The Type III Secretion Chaperone HpaB Controls the Translocation of Effector and Noneffector Proteins From *Xanthomonas campestris* pv. *vesicatoria*

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Accepted 2 August 2017.

Pathogenicity of the gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* depends on a type III secretion (T3S) system, which translocates effector proteins into plant cells. Effector proteins contain N-terminal T3S and translocation signals and interact with the T3S chaperone HpaB, which presumably escorts effectors to the secretion apparatus. The molecular mechanisms underlying the recognition of effectors by the T3S system are not yet understood. In the present study, we analyzed T3S and translocation signals in the type III effectors XopE2 and XopJ from *X. campestris* pv. *vesicatoria*. Both effectors contain minimal translocation signals, which are only recognized in the absence of HpaB. Additional N-terminal signals promote translocation of XopE2 and XopJ in the wild-type strain. The results of translocation and interaction studies revealed that the interaction of XopE2 and XopJ with HpaB and a predicted cytoplasmic substrate docking site of the T3S system is not sufficient for translocation. In agreement with this finding, we show that the presence of an artificial HpaB-binding site does not promote translocation of the noneffector XopA in the wild-type strain. Our data, therefore, suggest that the T3S chaperone HpaB not only acts as an escort protein but also controls the recognition of translocation signals.

Pathogenicity of many gram-negative bacteria depends on the translocation of bacterial effector proteins into eukaryotic cells. Effector proteins interfere with various host cellular processes, such as signaling cascades, gene expression, and defense responses, and promote pathogen survival and multiplication (Büttner 2016; Dean 2011; Raymond et al. 2013). Effector protein delivery is often mediated by the translocation-associated type III secretion (T3S) system, which is conserved in different

plant- and animal-pathogenic bacteria and is evolutionarily related to the bacterial flagellum (Diepold and Armitage 2015; He 1998). While the flagellar T3S system is linked to an extracellular filament, translocation-associated T3S systems are associated with a pilus (up to 2 µm long; in plant-pathogenic bacteria) or needle (40 to 80 nm long, in animal-pathogenic bacteria). T3S pili or needles presumably serve as transport channels for secreted proteins to the host-pathogen interface (Büttner 2012). A channel-like bacterial T3S translocon in the eukaryotic plasma membrane mediates the subsequent translocation of effector proteins (Galán et al. 2014; Mattei et al. 2011; Mueller et al. 2008).

Despite the differences in the extracellular appendages of flagellar and translocation-associated T3S systems, at least eight components of the membrane-spanning secretion apparatus are conserved in different bacterial species. According to a universal nomenclature, conserved components of T3S systems were designated Sct (secretion and cellular translocation) proteins in animal-pathogenic bacteria followed by a letter that refers to the Ysc proteins of the T3S system from *Yersinia* species (Hueck 1998). Sct proteins build the core structural elements of the secretion apparatus, which consists of ring structures in the inner and outer bacterial membrane and a predicted periplasmic inner rod (Büttner 2012; Deng et al. 2017). The inner membrane ring is associated with the export apparatus, which is assembled by members of five families of transmembrane proteins (SctU, V, R, S, and T) and presumably forms a transport channel for T3S substrates (Büttner 2012; Deng et al. 2017). Cytoplasmic components of the T3S system include the ATPase, which presumably provides the energy for, one or both, secretion or unfolding of T3S substrates, and the predicted cytoplasmic (C) ring, which is assembled by SctQ family members (Büttner 2012; Diepold et al. 2015; Hu et al. 2015, 2017).

One model organism for the analysis of T3S systems in plant-pathogenic bacteria is the tomato and pepper pathogen *Xanthomonas campestris* pv. *vesicatoria*, which is the causal agent of bacterial spot disease. The T3S system from *X. campestris* pv. *vesicatoria* is encoded by the chromosomal *hrp* (hypersensitive response and pathogenicity) gene cluster, which is activated in planta or when the bacteria are cultivated in specific minimal media (Bonas et al. 1991; Wengelnik and Bonas 1996; Wengelnik et al. 1996). Among the T3S substrates from *X. campestris* pv. *vesicatoria* are effector, translocon, and pilus proteins as well as the predicted inner rod protein HrpB2 (Büttner and Bonas 2010; Rossier et al. 2000; Weber et al. 2005). Given that the predicted inner rod and the pilus are essential for T3S, a hierarchy in the secretion of different T3S substrates has been proposed. Thus, the T3S substrate specificity likely switches from the early substrate HrpB2 to middle and late substrates including translocon and effector proteins. This predicted hierarchy in T3S

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*The e-Xtra logo stands for “electronic extra” and indicates that seven supplementary figures and one supplementary table are published online.

is presumably established by the cytoplasmic T3S substrate specificity switch (T3S4) protein HpaC, which suppresses the secretion of HrpB2 and promotes the secretion of translocon and effector proteins (Büttner et al. 2006; Lorenz et al. 2008b).

Translocation of translocon and effector proteins is controlled by the T3S chaperone HpaB and its secreted regulator HpaA. HpaB promotes the translocation of effectors, presumably after the assembly of the translocon (Büttner et al. 2004). Previous interaction studies suggested that HpaB interacts with effector proteins and targets them to the ATPase of the T3S system (Büttner et al. 2004; Lorenz and Büttner 2009). The absence of HpaB interferes with effector protein translocation but promotes the translocation of translocon proteins (Büttner et al. 2004). Proteins such as components of the translocon that are only translocated in the absence of HpaB will hereafter be referred to as noneffectors. Experimental evidence suggests that the activity of HpaB is suppressed during the assembly of the T3S system, by the control protein HpaA (Lorenz et al. 2008a). Secretion and translocation of HpaA likely liberates HpaB and, thus, activates HpaB-dependent effector protein secretion after the T3S substrate specificity switch (Lorenz et al. 2008a). The mechanisms underlying the control of effector and noneffector translocation by HpaB, however, are still unknown.

Secretion and translocation of T3S substrates depends on export signals, which are often present in the N-terminal 50 to 100 amino acids and are not conserved on the amino acid level (Arnold et al. 2009; Rüssmann et al. 2002; Schechter et al. 2004; Schesser et al. 1996; Sory et al. 1995). Sequence analyses revealed that the N-terminal regions of T3S substrates are often unstructured and contain specific amino acid compositions or patterns (Arnold et al. 2009; Buchko et al. 2010; Löwer and Schneider 2009; McDermott et al. 2011; Samudrala et al. 2009; Schechter et al. 2004). However, export signals are not exclusively located in N-terminal regions of T3S substrates but have also been identified in C-terminal protein regions or in 5'-untranslated regions of corresponding mRNAs (Allen-Vercos et al. 2005; Anderson and Schneewind 1997, 1999; Chiu et al. 2003; Kim et al. 2007; Majander et al. 2005; Morello and Collmer 2009; Niemann et al. 2013; Tomalka et al. 2012). mRNA-based secretion signals are controversially discussed because a cotranslational secretion presumably does not account for the fast rate of effector protein translocation (Enninga et al. 2005; Mills et al. 2008; Schlumberger et al. 2005; VanEngelenburg and Palmer 2008; Winnen et al. 2008). It was, therefore, proposed that secretion of T3S substrates is mediated by composite signals in the mRNA and the N- or C-terminal protein regions (Niemann et al. 2013; Schechter et al. 2012; Singer et al. 2014).

The mechanisms underlying the recognition of T3S signals by the secretion apparatus are not yet understood. Putative substrate docking sites include the predicted C ring of the T3S system, the cytoplasmic domains of SctU and SctV family members, and the cytoplasmic T3S ATPase, which was shown to interact with chaperone-substrate complexes (Akedo and Galán 2005; Büttner 2012; Deng et al. 2017; Lorenz and Büttner 2009; Thomas et al. 2005). Experimental evidence suggests that the T3S-ATPase dissociates chaperone-effector complexes and is involved in the unfolding of T3S substrates, which is a prerequisite for the passage of proteins across the inner secretion channel (Akedo and Galán 2005; Blocker et al. 2001; Galán et al. 2014; Lorenz and Büttner 2009). To date, experimental evidence for the recognition of export signal by components of the T3S system has been reported for the predicted inner rod protein HrpB2 from *X. campestris* pv. *vesicatoria* and the effector YopR from *Yersinia enterocolitica* (Scheibner et al. 2016; Sorg et al. 2006). In plant-pathogenic bacteria, however, export signals and their possible interactions with components of the T3S system have not yet been intensively studied.

