Secretion and translocation signals and DspB/F-binding domains in the type III effector DspA/E of Erwinia amylovora

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DspA/E is a type III effector of Erwinia amylovora, the bacterial pathogen that causes fire blight disease in roseaceous plants. This effector is indispensable for disease development, and it is translocated into plant cells. A DspA/E-specific chaperone, DspB/F, is necessary for DspA/E secretion and possibly for its translocation. In this work, DspB/F-binding sites and secretion and translocation signals in the DspA/E protein were determined. Based on yeast two-hybrid assays, DspB/F was found to bind DspA/E within the first 210 amino acids of the protein. Surprisingly, both DspB/F and OrfA, the putative chaperone of Eop1, also interacted with the C-terminal 1059 amino acids of DspA/E; this suggests another chaperone-binding site. Secretion and translocation assays using serial N-terminal lengths of DspA/E fused with the active form of AvrRpt2 revealed that at least the first 109 amino acids, including the first N-terminal chaperone-binding motif and DspB/F, were required for efficient translocation of DspA/E, although the first 35 amino acids were sufficient for its secretion and the presence of DspB/F was not required. These results indicate that secretion and translocation signals are present in the N terminus of DspA/E, and that at least one DspB/F-binding motif is required for efficient translocation into plant cells.

INTRODUCTION

Gram-negative bacterial pathogens of plants and animals have evolved a sophisticated arsenal of proteins that allows them to overcome their hosts’ defences. The type III secretion system (T3SS) is central to this capability. It is a syringe-like protein complex that facilitates the transport of many host defence-thwarting protein effectors across the inner and outer bacterial membranes into the cells of eukaryotic hosts (Stebbins, 2005).

The targeting of proteins to the T3SS for secretion (i.e. delivery outside the bacterial cell) or translocation (i.e. delivery into host cells) in animal bacterial pathogens is accomplished in part by a short N-terminal secretion signal often located within the first 15–20 amino acids of type III (T3)-secreted proteins (Lilic et al., 2006; Lloyd et al., 2002; Sorg et al., 2005). The exact means by which this N-terminal signal targets proteins for secretion and translocation is unclear. There is little consensus between secretion signals of different bacteria, and it is thought that the protein sequence of these N-terminal secretion signals possesses a common secondary structure that allows proteins to be recruited to the T3SS (Stebbins, 2005). However, the fact that frameshift mutations of the DNA sequence corresponding to the first 15 amino acids of certain effectors still allow protein secretion has led others to propose an RNA-mediated secretion signal for transcripts of effectors to be recruited and effectors to be translated and secreted simultaneously (Ramamurthi & Schneewind, 2003; Sorg et al., 2005).

T3-secreted proteins in plant-pathogenic bacteria also very likely carry secretion or translocation signals in their N termini. AvrPto and AvrB of Pseudomonas syringae require the first N-terminal 10–15 amino acids for their secretion, based on deletion analysis (Anderson et al., 1999). AvrBs2 of Xanthomonas campestris requires the first N-terminal 58 amino acids for its secretion and translocation into plant cells, based on an AvrRpt2 reporter assay (Mudgett et al., 2000). By comprehensive alignment of the first 40 amino acids in 28 putative effectors of P. syringae pathovars, six predictive rules for T3-secreted proteins have been proposed: (a) Ile, Leu, Val, Ala or Pro are found in
positions 3 or 4, but not in both, and often are preceded by a Pro, polar or basic amino acid; (b) position 5 is rarely occupied by a Met, Ile, Leu, Phe, Tyr or Trp; (c) Asp or Glu do not occur within the first 12 positions; (d) Cys rarely occurs after position 5; (e) the first 50 amino acids are amphipathic and rich in polar residues, especially Ser and Gln (only 40 residues shown); and (f) no more than three consecutive residues consisting of either Met, Ile, Leu, Val, Phe, Tyr or Trp occur in the first 50 residues (Petnicki-Ocwieja et al., 2002).

