New Type III Effectors from *Xanthomonas campestris* pv. *vesicatoria* Trigger Plant Reactions Dependent on a Conserved N-Myristoylation Motif

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Pathogenicity of the gram-negative plant pathogen *Xanthomonas campestris* pv. *vesicatoria* depends on a type III secretion system, which translocates bacterial effector proteins into the plant cell. In this study, we identified two novel type III effectors, XopE1 and XopE2 (*Xanthomonas* outer proteins), using the AvrBs3 effector domain as reporter. XopE1 and XopE2 belong to the HopX family and possess a conserved putative N-myristoylation motif that is also present in the effector XopJ from *X. campestris* pv. *vesicatoria* 85-10. XopJ is a member of the YopJ/AvrRxv family of acetyltransferases. Confocal laser scanning microscopy and immunocytochemistry revealed that green fluorescent protein fusions of XopE1, XopE2, and XopJ localized to the plant cell plasma membrane. Targeting to the membrane is probably due to N-myristoylation, because a point mutation in the putative myristoylated glycine residue G2 in XopE1, XopE2, and XopJ resulted in cytoplasmic localization of the mutant proteins. Results of hydroxylamine treatments of XopE2 protein extracts suggest that the proteins are additionally anchored in the host cell plasma membrane by palmitoylation. The membrane localization of the effectors strongly influences the phenotypes they trigger in the plant. *Agrobacterium*-mediated expression of *xopE1* and *xopJ* in *Nicotiana benthamiana* led to cell-death reactions that, for *xopJ*, were dependent on the N-myristoylation motif. In the case of *xopE1*(G2A), cell death was more pronounced with the mutant than with the wild-type protein. In addition, XopE2 has an avirulence activity in *Solanum pseudo-capsicum*.

Additional keyword: bacterial spot disease, pepper, tomato.

Plants have evolved effective strategies to recognize possible pathogens and trigger defense reactions against the invader (Nürnberger et al. 2004; Schornack et al. 2006). On the other hand, bacterial plant pathogens employ sophisticated mechanisms to conquer their hosts, i.e., they suppress basic plant defense reactions. Pathogenicity of most gram-negative, bacterial pathogens depends on a highly conserved type III secretion (T3S) system (Büttner and Bonas 2006). The T3S system translocates a large number of type III effector proteins directly into the host cell cytosol. Bacterial mutants in which type III translocation is abolished are nonpathogenic, indicating that the successful interaction with the host depends on effector protein translocation (Alfano and Collmer 2004). Many type III effectors were first identified as products of avirulence (*avr*) genes, which are specifically recognized in a resistant plant that expresses the corresponding resistance gene. Recognition most often results in the induction of the hypersensitive response (HR), a rapid, localized programmed cell-death reaction of the infected tissue that ultimately arrests bacterial growth (Klement 1982; Staskawicz 2001). However, it is now clear that the primary function of many Avr and other effector proteins is in the establishment of the infection, i.e., in virulence (Grant et al. 2006; Mudgett 2005). More recently, effector genes have been discovered by transposon-based screens using reporter-gene fusions (Guttman et al. 2002; Roden et al. 2004b), their coregulation with the T3S system, and bioinformatic approaches (Chang et al. 2005; Fouts et al. 2002; Noël et al. 2001, 2002, 2003; Schechter et al. 2006).

An important question concerns the localization and molecular function a given effector protein displays inside the plant cell. Several effectors have an enzymatic function (Grant et al. 2006; Mudgett 2005), e.g., AvrPtoB from *Pseudomonas syringae*, an E3-ubiquitin ligase with plant cell death suppressing activity (Abramovitch et al. 2006; Janjusevic et al. 2006). Another example is XopD (*Xanthomonas* outer protein D) from *Xanthomonas campestris* pv. *vesicatoria*, which belongs to the C48-family of SUMO (small ubiquitin-like modifier) cysteine proteases and localizes to the plant cell nucleus (Hotson et al. 2003). A number of type III effector proteins show a distinct subcellular localization in the plant cell, e.g., targeting to the plasma membrane. Examples are the *Pseudomonas* effectors AvrB, AvrRpm1, AvrPphB, AvrPto, and HopF2, the membrane localization of which depends on N-myristoylation motifs that mediate fatty acid modification of the effector in the eukaryotic cell (Nimchuk et al. 2000; Robert-Seilanianz et al. 2006; Shan

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Nucleotide sequence data for *Xanthomonas campestris* pv. *vesicatoria* 85-10 effectors XopE1, XopE2, and XopJ are available in the GenBank database under accession numbers CAJ21925, CAJ23957, and CAJ23833, respectively.

et al. 2000). Interestingly, the N-myristoylation motif of these effectors is required for their avirulence and virulence function (Nimchuk et al. 2000; Robert-Seilanianetz et al. 2006; Shan et al. 2000).

Our laboratory studies the T3S system and type III effector proteins from *Xanthomonas campestris* pv. *vesicatoria* (also designated *Xanthomonas axonopodis* pv. *vesicatoria* [Vauterin et al. 2000] or *Xanthomonas euvesicatoria* [Jones et al. 2004]), the causal agent of bacterial spot disease in pepper and tomato (Gürlebeck et al. 2006). The T3S system of *X. campestris* pv. *vesicatoria* is encoded by the 23-kb chromosomal *hrp* (hypersensitive response and pathogenicity) gene cluster (Gürlebeck et al. 2006). *hrp* mutants are unable to grow and to cause disease symptoms in susceptible plants or to induce the HR in resistant host and nonhost plants (Bonas et al. 1991). Expression of *hrp* and most effector genes is induced in planta (Noël et al. 2001, 2002, 2003; Schulte and Bonas 1992). Key regulators are the OmpR-family regulator HrpG and HrpX, an AraC-type transcriptional activator whose expression is induced by HrpG (Wengelnik and Bonas 1996; Wengelnik et al. 1996). HrpX binds to the *cis*-regulatory PIP (plant-inducible promoter) box in the promoter region of regulated genes (Koebnik et al. 2006).

Up to now, 15 type III effector proteins have been confirmed experimentally in *X. campestris* pv. *vesicatoria* 85-10 or closely related strains (Gürlebeck et al. 2006). Recently, the genome sequence of *X. campestris* pv. *vesicatoria* 85-10 (Thieme et al. 2005) was exploited to identify new type III effector candidates. In this study, we present two new *Xanthomonas* type III effector proteins, XopE1 and XopE2, which possess putative N-myristoylation motifs in their N-termini. Such a motif is also present in the effector XopJ, which we described previously (Noël et al. 2003). Subcellular localization studies of XopE1, XopE2, XopJ, and mutant versions thereof, using fusions to the green fluorescent protein (GFP), showed localization to the plant cell plasma membrane that is dependent on the N-myristoylation motif. The motif was also important for the induction of cell-death reactions in *Nicotiana* species triggered by XopE1 and XopJ. For XopE2 an avirulence activity in *Solanum pseudocapsicum* was uncovered that was independent of the N-myristoylation motif.