In the present study, we aimed at the localization and characterization of export signals in the effector proteins XopE2 and XopJ from *X. campestris* pv. *vesicatoria*. We identified separate N-terminal T3S and translocation signals as well as minimal translocation signals, which are suppressed in the wild-type strain by the T3S chaperone HpaB. In vivo translocation and in vitro interaction studies showed that N-terminal deletion derivatives of XopE2 and XopJ interact with HpaB and the putative T3S substrate docking protein HrcQ but are not translocated by the wild-type strain. Our data, therefore, suggest that the interaction of effectors with HpaB and the T3S system is not sufficient for translocation.

RESULTS

Localization of secretion signals in the effector proteins XopE2 and XopJ.

To localize T3S signals in the type III effector XopE2, C-terminally c-Myc epitope-tagged derivatives of XopE2 and derivatives thereof deleted in amino acids 2 to 5, 9 to 13, or 2 to 10 were analyzed for in vitro T3S in *X. campestris* pv. *vesicatoria* 85-10hrpG* (85*). Strain 85* is a derivative of the wild-type strain 85-10 and contains HrpG*, which is an active version of the key regulator HrpG and allows the constitutive expression of *hrp* genes (Wengelnik et al. 1999). When bacteria were incubated in secretion medium, XopE2-c-Myc and XopE2_{Δ2-5}-c-Myc were detected in the culture supernatant, suggesting that they were secreted (Noël et al. 2002; Thieme et al. 2007) (Fig. 1A). In contrast, secretion of XopE2-c-Myc derivatives deleted in amino acids 9 to 13 or 2 to 10 was significantly reduced or not detectable (Fig. 1A). Notably, however, stability or synthesis of XopE2_{Δ9-13}-c-Myc in the cell extracts was also reduced (Fig. 1A). All strains secreted the translocon protein HrpF, suggesting that the ectopic expression of *xopE2* derivatives did not interfere with T3S (Fig. 1A). The predicted inner membrane ring protein HrcJ was only detected in cell extracts but not in culture supernatants, indicating that no cell lysis occurred (Fig. 1A).

Comparative sequence analysis revealed that the N-terminal region of XopE2 is homologous to N-terminal regions of the effector proteins XopE1 and XopJ, which contain conserved N-myristoylation signals for membrane targeting in the plant cell (Thieme et al. 2007) (Fig. 1B). To confirm the contribution of the N-terminal 10 amino acids to secretion, we analyzed derivatives of XopJ-c-Myc deleted in amino acids 2 to 5, 2 to 10, or 9 to 13. When compared with XopJ-c-Myc, secretion of XopJ_{Δ2-5}-c-Myc was reduced, whereas XopJ_{Δ2-10}-c-Myc was not detected in the culture supernatant (Fig. 1C). This is in agreement with the results obtained for the corresponding XopE2 deletion derivatives (Fig. 1A). XopJ_{Δ9-13}-c-Myc was stably synthesized and secreted at wild-type levels (Fig. 1C). Taken together, these observations suggest that amino acids 6 to 8 are essential for secretion of XopE2 and XopJ.

The N-terminal 20 amino acids of XopE2 are sufficient for secretion.

To investigate whether the N-terminal regions of XopE2 and XopJ are sufficient for T3S and translocation, we generated fusion proteins between N-terminal effector regions and the AvrBs3Δ2 reporter protein. AvrBs3Δ2 is an N-terminal deletion derivative of the transcription activator-like (TAL) effector AvrBs3 from *X. campestris* pv. *vesicatoria*. TAL effectors bind, via specific amino acids in their central repeat region, to sequence motifs in the promoters of plant target genes and activate gene expression (Boch and Bonas 2010). AvrBs3Δ2 lacks the N-terminal 152 amino acids and, thus, the T3S and translocation signal. However, AvrBs3Δ2 contains the effector domain and, therefore, activates the expression of the resistance gene *Bs3* in AvrBs3-responsive

'Early Cal Wonder' (ECW)-30R pepper plants when delivered as fusion partner of a functional T3S and translocation signal (Boch et al. 2009; Noël et al. 2003; Szurek et al. 2002). *Bs3* expression leads to the initiation of plant defense responses, which are associated with a hypersensitive response (HR), i.e., a local cell death at the infection site (Dangl and Jones 2001; Römer et al. 2007).

We first analyzed the in vitro T3S of AvrBs3Δ2 fusion proteins containing the N-terminal 20, 30, 40, 50, or 150 amino acids of

XopE2 or the full-length protein. All fusion proteins were secreted and detected in the culture supernatants of strain 85* (Fig. 2A). Similar findings were observed for XopJ-AvrBs3Δ2 fusion proteins containing the N-terminal 50 amino acids or the full-length protein (Fig. 2B). It should be noted that all AvrBs3Δ2 fusions were reproducibly secreted; however, the relative secretion levels varied in different experiments. Thus, the N-terminal 20 and 50 amino acids of XopE2 and XopJ, respectively, are sufficient for secretion of the AvrBs3Δ2 reporter.

Fig. 1. Amino acids 6 to 8 of XopE2 and XopJ are essential for type III secretion (T3S). **A**, T3S assays with XopE2-c-Myc and derivatives thereof. Strain 85* containing XopE2-c-Myc or N-terminal deletion derivatives thereof lacking amino acids 2 to 5, 9 to 13, or 2 to 10 was incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting, using antibodies specific for the c-Myc epitope, the inner membrane ring protein HrcJ, and the translocon protein HrpF. **B**, The N-terminal regions of XopE1, XopE2 and XopJ share amino acid identities. The N-terminal amino acid sequences of XopE1, XopE2, XopJ, and XopA are shown. Numbers refer to amino acid positions. Gray boxes indicate identical amino acids. **C**, Deletion of amino acids 2 to 10 abolishes secretion of XopJ-c-Myc. Strain 85* containing XopJ-c-Myc, XopJ_{Δ2-5}-c-Myc, XopJ_{Δ9-13}-c-Myc, or XopJ_{Δ2-10}-c-Myc, as indicated, was incubated in secretion medium. TE and SN were analyzed as described in A.

Fig. 2. The N-terminal 20 amino acids of XopE2 are sufficient for type III secretion. **A**, In vitro secretion of XopE2-AvrBs3Δ2 fusion proteins. Strain 85* encoding XopE2₁₋₂₀, XopE2₁₋₃₀, XopE2₁₋₄₀, XopE2₁₋₅₀, XopE2₁₋₁₅₀, or XopE2_{FL}-AvrBs3Δ2 fusion proteins, as indicated, was incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting, using antibodies specific for AvrBs3 and the inner membrane ring protein HrcJ. **B**, The N-terminal 50 amino acids of XopJ target AvrBs3Δ2 for secretion. Strain 85* containing XopJ₁₋₅₀-AvrBs3Δ2 or XopJ_{FL}-AvrBs3Δ2 was incubated in secretion medium. TE and SN were analyzed by immunoblotting, using antibodies specific for AvrBs3 and the periplasmic protein HrpB1.

XopE2 and XopJ contain separate T3S and translocation signals.