Interestingly, the secretion signal at the N terminus of effectors in both animal- and plant-pathogenic bacteria is not necessarily the sole signal for T3 protein secretion: the chaperone-binding domain (CBD), a 50–100 amino acid long domain found after the initial N-terminal signal, but usually within the first 100 amino acids of an effector, also influences secretion of some proteins (Galán & Wolf-Watz, 2006; Schesser et al., 1996). This domain mediates the binding of chaperones, which are small acidic proteins that influence the secretion, regulation and stability of certain effectors (Luo et al., 2001).

The relationship between the N-terminal secretion signal and the CBD is complex, and may be different for different bacterial species or different effectors. The presence of the CBD can override secretion otherwise dictated by the presence of the N-terminal secretion signal. For example, in Salmonella spp., the absence of the chaperone for a secreted protein can preclude its secretion unless the CBD is also absent (Ehrbar et al., 2006; Lilic et al., 2006). If the CBD of an effector is deleted but the N-terminal secretion signal remains, secretion through the T3SS can be thwarted and the effector can be rerouted to the flagellar secretion apparatus, indicating that the chaperone and its CBD confer some specificity for T3 secretion on effectors (Lee & Galán, 2004; Lilic et al., 2006). However, this CBD-determined specificity does not exist in all bacterial systems. For example, in Yersinia spp., in the absence of the CBD, the N-terminal secretion signal is sufficient for secretion through the T3SS (Letzelter et al., 2006). Thus, although the CBD and its cognate chaperone can both exert considerable influence over whether a protein is secreted and, presumably, translocated into host cells to facilitate disease, the relative influence of the two signals can differ among species of bacteria.

Erwinia amylovora, a Gram-negative bacterial pathogen of rosaceous plants, has a small genome (3.8 Mb) and is minimal, but effective, arsenal of T3-secreted effectors (DebRoy et al., 2004) and causes cell death in both host and non-host plants (Boureau et al., 2006; Oh et al., 2007), thus enabling and promoting disease. DspA/E is a large (198 kDa) protein that belongs to the AvrE family of effectors and may have multiple functional domains on account of its size (Bogdanove et al., 1998; Gaudriault et al., 1997). Indeed, like many AvrE-family effectors, DspA/E requires a chaperone, DspB/F, for its secretion and intracellular stability (Gaudriault et al., 1997, 2002). However, while a dspA/E mutant of Er. amylovora lacks pathogenic ability, a dspB/F mutant is attenuated in virulence, suggesting a low level of secretion in the absence of the chaperone. In addition, DspA/E is translocated into plant cells, based on studies using the CyaA reporter system (Bocsanycz et al., 2006).

Although DspA/E is known to be secreted and translocated, and to have a chaperone important for its secretion, there are no reports concerning the regions of DspA/E required for its secretion or translocation, or the location of CBDs. In this study, we sought evidence of secretion and translocation signals and CBDs of DspA/E in its N terminus. Also, we determined whether these domains are necessary for secretion or translocation of DspA/E in the presence or absence of its chaperone, DspB/F. We found that the first 35 amino acids of DspA/E are sufficient for secretion. We also identified two DspB/F-binding motifs that are present in the first 210 amino acids and found that the first 109 amino acids, which include the DspB/F-binding motif, are required for efficient translocation.

**METHODS**

**Yeast two-hybrid assay.** The Matchmaker LexA Two-Hybrid System (Clontech) was used to determine the interaction between DspA/E and DspB/F, OrfA, OrfC and DspA/E itself. Constructs used in this assay are described in Supplementary Table S1. Various lengths of DspA/E were amplified with primers listed in Supplementary Table S2 by PCR and cloned into the bait vector pGilda, including DspA/E1-210, DspA/E1-35, DspA/E1-50, DspA/E1-70, DspA/E1-80, DspA/E1-109, DspA/E35-80, DspA/E35-109, DspA/E50-109, DspA/E80-109, DspA/E80-210, DspA/E80-150, DspA/E80-130 and DspA/E110-210. Additionally, DspA/E780-1838 in pGilda (W.-S. Kim and S. V. Beer, unpublished results), and OrfA in pGilda and DspB/F, OrfA, and OrfC in pB42AD (Asselin et al., 2006), were used in this study.

