DspA/E, a Type III Effector Essential for *Erwinia amylovora* Pathogenicity and Growth In Planta, Induces Cell Death in Host Apple and Nonhost Tobacco Plants

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Erwinia amylovora is responsible for fire blight, a necrotic disease of apples and pears. *E. amylovora* relies on a type III secretion system (TTSS) to induce disease on hosts and hypersensitive response (HR) on nonhost plants. The DspA/E protein is essential for *E. amylovora* pathogenicity and is secreted via the TTSS in vitro. DspA/E belongs to a type III effector family that is conserved in several phytopathogenic bacteria. In *E. amylovora*, DspA/E has been implicated in the generation of an oxidative stress during disease and the suppression of callose deposition. We investigated the fate of DspA/E in planta. DspA/E delivered artificially to apple or tobacco cells by agroinfection induced necrotic symptoms, indicating that DspA/E was probably injected via the TTSS. We confirmed that DspA/E acts as a major cell-death inducer during disease and HR, because the *dspA/E* mutant is severely impaired in its ability to induce electrolyte leakage in apple and tobacco leaves. Expression of the defense marker gene *PR1* was delayed when *dspA/E* was transiently expressed in tobacco, suggesting that DspA/E-mediated necrosis may be associated with an alteration of defense responses.

The gram-negative bacterium *Erwinia amylovora* causes fire blight, a necrotic disease responsible for considerable economic losses in plants of the family Rosaceae, especially those of the subfamily Maloidae, which include apple (*Malus* spp.) and pear (*Pyrus* spp.). The main disease symptom in host plants is black necrosis of shoots and dark browning of the leaves, resembling fire damage and accounting for the name given to the disease (Van der Swet and Beer 1995). Resistant cultivars exist, however their resistance is complex and cannot be accounted for by a major resistance (*R*) gene (Lespinasse and Aldwinckle 2000). Concomitantly, no avirulence gene has been described in *E. amylovora*. In nonhost plants, such as tobacco, *E. amylovora* elicits a hypersensitive reaction (HR) restricting the growth of the pathogen at the site of inoculation. Genetic studies have demonstrated that the ability of *E. amylovora* to cause disease in host plants and to elicit the HR in nonhost plants de-

pends on the presence of a functional type III secretion system (TTSS) encoded by the *hrp* (hypersensitive reaction and pathogenicity) gene cluster (Steinberger and Beer 1988; Barny et al. 1990).

The TTSS is widespread and its structure is well conserved among gram-negative pathogenic bacteria, whether they attack animals (*Yersinia*, *Salmonella*, EPEC spp.) or plants (*Pseudomonas*, *Ralstonia*, *Xanthomonas*, *Pantoea*, *Erwinia* spp.). The TTSS enables the pathogenic bacteria to inject effector proteins directly into the eukaryotic cell (Cornelis and Van Gijsegem 2000). Once inside the host cell, type III effector proteins modulate and divert the metabolism of the eukaryotic cell towards responses favorable to the pathogenic bacteria (Alfano and Collmer 2004). Recent studies showed that type III effectors of plant pathogenic bacteria can mask the presence of other effectors potentially recognized by the plant (Espinosa et al. 2003; Jackson et al. 1999; Jamir et al. 2004; Tsiamis et al. 2000), suppress basal defenses (DebRoy et al. 2004; Hauck et al. 2003; Keshavarzi et al. 2004), suppress the phenylpropanoid pathway (Venisse et al. 2002), or activate an oxidative burst required to induce disease (Venisse et al. 2001, 2003).