RESULTS

Identification and verification of the new type III effectors XopE1 and XopE2.

Homology searches of the genomic sequence of *X. campestris* pv. *vesicatoria* 85-10 (Thieme et al. 2005) identified new putative type III effector genes. Here, we describe the chromosomal genes *xopE1* (XCV0294) and *xopE2* (XCV2280), which are highly identical to each other (79% DNA sequence identity). The predicted proteins XopE1 and XopE2 share 69% amino-acid sequence identity and belong to the HopX2 subgroup of the HopX family of type III effector proteins (63 and 79%, respectively, amino-acid sequence identity to HopX2 from *P. syringae* pv. *maculicola*) (Guttman et al. 2002; Lindeberg et al. 2005; Rohmer et al. 2004). The HopX family includes homologs of AvrPphE (HopX1 subgroup) and HopPmaB (HopX2 subgroup) from *P. syringae*, *Ralstonia solanacearum*, and *Xanthomonas* spp. (Lindeberg et al. 2005; Rohmer et al. 2004). All proteins of the HopX2 subgroup possess a putative N-myristoylation motif, which has not yet been studied (discussed below) (Table 1).

To investigate whether XopE1 and XopE2 are indeed type III effectors, we tested for their secretion in vitro and translocation into the plant cell. For this, we generated translational fusions with the reporter protein AvrBs3Δ2, which lacks a T3S and translocation signal (Noël et al. 2003; Szurek et al. 2002). Fusion of a functional T3S and translocation signal to AvrBs3Δ2 leads to its translocation and thus the induction of the HR in Early Cal Wonder (ECW)-30R pepper plants, which harbor the corresponding resistance gene *Bs3* (Noël et al. 2003). The promoter and 5' coding regions of *xopE1* and *xopE2*, respectively, were fused to *avrBs3Δ2* in pL6GW356 (Noël et al. 2003). As controls, we used AvrBs3Δ2 (Szurek et al. 2002), AvrBs3₁₋₂₀₀-AvrBs3Δ2 (Noël et al. 2003), and HrpF₁₋₃₈₇-AvrBs3Δ2, which is secreted but not translocated (Büttner et al. 2002). The fusion constructs were conjugated into *X. campestris* pv. *vesicatoria* strains 85* (Wengelnik et al. 1999), the T3S mutant 85*Δ*hrcV*, which is deleted in a gene coding for a conserved inner membrane component of the T3S system (Rossier et al. 2000), and 85*Δ*hrpF*, which is T3S-competent but translocation deficient (Büttner et al. 2002). Strain 85* is a

Table 1. Conserved N-termini of effector proteins from *Xanthomonas* and *Pseudomonas* spp. harbor putative N-myristoylation signals

Organism ^a	Effector protein ^b	Accession number	N-terminal amino-acid sequence ^c	Putative function or family membership
<i>Xcv</i> 85-10	XopE1 ^d	CAJ21925	MGLCISKPAMSGSSVAA	HopX2 family
	XopE2 ^d	CAJ23957	MGLCSSKPSVVGSPVAGSPE	HopX2 family
	XopJ ^d	AAK72486	MGLCVSKPSVAGSPEHYAAH	acetyltransferase
<i>Xac</i> 306	AvrXacE1	AAM35178	MGLCVSRPATSGSSVAA	HopX2 family
	AvrXacE3*	AAM39257	MGLCSSKPSVAGSPVAGSPE	HopX2 family
	XAC3230	AAM38074	MGLCTSKPSVVGSPVAGSPE	ADP-ribosyl transferase
<i>Xcc</i>	AvrXccB*	AAM42989	MGLCNSKSVAGSVVGSPIAT	Acetyltransferase
ATCC33913	AvrXccC*	AAM41397	MGLCASKPSVAGSPARYTTH	AvrB1 homolog
	AvrXccE1	AAM40923	MGLCVSKPSVAGSPDHYATH	HopX2 family
<i>Xcc</i> 8004	XC_2004*	AAY49067	MGLCASKPSVAGSPARYTTH	AvrB1 homolog
	XC_2602	AAY49651	MGLCVSKPSVAGSPDHYATH	HopX2 family
	XC_3802*	AAY50842	MGLCNSKSVAGSVVGSPIAT	Acetyltransferase
<i>P. syringae</i>	HopX2 ^d	AAL84240	MGLCVSKGSTASSPQHYAVR	HopX2 family
	HopZ2 ^d	CAC16700	MGLCVSKPSVRHDYNYGR	Acetyltransferase

^a *X. campestris* pv. *vesicatoria* 85-10 (*Xcv* 85-10), *X. axonopodis* pv. *citri* 306 (*Xac* 306), *X. campestris* pv. *campestris* ATCC33913 and 8004 (*Xcc* ATCC33913, *Xcc* 8004), *P. syringae* pv. *maculicola* (HopX2) and *P. syringae* pv. *pisi* (HopZ2).

^b For *Pseudomonas* effectors the unified nomenclature was used (Lindeberg et al. 2005). Asterisks indicate a manual change of the predicted translational start position (GLIMMER-based gene prediction leads to approximately 40% wrongly assigned start codons [Zhu et al. 2004]). The here-predicted start codon for *avrXccC* was recently verified experimentally (Castañeda et al. 2005).

^c Characteristic features of the N-myristoylation signal. The putatively myristoylated glycine residue is labeled in black. Residues likely to interact with the active center of the eukaryotic N-myristoyl transferase are shaded in light gray. The conserved cysteine residue (white) might be palmitoylated. Residues that might interact with the surface of the N-myristoyl transferase are shaded in dark gray. The less well-conserved hydrophilic linkers are boxed.

^d Experimentally verified type III effectors: XopE1 and XopE2 (this study); XopJ (Noël et al. 2003); HopX2 (Guttman et al. 2002); HopZ2 (Arnold et al. 2001).