Saccharomyces cerevisiae EGY48 was co-transformed with the bait and prey vectors by the LiAc/PEG transformation method developed by Gietz & Woods (2002). Transformants were selected on SD triple-dropout medium (–Ura, –His, –Trp). Each prey and bait construct was also co-transformed into yeast with an empty prey or bait vector to provide negative controls for the assay.

Positive interactions were identified when yeast containing a particular bait and prey pair turned blue on SD triple-dropout X-Gal medium within 24 h of being streaked onto plates and was able to grow on SD quadruple-dropout medium (–Ura, –His, –Trp, –Leu). A yeast strain co-transformed with pLexA-53 and pB42AD-T, vectors containing sequences for the known interacting proteins murine p53 and SV40 large T-antigen, was used as a positive control for protein interaction.

**Recombinant DNA techniques.** Cloning and plasmid construction were performed as described by Sambrook et al. (1989). Plasmids were transformed into bacterial strains by electroporation using a Gene Pulser II (Bio-Rad). All constructs were sequenced at the Cornell University Biotechnology Program DNA Sequencing Facility.

**Construction of fusion genes with avrRpt2_80-255.** The truncated form of the avrRpt2 gene, avrRpt2_80-255, was amplified by PCR using primers listed in the supplementary tables. This PCR fragment was
cloned into the pBC SK(−) vector (Stratagene) and named pCPP1571. Partial genes encoding truncated DspA/E proteins and AvrPto as a putative positive control, including their own promoters, were amplified by PCR with gene-specific primers (see Supplementary Table S2). Xhol sites and BamHI sites were added into forward and reverse primers, respectively, except for the reverse primer for avrPto1-109, to which an Xhol site alone was added. These PCR fragments were cloned into pCPP1571 and named pCPP1585 (AvrPto1-109::AvrRpt280-255), pCPP1586 (DspA/E1-109::AvrRpt280-255), pCPP1627 (DspA/E1-70::AvrRpt280-255), pCPP1628 (DspA/E1-50::AvrRpt280-255) and pCPP1629 (DspA/E1-35::AvrRpt280-255), respectively, as shown in Supplementary Table S1. *dsp/B*, encoding a chaperone of DspA/E, was amplified by PCR with the primers listed in the supplementary data. Xhol and KpnI sites were added into forward and reverse primers, respectively, and cloned into appropriate plasmids, which were named pCPP1649 (DspA/E1-109::AvrRpt280-255, DspB/F), pCPP1652 (DspA/E1-70::AvrRpt280-255, DspB/F), pCPP1653 (DspA/E1-50::AvrRpt280-255, DspB/F), pCPP1654 (DspA/E1-35::AvrRpt280-255, DspB/F), pCPP1672 (DspA/E1-152::AvrRpt280-255, DspB/F) and pCPP1673 (DspA/E1-210::AvrRpt280-255, DspB/F), as listed in Supplementary Table S1.

Escherichia coli delivery system for secretion and translocation assays. Plasmid pCPP431, which carries the genes of the T3SS of *Er. amylovora* (Garr, 2001) (Fig. 3a), was transformed into *E. coli* strain MC4100. Each plasmid carrying each fusion construct was transformed into *E. coli* MC4100 (pCPP431). These strains were used for the secretion and translocation assays described below.

Secretion assays. Secretion assays were performed using a protocol adapted from one developed by Ham et al. (1998). Bacteria were grown for 12 h in Luria–Bertani (LB) broth at 37 °C, and then harvested by centrifugation for 10 min at 3400 g and washed three times in LB supplemented with the antibiotics chloramphenicol, kanamycin and spectinomycin, as appropriate. Washed cells were then centrifuged for 10 min at 3400 g at 4 °C and 1.0 was infiltrated into fully expanded leaves of *A. thaliana* ecotype Columbia with RPS2 and rps2 genetic backgrounds, and inoculum of OD600 1.0 was infiltrated into N. tabacum cv. Xanthi using a needleless syringe. Occurrences of the HR in inoculation sites were documented 24 h after infiltration.