In *E. amylovora*, four proteins secreted by the TTSS in vitro have been characterized, HrpA, HrpN, HrpW, and DspA/E. HrpA is the structural protein of the type III secretion pilus; it forms a hollow structure functioning as a conduit for protein delivery (Jin and He 2001; Jin et al. 2001; Li et al. 2002). HrpN and HrpW belong to the harpin family and are HR elicitors in tobacco (Gaudriault et al. 1998; Kim and Beer 1998; Wei et al. 1992). HrpN has been detected in the intercellular spaces of infected apple seedlings by immunocytochemistry, indicating that HrpN is secreted in planta (Perino et al. 1999). DspA/E is a major candidate effector. DspA/E is homologous to AvrE, a type III effector identified in *Pseudomonas syringae* pv. *tomato* that is recognized as an avirulence protein in soybean plant (Lorang and Keen 1995). Functional cross-complementation has been demonstrated between DspA/E and AvrE, although these proteins display only 21% identity (Bogdanove et al. 1998). This suggests that DspA/E and AvrE play the same basic role during disease. In *E. amylovora*, DspA/E is central to the disease process, as *dspA/E* mutants do not grow on host plants and cannot induce disease symptoms (Barny et al. 1990; Bogdanove et al. 1998; Gaudriault et al. 1997). It was recently shown that the *E. amylovora* *dspA/E* mutant and *P. syringae* pv. *tomato* Δ cel mutant (deleted of four characterized effector

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This paper is dedicated to the memory of our friend and colleague Igor Pucheu, who died on December 26, 2004, at the age of 34.

genes including *avrE*) are unable to suppress callose deposition during disease (DebRoy et al. 2004). DspA/E has also been implicated in the generation of the oxidative stress during disease on host plants, as the *dspA/E* mutant is severely affected in its ability to activate plant antioxidant enzymes (Venisse et al. 2003). Whether DspA/E acts alone during these processes remains an open question.

In the present study, we investigated the fate of DspA/E in host and nonhost plants.

RESULTS

DspA/E belongs to a family of type III effectors conserved in plant pathogenic bacteria.

DspA/E homologs were identified by PsiBlast analysis or were obtained from preliminary genome sequence data for *E. chrysanthemi* 3937. Homologs were identified in several gram-negative phytopathogenic bacteria and in the saprophytic bacterium *P. fluorescens* (Fig. 1). Three complete genome sequences are available for *Xanthomonas* spp. (*X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri*, and *X. campestris* pv. *campestris*), but none were found to contain a DspA/E homolog, suggesting that this type III effector is absent from *Xanthomonas* species.

To analyze the relationship between the various DspA/E homologs, we used the method of maximum parsimony to construct a phylogenetic tree. The phylogenetic tree was congruent with overall bacterial phylogeny, suggesting that DspA/E is not a target for frequent horizontal transfer (Fig. 1). Indeed, in all but two cases (*R. solanacearum* and *E. chrysanthemi*), the gene

encoding the *dspA/E* homolog was found to be adjacent to the genes of the type III secretion cluster, in the conserved effector locus as defined in *Pseudomonas* spp. (Alfano et al. 2000), suggesting this gene coevolved with the genes encoding the TTSS.

Depending on the plant-bacterium interaction considered, the effectors of the DspA/E family are important for bacterial fitness or are required for pathogenicity (Fig. 1). All the proteins of this family are large, ranging from 154 (*P. fluorescens*) to 198 kDa (*E. amylovora* and *E. pyrifoliae*) in size. Their sequences do not share homology to any protein of known biochemical function.

DspA/E can be immunodetected associated with the bacterial cell surface within infected plants.

In order to detect DspA/E during disease, we carried out immunolabeling with a polyclonal antibody against DspA/E in infected apple seedlings (Gaudriault et al. 2002).