Fig. 1. The N termini of XopE1 and XopE2 harbor type III secretion signals and target the reporter AvrBs3 Δ 2 into the plant cell. **A**, In vitro secretion assay of Xop-AvrBs3 Δ 2 fusion proteins. *X. campestris* pv. *vesicatoria* strains 85*, the T3S-deficient mutant 85* $\Delta hrcV$, and the translocation-deficient mutant 85* $\Delta hrpF$ expressing the AvrBs3 Δ 2 chimeras as indicated were incubated in secretion medium. Equal protein amounts of total extracts and supernatants were analyzed by immunoblotting, using an AvrBs3-specific antibody. Membranes were reprobed with a specific antibody against the intracellular protein HrcN to ensure that no bacterial lysis had occurred. **B**, In vivo translocation assay. *X. campestris* pv. *vesicatoria* strains 85*, 85* $\Delta hrcV$, and 85* $\Delta hrpF$ expressing the AvrBs3 Δ 2 fusion constructs as indicated were infiltrated into AvrBs3-responsive ECW-30R plants. Leaves were harvested two days postinoculation and bleached in ethanol for better visualization of the hypersensitive response. **C**, *xopE1* and *xopE2* belong to the Hrp regulon. Reverse transcription-polymerase chain reaction analysis of the type III effector genes *xopE1* and *xopE2*. *X. campestris* pv. *vesicatoria* strains 85-10, 85*, and 85* $\Delta hrpX$ were grown in NYG medium (Daniels et al. 1984). 16S rRNA was used as constitutive control. The DNA samples were separated on a 1.5% agarose gel and were stained with ethidium bromide. The results of one representative experiment are shown.

derivative of strain 85-10 and carries a point mutation in the regulatory gene *hrpG* leading to constitutive expression of the T3S system (Wengelnik et al. 1999). Expression of the fusion proteins in *Xanthomonas* was verified by Western blot analysis, using an AvrBs3-specific antibody (Fig. 1A). When the bacteria were incubated in secretion medium, XopE1₁₋₁₃₅ and XopE2₁₋₁₅₀-AvrBs3 Δ 2 were detected in the culture supernatants of strain 85* and 85* $\Delta hrpF$ but not of 85* $\Delta hrcV$ (Fig. 1A). To ensure that no bacterial lysis had occurred, the blots were reprobed with an antibody against HrcN, an intracellular component of the T3S system (Rossier et al. 2000) (Fig. 1A). These results demonstrate that XopE1 and XopE2 contain functional T3S signals in their N-terminal regions.

To test for type III-dependent translocation, strains 85*, 85* $\Delta hrcV$, and 85* $\Delta hrpF$ expressing AvrBs3 Δ 2 and the fusion constructs described above, respectively, were inoculated into leaves of ECW-30R plants and the near-isogenic susceptible pepper line ECW that lacks the *Bs3* resistance gene. Strain 85* derivatives expressing AvrBs3₁₋₂₀₀-AvrBs3 Δ 2, XopE1₁₋₁₃₅-AvrBs3 Δ 2, and XopE2₁₋₁₅₀-AvrBs3 Δ 2 fusions, respectively, induced the HR in ECW-30R (Fig. 1B) but not in ECW plants (data not shown). As expected, no HR induction was observed in plants infected with strain 85* expressing AvrBs3 Δ 2 (Fig. 1B), HrpF₁₋₃₈₇-AvrBs3 Δ 2 (data not shown) (Büttner et al. 2002), and strains 85* $\Delta hrcV$ and 85* $\Delta hrpF$ expressing the chimeric proteins (Fig. 1B). Taken together, the type III-dependent translocation demonstrates that XopE1 and XopE2 are indeed effector proteins.

xopE1 and *xopE2* are members of the HrpG regulon.

To analyze the regulation of expression of the new effector genes, we performed reverse transcription-polymerase chain reaction (RT-PCR) analyses of the *X. campestris* pv. *vesicatoria* wild-type strain 85-10, its derivative 85*, and the *hrpX* deletion mutant 85* $\Delta hrpX$ (Noël et al. 2001) grown in complex NYG medium (Daniels et al. 1984). As shown in Figure 1C, transcripts of *xopE1* and *xopE2* are highly abundant in strain 85* but not (*xopE1*) or in much lower amounts (*xopE2*) in strains 85-10 and 85* $\Delta hrpX$. This indicates that *xopE1* and *xopE2* are HrpG- and HrpX-dependently expressed and, thus, coregulated with the T3S system.

XopE1, XopE2, and XopJ harbor a conserved N-myristoylation motif and localize to the plant cell plasma membrane.

Inspection of the XopE1 and XopE2 amino-acid sequences revealed a putative N-myristoylation motif in both N-termini. These typical eukaryotic motifs suggest that XopE1 and XopE2 might be acylated in the host cell cytosol and targeted to the plasma membrane (Farazi et al. 2001) (Table 1). The N-myristoylation motif is conserved in other effectors from *Xanthomonas* and *Pseudomonas* spp. (Table 1) and suggests myristoylation of the N-terminal glycine residue (G2) in planta. Additionally, the conserved cysteine residue at position 4 could be palmitoylated (Linder and Deschenes 2007) (Table 1). XopE1, XopE2, and the effector XopJ (Noël et al. 2003) are the only proteins of *X. campestris* pv. *vesicatoria* 85-10 that possess this motif.

To investigate the subcellular localization of XopE1, XopE2, and XopJ, we used GFP as reporter. The three genes were translationally fused to *gfp* in a binary vector under control of the *Cauliflower mosaic virus* 35S promoter and transiently expressed in leaves of the solanaceous plant *Nicotiana benthamiana*, using *Agrobacterium*-mediated gene transfer. Immunoblot analysis of XopE1 and XopJ fusion proteins 30 h postinoculation (hpi) and of XopE2 fusions 48 hpi showed stable expression (Fig. 2A). Subcellular localization of the GFP

fusion proteins was determined by confocal laser scanning microscopy of lower epidermal cells. The GFP control was clearly detectable in the cytoplasm surrounding chloroplasts and nuclei, in the nuclei, and in cytoplasmic strands spanning the tonoplast (Fig. 3A and B). Nuclei were visualized by DAPI (4',6-diamidino-2-phenylindole) staining. In contrast to GFP, the fluorescence of the XopE1::GFP, XopE2::GFP, and XopJ::GFP fusions was clearly confined to the periphery of the cells and was not detectable in the nucleus or in the cytoplasm (Fig. 3A and B).

Localization of the XopE2::GFP and XopJ::GFP fusion proteins to the plasma membrane of *N. benthamiana* mesophyll cells could be confirmed by immunocytochemistry. XopE1 was not studied because of its low expression. Leaf sections of *Agrobacterium*-infected *N. benthamiana* were analyzed 30 hpi by immunofluorescence, using anti-GFP and fluorescent Alexa 488 antibodies. As shown in Figure 4A, XopE2::GFP and XopJ::GFP were only detected in the periphery of the mesophyll cells and not in the cytoplasm surrounding the chloroplasts or the DAPI-stained nucleus. Untreated leaf material and GFP were used as negative controls (Fig. 4A). Bright green fluorescence of immunolabeled GFP was detectable in the cytoplasm surrounding the chloroplasts and in the nucleus, as expected (Fig. 4A).