**RESULTS**

**A DspB/F-binding domain is located within the first 210 amino acids of DspA/E**

A yeast two-hybrid assay was used to determine the interaction between DspB/F and 14 different N-terminal DspA/E fragments, because chaperones generally bind to the N-terminal regions of effectors to help their secretion. All fragments of DspA/E in bait constructs were expressed well in yeast (Fig. 1). Our assays showed that a DspB/F-binding domain, which is mainly located in the amino acid 1–80 region, exists within the first 210 amino acids of the N-terminal of DspA/E.

In this binding domain, the first 70 amino acids are sufficient for interaction with DspB/F (Fig. 1), although the strength of the interaction between DspA/E and DspB/F increased dramatically when 10 more amino acids were added, which was apparent within 12 h of streaking strains onto X-Gal assay plates. In order to narrow down the binding domain, we tested the 35–80 fragment of DspA/E. Unexpectedly, this fragment did not show positive interaction with DspB/F. Interestingly, however, the 35–109 and 50–109 fragments, but not the 80–109 fragment, strongly interacted with DspB/F, just like the 1–80 fragment. These results suggest that the binding domain is very likely located in the 50–80 region of DspA/E.

In addition to the 1–80 region, the 80–210 region showed weak interaction with DspB/F in yeast. For this region, we tested the 80–150, 80–130 and 110–210 fragments of DspA/E.
Surprisingly, none of these showed positive interaction with DspB/F (Fig. 1).

After exploring the DspB/F-binding capabilities of the N-terminal 210 amino acids of DspA/E, we determined whether DspB/F could bind to the C-terminal 1059 amino acids of DspA/E. Amino acids 780–1838 of DspA/E had a strong positive interaction with DspB/F, indicating that there are one or more DspB/F-binding motifs or domains near the C terminus of DspA/E (Fig. 2).

The secretion signal is located within the first 35 amino acids of DspA/E

Next, we determined whether a CBD located within the first 210 amino acids of the N terminus of DspA/E is important for its secretion, and also the location of the secretion signal for the T3SS. For this, DNA fragments encoding DspA/E1-35, DspA/E1-50b, DspA/E1-70b, DspA/E1-109, DspA/E1-152 and DspA/E1-210 and its own hrp promoter were fused with a gene fragment encoding AvrRpt2280-255 in pCPP1571 with or without the full-length of the dspB/F gene. All fusion proteins were produced in E. coli MC4100(pCPP431), which carries hrp regulatory genes and genes for the Erwinia (Pectobacterium) chrysanthemi T3SS (Ham et al., 1998). For negative controls, only AvrPto1-109, DspA/E1-35 and DspA/E1-109 were produced in E. coli MC4100 not carrying pCPP431.

E. coli MC4000 not carrying pCPP431 did not secrete either AvrPto1-109 or DspA/E1-35 and DspA/E1-109 while E. coli MC4100(pCPP431) did (Fig. 3b). This indicates that the functional Er. amylovora T3SS is required for secretion of both effector proteins in E. coli. The N-terminal region of DspA/E was dissected in more detail, and all N-terminal portions of DspA/E tested in this study were secreted regardless of the presence of DspB/F (Table 1, Fig. 3c). These results indicate that the first 35 amino acids of DspA/E are sufficient for its secretion and that DspB/F is
dispensable for secretion of DspA/E in \textit{E. coli} containing the \textit{Er. amylovora} T3SS. In addition, AvrPto was secreted efficiently via the \textit{Er. amylovora} T3SS (Fig. 3b), indicating that \textit{Er. amylovora} T3SS can also secrete a heterologous effector protein in the same manner as the \textit{Er. chrysanthemi} T3SS.