For in vivo translocation assays, strain 85* containing AvrBs3 Δ 2 reporter fusions was infiltrated into leaves of AvrBs3-responsive pepper plants. XopE2_{FL}-AvrBs3 Δ 2 and XopE2₁₋₁₅₀-AvrBs3 Δ 2 induced the AvrBs3-specific HR, suggesting that they were translocated (Fig. 3A). In contrast, AvrBs3 Δ 2 fusion proteins containing the N-terminal 20, 30, 40, or 50 amino acids of XopE2 did not induce the HR when delivered by strain 85* (Fig. 3A). As a control, bacteria were infiltrated into leaves of AvrBs1-responsive pepper plants. All strains induced the AvrBs1-specific HR, suggesting that the ectopic expression of *avrBs3* Δ 2 fusions did not interfere with the delivery of AvrBs1 and, thus, with the activity of the T3S system (Supplementary Fig. S1). Given that all fusions were secreted (discussed above) (Fig. 2A), we assume that XopE2 contains separate secretion and translocation signals within the N-terminal 20 and 150 amino acids. Similarly, the N-terminal 50 amino acids of XopJ, which are sufficient for T3S, did not target the AvrBs3 Δ 2 reporter for translocation, suggesting that the T3S signal is not sufficient for translocation (Fig. 4A).

XopE2 and XopJ contain minimal translocation signals.

We also analyzed the translocation of AvrBs3 Δ 2 fusion proteins in strain 85* Δ hpaB, which lacks the T3S chaperone HpaB. Compared with the wild-type strain 85*, XopE2₁₋₁₅₀-AvrBs3 Δ 2 induced a significantly reduced HR when delivered by strain 85* Δ hpaB (Fig. 3A). In contrast, XopE2_{FL}-AvrBs3 Δ 2 was efficiently translocated in the absence of HpaB (Fig. 3A). In the

hrpG wild-type strain 85-10 Δ hpaB, however, the translocation of both fusions was only detectable when bacteria were infiltrated at high densities (Supplementary Fig. S2). This suggests that the overexpression of *hrp* genes in strain 85* can at least partially compensate for the absence of HpaB.

Notably, XopE2₁₋₄₀-AvrBs3 Δ 2 induced a weak HR when delivered by strain 85* Δ hpaB (Fig. 3A). This implies that the N-terminal 40 amino acids of XopE2 contain a translocation signal that is functional in the absence of HpaB. Translocation signals, which are recognized in *hpaB* deletion mutants but not in the wild-type strain, will hereafter be referred to as minimal translocation signals. Translocation of XopE2₁₋₄₀-AvrBs3 Δ 2 in the *hpaB* deletion mutant was type III-dependent because the translocation-deficient strain 85* Δ hpaB Δ hrpF containing XopE2₁₋₄₀-AvrBs3 Δ 2 failed to induce the HR (Fig. 3B). No HR was observed after inoculation with strain 85* containing XopE2₁₋₅₀-AvrBs3 Δ 2 (Fig. 3A). This was presumably not caused by a misfolding of the AvrBs3 Δ 2 reporter because XopE2₁₋₅₀-AvrBs3 Δ 2 induced the AvrBs3-specific HR when transiently expressed under control of the 35S promoter in AvrBs3-responsive ECW-30R pepper leaves (Fig. 3C). It cannot be excluded that the recognition of the minimal translocation signal in the N-terminal 40 amino acids of XopE2 depends on a certain protein conformation that is altered in the context of larger protein regions.

In addition to XopE2, we also identified a minimal translocation signal in the N-terminal region of XopJ. Thus, XopJ₁₋₅₀-AvrBs3 Δ 2 was translocated by strains 85* Δ hpaB and 85-10 Δ hpaB but not by strain 85* or the translocation-deficient strain 85* Δ hpaB Δ hrpF

Fig. 3. Localization of translocation signals in XopE2. **A**, In vivo translocation assays with XopE2_{1-x}-AvrBs3 Δ 2 fusion proteins. Strains 85*, 85* Δ hrpF (Δ hrpF), and 85* Δ hpaB (Δ hpaB) containing XopE2_{1-x}-AvrBs3 Δ 2 fusion proteins were infiltrated into leaves of AvrBs3-responsive ECW-30R pepper plants. Dashed lines indicate the infiltrated areas. Leaves were destained 3 days postinoculation (dpi). Equal amounts of cell extracts were analyzed by immunoblotting, using an AvrBs3-specific antiserum. Note that reduced amounts of XopE2₁₋₁₅₀-AvrBs3 Δ 2 were detected in the total cell extracts when compared with other XopE2_{1-x}-AvrBs3 Δ 2 fusion proteins. **B**, Translocation of XopE2₁₋₄₀-AvrBs3 Δ 2 is type III-dependent. Strains 85*, 85* Δ hpaB (Δ hpaB), and 85* Δ hpaB Δ hrpF (Δ hpaB Δ hrpF) encoding XopE2₁₋₄₀-AvrBs3 Δ 2 were infiltrated into leaves of AvrBs3-responsive pepper plants. Leaves were destained 3 dpi. Plant reactions and cell extracts were analyzed as described in A. **C**, Transient expression of *xopE2*₁₋₅₀-*avrBs3* Δ 2 in AvrBs3-responsive pepper plants induces the hypersensitive response. *Agrobacterium tumefaciens* GV2260 without expression construct (-) or containing a binary vector encoding XopE2₁₋₅₀-AvrBs3 Δ 2 under control of the 35S promoter was infiltrated, at a density of 8×10^8 CFU ml⁻¹, into leaves of AvrBs3-responsive ECW-30R and AvrBs1-responsive ECW-10R plants. Leaves of ECW-30R plants were destained in ethanol 3 dpi, leaves of ECW-10R plants 2 dpi.

(Fig. 4A and B; Supplementary Fig. S3). In contrast, XopJ_{FL}-AvrBs3Δ2 did not induce the HR when delivered by strain 85*Δ*hpaB*, suggesting that the translocation of the full-length XopJ protein depends on HpaB (Fig. 4A).

The N-terminal regions of XopE2 and XopJ are dispensable for translocation in *hpaB* deletion mutants.

To analyze the contribution of the minimal translocation signals to the delivery of XopE2 and XopJ, we generated XopE2- and XopJ-AvrBs3Δ2 fusion proteins lacking amino acids 2 to 40 and 2 to 50, respectively. Unexpectedly, XopE2_{Δ2-40}-AvrBs3Δ2 induced the AvrBs3-specific HR when delivered by strain 85*Δ*hpaB* but not by strain 85* (Fig. 4C). A partial HR induction was observed with strain 85-10Δ*hpaB* whereas strain 85* or the translocation-deficient strain 85*Δ*hpaB*Δ*hrpF* containing XopE2_{Δ2-40}-AvrBs3Δ2 failed to induce the HR (Fig. 4C and D). Similarly, XopJ_{Δ2-50}-AvrBs3Δ2 was translocated by strains 85*Δ*hpaB* and 85-10Δ*hpaB* but not by strains 85*, 85-10 and 85*Δ*hpaB*Δ*hrpF* (Fig. 4A and B). We, therefore, assume that the absence of HpaB allows the recognition of alternative T3S and translocation signals, which are located outside the N-terminal regions of XopE2 and XopJ. The finding that N-terminal deletion derivatives of XopE2 and XopJ are translocated by *hpaB* deletion mutants also indicates that the N-terminal T3S signals of XopE2 and XopJ are dispensable for protein translocation in the absence of HpaB.

N-terminal deletion derivatives of XopE2 and XopJ interact with the T3S chaperone HpaB and the predicted C ring protein HrcQ.

The mechanisms underlying the recognition of T3S and translocation signals are still largely unknown. Putative substrate docking sites include the ATPase HrcN, which interacts with HpaB-effector complexes, and the putative C ring protein HrcQ (Lorenz and Büttner 2009; Lorenz et al. 2012). To analyze a potential contribution of the N-terminal regions of XopE2 and XopJ to the interaction with HrcQ or HpaB, we performed in vitro glutathione-S-transferase (GST) pull-down assays. For this, GST, GST-XopE2, and GST-XopJ, as well as GST fusions of N-terminal deletion derivatives of XopE2 and XopJ were immobilized on glutathione sepharose and were incubated with bacterial lysates containing HrcQ-c-Myc or HpaB-c-Myc. HrcQ-c-Myc coeluted with all tested GST fusion proteins but was not or only weakly detectable in the eluate of GST (Fig. 5A and B). Similarly, HpaB-c-Myc coeluted with GST fusions of XopE2, XopE2_{Δ2-40}, XopJ, and XopJ_{Δ2-50} but not with GST alone (Fig. 5C and D). These findings suggest that the N-terminal regions of XopE2 and XopJ are dispensable for the interaction with one or both HpaB and HrcQ. The lack of secretion of N-terminal deletion derivatives of XopE2 and XopJ is, therefore, presumably not caused by a lack of interaction with the putative C ring or the T3S chaperone HpaB.