To confirm the localization on the ultrastructural level we carried out electron microscopy of *N. benthamiana* leaf tissue expressing XopJ::GFP. The protein was detected by immunolabeling with anti-GFP and gold particle-conjugated antibodies and showed a predominant localization to the plasma membrane (Fig. 4B).

Localization and modification of XopE1, XopE2, and XopJ in the plant cell depends on the N-terminal glycine residue.

To test whether the membrane localization of XopE1, XopE2, and XopJ is due to myristoylation, the glycine residue at amino-acid position 2 of each protein was substituted by alanine or glutamic acid, using site-directed mutagenesis. The mutant fusion proteins XopE1(G2A)::GFP, XopE2(G2A)::GFP, XopE2(G2E)::GFP, and XopJ(G2E)::GFP in *N. benthamiana* clearly localized to the cytoplasm, similarly to the GFP control (Fig. 3A; data not shown). Immunocytochemistry of mesophyll cells confirmed these results. XopE2(G2E)::GFP and XopJ(G2E)::GFP were detected in the cytoplasm and nucleus (Fig. 4A). The cytoplasmic localization of the XopJ(G2E)::GFP fusion protein was confirmed by electron microscopy (Fig. 4B).

Immunoblot analyses demonstrated that the fusion proteins had the expected molecular mass, indicating that the difference in localization was not due to protein instability (Fig. 2A; data not shown). Thus, the glycine residue at amino-acid position 2 of XopE1, XopE2, and XopJ is essential for localization to the plant cell plasma membrane.

Close inspection of the immunoblot in Figure 2A revealed a higher molecular mass of XopE2::GFP and XopJ::GFP compared with the respective myristoylation motif mutants. For XopE1, a size difference was barely detectable (Fig. 2A). Together with the localization studies (Figs. 3 and 4), these data strongly suggest that the wild-type XopE1, XopE2, and XopJ proteins are modified in planta. We reasoned that the post-translational modification could be covalent linkage of acyl chains subsequent to myristoylation, e.g., palmitoylation via thioester bonds (Linder and Deschenes 2007). To examine this possibility, protein extracts from *N. benthamiana* leaves expressing XopE2::GFP and XopE2(G2A)::GFP were treated with hydroxylamine to release thioester bonds (Linder and Deschenes 2007). Immunoblot analyses revealed two distinct signals that correspond in size to the untreated control ("Tris") and the XopE2(G2A)::GFP mutant version (Fig. 2B). These

findings strongly suggest that XopE2 is modified in the plant cell by myristoylation and subsequent palmitoylation.

XopE1, XopE2, and XopJ trigger cell death in different *Solanaceae* spp.

To study the contribution of *xopE1* and *xopE2* to bacterial virulence, nonpolar deletions of both genes were generated in *X. campestris* pv. *vesicatoria* 85-10. A *xopJ* mutant has been described earlier (Noël et al. 2003). The single and double *xopE1* and *xopE2* mutants were tested for symptom formation and growth in susceptible pepper plants (ECW) and for the HR elicitation in resistant pepper plants (ECW-10R). Strain 85-10 expresses the avirulence protein AvrBs1, which is recognized in ECW-10R, which harbors the *Bs1* resistance gene (Minsavage et al. 1990). Bacterial strains carrying single or double deletions in *xopE1* and *xopE2* showed no significant difference in disease symptoms and the HR induction when compared with wild-type strain 85-10, even at low inoculation densities (data not shown). Furthermore, growth of single *xopE1* and *xopE2* mutants and the double mutant in susceptible pepper plants was not significantly altered (Fig. 5). Similar results were obtained previously for a *xopJ* mutant (Noël et al. 2003).

Because no obvious virulence or avirulence activities were identified for XopE1, XopE2, and XopJ in the host plant *Cap-sicum annuum*, we inoculated 17 different solanaceous species with *Xanthomonas* wild-type and *xopE1/xopE2* double mutant strains and with *Agrobacterium* strains mediating the expression of *xopE1*, *xopE2*, *xopJ*, and *gfp*, respectively. Differential

Fig. 2. In planta expression of wild-type and mutant Xop::GFP fusions. **A**, XopE1::GFP, XopE2::GFP, and XopJ::GFP fusion proteins are stably expressed in planta. *Agrobacterium* strains that mediate T-DNA-based delivery of *xopE1::gfp*, *xopE1(G2A)::gfp*, *xopE2::gfp*, *xopE2(G2A)::gfp*, *xopE2(G2E)::gfp*, *xopJ::gfp*, *xopJ(G2E)::gfp*, and *gfp*, respectively, under control of the *Cauliflower mosaic virus* 35S promoter were infiltrated into *N. benthamiana* leaves. Samples were collected 30 (XopJ fusions) and 48 h postinoculation (XopE2 fusions), and equal protein amounts were analyzed by immunoblotting, using a GFP-specific antibody. Specific signals are marked with asterisks. **B**, XopE2 is acylated in the plant. XopE2::GFP and XopE2(G2A)::GFP samples prepared as in A were incubated in 0.5 M hydroxylamine, pH 7 (HA), or in 0.5 M Tris, pH 7 (Tris). Immunoblot analysis was carried out as in A.

Fig. 3. Membrane localization of Xop::GFP fusions depends on the putative N-myristoylation motifs. *Agrobacterium* strains that mediate T-DNA-based transfer of *gfp*, *xopE1::gfp*, *xopE1(G2A)::gfp*, *xopE2::gfp*, *xopE2(G2E)::gfp*, *xopJ::gfp*, and *xopJ(G2E)::gfp*, respectively, were infiltrated into *Nicotiana benthamiana* leaves. For confocal laser scanning microscopy of lower epidermal cells, samples were taken 30 (XopE1 and XopJ fusions) and 48 h postinoculation (XopE2 fusions). **A**, GFP fluorescence is shown in green (left panel) and diamidin-2-phenylindol (DAPI)-staining of nuclei (white) in the second panel. The rightmost panel is a merged view including chloroplast autofluorescence (red). The size bars correspond to 50 μ m. **B**, Magnifications of nucleus- and chloroplast-containing areas from the merged view in A, respectively. White arrows indicate cytoplasmic strands that span through the tonoplast. The size bars correspond to 20 μ m. Note that, for XopE1::GFP, a different section from the same experiment is shown.

phenotypes were analyzed further. The results of *Agrobacterium*-mediated expression assays could be reproduced with GFP-tagged and untagged protein variants.

As shown in Figure 6A, *X. campestris* pv. *vesicatoria* 85-10 triggered an HR in *Solanum pseudocapsicum*, whereas 85* Δ *hrcV* and the 85-10 *xopE1/xopE2* double mutant derivative did not. Complementation assays showed that the double mutant 85-10 Δ *xopE1* Δ *xopE2* could only be complemented by the *xopE2* gene (Fig. 6A).

