylation site (Nimchuk *et al.*, 2000), indicating that subsequent post-translational modification of AvrPphB *in planta* may target the effector to a different subcellular site where it acts on a plant substrate and, subsequently, triggers host defences.

Analysis of the putative secondary structure of the *Yersinia* YopJ effector revealed that YopJ and its family mem-

bers encode cysteine proteases with structural features similar to that of the adenovirus cysteine protease. A functional YopJ catalytic core is required for the inhibition of MAPK and NF- κ B signalling in animal cells (Orth *et al.*, 2000). Similarly, its *Xanthomonas* homologue AvrBsT requires a functional catalytic core to elicit plant defence responses. The mammalian substrates for YopJ are proposed to be SUMO-1 (small ubiquitin-like modifier) conjugated proteins, suggesting that the *Yersinia* protease mimics a host protease that removes SUMO-1 modifications. Therefore, YopT and YopJ effector families both appear to use proteolysis as the mechanism to alter host cell signalling events, presumably for the pathogen's benefit.

Recently, Noel *et al.* (2002) reported two novel TTSS effector proteins in *Xcv*. Upon further inspection, we discovered that one of the effectors, XopD, is a homologue of the ubiquitin-like protein protease, Ulp1, substrates of which are SUMO-conjugated proteins (Li and Hochstrasser, 1999). This striking homology further supports our hypothesis that a suite of *Xcv* TTSS effectors may be working *in planta* to disrupt the steady state of SUMO-conjugated proteins during infection.

SUMO is a member of a family of ubiquitin-related proteins that are covalently attached to eukaryotic proteins by a conjugation system that operates similarly to the ubiquitin conjugation system (Melchoir, 2000; Kurepa *et al.*, 2003). SUMO and SUMO proteases dynamically regulate protein conjugation and deconjugation of proteins and thus regulate a number of cellular processes. For example, SUMO modification controls nuclear transport, signal transduction, cell cycle progression and the stress response (Melchoir, 2000). In contrast to ubiquitin, SUMO stabilizes proteins and can be an antagonist of ubiquitin. It is thus becoming even more apparent that post-translational regulation of proteins by ubiquitin and ubiquitin-like proteins is analogous to the post-translational regulation of proteins by phosphorylation and is critical for maintaining cellular signalling and homeostasis (Orth, 2002).

Here, we show that XopD, an *Xcv* type III effector protein, encodes an active cysteine protease with plant-specific SUMO substrate specificity. This protein is injected into plant cells by the Hrp TTSS during *Xcv* pathogenesis and is then translocated to the plant nucleus to subnuclear foci. XopD's *in planta* substrates are SUMO-conjugated proteins. Our studies indicate that XopD mimics an endogenous plant SUMO isopeptidase(s), presumably to interfere with the regulation of host proteins during *Xcv* infection. We propose therefore that the pathogen uses SUMO protein deconjugation as a mechanism to alter plant signal transduction and, ultimately, plant physiology.

Results

XopD is a Ulp1 protein homologue

The XopD protein was recently identified as a novel type III secreted protein from *Xcv* (Noel *et al.*, 2002). XopD shares sequence similarity with the virulence factor PsvA from *Pseudomonas syringae* pv. *eriobotryae*; however, homologous proteins with known function were not reported (Noel *et al.*, 2002). Using BLAST (Altschul *et al.*, 1997) and PROSITE (Falquet *et al.*, 2002) analysis, we found that the C-terminus of XopD (amino acids 322–520) shares primary sequence similarity with the C48 family of cysteine peptidases (Fig. 1A). Moreover, amino acid positions 309–481 in the XopD polypeptide are most homologous to the C-terminal catalytic domain of the Ulp1 ubiquitin-like protease protein family (Li and Hochstrasser, 1999). Ulp1 is a cysteine protease that catalyses two critical reactions in the SUMO/Smt3 pathway. In the first, Ulp1 processes the invariant C-terminal sequence (–GG XXX) of SUMO to its mature form (–GG) so that the terminal glycine residue can be used as a substrate to modify target proteins covalently (Melchoir, 2000). In the second, Ulp1 deconjugates SUMO from its target protein by cleaving the isopeptide bond that links the C-terminus of SUMO to the epsilon-amine of a lysine residue on the target protein (Melchoir, 2000). Alignment of the C-terminal domains of XopD and Ulp1 revealed that the residues comprising the putative catalytic core in the XopD cysteine protease are H409, D421 and C470 (Fig. 1B). Based on information from the co-crystal structure of Ulp1 and SUMO, we observed that residues important for substrate recognition are also conserved in XopD (amino acids 338–341) (Mossessova and Lima, 2000). Analysis of the N-terminus of XopD failed to reveal significant homology to any protein in existing databases possessing a known functional domain or structural motif.

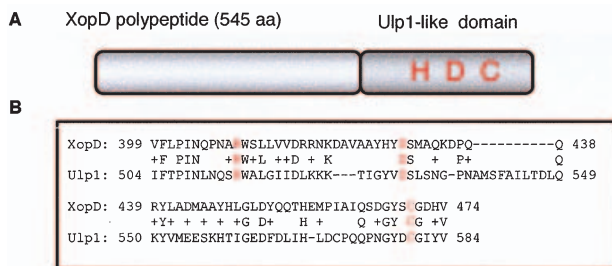


Fig. 1. Features of the *Xcv* XopD polypeptide.

A. Schematic representation of XopD. XopD encodes a 545-amino-acid protein. Amino acids 309–481 encompass the Ulp1-like protease domain. The putative catalytic core residues are His-409, Asp-421 and C470, depicted as H, D and C.

B. Sequence alignment of amino acids in the catalytic core of yeast Ulp1 and *Xcv* XopD.

Based on the striking sequence homology, we hypothesized that XopD encodes a ubiquitin-like protein protease that functions *in planta* during *Xcv* pathogenesis. We predicted previously that YopJ-like effector proteins in *Xcv* might similarly encode cysteine proteases with SUMO substrate specificity (Orth *et al.*, 2000). Although the YopJ-like effectors, including AvrBsT, AvrRxv and AvrXv4, share limited sequence similarity with amino cysteine proteases including Ulp1 and adenovirus protease AVP (Orth *et al.*, 2000; Staskawicz *et al.*, 2001), the catalytic domain of XopD possesses extensive sequence similarity with the catalytic core of Ulp1 (Fig. 1; MEROPS Protease Database, <http://merops.sanger.ac.uk/>). Sequence and structural analysis categorize AVP, Ulp1 and XopD, and YopJ and YopJ-like effectors in the CE clan of cysteine proteases characterized by a cysteine nucleophile and a catalytic core with the ordered sequence: H, E or D, and C. Within the CE clan of cysteine proteases, these proteins have been assigned to the C5, C48 and C55 peptidase families, respectively, based on additional structural information that distinguishes these polypeptides (MEROPS Protease Database). Thus, *Xcv* expresses two different families (C48 and C55) of cysteine proteases that possibly function as SUMO protease-like effectors.