Double labeling with α -DspA/E and α -lipopolysaccharide (LPS) was performed on leaf tissues infiltrated with the wild-type strain CFBP1430 or with the M52 *dspA/E* mutant as a control. Immunolabeling with the *E. amylovora* LPS antibody was used to detect bacterial cells within leaf tissues, as previously described (Perino et al. 1999). The red signal obtained with LPS labeling (Fig. 2A) and the green signal obtained with DspA/E labeling (Fig. 2B) overlapped on confocal micrographs of leaf tissues infiltrated with the wild-type strain. The resulting yellow signal demonstrates colocalization of the bacterial cells and DspA/E (Fig. 2C). Transmission electron microscopy (TEM) of leaves infected with the wild-type strain confirmed a faint DspA/E-labeling associated with the bacterial

10 species, 1338 sites (1022 informative).
Maximum Parsimony (6101 steps required)
500 bootstrap replicates

Phenotype of the mutant.	References (accession number)
Non Pathogenic	Gaudriault et al., 1997 Bogdanove et al., 1998 (CAA74156)
Not tested	(AAS45452)
Non Pathogenic	Frederick et al., 2001 (AAG01467)
Non Pathogenic	Mor et al., 2001 (AAF76343)
Reduced virulence (only for low inocula)	Holeva et al., 2004 (YP_050208)
Not tested	(&)
Reduced virulence	Lorang and Keen, 1994 (AAF71499)
Not tested	(ZP_00125692)
*	Preston et al., 2001 (AAK74145)
Not tested	Salanoubat et al., 2002 (NP_522840)

Fig. 1. Phylogenetic tree based on aligned amino acid sequences of DspA/E homologs, using the method of maximum of parsimony. The numbers at each node are bootstrap confidence values from 500 replicates. The importance of DspA/E homologs in pathogenicity of their respective bacteria and the accession numbers of the sequences used are indicated on the right. § = Sequences that have not yet been named. & = Sequence of the DspA/E homolog from *E. chrysanthemi* 3937 obtained from the preliminary genome sequence data (funded by the United States Department of Agriculture) and annotated with the ASAP system (Glasner et al. 2003). Community-contributed annotations are made available by the University of Wisconsin and The Institute for Genomic Research. *As *P. fluorescens* is a nonpathogenic strain, testing the importance of the RopE protein in pathogenicity is not relevant.

cell surface (Fig. 3B). No DspA/E labeling could be detected in plant cells, but this could simply reflect that the amount of DspA/E in the plant cell is below the detection threshold with the technique used.

Transient expression

of *dspA/E* induces necrosis on apple and tobacco leaves.

To investigate whether DspA/E had an effect when delivered intracellularly, we transiently expressed *dspA/E* into apple

Fig. 2. Immunodetection of DspA/E in infected leaves by confocal microscopy. Apple leaves were infiltrated with a bacterial suspension calibrated to an optical density at 600 nm of 2, and leaf tissues were harvested 4.5 hpi. for subsequent observation by confocal microscopy. **A, B, and C,** Experiments with the wild-type strain *Erwinia amylovora* CFBP 1430 showed that DspA/E was detected in plants (**B**) in association with the bacterial cells (**C**). **D, E, and F,** Control experiments with the *dspA/E* mutant strain M52 confirmed that no specific fluorescein isothiocyanate (FITC) immunofluorescence was detected in the absence of DspA/E (**E**). Texas Red immunofluorescence was used to detect lipopolysaccharides, indicating the position of bacteria in the sample (**A** and **D**). FITC immunofluorescence was used to detect DspA/E (**B** and **E**). The images obtained with Texas Red and FITC immunofluorescence were superimposed to compare staining patterns (**C** and **F**).

Fig. 3. Immunodetection of DspA/E at the ultrastructural level. Ultrathin transverse sections of leaves inoculated with **A**, the type III secretion mutant PMV6023, **B**, the wild-type strain CFBP1430, or **C**, the *dspA/E* mutant strain M52. Scale bar = 1 μm. Samples were harvested 4.5 hpi for subsequent observation by transmission electron microscopy. Sections were immunogold-labeled with DspA/E antiserum. Secondary antibodies were conjugated to ultrasmall gold, and labeling was silver-enhanced using the Silver Enhancement kit (Amersham). The silver-enhanced particles were restricted to the bacterial cytoplasm in the type III secretion mutant PMV6023 (**A**). In wild-type strain CFBP1430, silver-enhanced particles were observed in the intercellular space (**B**). No silver-enhanced particles were observed in control experiments carried out with the *dspA/E* mutant strain M52 (**C**). CW = cell wall and IS = intercellular space.

