MOLECULAR PLANT PATHOLOGY (2008) **9**(4), 425–434 DOI: 10.1111/J.1364-3703.2008.00471.X

HrpN of *Erwinia amylovora* functions in the translocation of DspA/E into plant cells

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SUMMARY

The type III secretion system (T3SS) is required by plant pathogenic bacteria for the translocation of certain bacterial proteins to the cytoplasm of plant cells or secretion of some proteins to the apoplast. The T3SS of *Erwinia amylovora,* which causes fire blight of pear, apple and other rosaceous plants, secretes DspA/E, which is an indispensable pathogenicity factor. Several other proteins, including HrpN, a critical virulence factor, are also secreted by the T3SS. Using a CyaA reporter system, we demonstrated that DspA/E is translocated into the cells of *Nicotiana tabacum* 'Xanthi'. To determine if other T3-secreted proteins are needed for translocation of DspA/E, we examined its translocation in several mutants of *E. amylovora* strain Ea321. DspA/E was translocated by both *hrpW* and *hrpK* mutants, although with some delay, indicating that these two proteins are dispensable in the translocation of DspA/E. Remarkably, translocation of DspA/E was essentially abolished in both *hrpN* and *hrpJ* mutants; however, secretion of DspA/E into medium was not affected in any of the mentioned mutants. In contrast to the more virulent strain Ea273, secretion of HrpN was abolished in a *hrpJ* mutant of strain Ea321. In addition, HrpN was weakly translocated into plant cytoplasm. These results suggest that HrpN plays a significant role in the translocation of DspA/E, and HrpJ affects the translocation of DspA/E by affecting secretion or stability of HrpN. Taken together, these results explain the critical importance of HrpN and HrpJ to the development of fire blight.

INTRODUCTION

Progress in understanding the genetics of pathogenesis of *Erwinia amylovora*, which causes the devastating disease known as fire blight in rosaceous plants, has proceeded rapidly in the past two decades (Oh and Beer, 2005). Several pathogenicity or virulence factors of *E. amylovora,* such as the type III secretion system (T3SS) (Desvaux *et al*., 2006), proteins secreted by it, extracellular polysaccharides and siderophores, have been described (Bereswill and Geider, 1997; Dellagi *et al*., 1998; Expert, 1999).

The T3SS provides a dedicated mechanism whereby extracellular bacteria can deliver proteins to the cytosol of host cells or the apoplast (Galán and Wolf-Watz, 2006; He *et al*., 2004). The T3SS of plant pathogenic bacteria consists mainly of Hrc proteins, encoded by conserved *hrc* (*hrp*-conserved) genes among plant pathogenic bacteria and Hrp proteins, encoded by *hrp* (hypersensitive response and pathogenicity) genes (Cornelis and Van Gijsegem, 2000). In *E. amylovora*, *hrc* and *hrp* genes are clustered in a pathogenicity island (PAI) (Oh *et al*., 2005), which also includes *dsp* (disease-specific) genes (Bogdanove *et al*., 1998a; Steinberger and Beer, 1988). In addition to the T3SS, *hrp* genes of *E. amylovora* encode T3-secreted proteins such as harpins, HrpN (Wei *et al*., 1992) and HrpW (Kim and Beer, 1998). DspA/E (Bogdanove *et al*., 1998a; Gaudriault *et al*., 1997), which is critical to disease development, and Eop1 [or EopB]; (Nissinen *et al*., 2007; Oh and Beer, 2005), a YopJ homologue, were also shown to be secreted through the T3SS. Recently, 12 proteins, including HrpN, HrpW, HrpK, HrpJ and DspA/E, were determined as T3-secreted proteins by proteomic analyses (Nissinen *et al*., 2007).

T3 secretion and translocation is thought to be a one-step process, whereby certain T3-secreted proteins, so-called effector proteins, are translocated directly into host cells through two bacterial membranes and the host plasma membrane. In the case of plant pathogens, effectors must also traverse plant cell walls. This process involves protein secretion machinery similar to the flagellar export system, an extracellular pilus or needle-like appendage, and particular T3-secreted proteins called translocators (Mota and Cornelis, 2005). Translocators are required for effector transport across the host membrane, either by forming pores in the membrane (Goure *et al*., 2005) or by forming a complex with the pore-formers (Holmstrom *et al*., 2001). For plant pathogenic bacteria, both HrpF of *Xanthomonas campestris* pv. *vesicatoria* (Büttner *et al*., 2002) and HrpK of *Pseudomonas*

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syringae pv. *tomato* ($HrpF_{xav}$ and $HrpK_{Ps}$) (Petnicki-Ocwieja *et al.*, 2005) have been characterized as putative pore-forming translocators.

Thus far, no proteinaceous translocators have been determined in *E. amylovora*. However, there are several likely candidates based on previous reports and homology to proteins known to be involved in effector translocation. Two harpins, HrpN and HrpW, are candidate translocators in *E. amylovora* because they are probably targeted to the plant apoplast, where these proteins elicit a hypersensitive response (HR) following infiltration into the intercellular spaces of leaf tissue (Kim and Beer, 1998; Wei *et al.*, 1992). More interestingly, HrpN of *E. amylovora*, unlike HrpW, is required for full virulence in plants (Barny, 1995; Wei *et al.*, 1992). HrpN of *E. amylovora* and $HrpZ_{Psph}$ from *P. syringae* pv. *phaseolicola* have been shown to form ion-conducting pores *in vitro* (Lee *et al.*, 2001; J. Lee and T. Nürnberger, personal communication).