XopD possesses Ulp1-like protease activity

To determine whether XopD possessed Ulp1-like protease activity, we cloned the *xopD* gene from *Xcv* strain 85-10 and constructed wild-type and mutant GST–XopD fusion proteins. GST–XopD and GST–Ulp1 fusion proteins were expressed in *Escherichia coli* BL21 cells, purified using standard GST–glutathione affinity chromatography and then analysed with *in vitro* protease assays using as substrates ³⁵S-labelled tomato and mammalian SUMO with a carboxy-terminal HA-epitope tag. [³⁵S]-T-SUMO-HA was incubated with buffer, GST–XopD_{1–545}, GST–XopD_{283–545} or GST–Ulp1 for 60 min and then analysed by SDS-PAGE and autoradiography.

Wild-type *Xcv* GST–XopD_{1–545} and yeast GST–Ulp1 cleaved the [³⁵S]-T-SUMO-HA substrate (SUMO-GlyGly-HA) *in vitro* after the invariant C-terminal –GlyGly residues generating [³⁵S]-T-SUMO (Fig. 2A). GST–XopD_{283–545}, a fusion protein containing only the Ulp1-like domain, also cleaved the [³⁵S]-T-SUMO-HA substrate. We confirmed that XopD cleaves the tomato SUMO polypeptide at the predicted cleavage site (after the invariant C-terminal –GlyGly residues) using tandem mass spectrometry. Recombinant T-SUMO-6×His purified from *E. coli* was incubated with purified GST–XopD_{283–545} *in vitro*, and then the amino acid composition of the XopD-cleaved T-SUMO and uncleaved T-SUMO-6×His polypeptides was determined (data not shown). Only T-SUMO-6×His substrate

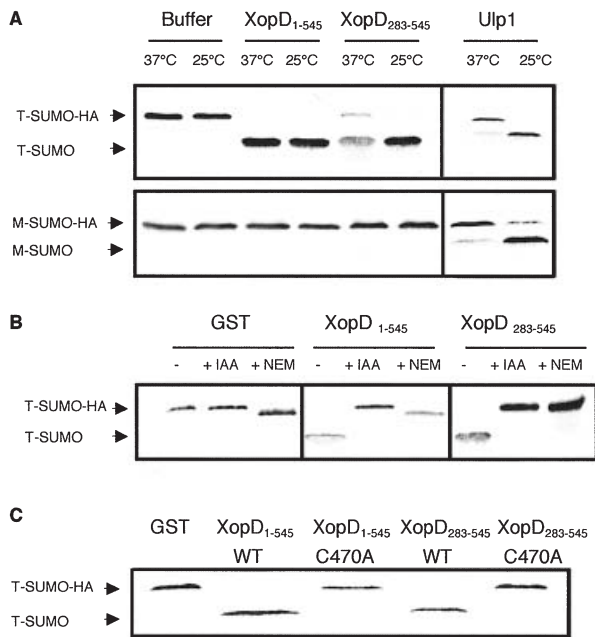


Fig. 2. *Xcv* XopD is a plant-specific SUMO cysteine protease. **A.** XopD exhibits peptidase activity similar to yeast Ulp1. [³⁵S]-Tomato-SUMO-HA and [³⁵S]-Mammalian-SUMO-HA were translated *in vitro* in a rabbit reticulocyte lysate and then incubated with buffer, 0.2 mg ml⁻¹ GST-XopD₁₋₅₄₅, 0.2 mg ml⁻¹ GST-XopD₂₈₃₋₅₄₅ or 0.4 mg ml⁻¹ GST-Ulp1 for 1 h at the indicated temperatures. SUMO species were resolved on a 15% SDS gel and visualized by autoradiography. **B.** XopD enzyme activity is inactivated by cysteine protease inhibitors. [³⁵S]-Tomato-SUMO-HA translated *in vitro* in a rabbit reticulocyte lysate was incubated for 1 h at 30°C with 0.2 mg ml⁻¹ GST, 0.2 mg ml⁻¹ GST-XopD₁₋₅₄₅ or 0.2 mg ml⁻¹ GST-XopD₂₈₃₋₅₄₅ treated with 10 mM IAA or 5 mM NEM. SUMO species were resolved on a 15% SDS gel and visualized by autoradiography. **C.** XopD C470A mutants are inactive proteases. [³⁵S]-Tomato-SUMO-HA translated *in vitro* in a rabbit reticulocyte lysate was incubated with 0.2 mg ml⁻¹ GST, 0.2 mg ml⁻¹ GST-XopD₁₋₅₄₅, 0.2 mg ml⁻¹ GST-XopD₁₋₅₄₅ C470A, 0.2 mg ml⁻¹ GST-XopD₂₈₃₋₅₄₅ or 0.2 mg ml⁻¹ GST-XopD₂₈₃₋₅₄₅ C470A for 1 h at 30°C. SUMO species were resolved on a 15% SDS gel and visualized by autoradiography.

incubated with purified XopD was cleaved after -GlyGly. These studies demonstrate that *Xcv* XopD possesses SUMO peptidase activity analogous to that observed for the yeast Ulp1 enzyme. SUMO peptidase activity is specifically localized to the predicted catalytic domain of XopD that shares homology with the catalytic domain of Ulp1. Moreover, the GST-XopD₂₈₃₋₅₄₅ enzyme is constitutively active and does not require any eukaryotic factors for its enzymatic activity.

XopD is a plant-specific SUMO cysteine protease

To characterize further the activity of XopD, we studied its activity by varying the substrate and temperature. GST-XopD₁₋₅₄₅ and GST-XopD₂₈₃₋₅₄₅ were incubated *in vitro* with a mammalian SUMO-1 substrate, [³⁵S]-M-SUMO-HA.

Interestingly, [³⁵S]-M-SUMO-HA was not cleaved by GST-XopD₁₋₅₄₅ or GST-XopD₂₈₃₋₅₄₅ (Fig. 2A), whereas yeast Ulp1 protease was able to cleave both the tomato and the mammalian SUMO-HA substrates. The specificity of XopD was not temperature dependent, as XopD was able to cleave tomato SUMO-HA at 37°C and 25°C, growth temperatures for some animal and plant hosts respectively (Fig. 2A). Although Ulp1 was able to cleave both tomato and mammalian SUMO-HA, the cleavage of tomato SUMO-HA by Ulp1 was more efficient at 25°C. These studies indicate that *Xcv* XopD has plant-specific SUMO substrate specificity, whereas yeast Ulp1 has broader host substrate specificity.