Agrobacterium-mediated expression of *xopE1* and *xopJ*, respectively, triggered a cell death reaction in *N. bentha-*

miana and *N. clevelandii* 3 to 4 days postinoculation (dpi) (Fig. 6B and C; data not shown). Interestingly, the mutant derivative *XopE1*(G2A) also triggered cell death in *Nicotiana* spp., but the reaction was faster and stronger (Fig. 6B). Immunoblot analyses showed that this phenotype was not due to higher expression levels of the *XopE1*(G2A) mutant version (Fig. 2A). These results suggest that cell death triggered by *XopE1* is normally suppressed when the protein is targeted to the plasma membrane. In contrast, the *XopJ*(G2E) mutant protein did not trigger cell death in *Nicotiana* spp. (Fig. 6C). Hence, the membrane localization

Fig. 4. Immunocytochemistry confirms N-myristoylation motif-dependent membrane localization of *XopE2*::GFP and *XopJ*::GFP fusions. *Agrobacterium* strains that mediate T-DNA-based transfer of *gfp*, *xopE2::gfp*, *xopE2*(G2E)::*gfp*, *xopJ::gfp*, and *xopJ*(G2E)::*gfp*, respectively, under control of the *Cauliflower mosaic virus* 35S promoter were infiltrated into *Nicotiana benthamiana* leaves. Samples were taken 30 h postinoculation. **A**, GFP and GFP fusion proteins were visualized in mesophyll cells by immunofluorescence using anti-GFP and fluorescent Alexa 488 antibodies. Background of an untreated leaf area (pale green, upper left panel) and detection of GFP and fusion proteins (bright green) is shown in the left panel. The second panel shows diamidin-2-phenylindol (DAPI) staining of the respective areas in the left panels. Nuclei appear blue. The size bars correspond to 50 μ m. **B**, Ultrastructural localization of *XopJ*::GFP and *XopJ*(G2E)::GFP fusion proteins by transmission electron microscopy in mesophyll cells of *N. benthamiana*. Tissue samples were cryo-substituted and incubated with polyclonal anti-GFP antibody and a gold particle-conjugated secondary antibody. For the sake of clarity gold particles were marked with blue arrows. The size bars correspond to 500 nm. (C = chloroplast, CW = cell wall, P = peroxisome).

of XopJ appears to be essential for the cell death reaction in *Nicotiana* spp.

DISCUSSION

In this study, we characterized two new type III effector genes, *xopE1* and *xopE2*, which increases the number of known type III effectors in *X. campestris* pv. *vesicatoria* 85-10 to a total of 17 proteins. Most type III effector genes discovered recently in *Xanthomonas* are coregulated with the T3S system (Noël et al. 2001, 2002, 2003). This is also true for *xopE1* and *xopE2*, the promoter regions of which contain a PIP box. This conserved promoter motif has recently been shown to be bound by the AraC-type transcriptional activator HrpX (Koebnik et al. 2006).

XopE1 and XopE2 belong to the HopX effector family, which includes AvrPphE, HopX2, and homologs from subspecies of the plant pathogen genera *Pseudomonas*, *Ralstonia*, and *Xanthomonas* (Lindeberg et al. 2005; Rohmer et al. 2004). Members of the HopX family are part of the transglutaminase superfamily, and the respective conserved amino-acid residues are important for their function (Nimchuk et al. 2007). However, the transglutaminase superfamily encompasses proteins with different enzymatic functions, e.g., proteases, peptide N-glycanases, and DNA repair proteins (Anantharaman et al. 2001; Makarova et al. 1999). Due to the functional diversity of the transglutaminase family, the enzymatic activity of XopE1 and XopE2 is unclear and has to await biological data, i.e., identification of plant targets of XopE1 and XopE2.

Interestingly, all members of the HopX2 subgroup of the HopX family, including XopE1 and XopE2, possess a putative N-myristoylation motif in their N-terminus (Table 1) that has not been studied so far. A homologous N-myristoylation motif was only found in one additional protein in *X. campestris* pv. *vesicatoria* 85-10, XopJ, which belongs to the YopJ/AvrRxv effector family (Noël et al. 2003). Members of this family were thought to function as SUMO proteases (Orth et al. 2000; Roden et al. 2004a). However, it was recently shown that YopJ

from *Yersinia* spp. acts as an acetyltransferase on host cell mitogen-activated protein kinases involved in innate immunity (Mukherjee et al. 2006).

Microscopy analyses revealed that XopE1, XopE2 and XopJ localize to the plant cell plasma membrane, which is most likely due to an N-terminal acylation of the proteins mediated by the N-myristoylation motif (Figs. 3 and 4). Recognition of the N-myristoylation motif in the eukaryotic host cell leads to

Fig. 5. Bacterial growth of *Xanthomonas campestris* pv. *vesicatoria* wild type and *xopE1* and *xopE2* mutant strains in susceptible pepper plants. *X. campestris* pv. *vesicatoria* 85-10 (wild type) and mutant strains 85-10Δ*xopE1*, 85-10Δ*xopE2*, and 85-10Δ*xopE1*Δ*xopE2* were infiltrated into pepper ECW plants. 85Δ*hrcV* served as a negative control. Bacterial multiplication was monitored over a period of eight days. Values represent the mean of three samples from three different plants. Error bars indicate the standard deviations. Results from one representative experiment are shown.

Fig. 6. XopE1, XopE2, and XopJ trigger cell death in different solanaceous plants. **A**, XopE2 triggers cell death in *Solanum pseudocapsicum*. *X. campestris* pv. *vesicatoria* 85-10, 85Δ*hrcV*, and the double mutant 85-10Δ*xopE1*Δ*xopE2* were infiltrated into *Solanum pseudocapsicum*. The latter strain was complemented using pDSK604 constructs that mediate *xopE1* or *xopE2* expression under control of their own promoters. Leaves were harvested 4 days postinoculation (dpi) and were bleached in ethanol for better visualization of the hypersensitive response. **B**, XopE1 triggers cell death in *Nicotiana benthamiana*. *Agrobacterium* strains that mediate T-DNA-based transfer of *gfp*, *xopE1*, and *xopE1*(G2A), respectively, under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter were infiltrated into *N. benthamiana* leaves. Leaves were harvested 4 and 7 dpi and were bleached in ethanol for better visualization of the cell death reactions. **C**, XopJ triggers cell death in *Nicotiana benthamiana*. *Agrobacterium* strains that mediate T-DNA-based transfer of *gfp*, *xopJ::gfp*, and *xopJ*(G2E)::*gfp*, respectively, under control of the CaMV 35S promoter were infiltrated into *N. benthamiana* leaves. Leaves were harvested 3 dpi and were bleached in ethanol for better visualization of the cell death reactions.