HrpW has a pectate-lyase domain (Kim and Beer, 1998), as does a homologue of the same name of *P. syringae* (Charkowski *et al.*, 1998) and several similar proteins that have been detected in other plant pathogenic enterobacteria (Bell *et al.*, 2004). The pectate lyase domain has not shown enzymatic activity in either *P. syringae* or *E. amylovora*, but HrpW of the former was shown to bind to calcium pectate, a major component of plant cell walls (Charkowski *et al.*, 1998).

Based on *in silico* analysis, *E. amylovora* HrpK ($HrpK_{Ea}$) has homology to $HrpK_{Ps}$. The similarity of $HrpK_{Ea}$ with the putative translocator $HrpK_{Ps}$ (Petnicki-Ocwieja *et al.*, 2005) suggests the possibility that $HrpK_{Ea}$ might function as a translocator in *E. amylovora*. $HrpK_{Ea}$ also has slight homology to HrpF, a T3-translocator protein from *X. campestris* pv. *vesicatoria* (Alfano and Collmer, 2004; Büttner *et al.*, 2002). However, unlike $HrpK_{Ps}$, $HrpK_{Ea}$ mutants are not affected in virulence of *E. amylovora* in either immature pear fruits or in apple shoots (Nissinen *et al.*, 2007; Oh *et al.*, 2005).

E. amylovora HrpJ ($HrpJ_{Ea}$) is a member of the HrpJ family identified in several Gram-negative plant pathogenic bacteria. When compared with other plant pathogen sequences, highest similarity is with homologous sequences in bacteria of the family Enterobacteriaceae. However $HrpJ_{Ea}$ is also similar to HrpJ of *P. syringae* pv. *tomato* ($HrpJ_{Pst}$), which has been shown to affect secretion of accessory T3 proteins and translocation of effectors (Fu *et al.*, 2006).

$HrpJ_{Ea}$ is also somewhat similar to YopN of the animal pathogen *Yersinia* spp. (Bogdanove *et al.*, 1996), which functions indirectly in effector translocation and prevents premature secretion of T3-secreted proteins (Ferracci *et al.*, 2005). Recently, $HrpJ_{Ea}$ was shown to be required for pathogenicity and elicitation of the HR, a rapid localized cell death response. In addition, $HrpJ_{Ea}$ controls secretion of HrpN because its secretion by an *hrpJ* mutant was reduced significantly relative to secretion of HrpN by the virulent strain Ea273 (Nissinen *et al.*, 2007). However, whether $HrpJ_{Ea}$ is involved in translocation of effector proteins remains to be determined.

DspA/E is a well-known protein of 198 kDa encoded by a gene of the *E. amylovora* PAI (Bogdanove *et al.*, 1998b; Gaudriault *et al.*, 1997), and it belongs to the AvrE family of effector proteins. DspA/E, as well as AvrE, has been shown to suppress basal defence by suppressing callose deposition (DebRoy *et al.*, 2004). In addition, members of our group have shown that DspA/E interacts with the intracellular portion of receptor kinases from apple (Meng *et al.*, 2006) and with preferredoxin in the apple cytoplasm (Bonasera *et al.*, 2006), indicating that DspA/E may function inside host plant cells. Moreover, transient expression of DspA/E in apple and tobacco cells induces an HR (Boureau *et al.*, 2006, Oh *et al.*, 2007). Although DspA/E is secreted via a T3SS (Bogdanove *et al.*, 1998a) and is thought to be delivered into plant cells, no definitive evidence that DspA/E is translocated into plant cells has been presented.

In the present study, we first show that DspA/E of *E. amylovora* is translocated into tobacco cells in a T3SS-dependent manner using the CyaA reporter system (Sory and Cornelis, 1994). Similarly, we demonstrate, using several mutant strains of *E. amylovora*, that HrpN and HrpJ are necessary for translocation of DspA/E. In addition, we show that HrpN is weakly translocated into tobacco plant cells.

RESULTS

$DspA/E_{1-733}$-CyaA is translocated into plant cells in a T3-dependent manner

To determine whether DspA/E is translocated into plant cells, we constructed plasmid pCPP1553, which expresses the catalytic domain of CyaA, by fusing a 733-amino-acid fragment of the N-terminus of DspA/E, resulting in $DspA/E_{1-733}$-CyaA (Table 1). All wild-type and mutant strains of Ea321 harbouring pCPP1553 produced cAMP only in the presence of calmodulin *in vitro* (data not shown). To find a suitable plant for assay of translocation of DspA/E *in vivo*, the strains harbouring pCPP1553 were inoculated into leaves of apple (*Malus* x *domestica* 'Gala'), *Nicotiana tabacum* 'Xanthi' and *N. benthamiana*. Unfortunately, leaves of apple, a natural host of *E. amylovora*, were consistently difficult to infiltrate due to their hardness and extensive wax/cutin layer (data not shown). Thus, we tested *Nicotiana* sp. plants, which frequently are used for translocation assays.