Ubiquitin-like proteases in the C48 family are cysteine peptidases with activity that depends on a functional catalytic triad (H, D, C) (MEROPS Protease Database, <http://merops.sanger.ac.uk/>). Members of this family are inactivated by thiol protease inhibitors, including iodoacetamide (IAA) and N-ethylmaleimide (NEM), or by single amino acid substitutions within the catalytic core. We next explored whether XopD, like Ulp1, was acting as a cysteine protease. GST, GST-XopD₁₋₅₄₅ and GST-XopD₂₈₃₋₅₄₅ were incubated with [³⁵S]-T-SUMO-HA at 25°C in the presence and absence of IAA and NEM. The GST-XopD₁₋₅₄₅ and GST-XopD₂₈₃₋₅₄₅ enzymes were specifically inhibited by IAA and NEM and were unable to cleave the T-SUMO-HA substrate (Fig. 2B). We consistently observed that NEM treatment alone slightly altered the migration of the T-SUMO-HA substrate whether or not enzyme was added. Furthermore, mutation of cysteine 470 to an alanine residue in the predicted XopD catalytic core completely inactivated the XopD peptidase activity (Fig. 2C). GST-XopD₁₋₅₄₅ C470A and GST-XopD₂₈₃₋₅₄₅ C470A were not able to cleave the T-SUMO-HA substrate. Collectively, these observations demonstrate that *Xcv* XopD encodes a plant-specific SUMO cysteine protease with peptidase activity.

XopD is translocated to plant cells during *Xanthomonas* infection

If XopD is a virulence protease that disrupts SUMOylation *in planta*, XopD must be targeted to plant cells during *Xcv* infection. XopD is secreted by the *Xcv* Hrp TTSS *in vitro*, suggesting that this protein is targeted to plant cells (Noel *et al.*, 2002). To confirm that *Xcv* translocates XopD via the Hrp TTSS into plant cells during infection, we used the calmodulin-dependent adenylate cyclase domain (Cya) of *Bordetella pertussis* cyclolysin as a sensitive reporter protein (Sory and Cornelis, 1994). Cya enzymatic activity requires eukaryotic calmodulin for the production of cAMP. Therefore, bacterially produced Cya must be translocated into eukaryotic cells to be activated. We constructed a translational C-terminal fusion of the Cya reporter to the

Table 1. cAMP^a produced by *Xcv* strains expressing XopD-cya enzymatic activity.

<i>In vitro</i> bacterial assay	–calmodulin	+calmodulin				
85-10 <i>hrpG</i> *	0.09 ± 0.16	274.24 ± 12.49				
85-10 <i>hrpG</i> *, Δ <i>hrpF</i>	0.00 ± 0.00	240.93 ± 24.50				

<i>In planta</i> assay	T = 0	T = 8 h	T = 16 h	T = 24 h	T = 32 h	T = 48 h
85-10 <i>hrpG</i> *	0.02 ± 0.02	1.15 ± 0.48	5.30 ± 1.70	7.70 ± 0.63	18.37 ± 3.23	27.12 ± 5.25
85-10 <i>hrpG</i> *, Δ <i>hrpF</i>	0.01 ± 0.00	0.04 ± 0.03	0.14 ± 0.03	0.27 ± 0.30	0.10 ± 0.00	0.58 ± 0.16

a. pmol cAMP mg⁻¹ total protein.

full-length XopD protein. Protein extracts isolated from *Xcv* strains expressing the XopD–Cya fusion protein in liquid culture did not possess endogenous Cya enzyme activity (Table 1). However, XopD–Cya enzyme activity could be reconstituted when calmodulin was added to the bacterial protein extract, demonstrating that the chimeric XopD–Cya fusion protein encodes a functional cyclase.

To study XopD–Cya translocation *in planta*, *Xcv hrpG** and *Xcv hrpG** Δ *hrpF* expressing XopD–Cya were inoculated into pepper leaves. Leaf tissue collected over 48 h was analysed for XopD–Cya activity by measuring the production of cAMP. The *Xcv hrpG** strain (Rossier *et al.*, 1999) constitutively expresses a functional TTSS, whereas the *Xcv hrpG** Δ *hrpF* strain constitutively expresses a secretion-competent and translocation-deficient TTSS (Buttner *et al.*, 2002; Casper-Lindley *et al.*, 2002). Pepper leaves infected with *Xcv hrpG** expressing XopD–Cya progressively accumulated cAMP (Table 1). Conversely, pepper leaves infected with *Xcv hrpG** Δ *hrpF* expressing XopD–Cya did not accumulate cAMP in the host during the course of the infection. Thus, as with other TTSS effector proteins, XopD is secreted via the TTSS out of the bacterium and translocated directly through the plant cell wall into the interior of the plant cell.

Xcv XopD protein is localized to the plant nucleus

To characterize further XopD's activity, we examined its intracellular location in host plant cells. The C-terminus of XopD contains a putative nuclear localization signal (NLS; KKKKXXK) at amino acid position 535–540, suggesting that XopD may be targeted to the plant nucleus during *Xanthomonas* infection. To visualize XopD in live plant cells, we constructed several EYFP (enhanced yellow fluorescent protein)-tagged XopD proteins (Fig. 3A) and expressed the respective gene fusions via the CaMV 35S promoter in tobacco, *Nicotiana benthamiana*, using the *Agrobacterium*-mediated transient expression system (Mudgett and Staskawicz, 1999). Protein expression was confirmed by immunoblot analysis with green fluorescent protein (GFP) antisera (data not shown). *Agrobacteria*-

infected leaves were analysed directly using epifluorescence light microscopy. EYFP–XopD_{1–545} protein was predominantly localized to the nucleus of infected *N. benthamiana* cells, whereas EYFP alone was distributed equally between the cytoplasm and the nucleus (Fig. 3B). Unlike EYFP, EYFP–XopD_{1–545} was localized to distinct foci within the plant nucleus. The number of nuclear dot-like structures varied in size during the course of the plant infection. Typically, many small dots were observed 24 h after inoculation. Fewer dots of a larger size were observed 48–72 h after inoculation, suggesting that the protein was accumulating at subnuclear foci.

The localization of EYFP–XopD_{1–534}, a protein lacking the putative XopD NLS, was similar to that of EYFP–XopD_{1–545}, indicating that the putative NLS was not required for nuclear import or subnuclear targeting (Fig. 3B). EYFP–XopD_{283–545} (Fig. 3B) and EYFP–XopD_{283–534} (data not shown) proteins containing only the C-terminal XopD protease domain were equally distributed in the plant cytoplasm and nucleus similar to EYFP localization. Considering that EYFP–XopD_{283–545} and EYFP–XopD_{283–534} encode a 57.4 kDa and 55.9 kDa polypeptide, respectively, their diffuse nuclear localization is likely to be the result of passive protein diffusion in and out of the plant nucleus. This suggested that the N-terminus of XopD was required for nuclear import and nuclear speckling. EYFP–XopD_{1–282}, a protein lacking the XopD protease domain, was localized specifically to subnuclear foci similar to EYFP–XopD_{1–545} and EYFP–XopD_{1–534} localization (Fig. 3B). Thus, the N-terminus of XopD is necessary and sufficient for XopD import and subnuclear localization.