the attachment of the 14-carbon saturated fatty acid myristate at the N-terminal glycine residue (Farazi et al. 2001). Indeed, the membrane localization of XopE1, XopE2, and XopJ was dependent on the N-terminal glycine residue (G2) (Figs. 3 and 4). Besides the myristoyl anchor, additional membrane attachment factors are often found, e.g., palmitoylation of a cysteine residue (Table 1). The latter modifications are required for stable binding of the target protein to the membrane (Maurer-Stroh and Eisenhaber 2004). Our finding that hydroxylamine treatment of plant extracts containing the XopE2::GFP protein resulted in a shift in molecular mass of XopE2 strongly suggests that palmitate is covalently linked by a thioester bond to the cysteine residue at amino-acid position 4 (Table 1), a modification which is also discussed for other effector proteins (Maurer-Stroh and Eisenhaber 2004). In animal cells, such dual acylation (myristoylation and palmitoylation) can direct proteins to lipid rafts, specialized membrane microdomains in which signaling molecules are often concentrated (Zacharias et al. 2002). Recently, lipid rafts were also described for plant plasma membranes (Borner et al. 2005), but their role in the interaction with pathogens has not been elucidated yet.

To address the biochemical function of bacterial effectors and the relevance of modifications like N-myristoylation, it is crucial to identify specific effects of the Xops in the plant. However, neither *xopE1* and *xopE2* single and double mutant strains nor a *xopJ* frameshift mutant (Noël et al. 2003) were affected in bacterial growth and symptom formation on susceptible pepper plants under the conditions tested. The same holds true for a mutant in the *X. campestris* pv. *campestris* HopX2 homolog *avrXccE1* (Castañeda et al. 2005). These results suggest subtle contributions of the effectors under study to bacterial virulence or functional redundancy, which is discussed for many type III effectors (Alfano and Collmer 2004). Because the host plant *C. annuum* did not show visible reactions triggered by the effectors tested here, we inoculated *Xanthomonas* mutant and *Agrobacterium* strains into 17 different solanaceous species. This approach identified cell death-triggering activities for all three effectors in different plant species. *Agrobacterium*-mediated expression of XopE1 and XopJ triggered a fast cell-death reaction in nonhost *Nicotiana* plants that was enhanced with the myristoylation-motif mutant XopE1(G2A) but abolished for the XopJ(G2E) mutant version. These results suggest that XopE1 and XopJ are recognized in *Nicotiana* species. In the case of XopE1, the membrane localization delays the detection by the plant surveillance system, which could be of importance for the pathogen. If an effector can escape detection until other effector proteins have suppressed plant defense, the former effector might not be recognized (Mudgett 2005; Grant et al. 2006). In contrast to XopE1, the recognition of XopJ in *Nicotiana* species is completely dependent on the localization at the plant plasma membrane or on its function at the membrane. Similar results were described for the *Pseudomonas* effectors AvrPto, AvrRpm1, AvrB, and HopF (Nimchuk et al. 2000; Robert-Seilanianz et al. 2006; Shan et al. 2000). For XopE2 an avirulence activity in the ornamental plant *S. pseudocapsicum* was identified. Based on our unpublished results, this reaction seems to be independent of the N-myristoylation motif. Specific recognition in the plant often but not always depends on the correct localization of the respective effector protein, e.g., the *Xanthomonas* effector AvrBs4 localizes to the nucleus but is recognized in the cytoplasm (Schornack et al. 2006).

How common is N-myristoylation of type III effectors? Membrane targeting in the host plant cell mediated by N-myristoylation was described previously only for some type III effectors from the plant pathogen *P. syringae* (Nimchuk et al. 2000; Robert-Seilanianz et al. 2006; Shan et al. 2000). How-

ever, analysis of recently published *Xanthomonas* and *Pseudomonas* spp. genome sequences revealed a number of bona fide or putative effectors with N-myristoylation signals that are homologous to the motif described here (Table 1). In conclusion, membrane localization of effectors due to N-myristoylation appears to be quite common. It is noteworthy that N-myristoylation motifs are often conserved in different effectors and that they overlap with the putative N-terminal T3S signal, which is usually not conserved (Alfano and Collmer 2004). This finding suggests that such effectors have evolved by terminal reassortment, a proposed shuffling process that mobilizes T3S signals in the genome and creates new chimeric effector genes (Stavrinides et al. 2006).

In conclusion, XopE1, XopE2, and XopJ are the first *Xanthomonas* effectors that are targeted to the plant plasma membrane, most likely mediated by N-myristoylation. The next challenge is the identification of plant target proteins of XopE1, XopE2, and XopJ, which is crucial to elucidate their molecular function.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Escherichia coli cells were cultivated at 37°C in lysogenic-broth medium, *A. tumefaciens* at 30°C in YEB medium (0.5% BACTO beef extract, 1% BACTO yeast extract, 0.5% BACTO peptone, 0.5% saccharose, 0.2% 1M MgSO₄, pH 7.2) and *X. campestris* pv. *vesicatoria* at 30°C in NYG or in minimal medium A (Ausubel et al. 1996) supplemented with sucrose (10 mM) and casamino acids (0.3%). Plasmids were introduced into *E. coli*, *A. tumefaciens*, and *X. campestris* pv. *vesicatoria* by electroporation using a Bio-Rad 'MicroPulser' (Bio-Rad Laboratories, Munich, Germany) according to manufacturer protocols. Plasmids were also introduced into *X. campestris* pv. *vesicatoria* by conjugation, using pRK2013 as a helper plasmid in triparental matings (Ditta et al. 1980; Figurski and Helinski 1979).

Antibiotics were added to the media at the following final concentrations: ampicillin, 100 µg/ml; chloramphenicol, 30 µg/ml; hygromycin 150 µg/ml; kanamycin, 25 µg/ml (for *Agrobacterium* 100 µg/ml); rifampicin, 100 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 10 µg/ml; cycloheximide, 500 µg/ml.

Plant material and inoculations.

Xanthomonas inoculation into the near-isogenic pepper cultivars ECW, ECW-10R, ECW-30R (Minsavage et al. 1990), and other plants were performed as described previously (Bonas et al. 1991). Translocation assays were carried out as described (Noël et al. 2003). Leaves were harvested 2 dpi and were bleached with ethanol to facilitate better visualization of the HR. To determine the in planta growth of *Xanthomonas* cells, the method described for *Pseudomonas syringae* (Katagiri et al. 2002) was adapted. Bacteria were infiltrated at 5×10^4 CFU/ml into ECW plants. Samples were collected 0, 2, 4, 6, and 8 dpi. For each strain, three leaf disks of 0.5 cm² were harvested and ball-milled (Rentsch GmbH, Haan, Germany) in 100 µl of 10 mM MgCl₂ at 30 Hz for 40 s, and 10 µl of 10-fold dilutions up to 10⁻⁸ were dropped onto selective NYG agar.