Fig. 4. Identification of minimal translocation signals in XopJ and XopE2. **A**, In vivo translocation assays with XopJ_{1-X}-AvrBs3Δ2 fusion proteins. Strains 85* and 85*Δ*hpaB* (Δ*hpaB*) without expression construct (–) or containing XopJ₁₋₅₀-AvrBs3Δ2, XopJ_{Δ2-50}-AvrBs3Δ2, or XopJ_{FL}-AvrBs3Δ2 were infiltrated into leaves of AvrBs3-responsive ECW-30R pepper plants. Dashed lines indicate the infiltrated areas. Leaves were destained 3 days postinoculation (dpi). The brown color of the leaf area infected with strain 85*Δ*hpaB* containing XopJ_{FL}-AvrBs3Δ2 is a background reaction that was also observed after infection with strain 85*Δ*hpaB* without expression construct. Equal amounts of cell extracts were analyzed by immunoblotting, using an AvrBs3-specific antiserum. **B**, Translocation of XopJ₁₋₅₀-AvrBs3Δ2 and XopJ_{Δ2-50}-AvrBs3Δ2 depends on the type III secretion (T3S) system. Strains 85*, 85*Δ*hpaB* (Δ*hpaB*) and 85*Δ*hpaB*Δ*hrpF* (Δ*hpaB*Δ*hrpF*) encoding XopJ₁₋₅₀-AvrBs3Δ2 or XopJ_{Δ2-50}-AvrBs3Δ2 were infiltrated into leaves of AvrBs3-responsive pepper plants, and leaves were destained 3 dpi. Strains containing XopJ_{Δ2-50}-AvrBs3Δ2 were infiltrated at an optical density of 8×10^8 CFU ml⁻¹. Plant reactions and cell extracts were analyzed as described in A. **C**, Deletion of amino acids 2 to 40 does not abolish translocation of XopE2. Strains 85* and 85*Δ*hpaB* (Δ*hpaB*) without expression construct (–) or encoding XopE2-AvrBs3Δ2 fusion proteins were infiltrated into leaves of AvrBs3-responsive pepper plants. Plant reactions and cell extracts were analyzed as described in A. Plant reactions on ECW-10R plants are shown in Supplementary Figure S4. **D**, Translocation of XopE2_{Δ2-40}-AvrBs3Δ2 depends on the T3S system. Strains 85*, 85*Δ*hpaB* (Δ*hpaB*), and 85*Δ*hpaB*Δ*hrpF* (Δ*hpaB*Δ*hrpF*) encoding XopE2_{Δ2-40}-AvrBs3Δ2 were infiltrated into leaves of AvrBs3-responsive pepper plants. Leaves were destained 3 dpi. Plant reactions and cell extracts were analyzed as described in A.

The noneffector XopA contains separate T3S and minimal translocation signals.

In addition to the effector proteins XopE2 and XopJ, we analyzed export signals in the noneffector protein XopA, which is secreted by the T3S system and contributes to bacterial virulence (Noël et al. 2002). Previous translocation assays suggested that XopA is translocated in the absence of HpaB but not in the wild-type strain (Büttner et al. 2004). To localize the T3S and minimal translocation signal in XopA, we generated c-Myc epitope-tagged derivatives of XopA as well as XopA-AvrBs3Δ2 fusion proteins. In vitro T3S assays showed that XopA-c-Myc was secreted by strain 85*, whereas secretion of an N-terminal deletion derivative lacking amino acids 2 to 5 was reduced (Fig. 6A). Deletion of amino acids 2 to 10 or 2 to 21 abolished the detectable secretion of XopA-c-Myc, which is in agreement with the results obtained for XopE2 and XopJ derivatives (Fig. 6A) (discussed above).

AvrBs3Δ2 fusions containing the N-terminal 20, 25, or 30 amino acids of XopA were secreted by strains 85* and 85*ΔhpaB,

suggesting that amino acids 6 to 20 contain the T3S signal of XopA (Fig. 6B). In vivo translocation assays showed that the N-terminal 30 amino acids of XopA target the AvrBs3Δ2 reporter for translocation in strain 85*ΔhpaB but not in the wild-type strain or the translocation-deficient strain 85*ΔhpaBΔhrpF (Fig. 6C; Supplementary Fig. S5). In contrast, the N-terminal 20 amino acids of XopA were not sufficient for translocation, suggesting that XopA contains separate T3S and translocation signals (Fig. 6C). Deletion of amino acids 2 to 10 but not of amino acids 2 to 5 abolished the translocation of XopA-AvrBs3Δ2 (Fig. 6D). This is in agreement with the finding that amino acids 2 to 5 are dispensable for secretion of XopA (Fig. 6A).

The presence of an artificial HpaB-binding site does not promote the translocation of the noneffector XopA in the wild-type strain.

In contrast to effector proteins, XopA does not interact with the T3S chaperone HpaB and is only translocated in the absence of HpaB (Büttner et al. 2004). To investigate whether the

Fig. 5. N-terminal deletion derivatives of XopE2 and XopJ interact with the predicted C ring protein HrcQ and the type III secretion chaperone HpaB. **A**, N-terminal deletion derivatives of XopE2 interact with HrcQ. Glutathione-S-transferase (GST), GST-XopE2 and GST-XopE2 Δ_{2-40} were immobilized on glutathione sepharose and were incubated with a bacterial lysate containing HrcQ-c-Myc. The total cell extract (TE) containing HrcQ-c-Myc and eluted proteins (eluates) were analyzed by immunoblotting, using antibodies against the c-Myc epitope or GST. GST and GST fusion proteins are marked with asterisks, lower bands represent degradation products. **B**, The N-terminal 50 amino acids of XopJ are dispensable for the interaction with HrcQ. Immobilized GST and GST fusion proteins were incubated with a bacterial lysate containing HrcQ-c-Myc. TE and eluates were analyzed as described in A. **C**, An N-terminal deletion derivative of XopE2 interacts with HpaB. Immobilized GST and GST fusion proteins were incubated with a bacterial lysate containing HpaB-c-Myc. TE and eluates were analyzed as described in A. **D**, The N-terminal 50 amino acids of XopJ are dispensable for the interaction with HpaB. Immobilized GST and GST fusion proteins were incubated with a bacterial lysate containing HpaB-c-Myc. TE and eluates were analyzed as described in A.

presence of an artificial HpaB-binding site would promote translocation of XopA in the wild-type strain, we generated a fusion protein between XopA and the HpaB-binding site of HpaA, which is the secreted regulator of HpaB. The HpaB-binding site was previously localized to the C-terminal 50 amino acids of HpaA (Lorenz et al. 2008a). The interaction of HpaB with the fusion protein consisting of XopA and the C-terminal 50 amino acids of HpaA was confirmed by in vitro GST pull-down assays. Figure 7A shows that HpaB-c-Myc

coeluted with GST-XopA-HpaA_{C50} but not with GST or GST-XopA. Similarly, a C-terminally c-Myc epitope-tagged derivative of XopA-HpaA_{C50} coeluted with GST-HpaB but not with GST (Fig. 7B). In vivo translocation assays revealed that a XopA-HpaA_{C50}-AvrBs3Δ2 reporter fusion induced the HR when delivered by strain 85*Δ*hpaB* but not by the wild-type strain or the translocation-deficient strain 85*Δ*hpaB*Δ*hrpF* (Fig. 7C and D). The results of all in vitro secretion and in vivo translocation assays are summarized in Table 1. Taken together,