XopD possesses plant SUMO isopeptidase activity

To elucidate the role of XopD in pathogenesis, we next determined whether XopD recognized plant proteins post-translationally modified by SUMO. In contrast to yeast and mammalian systems, little is known about the proteins that are modified by SUMO in plants. Thus, we first determined the extent of SUMO protein conjugation *in planta* using

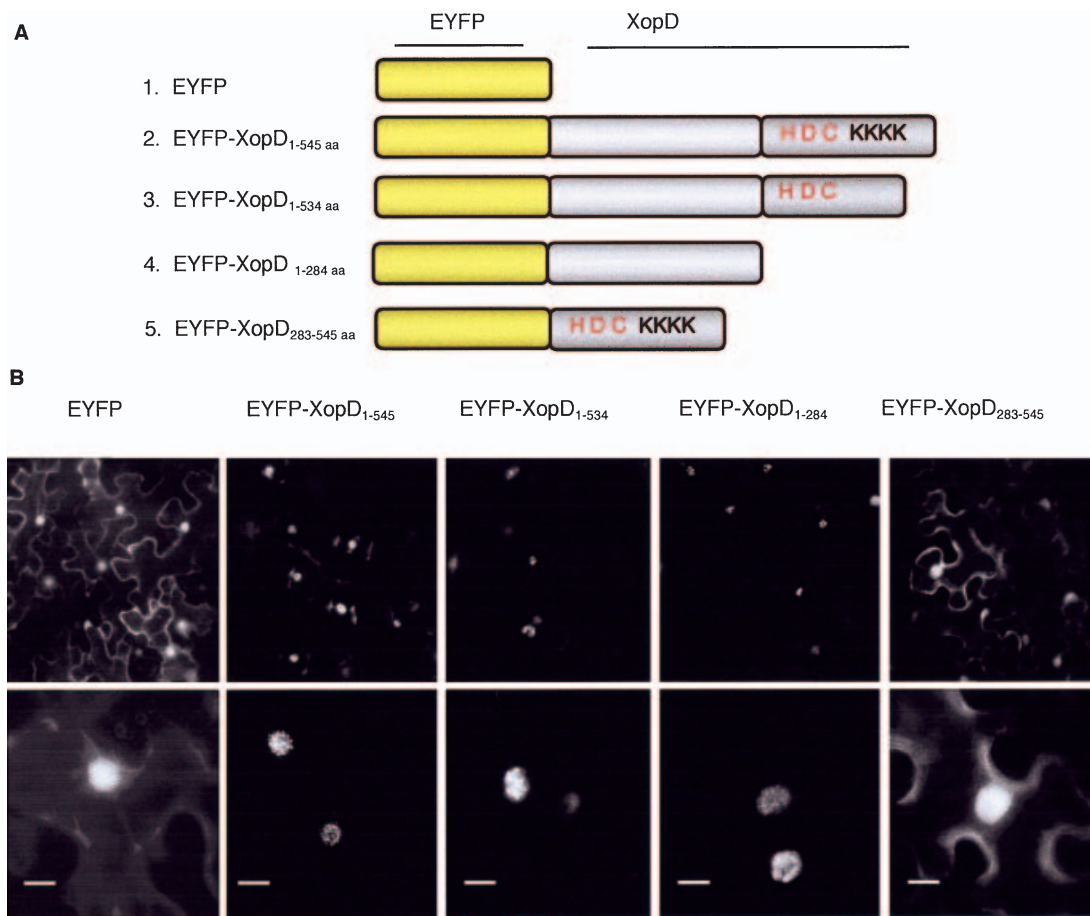


Fig. 3. XopD is localized to the plant nucleus.

A. Schematic representation of EYFP-XopD fusion proteins analysed *in planta*.

B. Cellular location of XopD protein transiently expressed *in planta*.

Nicotiana benthamiana leaves were inoculated with a 6×10^8 cells ml⁻¹ suspension of *A. tumefaciens* expressing EYFP, wild-type EYFP-XopD or mutant EYFP-XopD protein (see A). Epidermal cells in infected leaves were visualized live 48 h after inoculation by epifluorescence light microscopy at 20 \times (top) and 60 \times (bottom). Scale bar equals 10 μ m.

the *Agrobacteria* transient expression system to express tomato HA-SUMO in *N. benthamiana*. Total protein was extracted from infected *N. benthamiana* leaves 48 h after inoculation and analysed by immunoblot analysis using antisera specific for the HA epitope. Free tomato HA-SUMO and multiple HA-SUMO-conjugated proteins accumulated in the plant cells transiently expressing T-HA-SUMO (data not shown, Fig. 4), demonstrating that a number of plant proteins undergo SUMO post-translational modification in *N. benthamiana*.

We determined next whether the plant SUMO-conjugated proteins were substrates for XopD *in vitro*. Plant HA-SUMO-conjugated proteins were generated as described above and then used as substrates for GST-XopD₁₋₅₄₅, GST-XopD₁₋₅₄₅ C470A and GST-Ulp1. GST-XopD₁₋₅₄₅ was able to hydrolyse the SUMO substrates resulting in a significant reduction in the HA-SUMO-conjugated proteins (Fig. 4). The level of free HA-SUMO

(Fig. 4) and total plant protein (data not shown) was not significantly altered, indicating that XopD action did not cause random protein degradation. GST-Ulp1 also recognized the plant SUMO substrates; however, GST-Ulp1 isopeptidase activity was less efficient than GST-XopD₁₋₅₄₅. Mutant GST-XopD₁₋₅₄₅ C470A, as well as the GST control, exhibited no isopeptidase activity (Fig. 4). Conversely, the mutant GST-XopD₁₋₅₄₅ C470A protein often stabilized SUMO-conjugated proteins relative to the GST alone control (data not shown). These studies confirm that XopD possesses plant SUMO isopeptidase activity in addition to SUMO peptidase activity.