The composition of the first 50 N-terminal amino acids of DspA/E (Fig. 3d) was analysed to determine whether the secretion signal of DspA/E for the T3SS follows the six predictive rules proposed for \textit{Pseudomonas} effector proteins (see Introduction). This region carrying the secretion signal for DspA/E followed five (a, b, d, e and f) of the six predictive rules.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{DspA/E} & \textbf{Pellet} & \textbf{Supernatant} \\
\hline
1-35 & + DspB/F & - DspB/F & + DspB/F & - DspB/F \\
1-50 & N/A & N/A & N/A & N/A \\
1-70 & N/A & N/A & N/A & N/A \\
1-109 & N/A & N/A & N/A & N/A \\
1-152 & N/A & N/A & N/A & N/A \\
1-210 & N/A & N/A & N/A & N/A \\
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\end{tabular}
\end{table}

\textbf{Fig. 3.} Secretion of truncated forms of DspA/E in the presence or absence of DspB/F. (a) The gene structure of the \textit{Er. amylovora} hrp/dsp gene cluster. The \textit{dspA/E} gene is in red, regulatory genes are in blue and chaperone genes are in green. (b) \textit{E. coli} MC4100, with and without pCPP431 (containing a functional T3SS from \textit{Er. amylovora}) was grown in LB at 26 °C to express the fusion proteins AvrPto1-109::AvrRpt2\textsubscript{80-205}, DspA/E\textsubscript{1-109}::AvrRpt2\textsubscript{80-205} and DspA/E\textsubscript{1-35}::AvrRpt2\textsubscript{80-205}. Proteins were detected in cell pellets (non-secreted; 15 s exposure) and supernatants (secreted; 1 min exposure) via immunoblot analysis using an anti-AvrRpt2 antibody. On the left, protein reference positions are indicated by apparent molecular masses in kDa. Note that there are at least two faint bands (>37.1 and <25.9 kDa) that represent non-specific proteins recognized by the antisera, an issue previously encountered by McNellis \textit{et al.} (1998) and Mudgett & Staskawicz (1999). (c) The 35–210 amino acids portion from the N terminus of DspA/E was fused with AvrRpt2\textsubscript{80-205}, and a secretion assay was done as described in (b) with or without the presence of DspB/F. As above, Western blots of pellet and supernatant protein fractions were probed with anti-AvrRpt2 antibody. (d) The first N-terminal 50 amino acids of DspA/E.

\textbf{Translocation of DspA/E of \textit{Erwinia amylovora}}
Table 1. Translocation, secretion, and interaction with DspB/F of the N-terminal region of DspA/E

Table 1. Translocation, secretion, and interaction with DspB/F of the N-terminal region of DspA/E

<table>
<thead>
<tr>
<th>N-terminal length of DspA/E (aa)</th>
<th>Translocation*</th>
<th>Secretion†</th>
<th>DspB/F interaction in yeast‡</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>+ DspB/F</td>
<td>-DspB/F</td>
<td>+ DspB/F</td>
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<tr>
<td>35</td>
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<td>70</td>
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<tr>
<td>152</td>
<td>+ ND</td>
<td>ND</td>
<td>+ ND</td>
</tr>
<tr>
<td>210</td>
<td>+</td>
<td>ND</td>
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*Translocation was performed by infiltrating *N. tabacum* with *E. coli* strain MC4100 containing pCPP431 and each DspA/E-derived construct at OD600 1.0 and assessing HR after 24 h (+, strong HR; +, spotty HR; +/-, occasional, spotty HR; -, no HR).
†Secretion was determined by the method of Ham et al. (1998) and immunoblotting using an anti-AvrRpt2 antibody (+, secretion).
‡Interaction between N-terminal DspA/E and DspB/F in yeast was determined by blue colour on X-Gal medium and growth on SD quadruple-dropout medium (+, strong interaction; +, weak interaction; -, no interaction).

rules; rule (c) was not satisfied because there are two Glu residues within the first 12 amino acids. In accordance with rule (e), there is a Ser- and Gln-rich region within the first 50 amino acids that includes residues 29–38 (Fig. 3d).