Fig. 6. XopA contains separate type III secretion (T3S) and minimal translocation signals. **A**, Amino acids 2 to 5 are dispensable for secretion of XopA. Strain 85*, containing XopA-c-Myc and N-terminal deletion derivatives thereof lacking amino acids 2 to 5, 2 to 10, or 2 to 21, was incubated in secretion medium. Total cell extracts (TE) and culture supernatants (SN) were analyzed by immunoblotting, using antibodies specific for the c-Myc epitope, the inner membrane protein HrcJ, or the secreted translocon protein HrpF. **B**, Amino acids 1 to 20 of XopA target the AvrBs3Δ2 reporter for T3S. Strains 85* and 85*Δ*hpaB* containing XopA₁₋₂₀-AvrBs3Δ2, XopA₁₋₂₅-AvrBs3Δ2, or XopA₁₋₃₀-AvrBs3Δ2 were incubated in secretion medium. TE and SN were analyzed by immunoblotting, using antibodies specific for the c-Myc epitope, the periplasmic protein HrpB1, or the inner membrane protein HrcJ. **C**, Translocation of XopA depends on the N-terminal 30 amino acids. Strains 85* and 85*Δ*hpaB* (Δ*hpaB*), encoding XopA₁₋₂₀-AvrBs3Δ2, XopA₁₋₂₅-AvrBs3Δ2, or XopA₁₋₃₀-AvrBs3Δ2 under control of the *lac* or the native promoter (*pnat*), were infiltrated into leaves of AvrBs3-responsive ECW-30R pepper plants. For better visualization of the hypersensitive response, leaves were destained in ethanol 3 days postinoculation. Dashed lines indicate the infiltrated areas. Equal amounts of cell extracts were analyzed by immunoblotting, using an AvrBs3-specific antiserum. **D**, Amino acids 2 to 5 are dispensable for the translocation of XopA. Strains 85* and 85*Δ*hpaB* (Δ*hpaB*) encoding XopA-AvrBs3Δ2 fusion proteins were infiltrated into leaves of AvrBs3-responsive pepper plants. Plant reactions and cell extracts were analyzed as described in C.

our data suggest that the interaction of T3S substrates with HpaB is not sufficient for translocation.

DISCUSSION

In the present study, we provide experimental evidence that the effectors XopE2 and XopJ from *X. campestris* pv. *vesicatoria* contain composite export signals that control secretion and translocation in wild-type or *hpaB* mutant strains. The results of in vitro T3S assays suggest that the T3S signals of XopE2 and XopJ are located in the N-terminal 20 amino acids with amino acids 2 to 5 being dispensable for T3S. In contrast, amino acids 6 to 8 of XopE2 and XopJ are essential for secretion (Fig. 1). This is in agreement with the previous finding that amino acids 6 to 10 of T3S signals presumably contain critical features for T3S (Wang et al. 2013). The translocation signals of XopE2 and XopJ were previously localized in the N-terminal 150 and 155 amino acids, respectively (Noël et al. 2003; Thieme et al. 2007). Here, we show that the N-terminal 50 amino acids of both proteins are not sufficient for translocation in the wild-type strain, suggesting that XopE2 and XopJ contain separate signals for T3S and translocation (Fig. 3 and 4). Similar findings were reported for the effector protein AvrBs2 from *X. campestris* pv. *vesicatoria* as well as for effector proteins from the plant-pathogenic bacterium *Erwinia*

amylovora and the human pathogen *Yersinia* spp. (Mudgett et al. 2000; Oh et al. 2010; Schesser et al. 1996; Sory et al. 1995). The predicted inner rod protein HrpB2 from *X. campestris* pv. *vesicatoria*, however, presumably contains a joint secretion and translocation signal, suggesting that the organization of export signals can differ in early and late T3S substrates (Scheibner et al. 2016).

It was previously reported that the N-terminal regions of the effector proteins XopE1, XopE2, and XopJ contain N-myristoylation signals, which presumably target the proteins to the plant plasma membrane after translocation (Thieme et al. 2007). The N-myristoylation motifs are conserved in XopE1, XopE2 and XopJ; however, homologous amino acid sequences are absent in the N-terminal regions of other T3S substrates. This is in agreement with the general assumption that T3S signals are not conserved on the amino acid level (Arnold et al. 2009; Buchko et al. 2010; Löwer and Schneider 2009; McDermott et al. 2011; Samudrala et al. 2009; Schechter et al. 2004). Export signals of T3S substrates from plant- and animal-pathogenic bacteria were reported to contain characteristic amino acid compositions including a high content of polar (especially serine) residues (Greenberg and Vinatzer 2003; Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Schechter et al. 2004, 2006). Mutation of predicted characteristic N-terminal amino acids in the type III effector AvrPto from the plant-pathogenic

Fig. 7. XopA-HpaA_{C50} interacts with HpaB and targets the AvrBs3Δ2 reporter for translocation in *hpaB* deletion mutant strains. **A**, GST-XopA-HpaA_{C50} interacts with HpaB-c-Myc in vitro. Glutathione-S-transferase (GST), GST-XopA, and GST-XopA-HpaA_{C50} were immobilized on glutathione sepharose and were incubated with a bacterial cell lysate containing HpaB-c-Myc. The total cell extract (TE) and eluted proteins (eluates) were analyzed by immunoblotting, using antibodies specific for the c-Myc epitope and GST, respectively. **B**, GST-HpaB interacts with XopA-HpaA_{C50}-c-Myc in vitro. GST and GST-HpaB were immobilized on glutathione sepharose and were incubated with bacterial lysates containing XopA-c-Myc or XopA-HpaA_{C50}-c-Myc. TE and eluates were analyzed as described in A. **C**, XopA-HpaA_{C50}-AvrBs3Δ2 is translocated in the absence of HpaB. Strains 85* and 85*ΔhpaB (ΔhpaB) containing XopA-AvrBs3Δ2 or XopA-HpaA_{C50}-AvrBs3Δ2 were infiltrated into leaves of AvrBs3-responsive ECW-30R pepper plants. Dashed lines indicate the infiltrated areas. Leaves were destained in ethanol 3 days postinoculation. Plant reactions in AvrBs1-responsive ECW-10R plants are shown in Supplementary Fig. S6. Equal amounts of cell extracts were analyzed by immunoblotting, using antibodies specific for AvrBs3. **D**, Translocation of XopA-HpaA_{C50}-AvrBs3Δ2 depends on the translocon protein HrpF. Strains 85*, 85*ΔhpaB (ΔhpaB), and 85*ΔhpaBΔhrpF (ΔhpaBΔhrpF), encoding XopA-HpaA_{C50}-AvrBs3Δ2, were infiltrated into leaves of AvrBs3-responsive pepper plants. Plant reactions and cell extracts were analyzed as described in C.

bacterium *Pseudomonas syringae*, however, did not interfere with T3S and translocation, suggesting that the characteristic amino acids in the N-terminal region of AvrPto are dispensable for type III-dependent delivery (Schechter et al. 2012). The analysis of amino acid compositions of XopE2 and XopJ revealed that the N-terminal 40 and 50 amino acids, respectively, contain elevated amounts of serine, proline, and threonine residues and no arginine residues (Supplementary Fig. S7). The content of other polar amino acids was not significantly changed when compared with the full-length proteins. Increased levels of serine residues, however, are also present in the N-terminal region of the cytoplasmic T3S chaperone HpaB and might, therefore, not be specific for N-terminal T3S signals. The contribution of single N-terminal amino acids to the secretion and translocation of T3S substrates from *X. campestris* pv. *vesicatoria* will be in the focus of future studies.