seedlings. The *dspA/E* gene was cloned in pBIN under the control of the cauliflower mosaic virus (CaMV) 35S promoter; the resulting plasmid pAG1 was used for *Agrobacterium*-mediated transient expression of *dspA/E*. As a control, transient expression of the *gus* reporter gene cloned under the control of the CaMV 35S promoter was also performed (De Bondt et al. 1994). In the leaf area infiltrated with the CaMV 35S-*gus* construct, we detected β -glucuronidase activity. The weak, spotty staining observed shows that the efficiency of agroinfection in apple leaves was low. Furthermore, no macroscopic symptoms developed on apple leaves infiltrated with the CaMV 35S-*gus* construct, indicating that expression of the *gus* transgene under the control of the CaMV 35S promoter following agroinfection was not deleterious in apple leaves (Fig. 4A). In contrast, necrotic spots appeared within the infiltrated area on leaves inoculated with the CaMV 35S-*dspA/E* construct (Fig. 4A). Thus, *DspA/E* appears to induce cell death following its ectopic production in apple cells.

On tobacco leaves infiltrated with *A. tumefaciens* containing the CaMV 35S-*dspA/E* construct, necrosis developed over the entire infiltrated area within 48 h (Fig. 4B). The first detectable macroscopic symptom was faint glazing on the tobacco abaxial leaf surface 30 h after inoculation, which progressed to tissue collapse and became confluent 36 h postinoculation (hpi). At this timepoint (36 hpi), β -glucuronidase activity was evenly spread throughout leaves infiltrated with *A. tumefaciens* containing the CaMV 35S-*gus* construct, demonstrating a high efficiency of agroinfection in tobacco (Fig. 4B). Leaf tissues infiltrated with *A. tumefaciens* containing the CaMV 35S-*gus* construct yellowed slightly (Fig. 4B), due to a nonspecific response induced by *A. tumefaciens*, as previously described in similar experiments on pepper (Van den Ackerveken et al. 1996).

The high efficiency of agroinfection in tobacco allows monitoring of the response to *dspA/E* before the appearance of any macroscopic symptoms. Tobacco leaf tissues transiently expressing *dspA/E* or *gus* were studied under a light microscope

Fig. 4. Symptoms induced on host (apple) and nonhost (tobacco) plants by transient *dspA/E* expression. **A**, Apple leaves infiltrated with *Agrobacterium tumefaciens* EHA105 pAG1 for transient expression of *dspA/E*; necrotic spots developed in the infiltrated area two days after inoculation. These spots gradually increased in number until seven days after inoculation. As a control, apple leaves infiltrated with EHA 101 pFAJ3000, for transient expression of the *gus* reporter gene, did not develop necrotic symptoms. Only few spots on the leaf displayed the typical blue staining characteristic of β -glucuronidase activity, indicating that agroinfection was not very efficient in apple. These blue spots appeared on the leaves agroinfected with *dspA/E*. **B**, Tobacco leaves infiltrated with *A. tumefaciens* EHA105 pAG1 (*DspA/E*; D), *A. tumefaciens* EHA101 pFAJ3000 (*Gus*; G), and mock-infiltrated (M). Necrosis of the whole area agroinfected with EHA105 pAG1 is observed 48 hpi, with the first macroscopic symptoms appearing at 30 hpi. β -glucuronidase activity in tissues infected with EHA101 pFAJ3000 showed agroinfection to be highly efficient in tobacco, as the blue staining appeared in most of the infiltrated area. **C**, Topographical staining with toluidine blue of a section of tobacco leaf infiltrated with EHA101 pFAJ3000, 24 h after agroinfiltration. The shape of cells is well conserved, organites are pressed against the cell wall by intact vacuoles, and the staining of cell walls is well contrasted, demonstrating that tissues are healthy. **D**, Topographical staining with toluidine blue of a section of tobacco leaf infiltrated with EHA105 pAG1, 24 h after agroinfiltration. Cells are disorganized, with more organites loose in the cytoplasm than in panel C, and cell walls are swollen. IS = intercellular space, At = *A. tumefaciens* cells, ch = chloroplast, n = nucleus, and X = xylem.