E. amylovora induces HR in both *N. tabacum* and *N. benthamiana* (Oh and Beer, 2005). The HR reaction in *N. benthamiana* developed between 5 and 7 h after infiltration of moderate concentrations (5×10^7 cfu/mL) of *E. amylovora*. In *N. tabacum*, visible collapse of the infiltrated areas occurred approximately 8 h after infiltration with bacterial suspensions of 4×10^8 cfu/mL (data not shown). As collapse and necrosis of the tissue due to

Table 1 Bacterial strains and plasmids used in this study.

Strain	Characteristics	Reference
Ea321	Wild-type *E. amylovora*, moderately virulent on apple and pear	ATCC49947
Ea321-*hrcN*	Secretion mutant. Ea 321::Tn10 mini-Km in *hrcN* (Ea321K178)	Wei and Beer (1993)
Ea321-*hrpK*	Tn5 inserted in *hrpK* gene	Oh *et al*. (2005)
Ea321-*hrpW*	*hrpW*::TnphoA (Ea321-P110)	Kim and Beer (1998)
Ea321Δ*hrpJ*	777-bp deletion in frame of *hrpJ* gene	A. Bogdanove, unpublished data
Ea321-*hrpN*	Tn5Tac insert in 1.3-kb *hrpN* gene (Ea321-Tn5tac1)	Wei *et al*. (1992)
Ea321*hrpN*Δ*hrpJ*	Double mutant of *hrpN* and *hrpJ*	Present study
Ea321Δ*hrpN-W*	Deletion of *hrpN*, *orfa*, *eop1*, *orfC* and *hrpW*. Region replaced with *nptII*	J.-F. Kim, unpublished data
Ea273	Wild-type *E. amylovora*, highly virulent on apple and pear	ATCC 49946
Ea273-*hrcN*	Secretion mutant of Ea273 equivalent to Ea321-*hrcN*	Present study
E. coli DH5α	*Escherichia coli*, F^- φ80d*lac*Z M15 (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1	Invitrogen (Carlsbad, CA)
Plasmid		
pCPP1553	Codons 2–406 of Cya from pMJH20 cloned in *Hind*III site of pCPP1249 to yield fusion protein DspA/$E_{1\text{-}733}$-CyaA	Present study
pMJH20	pWSK29 containing codons 2–406 of CyaA in *Sma*I/*Eco*RI site	Miao *et al*. (1999)
pCPP1249	2.2-kb of N-terminal fragment of *dspE* cloned in a pML122 vector (Labes *et al*., 1990) with a *hrpL* promoter	A. Bogdanove, unpublished data
pCPP1084	*hrpN* gene under the control of T7 in M13KS+, expresses HrpN at low levels	Wei *et al*. (1992)
pCPP1729	Codons 1–323 of *hrpN* from pCPP1084 cloned in *Bam*HI–*Sma*I sites of pMJH20 to yield fusion protein $HrpN_{1\text{-}323}$-CyaA	Present study
pET-24a(+)	Expression vector. It carries an N-terminal T7•Tag® plus an optional C-terminal His•Tag®. Km^r	EMD Chemicals Inc. (San Diego, CA)
pCPP1031	Complete *hrpJ* gene in pMB1 replicon	A. Bogdanove, unpublished data
pCPP1763	Complete *hrpJ* gene in pET-24a(+)	Present study

HR would affect cytosolic proteins and cAMP, and thus affect the translocation assays (Casper-Lindley *et al*., 2002), we sampled the tissues before visible HR appeared. Thus, we used *N. tabacum* 'Xanthi' for translocation assays, because we considered the time for sample collection to be less restrictive with it. In addition, we were able to infiltrate higher concentrations of bacteria. Samples for determination of cAMP were collected 7 h after infiltration, just before visible collapse occurred.

To rule out the possibility that expression of DspA/$E_{1\text{-}733}$-CyaA interferes with secretion or translocation of other proteins, we compared the wild-type strain, with and without pCPP1553, for pathogenesis in apple and induction of HR in tobacco. We detected no difference in either the severity or the timing of development of the HR or disease symptoms based on the presence of pCPP1553 (data not shown). These observations confirmed that DspA/$E_{1\text{-}733}$-CyaA did not affect the relevant phenotypes of the strains studied.

To measure the translocation of DspA/$E_{1\text{-}733}$-CyaA into plant cells, we followed accumulation of cAMP in tobacco leaves inoculated with Ea321 and an Ea321-*hrcN* mutant harbouring pCPP1553 as a negative control. The concentrations of cAMP from wild-type Ea321, expressing DspA/$E_{1\text{-}733}$-CyaA, were significantly (30–500 times) higher than the values from the buffer control and Ea321-*hrcN*. These results indicate that DspA/$E_{1\text{-}733}$-CyaA is translocated into tobacco cells, and the translocation of DspA/$E_{1\text{-}733}$-CyaA is dependent on a functional T3SS (Fig. 1).