For *A. tumefaciens*-mediated transient expression studies, we used strain GV3101 (Van Larebeke et al. 1974). *Agrobacterium* suspensions (OD₆₀₀ = 0.8) were incubated for 3 h at room temperature in infiltration medium (Van den Ackerveken et al. 1996), prior to inoculation into leaves with a needleless syringe.

Plants were kept at 20°C, 70% relative humidity and with 16 h light. Tested solanaceous species included *Brugmansia* spp., *Capsicum annuum*, *Lycopersicon esculentum* MM, *Ly-*

copersicon pennellii LA2963, *Mandragora* spp., *Nicandra physaloides*, *Nicotiana benthamiana*, *N. clevelandii*, *N. tabacum* cv. Havanna, *Petunia* spp., *Physalis alkekengi*, *Physalis peruviana*, *Solanum aviculare*, *S. nigrum*, *S. americanum*, *S. pseudocapsicum*, *S. suaveolens*, and *S. tuberosum*. Each species was tested twice and the development of the HR and disease symptoms were monitored over a period of 8 dpi.

Construction of AvrBs3Δ2 fusion proteins.

To create translational fusions with *avrBs3Δ2*, the promoters and 5' coding sequences of *xop* genes were amplified by PCR from genomic DNA of *X. campestris* pv. *vesicatoria* 85-10, were cloned into pENTR/D-TOPO (Invitrogen, Karlsruhe, Germany), and were recombined into pL6GW356 (Noël et al. 2003). The PCR products contained the first 135 codons and 1,001-bp upstream sequence of *xopE1* and the first 150 codons and 1,018-bp upstream sequence of *xopE2*, respectively. Oligonucleotide sequences are listed in Table 2.

Protein analyses.

Xanthomonas in vitro secretion experiments and Western blot analyses were performed as described (Rossier et al. 1999). For *Agrobacterium*-mediated expression studies, samples (two 0.8-cm² leaf disks) were collected 30 to 48 h postinoculation, were frozen in liquid nitrogen, were ground and resuspended in 120 µl of 8 M urea and 30 µl of 5× Laemmli buffer, and were boiled for 10 min. Proteins were separated on a 12% polyacrylamide gel and were blotted to a PROTRAN membrane (Schleicher and Schuell, Dassel, Germany). For detection of AvrBs3 (Knoop et al. 1991), HrcN (Rossier et al. 2000) and GFP (Invitrogen, Karlsruhe, Germany) specific polyclonal antisera and horse-radish peroxidase-labeled goat α-rabbit antiserum (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) were used. Reactions were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

For the release of thioester bonds, hydroxylamine (pH 7) was added to the plant protein extracts to a final concentration of 0.5 M. Samples were incubated for 2 h at 22°C before loading.

RNA analyses.

RNA extraction, cDNA synthesis, and RT-PCR experiments were performed as described (Noël et al. 2001). Experiments were performed at least three times for each gene with two independent cDNA preparations each. Oligonucleotide sequences are listed in Table 2.

Generation of *xopE1* and *xopE2* mutants and complementation studies.

To mutate *xopE1*, a deletion of 1,194 bp encompassing the complete gene with exception of the start and stop codon was generated. For this, a 1,000-bp fragment upstream and a 998-bp fragment downstream of *xopE1* were amplified from genomic DNA of *X. campestris* pv. *vesicatoria* 85-10 using oligonucleotides (Table 2) harboring appropriate restriction sites. To generate a 1,071-bp deletion encompassing the *xopE2* gene between start and stop codon, a 999-bp fragment upstream and a 1,000-bp fragment downstream were amplified from *X. campestris* pv. *vesicatoria* 85-10 genomic DNA (Table 2). The PCR fragments were cloned into pBluescriptIIKS giving pKSΔ*xopE1* and pKSΔ*xopE2*. The 2-kb *SalI/XbaI* deletion fragments of pKSΔ*xopE1* and pKSΔ*xopE2* were ligated into the suicide vector pK18mob::sacB (Schäfer et al. 1994), resulting in pK18E1 and pK18E2, and were electroporated into *X. campestris* pv. *vesicatoria* 85-10. The deletions were ultimately introduced via double crossing-over into the chromosome. The resulting strains (85-10Δ*xopE1*, 85-10Δ*xopE2*, and 85-10Δ*xopE1ΔxopE2*) were identified by PCR and Southern blot

Table 2. Oligonucleotides used in this study

Name	Sequence	Features ^a
Construction of AvrBs3Δ2 fusions		
xopE1 forward	caccTGCCGTGATGGAAG GCGGCAC	Directional TOPO site
xopE1 reverse	AGACAGGTAACCTACAG GTGGAG	
xopE2 forward	caccCCTTCCTGGGGTTCA GCCGTTC	Directional TOPO site
xopE2 reverse	GCTGCCCGCCAGTAAAT TTTC	...
Reverse-transcription-polymerase chain reaction analyses		
RT_xopE1 forward	CATTTCAAAGCCGGCGA TGTC	...
RT_xopE1 reverse	GTACATGTGGGCGCACG CTTG	...
RT_xopE2 forward	AGTTCAAAGCCGAGCGT GGTG	...
RT_xopE2 reverse	GCTGCCCGCCAGTAAAT TTTC	...
RT_16SrRNA forward	TACGCTAATACCGCATA CGAC	...
RT_16SrRNA reverse	TGGCACGAAGTTAGCCG GTG	...
Generation of mutations in <i>xopE1</i> and <i>xopE2</i>		
ΔxopE1_1	ACGcgtcgacGTGATGGAA GGCGGCACCGTC	SalI site
ΔxopE1_2	CCCaagcttCATGTCGCTCT CCGATCATG	HindIII site
ΔxopE1_3	CCCaagcttAGATGAACGC CCCTTCGATC	HindIII site
ΔxopE1_4	CGCggatccCAGGCCACCA GCAGCCACAAC	BamHI site
ΔxopE2_1	ACGcgtcgacCTGGGGTTC AGCCGACATAC	SalI site
ΔxopE2_2	CCCaagcttCATCTTGATCT CCTGCAGTGAC	HindIII site
ΔxopE2_3	CCCaagcttTGACGATGGA TCAAGCCGGATG	HindIII site
ΔxopE2_4	CGCggatccCGCCTGGACG AACTCGCCCAAG	BamHI site
Complementation of the <i>xopE1xopE2</i> double mutant		
xopE1 promoter forward	GgaattcTGCCGTGATGGA AGCGGCAC	EcoRI site
xopE1 reverse	CCCaagcttTCATCTCGCCA CCGTGACAGG	HindIII site
xopE2 promoter forward	GgaattcCTTCCTGGGGTTC AGCCGTTC	EcoRI site
xopE2 reverse	CCCaagcttTCACCAACTC AAGGGTGGGCG	HindIII site
Generation of XopE1 and XopE2 GFP fusions		
xopE1 forward	caccATGGGACTATGCAT TTCAAAG	Directional TOPO site
xopE1 reverse	TCTCGCCACCGTGACAG GCGG	
xopE1(stop) reverse	CGTTCATCTCGCCACCG TGAC	
xopE2 forward	caccATGGGGCTATGCAG TTCAAAG	Directional TOPO site
xopE2 reverse	CCAACCTCAAGGGTGGGC GAC	
xopE2(stop) reverse	GATCCATCGTCACCAAC TCAAG	Directional TOPO site
xopJ forward	caccATGGGTCTATGCGT TTCAAAGCCGAG	Directional TOPO site
xopJ reverse	TGACTGGCGATCAGAGA TAGCTGTCTCCAG	
xopE1(G2A) forward	caccATGGcCTATGCATT TCAAAGCC	Directional TOPO site
xopE2(G2A) forward	caccATGGcGCTATGCAGT TCAAAG	Directional TOPO site
xopE2(G2E) forward	caccATGGaGCTATGCAGT TCAAAGCCGAG	Directional TOPO site
xopJ(G2E) forward	caccATGGaGCTATGCGTT TCAAAGCCGAG	Directional TOPO site