to determine whether a phenotype in terms of response to DspA/E could be detected less than 30 h after inoculation. Topographic staining with toluidine blue revealed a clear collapse of the mesophyll cells, disorganized nuclei, and swelling of the plant cell wall 24 h after inoculation, in response to the transient expression of *dspA/E* (Fig. 4D). No such response was observed following the transient expression of *gus* (Fig. 4C). In some cases, the plant cell wall was found to have swollen in response to transient expression of *dspA/E* 18 h after inoculation, suggesting that the disorganization of cell structures had already begun 18 hpi

We tried to determine exactly when transient expression began by i) using immunocytochemistry and microscopy to visualize the appearance of DspA/E and ii) amplifying the *dspA/E* transcript by reverse transcriptase-polymerase chain reaction (PCR) (from both the 5' and 3' ends of the transcript) from the *A. tumefaciens*-infected tissues. We were unable to visualize the protein or its transcript, suggesting that *dspA/E* mRNA and protein are rapidly degraded during transient expression (data not shown).

Altogether, results show that transient expression of *dspA/E* induced a necrosis both on apple seedlings and tobacco leaves, although the agroinfection was less efficient in apple seedlings

than in tobacco leaves. It seems likely that very little DspA/E is required to induce necrosis, because neither the protein nor the mRNA were detected, despite use of the CaMV 35S promoter to drive *dspA/E* expression.

DspA/E induces electrolyte leakage in apple and tobacco plants.

We found that *dspA/E* induced necrosis when ectopically expressed in tobacco. However, a mutant *dspA/E* strain has also been shown to induce a HR in tobacco (Barny et al. 1990). To assess the importance of DspA/E for cell death, we compared the electrolyte leakage induced in tobacco and apple leaves in response to inoculation with the *E. amylovora* wild-type strain CFBP1430, the *dspA/E* mutant M52, and the type III secretion mutant PMV6023.

Inoculation with wild-type strain CFBP1430 led to a significant increase in electrolyte leakage in both host and nonhost plants. The increase began 4 hpi and was exponential until 16 hpi, when a plateau was reached (Fig. 5). The leakage observed in both apple and tobacco was dependent on type III secreted or injected proteins, as no significant increase in electrolyte leakage was recorded in the secretion mutant strain PMV6023 (Fig. 5).

In apple, *dspA/E* mutant strain M52 induced no significant increase in ion leakage (Fig. 5B). Thus, in apple, the increase in electrolyte leakage is dependent on DspA/E. This is consistent with the absence of symptom induction by *dspA/E* mutants in apple plants (Barny et al. 1990; Gaudriault et al. 1997).

The electrolyte leakage elicited by the *dspA/E* mutant strain M52 was only one third that induced by the wild-type strain CFBP1430 (Fig. 5A). DspA/E, therefore, plays a major role in inducing cell death during interactions between *E. amylovora* and the nonhost plant tobacco. The residual electrolyte leakage observed may be attributable to other type III secreted proteins, including harpins, which are known to induce HR efficiently in tobacco (Gaudriault et al. 1998; Kim and Beer 1998; Wei et al. 1992). This is consistent with the observation that *dspA/E* mutants trigger a HR in tobacco (Barny et al. 1990).

Altogether, our electrolyte leakage data indicate that DspA/E is a major cell-death inducer in both tobacco and apple.

Transient expression of *dspA/E* inside tobacco leaves leads to delayed expression of PR1.