HrpN and HrpJ are required for translocation of DspA/$E_{1\text{-}733}$-CyaA into plant cells

E. amylovora secretes several proteins, including HrpK, HrpJ and the two harpins, HrpN and HrpW, which may function in translocation. To determine if any of these proteins is involved in the translocation of DspA/E, we assayed for translocation of DspA/E in strains that had been mutated in genes encoding these proteins. The concentrations of cAMP detected from both Ea321Δ*hrpJ* and Ea321-*hrpN* mutants 7 h after infiltration were significantly reduced and statistically similar to those obtained from the secretion mutant and the buffer treatment (Fig. 1). In contrast, no significant change in cAMP concentration was detected for the *hrpW* mutant of Ea321, as compared with the Ea321 wild-type (Fig. 1). Levels of cAMP for the *hrpK* mutant of Ea321 were higher in half of our experiments (Fig. 1), and not affected in the two other experiments (Fig. 2). Levels of cAMP accumulation from the double mutant, lacking both *hrpN* and *hrpJ*, were statistically similar to those of the single mutants in *hrpN* or *hrpJ* (data not shown). These results indicate that both HrpJ and HrpN are required for efficient translocation of the

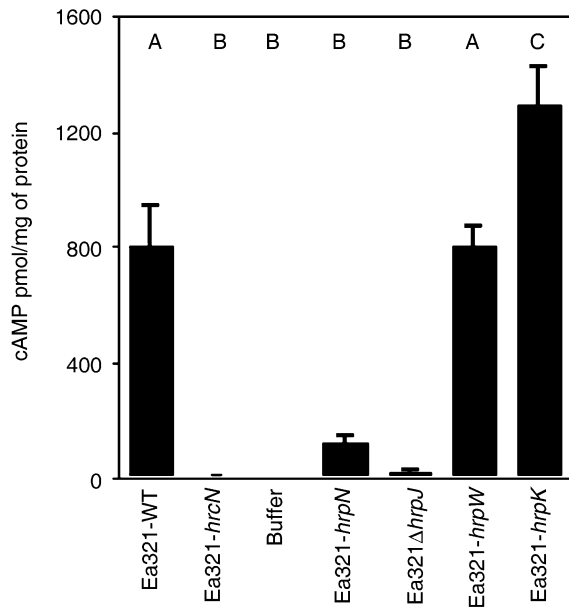

Fig. 1 Translocation of DspA/$E_{1\text{-}733}$-CyaA into plant cells by Ea321 and several mutants. Plants were infiltrated with suspensions of strains of Ea321 harbouring pCPP1553 at OD_{600} = 0.4. Leaf samples were taken 7 h after infiltration and the amount of cAMP was determined. cAMP is expressed as pmol/mg of total protein. The values for each strain are the means of 12 samples from one experiment. The bars represent standard errors. Values denoted by the same capital letters do not differ significantly at the $P = 0.05$ level. WT, wild-type.

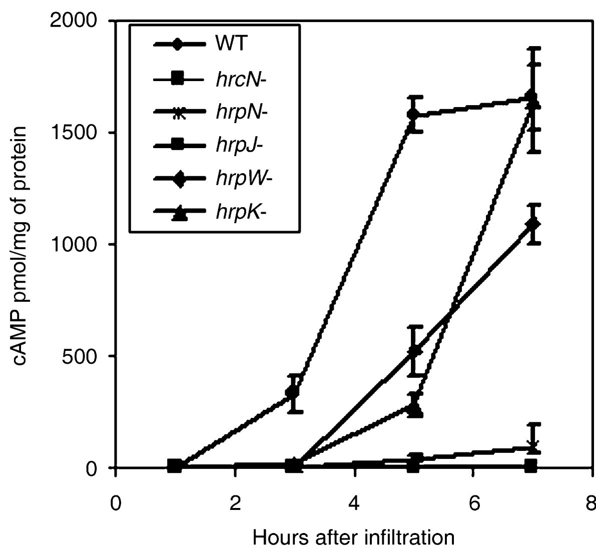

Fig. 2 Time-course translocation of DspA/$E_{1\text{-}733}$-CyaA. Plants were infiltrated with suspensions of strains of Ea321 harbouring pCPP1553 at OD_{600} = 0.4. Leaf samples were taken 1, 3, 5 and 7 h after infiltration and the amount of cAMP was determined. cAMP is expressed as pmol/mg of total protein. The values for each strain are the means of four samples from one experiment. The bars represent standard errors. WT, wild-type.

DspA/E into plant cells. Similarly, the levels of accumulation from a mutant with a deletion that includes the two genes encoding the harpins HrpW and HrpN, as well as the genes encoding the putative effector Eop1 and its chaperone, were similar to those of the single mutant in *hrpN* (data not shown). This result rules out any contribution that HrpW might make to the translocation of DspA/$E_{1\text{-}733}$-CyaA.

To determine translocation of DspA/$E_{1\text{-}733}$-CyaA from the wild-type Ea321 and mutant strains earlier than 7 h after infiltration, levels of cAMP accumulation were monitored at 1, 3, 5 and 7 h after infiltration (Fig. 2). cAMP was first detected 3 h after infiltration and reached a peak in wild-type Ea321 5 h after infiltration. Very little accumulation of cAMP was detected consistently from the *hrpN* and *hrpJ* mutants at all assay times (Fig. 2). Interestingly, similar amounts of cAMP were detected for the wild-type strain at 3 or 5 h after infiltration as were detected in both the *hrpK* and the *hrpW* mutants at 5 or 7 h after infiltration, respectively. These results suggest that translocation of DspA/E might be delayed in the absence of either HrpK or HrpW, but unlike HrpN and HrpJ, the former two proteins are not critical for translocation of DspA/E.