^a Special features of the oligonucleotides are indicated by lower case letters.

analysis. For complementation, fragments containing the promoters and coding sequences of *xopE1* and *xopE2* were amplified by PCR from genomic DNA of *X. campestris* pv. *vesicatoria* 85-10 and were cloned into pDSK602 (Murillo et al. 1994) using the *EcoRI* and *HindIII* site introduced by the oligonucleotides (Table 2). The resulting plasmids were conjugated into strain 85-10Δ*xopE1*Δ*xopE2*.

Generation of *xopE1*, *xopE2*, and *xopJ* expression and GFP fusion constructs.

To create expression constructs and translational fusions to GFP the *xopE1*, *xopE2*, and *xopJ* genes were amplified with and without stop codons from genomic DNA of *X. campestris* pv. *vesicatoria* 85-10, were cloned into pENTR/D-TOPO (Invitrogen), and were sequenced and recombined into pGWB5 (T. Nakagawa, Research Institute of Molecular Genetics, Matsue, Japan) or pK7FWG2 (Karimi et al. 2002), which, for fragments without stop codon, results in a C-terminal GFP-tag. Point mutations in *xopE1*, *xopE2*, and *xopJ* were introduced using modified forward oligonucleotides (Table 2).

Confocal laser scanning microscopy and immunocytochemistry of Xop::GFP fusions.

Subcellular localization of GFP and GFP fusions in *N. benthamiana* lower epidermal cells were inspected using the confocal laser scanning microscope LSM 510 and the software LSM Image Browser (Carl Zeiss GmbH, Göttingen, Germany) according to protocols from the manufacturer. To visualize plant cell nuclei, samples were treated with a 0.1% DAPI solution.

For immunofluorescence, leaf segments were fixed for 2 h at room temperature in phosphate buffered saline (PBS) containing 3% paraformaldehyde and 0.05% Triton, were dehydrated in ethanol, and were embedded in polyethylene glycol 1500 at 46°C. Thin sections (3 μm) were transferred to poly-L-lysine coated slides, were washed with PBS, and were blocked with 0.1 M NH₄Cl. After additional washing with PBS and blocking with 5% bovine serum albumin, the slides were incubated overnight at 4°C with polyclonal α-GFP antibody (Clontech-Europe, St. Germain, France). Sections were washed and incubated with secondary antibody (goat α-rabbit immunoglobulin G conjugated to Alexa Fluor 488; Invitrogen) for 90 min at 37°C. Sections were stained with DAPI, were enclosed with Citifluor/Glycerol, and were inspected with a fluorescence microscope (Axioskop 20, Carl Zeiss, Jena, Germany).

For ultrastructural localization, 2-mm leaf disks were prepared with a biopsy punch, were vacuum-infiltrated with 8% methanol, were transferred into aluminum planchettes, and were frozen under high pressure with an HPM 10 (BAL-TEC, Balzers, Lichtenstein). The specimens were cryo-substituted for 4 days at -80°C in acetone containing 0.25% glutaraldehyde and 0.1% uranyl acetate, using an FSU (BAL-TEC). After embedding in HM20 (Polysciences Europe, Eppelheim, Germany), ultrathin sections were immunolabeled using a polyclonal α-GFP antibody and goat α-rabbit IgG conjugated to 10-nm gold particles (Sigma, Taufkirchen, Germany). After staining with uranyl acetate and lead citrate in an EM-Stain apparatus (Leica, Bensheim, Germany), sections were examined using an EM 900 transmission electron microscope (Carl Zeiss NTS, Oberkochen, Germany). Micrographs were taken with a Variospeed SSCCD (TRS, Dünzelbach, Germany).

Bioinformatic approaches.

Sequence homology searches were conducted using BLAST (Ye et al. 2006). For motif searches, we used MEROPS (Rawlings et al. 2006) and InterPro (Mulder et al. 2007). N-myristoylation motifs were predicted using NMT (Eisenhaber et al. 2003) and Myristoylator (Bologna et al. 2004).

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In the article “New Type III Effectors from *Xanthomonas campestris* pv. *Vesicatoria* Trigger Plant Reactions Dependent on a Conserved N-Myristoylation Motif” by F. Thieme, R. Szczesny, A. Urban, O. Kirchner, G. Hause, and U. Bonas, Caption text has been added to reflect that Figure 3B shows a magnification of the nucleus- and chloroplast-containing areas from Figure 3A but, for XopE1-GFP, the magnified area does not correspond to the area shown in Figure 3A.

Revised text for Figure 3B

B, Magnifications of nucleus- and chloroplast-containing areas from the merged view in A, respectively. White arrows indicate cytoplasmic strands that span through the tonoplast. The size bars correspond to 20 μm . Note that, for XopE1::GFP, a different section from the same experiment is shown.

Previously Published version

B, Magnifications of nucleus- and chloroplast-containing areas from the merged view in A, respectively. White arrows indicate cytoplasmic strands that span through the tonoplast. The size bars correspond to 20 μm .