XopE2 and XopJ contain not only T3S and translocation signals but, also, minimal translocation signals in- and outside the N-terminal regions. Minimal translocation signals specifically promote translocation in the absence of HpaB but not in

the wild-type strain and were previously identified in the non-effectors XopA and HrpF (Büttner et al. 2004). It was, therefore, speculated that HrpF and XopA are translocated prior to the HpaA-mediated activation of HpaB. Similarly, the presence of minimal translocation signals in effector proteins might promote their translocation before the activation of HpaB. Notably, however, despite the presence of minimal translocation signals, the full-length XopJ protein is not translocated in the absence of HpaB (Fig. 4). Previous *in vivo* translocation assays already revealed that effector proteins likely differ in their HpaB-dependent translocation. Thus, some effectors, including XopE2, are translocated by *hpaB* deletion mutants, albeit in reduced amounts (Büttner et al. 2006; Schulze et al. 2012). Similar findings were reported for effectors from the related pathogen *Ralstonia solanacearum* (Lonjon et al. 2016). The HpaB-independent secretion and translocation of effectors is indicative of a temporal regulation of effector delivery. Thus, it was speculated that HpaB-independent effectors are translocated during early stages of the host-pathogen interaction (Büttner et al. 2006). A hierarchy in effector protein delivery was previously reported for animal-pathogenic bacteria (Enninga et al. 2005; Mills et al. 2008; Schlumberger et al. 2005; VanEngelenburg and Palmer 2008; Winnen et al. 2008) and might be achieved by differences in the expression of effector genes, specific export signals, or different affinities of effector-chaperone complexes to the T3S system (Deng et al. 2017; Sorg et al. 2007).

Table 1. Summary of the results of *in vitro* type III secretion (T3S) and *in vivo* translocation assays

Protein	Results of T3S assays ^a		Results of translocation assays ^b	
	85*	85*Δ <i>hpaB</i>	85*	85*Δ <i>hpaB</i>
XopE2 derivatives				
XopE2-c-Myc	+	n.d.	n.d.	n.d.
XopE2 _{Δ2-5} -c-Myc	+	n.d.	n.d.	n.d.
XopE2 _{Δ2-10} -c-Myc	–	n.d.	n.d.	n.d.
XopE2 _{Δ9-13} -c-Myc ^c	–	n.d.	n.d.	n.d.
XopE2-AvrBs3Δ2	+	n.d.	+	(+)
XopE2 ₁₋₂₀ -AvrBs3Δ2	+	n.d.	–	–
XopE2 ₁₋₃₀ -AvrBs3Δ2	+	n.d.	–	–
XopE2 ₁₋₄₀ -AvrBs3Δ2	+	n.d.	–	+
XopE2 ₁₋₅₀ -AvrBs3Δ2	+	n.d.	–	–
XopE2 ₁₋₁₅₀ -AvrBs3Δ2	+	n.d.	+	(+)
XopE2 _{Δ2-40} -AvrBs3Δ2	n.d.	n.d.	–	+
XopJ derivatives				
XopJ-c-Myc	+	n.d.	n.d.	n.d.
XopJ _{Δ2-5} -c-Myc	(+)	n.d.	n.d.	n.d.
XopJ _{Δ2-10} -c-Myc	–	n.d.	n.d.	n.d.
XopJ _{Δ9-13} -c-Myc	+	n.d.	n.d.	n.d.
XopJ-AvrBs3Δ2	+	n.d.	+	–
XopJ ₁₋₅₀ -AvrBs3Δ2	+	n.d.	–	+
XopJ _{Δ2-50} -AvrBs3Δ2	n.d.	n.d.	–	+
XopA derivatives				
XopA-c-Myc	+	n.d.	n.d.	n.d.
XopA _{Δ2-5} -c-Myc	(+)	n.d.	n.d.	n.d.
XopA _{Δ2-10} -c-Myc	–	n.d.	n.d.	n.d.
XopA _{Δ2-21} -c-Myc	–	n.d.	n.d.	n.d.
XopA-AvrBs3Δ2	n.d.	n.d.	–	+
XopA ₁₋₂₀ -AvrBs3Δ2	+	+	–	–
XopA ₁₋₂₅ -AvrBs3Δ2	+	+	–	–
XopA ₁₋₃₀ -AvrBs3Δ2	+	+	–	+
XopA _{Δ2-5} -AvrBs3Δ2	n.d.	n.d.	–	+
XopA _{Δ2-10} -AvrBs3Δ2	n.d.	n.d.	–	–
XopA _{Δ2-21} -AvrBs3Δ2	n.d.	n.d.	–	–
XopA-HpaA _{C50} -AvrBs3Δ2	n.d.	n.d.	–	+

^a Proteins were analyzed as C-terminal c-Myc epitope-tagged derivatives for *in vitro* T3S in *X. campestris* pv. *vesicatoria* strains 85* and 85*Δ*hpaB*. n.d. = not determined, + = secreted, – = secretion not detectable, (+) = reduced secretion.

^b Proteins were analyzed for *in vivo* translocation as N-terminal fusion partners of the AvrBs3Δ2 reporter. + = translocation, – = no translocation observed, (+) = reduced translocation.

^c Reduced amounts of XopE2_{Δ9-13}-c-Myc were detected in the cell extracts when compared with XopE2-c-Myc.

Fig. 8. Model of the HpaB-mediated control of protein translocation in *Xanthomonas campestris* pv. *vesicatoria*. The type III secretion (T3S) system consists of a membrane-spanning secretion apparatus and is associated with an extracellular pilus and a translocon in the plant plasma membrane. T3S substrates include extracellular components of the secretion apparatus, such as pilus and translocon proteins, which contain T3S and minimal translocation signals and are referred to as noneffectors. Noneffectors are secreted after the assembly of the membrane-spanning secretion apparatus and are, presumably, translocated into the plant cell in the absence of an active HpaB chaperone (indicated as B). HpaB associates with the T3S system and is activated after a switch in T3S substrate specificity upon secretion and translocation of its regulator HpaA (indicated as A). The activation of HpaB allows the recognition of translocation signals and, thus, the translocation of effector proteins into the plant cell. Given that HpaB interacts with effectors and components of the T3S system, it was proposed to act as an escort protein that targets effectors to the secretion apparatus. Notably, however, some effectors are even translocated before the activation of HpaB, albeit in reduced amounts (indicated by a dashed arrow). Activation of HpaB upon secretion of HpaA suppresses the recognition of minimal translocation signals by the T3S system. The model suggests a role of HpaB in the recognition of translocation signals in addition to its predicted function as an escort protein. IM = inner membrane, OM = outer membrane, and PM = plasma membrane of plant cell.

T3S chaperones including HpaB from *X. campestris* pv. *vesicatoria* were previously reported to interact with effectors and components of the T3S system and were proposed to act as escort proteins that target effectors to the secretion apparatus (Büttner 2012; Chen et al. 2013; Thomas et al. 2012). However, given our finding that N-terminal deletion derivatives of XopE2 and XopJ, which interact with HpaB, are not translocated by the wild-type strain, the interaction of XopE2 and XopJ with HpaB is presumably not sufficient for translocation. In agreement with this finding, the presence of a HpaB-binding site did not promote translocation of XopA in the wild-type strain (Fig. 7). Our translocation assays revealed that HpaB suppresses the translocation of XopA fusion proteins or N-terminal deletion derivatives of XopE2 and XopJ, despite its interaction with these proteins (Figs. 4 and 7). Therefore, we assume that the HpaB-mediated control of translocation is determined by the export signal and not by the presence of a HpaB-binding site. Thus, HpaB likely controls the recognition of translocation signals independently of its interaction with T3S substrates. In line with this hypothesis, we previously reported that the deletion of the HpaB-binding site in HpaA did not affect its HpaB-dependent secretion (Lorenz et al. 2008a). HpaB might be directly involved in the recognition of translocated proteins at the secretion apparatus, possibly by controlling a yet-unknown substrate acceptor site at the inner membrane that is specific for translocated proteins when associated with HpaB (Fig. 8). The absence of HpaB would thus allow the alternative recognition of minimal translocation signals.