HrpN and HrpJ are not needed for T3-dependent secretion *in vitro* of DspA/$E_{1\text{-}733}$-CyaA

To determine whether mutation in *hrpN* or *hrpJ* affects secretion of DspA/$E_{1\text{-}733}$-CyaA from bacterial cells, expression and secretion of the fusion protein under *hrp*-inducing conditions was examined by immunoblotting with a CyaA antibody. DspA/$E_{1\text{-}733}$-CyaA was expressed by all the tested strains, and it was secreted by all the strains except for the T3 secretion mutant Ea321-*hrcN* (Fig. 3). These results indicate that neither HrpN nor HrpJ are needed for expression and secretion of DspA/$E_{1\text{-}733}$-CyaA, and that HrpJ may affect translocation of DspA/$E_{1\text{-}733}$-CyaA by facilitating HrpN secretion.

HrpJ protein is secreted by an *hrpN* mutant

Our laboratory has shown previously that mutation in *hrpJ* significantly reduces secretion of HrpN in *E. amylovora* strain Ea273 (Nissinen *et al*., 2007). First, we determined secretion of HrpN in an Ea321Δ*hrpJ* mutant. In our experiment, no secreted HrpN protein was detected in this mutant strain, in contrast to the reduced secretion shown previously for an Ea273 *hrpJ* mutant (data not shown). Next, to examine if mutation of the *hrpN* gene affects HrpJ secretion, HrpJ proteins were assayed in culture supernatant, using a polyclonal HrpJ antibody raised in this study. Interestingly, HrpJ proteins were secreted by the *hrpN* mutant, just as with the wild-type strain (Fig. 4). These results suggest that HrpJ could function as a chaperone-like protein for secretion of HrpN.

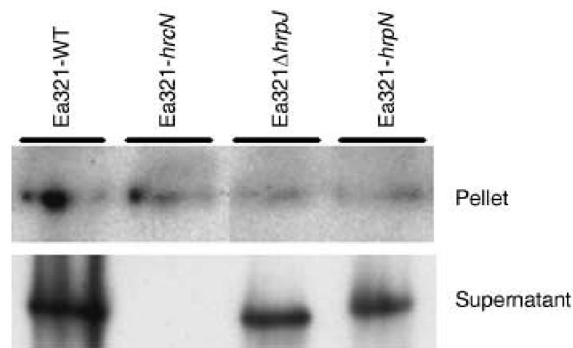

Fig. 3 Expression and secretion of DspA/E_{1-733}-CyaA by several strains of *E. amylovora* under *hrp*-inducing conditions. Strains of Ea321 (pCPP1553) indicated in the figure were grown in *hrp* gene-inducing medium at 18 °C for 36 h. Total proteins from cell pellet and supernatant were collected separately and loaded onto 8% SDS-PAGE gels. The DspA/E_{1-733}-CyaA fusion protein was detected with CyaA antibody. WT, wild-type.

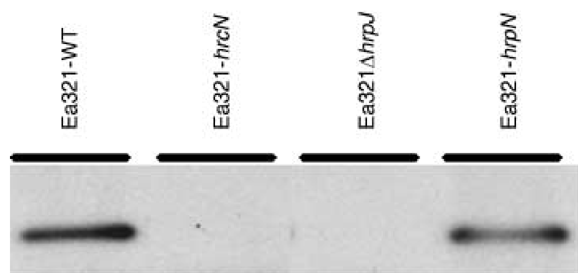

Fig. 4 Secretion of HrpJ by several strains of *E. amylovora* under *hrp* gene-inducing conditions. Strains of Ea321 (pCPP1553) indicated in the figure were grown in *hrp*-inducing medium at 18 °C for 36 h. Proteins from supernatant were collected and loaded in 8% SDS-PAGE gels; detection by Western blotting was carried out with HrpJ polyclonal antibody. WT, wild-type.

$HrpN_{1-323}$-CyaA is weakly translocated into plant cells in a T3-dependent manner

Because the putative translocator HrpK of *P. syringae* had been shown to be translocated into plant cells (Petnicki-Ocwieja *et al.*, 2005), we tested whether HrpN is also translocated into plant cells. A portion of the *hrpN* gene that encodes the first 323 amino acids (80%) from the N-terminus of the protein was fused with the catalytic domain of CyaA in a low-copy-number vector with a *lacZ* promoter (pCPP1729; Table 1). cAMP accumulation in tobacco cells was measured following infiltration of the highly virulent strain Ea273, as described previously (Schecter *et al.*, 2004). cAMP levels from wild-type Ea273, expressing $HrpN_{1-323}$-CyaA, was significantly higher (32-fold) than those from the T3-secretion mutant (Fig. 5). However, the absolute levels of cAMP accumulation were lower (59 pmol/mg) than those for