The AvrRpt2 reporter system as an assay for translocation using *E. coli* for delivery of the protein

Although the AvrRpt2 reporter system has been used to indicate the translocation of proteins from plant-pathogenic bacteria such as *P. syringae* into plant cells (Chang et al., 2005; Mudgett et al., 2000; Zwiesler-Vollick et al., 2002), attempts to use *E. coli* to deliver plant pathogen proteins into cells of *A. thaliana* have not been described. To determine whether *A. thaliana* is suitable for this assay, *E. coli* MC4100(pCPP431) carrying the same constructs as used for our secretion assays was infiltrated into leaves. Two attempts of six series of leaf infiltrations of *A. thaliana* did not yield HR-like responses in *rps2*-negative control plants or in *RPS2* plants using pCPP1571 as a negative control. Of these, only one series also yielded consistent HR-like responses for the positive control, the AvrPto1-109::AvrRpt280-255 fusion construct.

Because *A. thaliana* was not effective for use of *E. coli* as a delivery system for proteins, we tested *N. tabacum* cv. *Xanthi*. *N. tabacum* worked better than *A. thaliana*; however, the outcome of the assay depended on the growth stage and health of the plants used. In several assays, when the test plants used had small, pale leaves and widely spaced internodes, spotty and inconsistent HRs that varied from leaf to leaf developed in response to the positive control strain (Fig. 4). As a result, the assay may have yielded false-negative results for translocation. Another unexpected result pertinent to this assay in tobacco was the development of chlorosis associated with all inoculation sites (Fig. 4). Similar results have often been observed when *E. coli* containing the vector pCPP431 is present in tobacco (A. M. Bocsanczy, personal communication).

The translocation signal located within the first 109 amino acids of DspA/E; DspB/F is necessary for translocation of DspA/E

We showed that the first 35 amino acids of DspA/E are sufficient for its secretion and that there are two DspB/F-binding motifs within the first 210 N-terminal amino acids. To determine the location of the translocation signal and whether DspB/F is necessary for translocation of DspA/E, we carried out translocation assays in *N. tabacum*, using an *E. coli* delivery system combined with the AvrRpt2 reporter system. As expected, AvrPto1-109::AvrRpt280-255 was translocated though the *Er. amylovora* T3SS that was expressed in *E. coli* to cause an HR in tobacco leaves (Fig. 4a). Therefore, we could use the AvrPto fusion construct as a positive control to determine the minimal N-terminal region of DspA/E sufficient for its translocation into plant cells.

The three shortest constructs of DspA/E, DspA/E1-35::AvrRpt280-255, DspA/E1-50::AvrRpt280-255 and DspA/E1-70::AvrRpt280-255, were not translocated in the presence or absence of DspB/F, although all three were secreted and DspA/E1-70 also had DspB/F-binding activity in yeast (Fig. 1). The strain containing DspA/E1-109::AvrRpt280-255 and DspB/F resulted in the strongest and most consistent HR of any experimental or control strain tested, and the strain containing DspA/E1-109::AvrRpt280-255 without DspB/F resulted in occasional spotty HRs in several repetitions of this assay (Fig. 4b). DspA/E1-109 was the only fragment tested that resulted in spotty HRs in the absence of DspB/F.
Based on yeast two-hybrid results, this sequence includes the first N-terminal DspB/F-binding motif of DspA/E (Fig. 1, Table 1). DspA/E1-109::AvrRpt280-255 also appeared to be translocated in the presence of DspB/F, but the strains containing these constructs resulted in weaker, spottier HRs than the one carrying the construct for DspA/E1-109::AvrRpt280-255 (Fig. 4b). Our results indicate that the first 109 amino acids of DspA/E contain the entire signals for its secretion and translocation and that the presence of DspB/F strongly enhances translocation of the first 109 amino acids of DspA/E.

Chaperones bind at the C terminus of DspA/E

Because preliminary experiments by others have suggested that the absence of the putative chaperone OrfA affects the secretion of DspA/E (J. E. Asselin and S. V. Beer, unpublished results), whether DspA/E could bind to other putative chaperones of the pathogenicity island of Erwinia amylovora, namely OrfA and OrfC (Oh et al., 2005), was examined. Although neither OrfA nor OrfC bound the N-terminal 250 amino acids of DspA/E, OrfA bound the C-terminal 1059 amino acids of DspA/E (Fig. 2). These results indicate that DspA/E binds to more than one chaperone, which may explain why the dspB/F mutant strain of Erwinia amylovora still exhibits some virulence activity, although it is very low.