We monitored the pattern of expression of the classical defense marker *PR1* during the transient expression of *gus* or *dspA/E* in tobacco leaves. Surprisingly, *PR1* expression was stronger and occurred earlier in leaf tissues inoculated with *A. tumefaciens* containing the CaMV 35S-*gus* construct than in leaf tissues inoculated with *A. tumefaciens* containing the CaMV 35S-*dspA/E* construct (Fig. 6). In leaf tissues transiently expressing *gus*, *PR1* expression was clearly detected 24

Fig. 5. Monitoring of electrolyte leakage in apple and tobacco leaf disks. Electrolyte leakage was measured every 2 h on leaf disks infiltrated with bacterial suspensions at a density of 10^9 CFU ml^{-1} . ■ = *E. amylovora* CFBP1430 (wild-type strain), ▲ = *E. amylovora* M52 *dspA/E* mutant strain, □ = *E. amylovora* PMV6023 type III secretion mutant strain, and x = mock infection. Experiments were performed on **A**, tobacco and **B**, apple leaf disks.

Fig. 6. Transient expression of *dspA/E* delays *PR1* expression in tobacco. Semiquantitative reverse transcription-polymerase chain reaction was performed on mRNAs isolated from plant tissues transiently expressing *gus* (G) or *dspA/E* (D) 12 h, 18 h, 24 h, 30 h, and 36 h after agroinfiltration. EF1 was used as a loading control.

hpi and was maximal 30 and 36 hpi. In leaf tissues transiently expressing *dspA/E*, *PR1* expression was clearly detectable only after 30 hpi; the signal was stronger at 36 hpi but, nevertheless, remained weaker than that recorded in *gus*-expressing tissues (Fig 6). Similar results were obtained in three independent experiments on different plants. Thus, the transient expression of *dspA/E* delays *PR1* expression in tobacco.

DISCUSSION

In the present paper, we show that we could detect DspA/E associated with the bacterial cell surface in the intercellular spaces of apple leaves. However, we have been unable to detect DspA/E in plant cells. This could reflect technical limitations or biological reasons, as DspA/E could be masked in a multicomponent protein complex once inside the plant cell or could be degraded and processed very rapidly within plant cells.

Although the electron microscopy studies gave inconclusive results, the target of DspA/E is probably located inside the plant cell, since DspA/E induces necrotic symptoms once artificially delivered into apple plant cells via agroinfection. Thus, during disease development, DspA/E is probably injected via the TTSS and induces cell death alone, without the assistance of other bacterial effectors. The necrosis observed following agroinfection of *dspA/E* is particularly interesting because it mimics the main disease symptoms elicited by *E. amylovora*. The involvement of DspA/E in cell death was confirmed by electrolyte leakage measurements, as a *dspA/E* mutant was unable to induce electrolyte leakage in apple plants. As the *dspA/E* mutant is not pathogenic and cannot grow in planta (Barny et al. 1990), *E. amylovora* appears to be dependent on DspA/E-mediated cell death for disease development. Such a behavior differs from that of *P. syringae*, which deploys many different TTSS effectors to suppress cell death. In that sense, *E. amylovora* behaves as a model bacterial necrotrophic pathogen.

DspA/E belongs to a family of conserved bacterial effectors found in many plant pathogenic bacteria. The genes encoding these effectors are located in the conserved effector locus next to the type III secretion cluster (Alfano et al. 2000). Functional cross-complementation between *dspA/E* and its *P. syringae* homolog *avrE* has been previously demonstrated (Bogdanove et al. 1998), suggesting that DspA/E homologs have similar basic roles to DspA/E during disease. However the importance of each homolog during disease development depends on the bacteria concerned. For example, mutations in *wisE* and *dspE*—the *dspA/E* homologs of *Pantoea stewartii* and *Pantoea agglomerans* pv. *gypsophylae*—lead to major change in virulence (Frederick et al. 2001; Mor et al. 2001). In contrast, mutations in the DspA/E homolog of *Erwinia carotovora* subsp. *atroseptica*, the pathogenicity of which depends mainly on cell-wall-degrading enzymes secreted through the TTSS (Thomson et al.