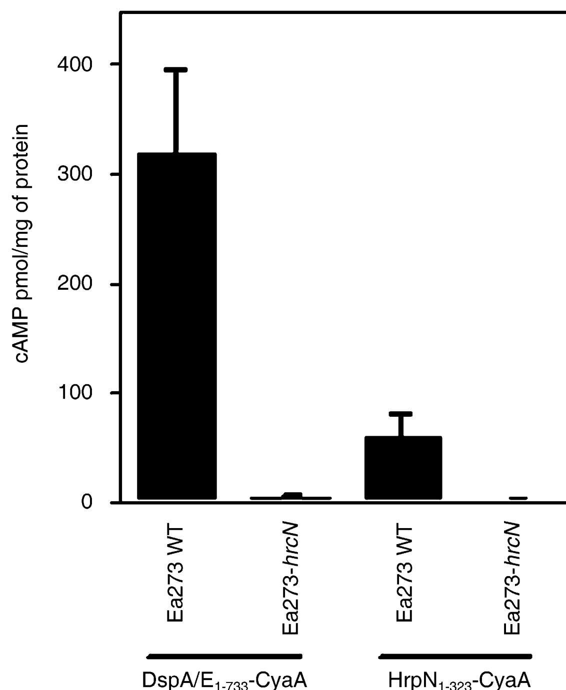

Fig. 5 Translocation of $HrpN_{1-323}$-CyaA into tobacco cells. Plants were infiltrated with bacterial suspensions of the wild-type Ea273 and T3SS-deficient mutant, Ea273-*hrcN*, harbouring pCPP1553 or pCPP1729 at $OD_{600} = 0.4$. Leaf samples were taken 7 h after infiltration and the amount of cAMP was determined. cAMP is expressed as pmol/mg of total protein. The values for each strain are the means of six samples from one experiment. The bars represent standard errors. WT, wild-type.

DspA/E_{1-733}-CyaA (318 pmol/mg) (Fig. 5). These results indicate that $HrpN_{1-323}$-CyaA is translocated into tobacco cells.

DISCUSSION

Based on indirect evidence (Boureau *et al.*, 2006; Meng *et al.*, 2006; Oh *et al.*, 2007), the major effector protein of *E. amylovora*, DspA/E, has been considered to function inside plant cells. In the present study, we provide direct biochemical evidence that DspA/E of *E. amylovora* is translocated into plant cells by the T3SS. Furthermore, we show that the first 733 N-terminal amino acids of the 1837-amino-acid protein are sufficient for both secretion and translocation. Translocation is detectable as soon as 3 h after inoculation (Fig. 2), indicating that the translocation process starts in the first few hours after bacteria invade plant tissue.

The translocation of T3 effectors from plant pathogenic bacteria through plant plasma membranes is poorly understood. For translocation of effectors into mammalian cells, Mota *et al.* (2005) proposed that a dedicated set of proteins, termed translocators, is needed to mediate the delivery of effector proteins through host plasma membranes. A subset of translocators is

thought to insert themselves into the plasma membranes of host cells and form pores through which the effectors can traverse the host plasma membrane to the cytosol (Büttner *et al.*, 2002; Marenne *et al.*, 2003; Meyer *et al.*, 2006; Petnicki-Ocwieja *et al.*, 2005). Translocators are needed for translocation, but they are not needed for secretion of the effector proteins (Mota *et al.*, 2005); the translocators themselves are secreted via the T3SS (Hakansson *et al.*, 1996; Sory and Cornelis, 1994), and they also can be translocated into host cells (Francis and Wolf-Watz, 1998; Petnicki-Ocwieja *et al.*, 2005).

The critical role of HrpN in the virulence of the fire blight pathogen has been a mystery since its characterization (Wei *et al.*, 1992) and further study (Barny, 1995). Here, we have shown that HrpN is required for translocation of DspA/E from the bacterial cytoplasm to the plant cytoplasm. Thus, the function of HrpN in the translocation of DspA/E, an essential pathogenicity effector of *E. amylovora*, is fully consistent with its importance for disease development. Although several other harpins, including HrpW, have been characterized for plant pathogenic bacteria, their precise role in virulence remains unclear. Our observation that $HrpN_{1\text{-}323}$-CyaA induced significantly more cAMP accumulation than the negative control (Fig. 5) suggests that HrpN itself is translocated into host plant cells. However, cAMP accumulation by $HrpN_{1\text{-}323}$-CyaA was much lower than for the $DspA/E_{1\text{-}733}$-CyaA fusion protein, suggesting that HrpN proteins are perhaps embedded in plasma membranes rather than fully translocated into the plant cytoplasm as is the $DspA/E_{1\text{-}733}$-CyaA fusion protein. This assumption would be consistent with the presence of two putative transmembrane helices in the N-terminal portion of HrpN, predicted by the 'TMpred' tool, available at the ExPASy proteomics server of the Swiss Institute of Bioinformatics. Interestingly, HrpN has also been shown to function as a pore-former (J. Lee and T Nürnberger, personal communication), as does HrpZ1, a harpin of *P. syringae* pv. *phaseolicola* (Lee *et al.*, 2001).

Although HrpN of *E. amylovora* is required for translocation of normal levels of DspA/E, other proteins could be involved in the translocation process. Accumulation of cAMP by the $DspA/E_{1\text{-}733}$-CyaA in the *hrpN* mutant of Ea321 is greater than in the *hrcN* mutant or the buffer control (Figs 1 and 2). This suggests that other proteins might contribute to the translocation of DspA/E. This is consistent with the results of virulence tests in apple shoots, where *hrpN* mutants show severely reduced virulence, but are still pathogenic (this study, results not shown; and Barny, 1995).

In addition to HrpN, HrpJ is necessary for translocation of DspA/E, although it is not required for secretion of DspA/E. However, depending on the aggressiveness of the strain, secretion of both harpins, HrpN and HrpW, is reduced or abolished in *hrpJ* mutants (Nissinen *et al.*, 2007; this study). These data suggest that HrpJ acts indirectly on the translocation of DspA/E by facilitating the secretion of HrpN. Interestingly, we observed interaction of HrpJ with both HrpN and HrpW in yeast (see supplementary Fig. S1). These interactions support the hypothesis that HrpJ might be involved in secretion of HrpN and/or HrpW. We also observed strong interaction of HrpJ with itself (supplementary Fig. S1), which suggests that HrpJ might function in a complex. Finally, we observed that there is no interaction between HrpJ and DspA/E in yeast (results not shown), consistent with our other results suggesting that HrpJ does not function directly on the effector DspA/E but indirectly through interaction with the putative translocator HrpN.