DISCUSSION

In this study, we found that the secretion and translocation signals of DspA/E are located in its N-terminal region. The DspB/F-binding domain of DspA/E is located within the first 210 amino acids. The presence of DspB/F, the chaperone of DspA/E, strongly enhances translocation of DspA/E into plant cells.

CBDs of DspA/E

Yeast two-hybrid results suggested that the DspB/F-binding domain is present between amino acids one and 80 of DspA/E. Triplett et al. (2009) found one DspB/F-binding domain within the first 200 amino acids of DspA/E, between amino acids 51 and 100, using a yeast two-hybrid approach. This binding domain includes a region of amino acids very close to the first DspB/F-binding domain located in this study. However, in the present study, we found that the 80–210 region of DspA/E interacted weakly with DspB/F, which was not detected by Triplett et al. (2009). This discrepancy may have occurred due to differences in the lengths of the DspA/E fragments used in the two experiments. In our study, we found that the addition of 10 more amino acids significantly affected interaction between DspA/E fragments and DspB/F (DspA/E1-152 vs DspA/E1-150). In addition, Triplett et al. (2009) found that the 1–810 fragment lacking the 50–100 region showed a positive interaction with DspB/F, which supports the presence of another DspB/F-binding domain. More comprehensive deletion analysis, including site-specific mutagenesis, might narrow down the DspB/F-binding region more precisely.

We are unaware of examples of multiple CBDs in the N-terminal regions of translocated proteins, but single CBDs with multiple chaperone-binding motifs have been
described (Stebbins & Galán, 2001). Multiple chaperone-binding motifs help effectors to associate with chaperone dimers; DspA/E may bind to a DspB/F homodimer through two or more DspB/F-interacting domains. Whether DspB/F can form a homodimer remains to be determined.

It was interesting and unexpected that yeast two-hybrid assays revealed binding of DspB/F to the C-terminal 1059 amino acids of DspA/E; this seems to indicate the presence of a CBD elsewhere in the protein. Most CBDs of effectors characterized to date have been located in the N-terminal regions of the effectors, immediately downstream of secretion signals. However, some chaperones, such as ShcO1, ShcS1 and ShcS2, apparently bind to the middle of the cognate effector protein, e.g. HopO1-1 of P. syringae pv. tomato (Guo et al., 2005). In addition, it is conceivable that because DspA/E is such a large effector, it requires more than one CBD for translocation into plant cells. One of the main hypothesized functions of chaperones is to prevent protein folding before the protein passes through the T3SS (Galán & Wolf-Watz, 2006). A large protein such as DspA/E might spontaneously fold at its C terminus if unprotected by a chaperone. The binding of a chaperone to the C-terminal half of the protein might prevent spontaneous folding until DspB/E passes safely through the T3SS.

The binding of OrfA, the putative chaperone of the effector Eop1 (Asselin et al., 2006), to the C-terminal 1059 amino acids of DspA/E is difficult to explain. However, some examples of chaperones binding to multiple substrates have been reported (van Eerde et al., 2004), so promiscuity in the chaperones of effectors from Er. amylovora would not be implausible. In addition, binding of OrfA to DspA/E might explain why a dspB/F mutant strain exhibits some virulence, unlike a dspA/E mutant strain, which is completely non-pathogenic (Gaudriault et al., 2002). If DspB/F were the sole chaperone of DspA/E, a dspB/F mutant strain should show the same phenotype as a dspA/E mutant strain. Although there was no overlap in the binding specificity of the CBDs at the N terminus of DspA/E, since only DspB/F was bound, OrfA seemed to bind in the C-terminal 1059 amino acids of DspA/E. The yeast two-hybrid constructs available for immediate testing did not permit the determination of whether the particular motifs that bound OrfA in the C-terminal portion of DspA/E were the same motifs that caused DspB/F binding. However, as suggested above, it is possible that there are motifs in that length of DspA/E that resemble a CBD. The question of whether DspB/F and OrfA coordinate to regulate the secretion and translocation of DspA/E or Eop1 remains to be addressed.