One known T3S substrate docking site is the putative C ring protein HrcQ, which interacts with T3S substrates and HpaB (Lorenz et al. 2012). The results of our interaction studies revealed that HrcQ interacts with N-terminal deletion derivatives of XopE2 and XopJ, suggesting that the T3S signal is dispensable for the targeting of T3S substrates to the predicted C ring. Similar findings were previously reported for the interaction of the effector protein AvrBs3 with HrcQ as well as with the cytoplasmic domain of the inner membrane protein HrcV, which is a component of the export apparatus (Hartmann and Büttner 2013; Lorenz et al. 2012). In line with these observations, T3S signals were previously reported to be dispensable for the interaction of chaperone-effector complexes with the ATPase of the T3S system (Chen et al. 2013; Rodgers et al. 2008). Translocation signals might, therefore, be recognized after the initial binding of T3S substrates to components of the T3S system, presumably upon activation of HpaB. In summary, our data suggest that the T3S chaperone HpaB is involved in the recognition of translocation signals independently of its predicted role as an escort protein for effector proteins. The identification of specific recognition sites of the T3S system for secretion and translocation signals remains a challenging topic for future studies.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids are listed in Table 2. *Escherichia coli* was cultivated at 37°C in lysogeny broth, *X. campestris* pv. *vesicatoria* and *Agrobacterium tumefaciens* strains at 30°C in nutrient-yeast extract-glycerol (NYG) medium (Daniels et al. 1984). For the analysis of in vitro T3S, *X. campestris* pv. *vesicatoria* was grown in minimal medium A (Ausubel et al. 1996) supplemented with sucrose (10 mM final concentration) and casamino acids (0.3% final concentration).

Plant material and plant inoculations.

Infection assays were performed with *X. campestris* pv. *vesicatoria* strains and the near-isogenic pepper cultivars ECW-10R and ECW-30R, which contain *Bs1* and *Bs3* resistance genes,

respectively. Bacteria were resuspended in 1 mM MgCl₂ and were infiltrated at concentrations of 4 × 10⁸ CFU ml⁻¹, if not stated otherwise (Bonas et al. 1991; Kousik and Ritchie 1998; Minsavage et al. 1990). After infection, pepper plants were incubated for day cycles of 16 h of light at 28°C and 65% humidity and 8 h of darkness at 22°C and 65% humidity. The appearance of plant reactions was scored over a period of 1 to 3 days post-inoculation. For the better visualization of the HR, leaves were destained in 70% ethanol. For each experiment, representative plant reactions, which were reproduced at least twice in independent experiments, are shown. For each construct, experiments were always performed with at least two different *X. campestris* pv. *vesicatoria* transformants, obtained by at least two independent conjugations or electroporations. *Agrobacterium tumefaciens* strains were resuspended in 1 mM MgCl₂ and were infiltrated at a density of 8 × 10⁸ CFU ml⁻¹ into leaves of ECW-10R and ECW-30R pepper plants.

Conjugation and electroporation of plasmids into *X. campestris* pv. *vesicatoria*.

Plasmids were introduced into *X. campestris* pv. *vesicatoria* by conjugation or electroporation. For conjugations, pRK2013 was used as a helper plasmid in triparental matings (Figurski and Helinski 1979). For electroporation of plasmids into *X. campestris* pv. *vesicatoria*, bacteria were resuspended in cold sterile water at a density of 10⁸ CFU ml⁻¹, were washed three times with 1 volume of ice-cold water, and were resuspended in 50 µl of sterile 10% glycerol. Plasmid DNA (50 to 100 ng) was electroporated at a voltage of 2.5 kV. After addition of 400 µl of NYG medium, cells were plated on NYG agar plates with appropriate antibiotics. The stable synthesis of fusion proteins was analyzed by immunoblot analysis of cell extracts of at least three different transformants.

Generation of expression constructs.

For the generation of expression constructs encoding C-terminally c-Myc epitope-tagged derivatives of XopA, XopE2, and XopJ, corresponding gene fragments were amplified by polymerase chain reaction (PCR) from *X. campestris* pv. *vesicatoria* 85-10 and were cloned into the *BsaI* sites of plasmid pBRM via Golden Gate cloning (Engler et al. 2008). pBRM is a derivative of the broad-host range vector pBBRMCS-5 and contains the *lac* promoter, the *lacZ alpha* fragment flanked by *BsaI* sites, and a triple c-Myc epitope-encoding sequence (Szczesny et al. 2010) (Table 2). To obtain a *xopA-hpaAC50-c-myc* expression construct, *xopA* and *hpaAC50* were individually amplified by PCR, using primers with *BsaI* recognition sites. The overhangs resulting from *BsaI* digestion allowed the unidirectional ligation of both gene fragments into pBRM in a single restriction-ligation reaction. For the generation of an expression construct encoding GST-XopA-HpaAC50, *xopA* and *hpaAC50* were amplified by PCR and were cloned into the *EcoRI* and *XhoI* sites of plasmid pGEX-2TKM. The pBRM derivative encoding XopE2_{Δ9-13}-c-Myc was generated by PCR, using pBxopE2 as template and phosphorylated primers annealing back to back to the flanking regions of the deletion. Similarly, pBxopJ_{Δ9-13} was generated by PCR amplification of pBxopJ, using primers that annealed to the flanking regions of the deletion and contained a *BsaI* site with a complementary overhang.

All constructs encoding AvrBs3Δ2 fusion proteins under control of the *lac* promoter are derivatives of plasmid pBR356, which contains the *lac* promoter, the *lacZ alpha* fragment flanked by *BsaI* sites, and the *avrBs3Δ2* reporter gene (Scheibner et al. 2016). Gene fragments encoding the AvrBs3Δ2 fusion partners were amplified by PCR and were cloned into the *BsaI* sites of pBR356 in a Golden Gate cloning reaction.

To obtain an expression construct encoding XopA₁₋₃₀-AvrBs3Δ2 under control of the native *xopA* promoter, 678 bp

upstream and the first 30 codons of *xopA* were amplified by PCR. The PCR product was cloned into the *EcoRI* site of plasmid pBS356F, which contains *avrBs3Δ2*. The resulting insert was subsequently excised via *EcoRI* and *HindIII* digestion and was cloned into pLAFR6, giving pL6xopA₁₋₃₀-356.

For the generation of a binary expression construct, *xopE2*₁₋₅₀ was amplified by PCR and was ligated with modules encoding N-terminal, central, and C-terminal regions of AvrBs3Δ2 into the binary vector pGGA3 downstream of the 35S promoter. The primers used in this study are listed in Supplementary Table S1.