Considering that XopD functions as both a peptidase and an isopeptidase, we next assessed the effect of XopD action on SUMO post-translational modification *in planta*. XopD-HA and T-HA-SUMO were co-expressed transiently in *N. benthamiana* leaves, and then HA-SUMO conjugates were analysed by immunoblot analysis. Wild-type

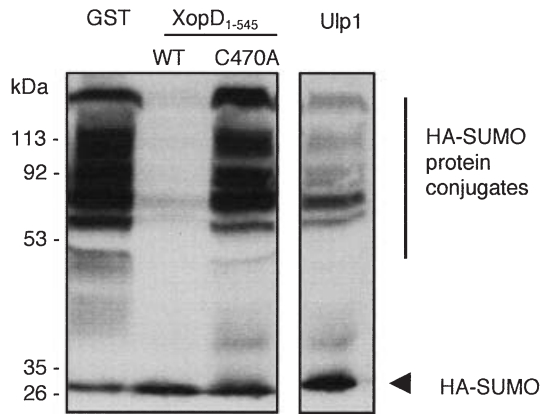


Fig. 4. XopD exhibits tomato SUMO isopeptidase activity *in vitro*. *N. benthamiana* leaves were inoculated with a 6×10^8 cells ml^{-1} suspension of *A. tumefaciens* expressing tomato HA-SUMO. Forty-eight hours after inoculation, total protein was extracted from infected leaves and incubated with 0.2 mg ml^{-1} GST, 0.2 mg ml^{-1} GST-XopD₁₋₅₄₅, 0.2 mg ml^{-1} GST-XopD₁₋₅₄₅ C470A or 0.4 mg ml^{-1} GST-Ulp1 for 1 h at room temperature. Protein was analysed by immunoblot analysis using HA antisera. Free HA-SUMO (arrow) and HA-SUMO conjugated to proteins (line) are indicated.

XopD-HA expression *in planta* led to a significant reduction in SUMO-modified proteins (Fig. 5, lane 6). The level of free HA-SUMO was not significantly altered, indicating that XopD action *in planta* was not affecting the expression or accumulation of HA-SUMO. Not all potential SUMO substrates were hydrolysed, indicating that XopD did not completely reduce protein SUMOylation. The mutant XopD-HA C470A was not able to hydrolyse the SUMO substrates (Fig. 5, lane 7). As with the *in vitro* analysis, we observed an increase in SUMO-conjugated

proteins when XopD-HA C470A was co-expressed with T-HA-SUMO *in vivo* (Figs 4 and 5).

Our *in planta* analysis revealed that XopD targets a number of SUMO-modified proteins and may therefore affect several plant signalling pathways during *Xcv* infection. Because individual plant proteins modified by SUMO have not yet been reported, we tested a major target of SUMO in higher eukaryotes, RanGAP1 (Matunis *et al.*, 1996). Initially, we used reticulocyte lysate to produce *in vitro*-translated, radiolabelled mammalian RanGAP1, which is known to produce both the [³⁵S]-RanGAP1 protein and the [³⁵S]-SUMO-RanGAP1 conjugate (Matunis *et al.*, 1998). We similarly observed the disappearance of the [³⁵S]-M-SUMO-RanGAP1 band when the translation reaction was incubated with purified Ulp1, indicative of Ulp1's isopeptidase activity (Fig. 6A; Li and Hochstrasser, 1999). However, no isopeptidase activity was observed when the reticulocyte lysate translation reaction was incubated with GST or purified GST-XopD₂₈₃₋₅₃₄ (Fig. 6A). These results were not surprising based on our previous observations that XopD was able to cleave tomato SUMO but unable to recognize mammalian SUMO as a substrate (Fig. 2A). Therefore, to test whether XopD has isopeptidase activity on a plant-derived SUMO-RanGAP1 substrate, we used a wheat germ lysate to translate mammalian RanGAP1 *in vitro*. We observed that the wheat germ extract, like the reticulocyte lysate, contains the enzymatic machinery that is sufficient to SUMOylate mammalian RanGAP1 (Fig. 6B). Wheat germ-generated [³⁵S]-SUMO-RanGAP1 was then incubated with purified GST, GST-XopD XopD₂₈₃₋₅₃₄, or GST-XopD XopD₂₈₃₋₅₃₄ C470A for 1 h, and we observed that incubation with

Empty Vector	+	-	-	-	+	-	-
HA-SUMO	-	+	-	-	+	+	+
XopD-HA	-	-	+	-	-	+	-
XopD-HA C470A	-	-	-	+	-	-	+
	1	2	3	4	5	6	7

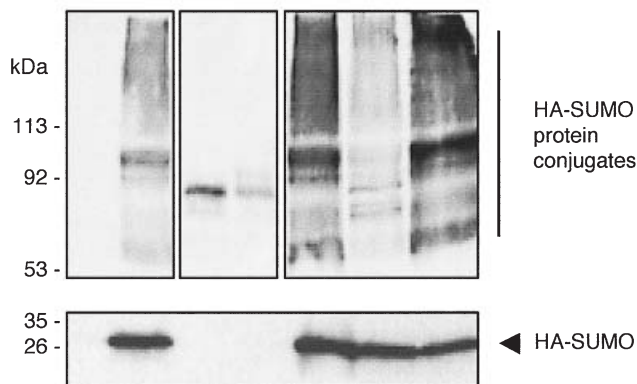


Fig. 5. XopD functions as a SUMO isopeptidase *in planta*. *N. benthamiana* leaves were inoculated with one or two *A. tumefaciens* strains expressing the vector control, tomato HA-SUMO, XopD-HA or XopD-HA C470A. Individual strains were inoculated at 6×10^8 cells ml^{-1} . For co-inoculations, strains were mixed equally and injected into the leaf at a final density of 1.2×10^9 cells ml^{-1} . Forty-eight hours after inoculation, total protein was extracted from infected leaves and analysed by immunoblot analysis using HA antisera.

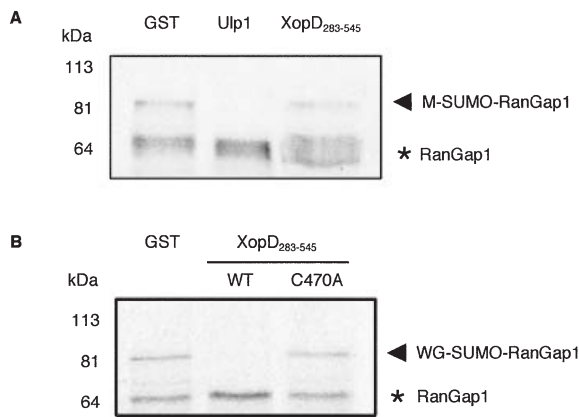


Fig. 6. XopD hydrolyses WG-SUMO-RanGAP1 and not M-SUMO-RanGAP1 *in vitro*. A. [35 S]-mammalian-RanGAP1 was translated *in vitro* in a rabbit reticulocyte lysate and then incubated with 0.2 mg ml $^{-1}$ GST, 0.4 mg ml $^{-1}$ GST-Ulp1 or 0.2 mg ml $^{-1}$ GST-XopD $_{283-545}$ for 1 h at 30°C. Products were resolved on a 10% SDS gel and visualized by autoradiography. M-SUMO-RanGAP1 (arrow) and RanGAP1 (asterisk) are indicated. B. [35 S]-mammalian-RanGAP1 was translated *in vitro* in a wheat germ lysate and then incubated with 0.2 mg ml $^{-1}$ GST, 0.2 mg ml $^{-1}$ GST-XopD $_{283-545}$ or 0.2 mg ml $^{-1}$ GST-XopD $_{283-545}$ C470A for 1 h at 30°C. Products were resolved on a 10% SDS gel and visualized by autoradiography. WG-SUMO-RanGAP1 (arrow) and RanGAP1 (asterisk) are indicated.