1999), was found to affect virulence only if very low levels of bacterial inoculum were used (Holeva et al. 2004). Similarly, the AvrE mutant of *P. syringae* pv. *tomato* is pathogenic but generates much less severe symptoms and displays lower rate of growth than the wild-type strain in tomato leaves (Lorang et al. 1994). This phenotype may be explained by the extensive redundancy of the type III effectors secreted by *P. syringae* (Collmer et al. 2002; Greenberg and Vinatzer 2003). For example, *P. syringae* pv. *tomato* DC3000 uses at least two different type III effectors, HopPtoM and the DspA/E homolog AvrE, to induce necrosis in its host plant (DebRoy et al. 2004).

Agroinfection of *dspA/E* induced necrosis on nonhost tobacco plants. This raises the question as to whether the necrosis elicited on nonhost tobacco plants results from the same biological process as the necrosis observed in host apple plants or whether tobacco contains a major *R* gene recognizing DspA/E and eliciting a HR. Indeed, nonhost *R* genes have been identified in interactions between *Pseudomonas* spp. or *Xanthomonas* spp. and plants (Simonich and Innes 1995; Warren et al. 1999; Zhao et al. 2004). However, it is generally accepted that the development of a hypersensitive reaction and disease necrosis in response to these pathogens must be considered as similar processes progressing with different timings (Klement 1982; Lamb et al. 1992; Tao et al. 2003). Several studies conducted with *Pseudomonas* spp. suggested that *R* proteins accelerate overall defense responses whereas many type III effectors act as suppressors of plant cell death and delay plant defense reactions (Bretz et al. 2003; Chang et al. 2004; Espinosa et al. 2003; Jackson et al. 1999; Jamir et al. 2004; Lopez-Solanilla et al. 2004; Tsiamis et al. 2000). The situation is quite different with *E. amylovora*–plant interactions, because the timing of the local necrosis induced on host and nonhost plants is similar. Both reactions were mediated by a sustained production of superoxide anion, lipid peroxidation, electrolyte leakage, and concomitant increases in the level of several antioxidant enzymes (ascorbate peroxidases, glutathione reductases, glutathion-*S*-transferases, and peroxidases) at similar rates and over similar timecourses (Venisse et al. 2001). These local necrotic reactions were heavily dependent on *dspA/E* expression, because the electrolyte leakage induced in both plants by a *dspA/E* mutant was much less severe than that induced by the wild-type strain. This suggests that DspA/E mediated the same basic attack mechanism in host and nonhost plants.

In tobacco, *PR1* was expressed later in plants infiltrated with *A. tumefaciens* containing *dspA/E* than in plants infiltrated with *A. tumefaciens* containing *gus*. This suggests that DspA/E-mediated necrosis may be associated with the inhibition of plant defense response. This recalls the action of the DspA/E homolog AvrE, which was recently involved in suppression of salicylic acid-dependent callose deposition during disease in *Arabidopsis thaliana* (DebRoy et al. 2004) and, once again, suggests that both proteins have the same basic role.

Table 1. List of strains and plasmids used in this study

Strain or plasmid	Characteristics ^a	Reference
<i>Erwinia amylovora</i>		
CFBP1430	Wild-type strain	Paulin and Samson 1973
PMV6023	CFBP1430 <i>hrcV</i> ::MuPR13	Barny et al. 1990
M52	CFBP1430 <i>dspA/E</i> :: <i>uidA</i> Km ^r	Gaudriault et al. 1997
<i>Agrobacterium tumefaciens</i>		
EHA 101	Strain with high transformation efficiency	Hood et al. 1986
EHA105	Strep ^r derivative of EHA101	Hood et al. 1993
pFAJ3000	<i>gus</i> gene cloned in pBIN under the control of the CaMV 35S promoter	De Bondt et al. 1996
pMAB79	8.0-kb <i>HindIII</i> to <i>PstI</i> fragment of the <i>dsp</i> region cloned into pT7-6	Gaudriault et al. 1997
pAG1	<i>dspA/E</i> gene cloned in pBIN under the control of the CaMV 35S promoter	This study
pTB4	<i>dspA/E</i> gene cloned into pUC19	This study

^a Km^r and Strep^r indicate kanamycin or streptomycin resistant; CaMV = *Cauliflower mosaic virus*.