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>Xanthomonas campestris</i> pv. <i>vesicatoria</i>		
85-10	Pepper-race 2; wild type; Rif ^r	Canteros 1990; Kousik and Ritchie 1998
85*	85-10 derivative containing the <i>hrpG</i> * mutation	Wengelnik et al. 1999
85*Δ <i>hpaB</i>	<i>hpaB</i> deletion mutant of strain 85*	Büttner et al. 2004
85-10Δ <i>hpaB</i>	<i>hpaB</i> deletion mutant of strain 85-10	Büttner et al. 2004
85*Δ <i>hpaB</i> Δ <i>hrpF</i>	<i>hpaB/hrpF</i> deletion mutant of strain 85*	Büttner et al. 2004
<i>Agrobacterium tumefaciens</i>		
GV2260	Contains Ti plasmid pGV2260, Rif ^r , Ap ^r	Deblaere et al. 1985
<i>Escherichia coli</i>		
DH5α	F ⁻ <i>recA hsdR17(r_k⁻,m_k⁺) Φ80dlacZ DM15</i>	Bethesda Research Laboratories, Bethesda, MD, U.S.A.
DH5α λpir	F ⁻ <i>recA hsdR17(r_k⁻,m_k⁺) Φ80dlacZ DM15 [λpir]</i>	Ménard et al. 1993
BL21(DE3)	F ⁻ <i>ompT hsdS20(r_bm_b) gal</i>	Stratagene, Heidelberg, Germany
Plasmids		
pBBR1MCS-5	Broad-host-range vector; <i>lac</i> promoter; Gm ^r	Kovach et al. 1995
pBRM	Golden Gate-compatible derivative of pBBR1MCS-5	Szczesny et al. 2010
pBR356	Derivative of pBBR1MCS-5 containing <i>avrBs3Δ2</i> downstream of the <i>lac</i> promoter and the <i>lacZα</i> fragment, which is flanked by <i>BsaI</i> sites	Scheibner et al. 2016
pBS356F	Derivative of pBluescript(II) KS containing <i>avrBs3Δ2</i>	Büttner et al. 2004
pGEX-2TKM	GST expression vector; p _{lac} GST <i>lacI</i> ^r pBR322 <i>ori</i> ; Ap ^r , derivative of pGEX-2TK with polylinker of pDSK604	Stratagene; Escobar et al. 2001
pLAFR6	RK2 replicon, Mob ⁺ Tra ⁻ , multicloning site flanked by transcription terminators; Tc ^r	Bonas et al. 1989
pRK2013	ColE1 replicon, TraRK ⁺ Mob ⁺ ; Km ^r	Figurski and Helinski 1979
pUC57Δ <i>BsaI</i>	Derivative of pUC57 with mutated <i>BsaI</i> site	Morbitzer et al. 2011
pBxopA ₁₋₂₀ -356	Derivative of pBR356 encoding XopA ₁₋₂₀ -AvrBs3Δ2	This study
pBxopA ₁₋₂₅ -356	Derivative of pBR356 encoding XopA ₁₋₂₅ -AvrBs3Δ2	This study
pBxopA ₁₋₃₀ -356	Derivative of pBR356 encoding XopA ₁₋₃₀ -AvrBs3Δ2	This study
pBxopA _{Δ2-5} -356	Derivative of pBR356 encoding XopA _{Δ2-5} -AvrBs3Δ2	This study
pBxopA _{Δ2-10} -356	Derivative of pBR356 encoding XopA _{Δ2-10} -AvrBs3Δ2	This study
pBxopA _{Δ2-21} -356	Derivative of pBR356 encoding XopA _{Δ2-21} -AvrBs3Δ2	This study
pBxopA _{FL} -356	Derivative of pBR356 encoding XopA _{FL} -AvrBs3Δ2	This study
pBxopA _{HpaA_{C50}} -356	Derivative of pBR356 encoding XopA-HpaA _{C50} -AvrBs3Δ2	This study
pBRMxopA	Derivative of pBRM encoding XopA-c-Myc	Lorenz and Büttner 2011
pBRMxopA _{Δ2-5}	Derivative of pBRM encoding XopA _{Δ2-5} -c-Myc	This study
pBRMxopA _{Δ2-10}	Derivative of pBRM encoding XopA _{Δ2-10} -c-Myc	This study
pBRMxopA _{Δ2-21}	Derivative of pBRM encoding XopA _{Δ2-21} -c-Myc	This study
pBRMxopA _{HpaA_{C50}}	Derivative of pBRM encoding XopA-HpaA _{C50} -c-Myc	This study
pBRMxopE2	Derivative of pBRM encoding XopE2-c-Myc	Lorenz and Büttner 2011
pBRMxopE2 _{Δ2-5}	Derivative of pBRM encoding XopE2 _{Δ2-5} -c-Myc	This study
pBRMxopE2 _{Δ2-10}	Derivative of pBRM encoding XopE2 _{Δ2-10} -c-Myc	This study
pBRMxopE2 _{Δ9-13}	Derivative of pBRM encoding XopE2 _{Δ9-13} -c-Myc	This study
pBxopE2 ₁₋₂₀ -356	Derivative of pBRM encoding XopE2 ₁₋₂₀ -AvrBs3Δ2	This study
pBxopE2 ₁₋₃₀ -356	Derivative of pBRM encoding XopE2 ₁₋₃₀ -AvrBs3Δ2	This study
pBxopE2 ₁₋₄₀ -356	Derivative of pBRM encoding XopE2 ₁₋₄₀ -AvrBs3Δ2	This study
pBxopE2 ₁₋₅₀ -356	Derivative of pBRM encoding XopE2 ₁₋₅₀ -AvrBs3Δ2	This study
pBxopE2 ₁₋₁₅₀ -356	Derivative of pBRM encoding XopE2 ₁₋₁₅₀ -AvrBs3Δ2	This study
pBxopE2 _{FL} -356	Derivative of pBRM encoding XopE2 _{FL} -AvrBs3Δ2	This study
pBxopE2 _{Δ2-40} -356	Derivative of pBRM encoding XopE2 _{Δ2-40} -AvrBs3Δ2	This study
pBRMxopJ	Derivative of pBRM encoding XopJ-c-Myc	Lorenz and Büttner 2011
pBRMxopJ _{Δ2-5}	Derivative of pBRM encoding XopJ _{Δ2-5} -c-Myc	This study
pBRMxopJ _{Δ2-10}	Derivative of pBRM encoding XopJ _{Δ2-10} -c-Myc	This study
pBRMxopJ _{Δ9-13}	Derivative of pBRM encoding XopJ _{Δ9-13} -c-Myc	This study
pBxopJ ₁₋₅₀ -356	Derivative of pBRM encoding XopJ ₁₋₅₀ -AvrBs3Δ2	This study
pBxopJ _{FL} -356	Derivative of pBRM encoding XopJ _{FL} -AvrBs3Δ2	This study
pBxopJ _{Δ2-50} -356	Derivative of pBRM encoding XopJ _{Δ2-50} -AvrBs3Δ2	This study
pDhpaB	Derivative of plasmid pDSK604 encoding HpaB-c-Myc	Büttner et al. 2004
pGGA3xopE2 ₁₋₅₀ -356	Binary vector containing <i>xopE2</i> ₁₋₅₀ - <i>avrBs3Δ2</i> downstream of the 35S promoter	This study; S. Thieme and U. Bonas, <i>unpublished</i>
pGhpaB	pGEX-2TKM derivative encoding GST-HpaB	This study
pGxopA	pGEX-2TKM derivative encoding GST-XopA	Büttner et al. 2004
pGxopA _{HpaA_{C50}}	pGEX-2TKM derivative encoding GST-XopA-HpaA _{C50}	This study
pL6xopA ₁₋₃₀ -356	Derivative of pLAFR6 encoding XopA ₁₋₃₀ -AvrBs3Δ2 under control of the native <i>xopA</i> promoter	This study

^a Ap = ampicillin, Km = kanamycin, Rif = rifampicin, Sm = spectinomycin, Tc = tetracycline, ^r = resistant.

Preparation of protein extracts and in vitro secretion assays.

For the analysis of protein synthesis, bacteria were cultivated overnight in liquid NYG medium and cells were harvested by centrifugation. Equal amounts of proteins adjusted according to the optical density of the culture were analyzed by immunoblotting, using AvrBs3-specific antibodies (Knoop et al. 1991). Secretion assays were performed in minimal medium A (pH 5.3) in the presence of BSA and thiamine, as described previously (Rossier et al. 1999). Equal amounts of bacterial total cell extracts and culture supernatants (adjusted according to the optical density of the cultures) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, using antibodies specific for the c-Myc epitope (Roche), GST (Amersham Pharmacia), AvrBs3, the predicted inner membrane ring protein HrcJ, the predicted periplasmic inner rod protein HrpB1, and the secreted translocon protein HrpF, as indicated (Büttner et al. 2002; Knoop et al. 1991; Rossier et al. 2000). Horseradish peroxidase-labeled anti-rabbit or antimouse antibodies (GE Healthcare) were used as secondary antibodies. Results were reproduced at least twice in independent experiments.

GST pull-down assays.

GST pull-down assays were performed as described previously (Hartmann et al. 2012). Briefly, *E. coli* cells containing GST or GST fusion proteins were broken with a French press and cell debris were removed by centrifugation. GST and GST fusion proteins were immobilized on glutathione sepharose and were incubated with *E. coli* lysates containing the putative interaction partner. Unbound proteins were removed by washing steps and proteins were eluted with 10 mM reduced glutathione. Total protein lysates and eluted proteins were analyzed by SDS-PAGE and immunoblotting, using antibodies specific for the c-Myc epitope and GST (Roche Applied Science), respectively. Results were reproduced at least twice in independent experiments.

ACKNOWLEDGMENTS

We are grateful to U. Bonas for helpful comments on the manuscript and for providing the AvrBs3-specific antibody and to M. Jordan for technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft (BU2145/5-1 and CRC 648 “Molecular mechanisms of information processing in plants”) to D. Büttner.

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