GST-XopD XopD $_{283-534}$, but not with GST or GST-XopD XopD $_{283-534}$ C470A, led to the disappearance of [35 S]-WG-SUMO-RanGAP1. These *in vitro* results support our *in vivo* observations that XopD is an isopeptidase that can hydrolyse plant SUMO protein conjugates. Moreover, these results confirm that XopD isopeptidase, as well as peptidase, activity is plant SUMO specific.

Discussion

These studies reveal that the *Xcv* XopD effector encodes an active Ulp1-like cysteine protease that functions inside plant cells to cleave SUMOylated proteins. Two other studies have linked SUMO and SUMO-like proteases to signal transduction events controlling plant defence responses revealing that the SUMO conjugation system may be a key target of plant pathogens (Hanania *et al.*, 1999; Orth *et al.*, 2000). Although SUMO protein targets in plant cells await discovery, a recent report revealed that the SUMO conjugation systems operate in plants (Kurepa *et al.*, 2003). Characterization of the SUMO pathway in *Arabidopsis* shows that this conjugation system is more complex than any other system characterized to date. *Arabidopsis* is predicted to use eight SUMOs and 12 SUMO proteases, revealing that SUMOylation and deSUMOylation of proteins are pivotal regulatory steps in protein signal transduction in plants (Kurepa *et al.*, 2003). A diverse array of SUMO-conjugated proteins exists *in planta*, and some of these are SUMO isoform specific.

Moreover, SUMO protein conjugation is induced by heat, H $_2$ O $_2$, ethanol and the amino acid analogue canavanine. Rapid and reversible modification by SUMO clearly reveals that plants use SUMOylation as an important regulator of the stress response (Kurepa *et al.*, 2003).

Importantly, our work shows that *Xcv* can reduce SUMO protein conjugation *in planta*. Furthermore, the key substrate for XopD is plant SUMO and not mammalian SUMO, indicating that this enzyme is not a general eukaryotic SUMO protease. The co-crystal structure of Ulp1 and yeast SUMO reveals key residues that are important for enzyme:substrate recognition (Mossessova and Lima, 2000). We observe that some of these contact residues in mammalian SUMO encode charge reversals in tomato SUMO, thereby supporting the proposal that the variability in these residues is playing a role in enzyme substrate specificity. SUMO cleavage by SUMO proteases may be distinct for different SUMO isoforms and/or for SUMOs from different organisms. Similarly, the enzyme structure for distinct SUMO proteases is expected to compensate for distinct SUMO structural features. We hypothesize that the target proteins affected by XopD proteolysis may be plant SUMO isoform specific. The identification of SUMOylated plant targets will be paramount to understanding the complexity of cellular pathways modulated by these proteases during plant pathogenesis.

We surveyed the MEROPS Protease Database (<http://merops.sanger.ac.uk/>) and found that proteins containing a protease domain similar to XopD (i.e. members of the C48 peptidase family) are found only in eukaryotic genomes and in bacteria known to associate with plant hosts. The plant pathogens *Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringae* pv. *eriobotryae* and the plant symbiont *Mesorhizobium loti* each possess proteins with the invariant H/D/C residues in their putative protease domains, suggesting that these proteins are also cysteine proteases. HopPmal from the plant pathogen *Pseudomonas syringae* pv. *maculicola* shares limited homology with the N-terminal domain of XopD but lacks the protease domain. Although HopPmal is not predicted to be a protease, the N-terminal domain shared by HopPmal and XopD may play a significant, yet to be identified, role in bacterial-plant interactions.

The XopD effector does not appear to be essential for pathogenicity considering that an *Xcv* mutant strain lacking the *xopD* gene is still virulent on susceptible pepper hosts (Noel *et al.*, 2002). It is not yet known whether *Xcv* has functionally redundant XopD-like proteins that can compensate for XopD action in *Xcv*. However, this strain of *Xcv* does contain the YopJ-like effector AvrRxv (Whalen *et al.*, 1988) that is predicted to function as a SUMO protease (Staskawicz *et al.*, 2001). We suspect that XopD may play a role in the establishment of *Xcv* colonization in the plant apoplast by modulating SUMO-dependent

signal transduction, possibly defence signalling, in plant cells. It is noteworthy that the aggressiveness of the plant pathogen *Ralstonia solanacearum* is modulated by the host specificity factor PopP1, a protein with homology to YopJ-like effectors (Lavie *et al.*, 2002). The precise contribution of the XopD effector in *Xcv* pathogenesis remains to be established.

We did, however, observe that leaves accumulating the wild-type EYFP–XopD active protease in subnuclear foci die by 7 days after transient infection (data not shown). Conversely, tissue necrosis was never observed for leaves infected with the mutant EYFP–XopD C470A protein (data not shown). Considering that *N. benthamiana* plants do not recognize the XopD effector (i.e. the plant is susceptible), the leaf necrosis observed is probably the consequence of XopD-specific proteolysis within the nucleus rather than a defence response triggered by the host (e.g. the hypersensitive cell death response). The deconjugation of SUMO targets in the nucleus is thus likely to play a central role in the control of a number of cellular processes. XopD SUMO protease activity and its localization to subnuclear foci indicate that *Xcv*, and possibly other pathogens, can disrupt the regulation of several proteins modified by SUMO, interfering with the homeostasis of plant cellular signalling.