The function of HrpJ seems to be similar to that of InvE of *Salmonella enterica* sv. *typhimurium*, which is required for normal accumulation of the extracellular translocator proteins SipB, SipC and SipD (Kubori and Galán, 2002). Similarly, HrpJ of *P. syringae* pv. *tomato* recently was shown to be required for secretion of HrpZ1 *in vitro* and for the translocation of several effector proteins (Fu *et al.*, 2006).

In contrast to HrpN and HrpJ, our tests of HrpW and HrpK indicate that these proteins are not critical to either secretion (Fig. 3) or translocation of DspA/E (Fig. 1) in Ea321. However, our time-course measurements of cAMP accumulation imply that both proteins have some effect on translocation of DspA/E because accumulation of cAMP was delayed significantly for up to 2 h (Fig. 2). This is an interesting observation, as one would expect a delay in translocation to be reflected in virulence. However, *hrpW* and *hrpK* mutants of *E. amylovora* are as virulent as the wild-type strain in host plants (Kim and Beer, 1998; Nissinen *et al.*, 2007; Oh *et al.*, 2005). This could be either because a delay of 2 h is insufficient to affect virulence or because our testing methods cannot measure a difference in virulence over such short periods.

HrpW does not contribute to the translocation of DspA/E based on our observations that a deletion mutant spanning the genes encoding both harpins, the putative effector Eop1 and its chaperone, produced a level of cAMP accumulation similar to that of the *hrpN* single mutant (data not shown).

HrpK is a T3-secreted protein of *E. amylovora* (Nissinen *et al.*, 2007), and it is similar to HrpK of *Pseudomonas* sp. Interestingly, HrpK of *P. syringae* pv. *tomato* DC3000 has been shown to contribute to virulence in host plants, translocation of effector proteins and HR elicitation (Petnicki-Ocwieja *et al.*, 2005). In contrast, *hrpK* mutants in *E. amylovora* Ea273, as well as Ea321, are fully virulent and HR-positive (Nissinen *et al.*, 2007; this study). Moreover, an *hrpK* mutant of Ea321 translocated DspA/E as well (Fig. 2) or better (Fig. 1) than the wild-type strain. The only indication that HrpK could be involved in translocation of DspA/E is the delay in onset of translocation (Fig. 2). Despite the similarity of $HrpK_{Ea}$ and $HrpK_{Pst}$, their phenotypes are quite different, possibly because they translocate different effectors. While the effectors of *P. syringae* that $HrpK_{Pst}$ putatively translocate are important for development of disease in its hosts (Petnicki-Ocwieja *et al.*, 2005), the effectors associated with $HrpK_{Ea}$ in

E. amylovora might have a redundant function. Under this assumption we cannot dismiss the possibility that HrpK or HrpW are possible translocators of other effector(s), although they clearly do not play a major role in translocation of DspA/E. To ascertain the possible roles of HrpW and HrpK, further translocation tests would be needed with other putative effectors, several of which have been identified in *E. amylovora* recently (Zhao *et al*., 2006).

The mechanistic role of HrpN of *E. amylovora* in the fire blight disease has been an intriguing question since the early 1990s when the protein was characterized and *hrpN* mutants of *E. amylovora* were found to be drastically reduced in virulence to host plants such as apple and pear (Wei *et al*., 1992). From the present study, it is clear that HrpN plays a pivotal role in disease development by facilitating translocation of DspA/E into plant cells. In the absence of HrpN, translocation of the critical disease-specific protein DspA/E into host cells is dramatically reduced, and consequently the *hrpN* mutant is essentially non-virulent.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used were grown in Luria–Bertani (LB) broth or LB agar with appropriate antibiotics for selection (Table 1). The DspA/$E_{1\text{-}733}$-CyaA fusion protein was constructed by cloning a fragment encoding the catalytic domain of *CyaA* from *Bordetella pertussis* from plasmid pMJH20 (Miao *et al*., 1999) into pCPP1249 (A. J. Bogdanove, unpublished data), in frame, after codons for the first 733 amino acids of DspA/E. The N-terminal fragment of DspA/E contains the putative secretion signal necessary for secretion and presumably for translocation. pCPP1249 is a low-copy vector with the native promoter sequence for *dspA/E* under control of the T3-specific sigma factor *hrpL* (Wei and Beer, 1995); it also includes the *nptII* promoter. The resulting plasmid, pCPP1553, which expresses the fusion protein, was transformed into several strains of *E. amylovora* (Table 1). The $HrpN_{1\text{-}323}$-CyaA fusion was constructed by cloning the fragment encoding the first 323 amino acids (80% of the protein) from pCPP1084 (Table 1) into pMJH20, in frame with the catalytic domain of *CyaA*. The fusion protein in pMJH20 is expressed under control of the *lacZ* promoter, which is inducible with isopropyl β-D-1-thiogalactopyranoside (IPTG).