**Secretion and translocation of DspA/E with and without DspB/F**

Gaudriault et al. (1997, 2002) have observed that DspB/F is required for the secretion and intracellular stability of DspA/E in its entirety. In this study, we showed that short lengths of the N-terminal portion of DspA/E could be secreted in the absence of DspB/F, because all DspA/E:AvrRpt2<sub>80-255</sub> fusion proteins were secreted from E. coli expressing the Er. amylovora T3SS in the presence or absence of DspB/F. Thus, the size of the DspA/E moiety used in the two studies may very well account for the different results.

Although all lengths of DspA/E were secreted in the presence or absence of DspB/F, this ubiquity did not extend to the translocation of the same fusion proteins. Instead, the first 109 amino acids of DspA/E, a section of DspA/E containing the CBD of DspB/F, as determined by yeast two-hybrid assay, and the presence of DspB/F were required to effect significant translocation. Shorter constructs of DspA/E were not translocated. This reflects the sufficiency of the first 109 amino acids of DspA/E to effect translocation in the presence of the chaperone DspB/F, and indicates that the first 109 amino acids of DspA/E contain most of the CBD for DspB/F. This result seems somewhat different from the results reported by workers in Michigan (Tripplett et al., 2009). They found that the first 51 amino acids, lacking the DspB/F-binding motif, were sufficient for moderate (about ninefold increase over control) levels of translocation of DspA/E. This may be due to the sensitivity of their CyaA translocation assay. However, based on their entire dataset, it appears that the inclusion of the first 150 amino acids contributes to even further increases (~14-fold over control) in DspA/E translocation. This observation approximates translocation levels for the full-length protein (~17-fold over control) and is consistent with the results presented here.

Interestingly, the first 109 amino acids of DspA/E were sometimes translocated in the absence of DspB/F, as indicated by an occasional minute HR that developed at infiltration sites in tobacco (data not shown). This was unexpected, especially considering that in other studies the presence of a CBD in the absence of the chaperone has thwarted translocation (Ehrbar et al., 2006). Preliminary tests in Arabidopsis also sometimes resulted in a spotty and weak HR for the 109-amino-acid construct in RPS2 and not rps2 plants in the absence of DspB/F, suggesting that the construct is indeed translocated into plant cells under some conditions, probably mainly depending on plant conditions (data not shown).

**E. coli delivery system to determine secretion and translocation of effectors**

In this study, we used an E. coli delivery system expressing the Er. amylovora T3SS, instead of the original bacterial strain, to secrete or deliver DspA/E fusion proteins into plant cells. As with the Er. chrysanthemi T3SS expressed in E. coli (Ham et al., 1998), the Er. amylovora T3SS in E. coli secreted, and more importantly translocated, AvrPto into plant cells. This indicates that this heterologous delivery system is a useful tool for determining effector translocation in planta. The E. coli system has several advantages.
(1) Although plant-pathogenic bacteria often induce an HR in planta, E. coli does not induce an HR in most plant species, including Arabidopsis and tobacco. Thus, an HR induced by E. coli translocating AvrRpt2 fused with certain effector proteins can be observed easily. (2) Many different effectors of plant-pathogenic bacteria are active in planta, but the E. coli strain used in this study has none. Thus, interference by other effectors in certain effector translocation studies is precluded. (3) Mutagenesis of strains is not needed to examine the effects of certain genes on effector translocation. Instead, we can easily add or remove genes by cloning into certain vectors and transforming E. coli. (4) Growth of E. coli in hrp-inducing medium is not necessary to induce expression of hrp (hypersensitive response and pathogenicity) genes because the assay works well with E. coli grown in LB.

This study revealed the location and characteristics of the N-terminal CBD of DspA/E. However, our results raise additional questions about the regulation of DspA/E translocation by the activity of two heterologous chaperones; also, the influence of OrfA on DspA/E translocation has not yet been established. Future studies might include a more comprehensive analysis of the influence of the C terminus of DspA/E on secretion and translocation and its interaction with chaperones, and a more detailed structural analysis of the interaction between chaperone-binding motifs of DspA/E and the chaperone DsbB/F.

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