The identification of specific SUMO targets in the plant nucleus may permit the elucidation of the biological consequence of XopD proteolysis during *Xcv* pathogenesis. So far, only a few plant proteins have been shown to be localized to subnuclear foci. These include: LAF1, a MYB transcriptional activator; COP1, a negative regulator of photomorphogenic development; PhyA to PhyE, the phytochromes; CRY2, a blue light photoreceptor; and RPN, a component of the proteasome (Stacey and von Arnim, 1999; Yamaguchi *et al.*, 1999; Mas *et al.*, 2000; Ballesteros *et al.*, 2001; Nagy *et al.*, 2001; Peng *et al.*, 2001; Kircher *et al.*, 2002). It is intriguing to speculate that one of these proteins is a XopD substrate. However, none of these proteins has been shown to be SUMOylated despite the fact that LAF1 and COP1 contain sequences similar to the minimal SUMO-specific consensus sequence (Melchoir, 2000). We speculate that some of these proteins may be affected by XopD action in the nucleus considering that light-induced signalling pathways control nuclear localization (Yamaguchi *et al.*, 1999; Nagy *et al.*, 2001; Kircher *et al.*, 2002) and interact with pathogen-dependent salicylic acid signal transduction pathways controlling defence responses in *Arabidopsis* (Genoud *et al.*, 2002).

Overall, our studies support a role for the XopD effector during *Xcv*–plant interactions. We show that XopD is an active cysteine protease with plant-specific SUMO specificity. This is the first evidence demonstrating a functional role for a phytopathogenic bacterial TTSS effector *in*

planta. Moreover, our studies reveal a novel mechanism used by *Xanthomonas*, and possibly other plant-associated microbes, to modulate plant physiology during infection. Similar to what has been observed for other bacterial effector proteins (e.g. YopH, YopE, YopJ and YpkA) (Cornelis, 2002; Orth, 2002), we hypothesize that *Xcv* has usurped the activity of a eukaryotic isopeptidase and uses this activity to alter directly SUMO protein targets in the plant nucleus that control plant susceptibility and/or plant defence. Based on these observations, we can explore candidate proteins that are regulated by plant SUMO modification and study their importance in plant–microbe interactions.

Experimental procedures

Constructs

Polymerase chain reaction (PCR) was used to clone tomato *SUMO-1* and *Xcv xopD* and to construct gene fusions. PCR-generated DNA fragments were cloned into pCR-BluntII-TOPO or pCRII-TOPO (Invitrogen). Primers, conditions used for PCR and cloning details will be available on request. The sequence of DNA constructs was verified by cycle sequencing.

Tomato *SUMO-1* (1–131 bp) (Hanania *et al.*, 1999) was amplified from a tomato cDNA library (a gift from W. Gruissem) and cloned to create pCRII(*HA-SUMO-1*) and pCRII(*SUMO-1-HA*). For transient expression *in planta*, tomato *HA-SUMO-1* was subcloned as an *XbaI*–*SacI* fragment into pATC940 (a gift from S. Gelvin) containing the superpromoter (Ni *et al.*, 1995), creating pATC940(*HA-SUMO-1*). For *in vitro* transcription and translation, tomato *SUMO-1-HA* was cloned as a *NdeI*–*XhoI* fragment into pET15b (Novagen) creating pET15b(*T-SUMO-1-HA*). Tomato *SUMO-1* 1–131 bp was amplified from pET15b(*T-SUMO-1-HA*) and subcloned as a *BamHI*–*HindIII* fragment into pT7-LO (a gift from J. Clemens) to create T-SUMO(1–96) followed by Ala–Thr–Val–Asn–6×His. Human SUMO-1 was amplified from EST# 2578604 (Research Genetics) and cloned in pET15b (Novagen) as an *NdeI*–*XhoI* fragment creating pET15b(*M-SUMO1-HA*). *RanGAP1* (a gift from N. R. Yaseen) was amplified and cloned into pcDNA3 (Invitrogen) as an *XbaI*–*EcoRI* fragment creating pcDNA3(*HA-RanGAP1*).

The *xopD* open reading frame (ORF; 1–1638 bp) (Noel *et al.*, 2002) was amplified from *Xcv* strain 85-10 genomic DNA by PCR and cloned, creating pCRII(*xopD1-546*). The promoter (815 bp 5' of the predicted ATG) (Noel *et al.*, 2002) and the 5' region of the *xopD* ORF to the *Clal* site (1–945 bp) were similarly amplified and cloned creating pCRII(*P-xopD1-315*). The cloned *xopD* gene fragments were used to generate the following *xopD* variants: pCRII(*P-xopD1-545*), pCRII(*xopD1-545-HA*), pCRII(*xopD1-545-HA C470A*), pCRII(*xopD1-534*), pCRII(*xopD1-282*), pCRII(*xopD283-546*) and pCRII(*xopD283-534*). Numbering refers to gene codon unless specified. For C-terminal deletion constructs, stop codons were introduced after the last coding codon.

To construct pVSP61(*P-xopD-cya*), *P-xopD1-545* was subcloned as a *BglII* fragment into pMS107(*cya*) (Sory and

Cornelis, 1994) creating pMS107(P-*xopD-cya*). For expression in *Xcv*, this P-*xopD-cya* was subcloned as a *HindIII* fragment into pVSP61 (DNA Plant Technology) creating pVSP61(P-*xopD-cya*). For transient expression *in planta*, constructs were cloned into pMDD1 (Mudgett *et al.*, 2000) or pEZRK-LCY (a gift from D. Ehrhardt); both are binary vectors containing the CaMV 35S promoter. pMDD1(*xopD*₁₋₅₄₅-HA) and pMDD1(*xopD*₁₋₅₄₅-HA C470A) were created by subcloning the respective *BamHI*-*XhoI* fragments. EYFP protein fusions were constructed in pEZRK-LCY by subcloning the respective *xopD* *BamHI*-*XbaI* fragments to create pEZRK(EYFP-*xopD*₁₋₅₄₆), pEZRK(EYFP-*xopD*₁₋₅₃₄), pEZRK(EYFP-*xopD*₁₋₂₈₂) and the respective *xopD* *EcoRI* fragments to create pEZRK(EYFP-*xopD*₂₈₃₋₅₄₆) and pEZRK(EYFP-*xopD*₂₈₃₋₅₃₄).

GST-XopD fusion protein was constructed by subcloning the *BamHI*-*XhoI* fragment from pCRII(*xopD*₁₋₅₄₆) into pGEX-5X-3 (Amersham Biosciences). Ulp1 was amplified by PCR from yeast genomic DNA and cloned into pGEX-KG as a *BamHI*-*EcoRI* fragment creating pGEX-KG(GST-Ulp1). The catalytic mutant of XopD was generated using the Stratagene QuikChange™ site-directed mutagenesis kit by replacing cysteine codon 470 (TGC) with an alanine codon (GCC), and mutants were confirmed by DNA sequence analysis.