Production of polyclonal HrpJ antibodies

Polyclonal antisera against HrpJ was raised at the College of Veterinary Medicine, Cornell University, by injecting intramuscularly 100 μg of purified HrpJ into a rabbit three times at 3-week intervals. Blood was collected 2 weeks after the final injection. The immunoglobulin fraction was cross-absorbed with heat-treated lysate of *Escherichia coli* BL21 (DE3) [pET24a)(+)]. To produce the HrpJ protein for immunization, the *hrpJ* gene was amplified from pCPP1031 and cloned into pET-24a(+) tagging it with the HIS-tag at HrpJ C-terminus. HrpJ was produced in high quantities by *E. coli* BL21 (DE3), and purified with TALON® Metal Affinity Resins (CLONTECH, Palo Alto, CA).

Protein secretion assay

Strains of *E. amylovora* Ea321 harbouring pCPP1553 or mutant strains were grown in Hyunh's minimal medium (Huynh *et al*., 1989), for 36 h at 18 °C on a shaker at 200 r.p.m. to induce T3-dependent protein secretion. Suspensions were treated with 0.5 mM phenyl methylsulfonyl fluoride (PMSF) to avoid protein degradation, centrifuged and the supernatants were concentrated 2500 times with Centriplus Centrifugal Filters YM-10 (Millipore, Bedford, MA). Cell pellets, recovered after centrifugation, were resuspended in one-tenth of the original volume of SDS loading buffer. Samples were electrophoresed in 8% SDS-PAGE gels, then proteins were transferred to PVDF membranes. DspA/$E_{1\text{-}733}$-CyaA was detected by adenylate cyclase murine monoclonal antibody 3D1 (List Biological Laboratories, Campbell, CA), and HrpN antibody (Wei *et al*., 1992) and HrpJ antibody (this study) were used to detect HrpN and HrpJ, respectively.

Quantification of cAMP *in planta*

Strains of *E. amylovora* were infiltrated at OD_{600} = 0.4 into leaf panels of *N. tabacum* 'Xanthi' as previously described (Wei *et al*., 1992). Leaf discs (8 mm in diameter) were harvested from the infiltrated areas 7 h after infiltration, placed individually in microfuge tubes, immediately frozen in liquid nitrogen and stored at –80 °C for later processing. The development of HR in infiltrated areas was assessed 24 h after infiltration. Frozen leaf discs were prepared for cAMP quantification as per Schechter *et al*. (2004), and the cAMP content in samples was determined with the Correlate-EIA Direct Cyclic AMP Enzyme Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI), following the manufacturer's instructions. Protein concentration in the samples was determined with a bicinchoninic acid (BCA) test kit (Pierce, Rockford, IL).

Quantification of cAMP *in vitro*

Strains of *E. amylovora* were grown overnight at 26 °C with shaking in LB with appropriate antibiotics. One hundred microlitres of overnight culture were transferred into fresh LB with appropriate antibiotics. The bacterial strains were grown to OD_{600} = 0.4. Bacterial suspensions were centrifuged and the pellet was resuspended in 20 mM Tris containing 10 mM $MgCl_2$ at pH 8. The cells were lysed by sonication for 90 s with a micro-tip (Model 550 Sonic Dismembrator, Fisher Scientific, Pittsburg, PA).

The lysate sample was centrifuged at 4600 ***g*** for 10 min and the supernatant was stored in microfuge tubes at –20 °C until further processing. The CyaA assay reaction mixture was prepared as previously described (Schechter *et al*., 2004; Sory and Cornelis, 1994) with and without calmodulin (0.2 μM). The mixture was incubated at 30 °C for 10 min before determining the content of cAMP, as described for quantification of cAMP *in planta* (above).

Statistical tests

The program S-PLUS version 7.0.0 for Windows (Insightful Corp., Seattle, WA.) was used for the statistical analysis. For the ANOVA, a fixed-effects model and the multiple comparisons (Tukey option) were used to compare the mean values for cAMP accumulated by the different strains in each *in planta* experiment.

ACKNOWLEDGEMENTS

We thank Lisa Schechter and Alan Collmer for providing protocols and helpful comments on the CyaA reporter system. We thank Kent Loeffler for photography. We thank Jacqueline Nock and Christopher Watkins for storing, in controlled atmospheres, the immature pear fruits used in this study. We thank Sam Cartinhour and James Giovannoni for critically reading the manuscript. This work was supported, in part, by USDA-CSREES Special Grants for research on fire blight of apple, an USDA-NSF Microbial Genome Sequencing grant, and royalty funds of Eden Biosciences Corporation to S.V.B.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Fig. S1 Direct interaction in yeast between HrpJ and HrpN, HrpW and itself. The *hrpJ* gene in the bait vector (pGilda) and *hrpN*, *hrpW* and *hrpJ* in the prey vector (pB42AD; EV) were co-transformed into the yeast strain, EYG48(p8oplacZ), using LiAc/PEG method (*Yeast Protocols Handbook*; Clontech, Mountain View, CA). Yeast transformants were screened on synthetic Drop-Out medium (SD) with glucose, but without histidine, tryptophan

and uracil (-HTU). Ten-microlitre cell suspensions at $OD_{600} = 0.2$ and 10-fold dilutions were plated and incubated for 5 days on SD-HTU with galactose in the presence or absence of leucine (Leu) to determine protein–protein interaction. Growth on plates regardless of the presence of leucine represents positive interactions. pLexA-p53 (LexA-p53) and pB42AD-T (T-antigen) were used as positive controls.

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