Protein expression and purification

GST-XopD₁₋₅₄₅, GST-XopD₁₋₅₄₅ C470A, GST-XopD₂₈₃₋₅₄₅, GST-XopD₂₈₃₋₅₄₅ C490A and GST-Ulp1 were expressed in *E. coli* BL21/DE3 cells and then purified by standard GST affinity chromatography (Zhou *et al.*, 1995). Briefly, cells were grown to an optical density of 0.6–0.8 in 2× YT media and then induced with 400 μM IPTG (Roche) for 4 h at room temperature (XopD) or at 37°C (Ulp1). Cells were lysed in PBS, pH 8, 1% Triton X-100 (Fisher Biotech), 0.1% β-mercaptoethanol (βME; Bio-Rad) and 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma) with a cell disrupter (Emulsiflex C5). Protein was bound to glutathione agarose beads and then eluted with 10 mM reduced glutathione as described previously (Zhou *et al.*, 1995). Purified GST fusion proteins were analysed by SDS-PAGE and quantified using a modification of the Lowry procedure (Bailey, 1967). For purification of recombinant T-SUMO-6×His, cells were lysed as described above in 50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 10 mM imidazole (Sigma), 0.05% βME (Bio-Rad) and 1 mM PMSF (Sigma) and purified as specified by Qiagen using Ni-NTA resin chromatography. After dialysis into 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% βME, recombinant T-SUMO-6×His was further purified using a Hi-Trap MonoQ column and a Pharmacia AKTA FPLC.

In vitro protease assays

Tomato SUMO-1-HA, mammalian SUMO-1-HA and mammalian HA-RanGAP were *in vitro* translated in the TNT coupled rabbit reticulocyte lysate system (Promega) with L-[³⁵S]-methionine (Amersham Pharmacia). Mammalian HA-RanGAP was similarly *in vitro* translated in TNT T7 coupled wheat germ extract system (Promega). For each *in vitro* assay, 2 μl of the ³⁵S-labelled translation reaction mixture

was added to 18 μl of either glutathione elution buffer or purified protein at the indicated concentrations in glutathione elution buffer and incubated at the indicated temperatures for 1 h. Samples were resolved by SDS-PAGE, and then the gels were incubated with Amplify fluorographic reagent (Amersham Biosciences) and analysed by autoradiography.

Cysteine protease inhibitors, N-ethylmaleimide (NEM; Sigma) or iodoacetamide (IAA; Sigma), were used for inhibition assays. In these assays, purified protein was exchanged for 50 mM Hepes buffer, pH 7, that did not contain βME or reduced glutathione. For the NEM inhibition assays, 5 mM NEM was added to the purified GST-XopD protein and incubated at room temperature for 20 min followed by the addition of 2 μl of 100 mM L-cysteine and 2 μl of 100 mM βME to stop the reaction. For the IAA inhibition assays, 10 mM IAA was added to purified GST-XopD protein (0.2 mg ml⁻¹) in 50 mM Hepes buffer, pH 7, and incubated at 37°C for 40 min. The inhibited enzymes were tested for *in vitro* proteolytic activity as described above.

To generate SUMO-protein conjugates *in planta*, tomato HA-SUMO-1 was expressed in *N. benthamiana* using the *Agrobacteria* transient expression assay. Forty-eight hours after inoculation, leaves were collected, frozen in liquid nitrogen and ground in a mortar. Tissue was suspended in extraction buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1 mM DTT and 1 mM PMSF) and centrifuged at 4°C for 15 min to remove insoluble material. Crude protein homogenate was incubated with purified protein in glutathione elution buffer and incubated at room temperature for 1 h. Protein samples were resolved by SDS-PAGE and transferred from gels to nitrocellulose (Osmonics) by electroblotting. HA-SUMO and HA-SUMO protein conjugates were detected by immunoblot analysis using mouse monoclonal HA.11 antisera (Covance) and horseradish peroxidase-conjugated secondary antisera (Bio-Rad) by chemiluminescence (Amersham Biosciences).

Mass spectrometry analysis

Purified recombinant T-SUMO-6×his was incubated with purified GST-XopD, and then XopD-cleaved T-SUMO and uncleaved T-SUMO-6×His protein bands were excised from the Colloidal blue-stained SDS gels and digested with trypsin. The tryptic peptides were extracted and analysed by nano-electrospray tandem mass spectrometry using methods similar to those described previously (Shu *et al.*, 2003). Proteins and peptides were identified by database searching of the MS and MS/MS spectra against the NCBI non-redundant databases using KNEXUS software.

CyA protein translocation assay

To determine XopD-Cya enzyme activity *in vitro*, total protein was isolated from *Xcv* 85-10 *hrpG** (Rossier *et al.*, 1999) and *Xcv* 85-10 *hrpG** Δ *hrpF* (Casper-Lindley *et al.*, 2002) strains carrying pVSP61(*xopD-cya*) and then assayed as described previously (Casper-Lindley *et al.*, 2002). To determine XopD-Cya enzyme activity *in vivo*, *Xcv* strains were hand infiltrated into pepper leaves (cultivar Early Calwonder; genotype *bs2*,

bs2) through a small wound using a 1 cc syringe. Leaves were inoculated with a 5×10^8 cells ml^{-1} suspension of bacteria in 1 mM MgCl_2 . Four leaf discs (0.7 cm^2 each) were collected in a microfuge tube, frozen in liquid nitrogen and then processed as described previously (Casper-Lindley *et al.*, 2002). cAMP was measured using the cAMP Biotrak enzyme immunoassay system (Amersham Biosciences). A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein after precipitation with 1 ml of 10% trichloroacetic acid. XopD-Cya enzyme activity is expressed as nmol of cAMP mg^{-1} total protein.

Agrobacterium-mediated transient expression assay

Agrobacterium tumefaciens strain C58C1 pCH32 (Tai *et al.*, 1999) was used for transient expression *in planta*. Strains were grown overnight at 28°C on Luria agar medium containing 100 $\mu\text{g ml}^{-1}$ rifampicin, 5 $\mu\text{g ml}^{-1}$ tetracycline and 35 $\mu\text{g ml}^{-1}$ kanamycin. Bacteria were collected and incubated in induction media (10 mM MES, pH 5.6, 10 mM MgCl_2 and 150 μM acetosyringone; Acros Organics) for 2 h before inoculation. *Nicotiana benthamiana* leaves were hand inoculated with a 6×10^8 cells ml^{-1} suspension of bacteria in induction media. Plants were incubated at room temperature under continuous low light for 2–4 days. Proteins were detected by immunoblot analysis as described previously (Mudgett and Staskawicz, 1999).

Microscopy

Agrobacterium tumefaciens-infected *N. benthamiana* leaves were analysed 48 h after inoculation. Leaf discs were placed on a slide and visualized using a 20 \times and 60 \times objective lens on an inverted microscope (TE200, Nikon). Fluorescence microscopy was performed with a trichroic filter set (Chroma 86006) using excitation at 493 ± 17 nm with image collection through band emission filter 530 ± 40 nm. Images were collected with a cooled CCD camera (model 1300; Princeton Instruments) at 12-bit precision (Diagnostic Instruments).

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