It was previously shown that DspA/E plays a major role in the induction of oxidative stress in planta (Venisse et al. 2003). Here, we show that DspA/E is a major inducer of cell death. How DspA/E achieves its goal remains unknown. Future work aims to identify molecules interacting with DspA/E in plants and to identify DspA/E biochemical function.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Bacterial strains and plasmids are listed in Table 1 or in the text. Luria-Bertani broth (LB) medium was routinely used for bacterial growth. When necessary, the following antibiotics were added to the medium ($\mu\text{g ml}^{-1}$): ampicillin (Ap), 100; kanamycin (Km), 50; and chloramphenicol, 20.

Cloning of *dspA/E* into pBIN and pUC19.

The full-length *dspA/E* gene was amplified by PCR with the Roche expand high fidelity system, using pMAB79 as a template (Gaudriault et al. 1997). For amplification, PCR conditions were as follows: 94°C for 2 min, followed by 15 cycles of 94°C for 10 s, 48°C for 30 s, 68°C for 4 min and 10 cycles of 94°C for 10 s, 48°C for 30 s, 68°C for 4 min in the first cycle and increasing by 20 s in each subsequent cycle. Primers for the amplification of *dspA/E* were designed based on the Y13831 sequence. An *Xba*I restriction site was inserted in the forward primer and a *Sac*I site in the reverse primer to facilitate subsequent cloning. The sequence of the primers used were as follows (restriction sites are shown in bold): *dspA/E* - *Xba*I 5'-GCTCTAGAAATGGAATTAAAATCAC-3', and *dspA/E* - *Sac*I 5'-CGCGAGCTCTTAGCTCTTCATTTTC-3'.

PCR products were cloned into pGEM-T Easy (Promega, Charbonnières-les-bains, France) and were used to transform *Escherichia coli* DH5- α . We checked that the insert was present in putative transformants, by restriction analysis with *Xba*I and *Sac*I of plasmid DNA isolated from the positive clones (white colonies, Ap^r).

To subclone the PCR-amplified *dspA/E* into pBIN, a vector suitable for transient expression experiments, the pGEM-*dspA/E* and pBIN-mGFP4 (Haseloff et al. 1997) were digested with *Xba*I and *Sac*I. Restriction products were bulked and ligated using standard procedures. Ligation products were introduced into *Escherichia coli* DH5- α and Km^r/Ap^r transformants were selected on LB containing Km (pBIN). Positive clones were identified by PCR on DNA from colonies. The pBIN derivative containing full-length *dspA/E* under the control of the CAMV35S promoter was called pAG1. The full-length *dspA/E* gene was then extracted from pAG1 and inserted into pUC19, a vector suitable for expression in bacteria; pAG1 and pUC19 were digested with *Xba*I and *Sac*I. Restriction products were bulked and ligated using standard procedures. Positive Ap^r clones were identified by restriction analysis with *Xba*I and *Sac*I. The plasmid obtained was named pTB4. In pTB4, the expression of *dspA/E* is driven by the *lac* promoter. We used pAG1 to electroporate the EHA105 strain of *A. tumefaciens* (Hood et al. 1993), and pTB4 was used to electroporate the *E. amylovora dspA/E* mutant M52 (Gaudriault et al. 1997). We demonstrated that the PCR-amplified copy of *dspA/E* encoded a functional DspA/E protein by showing pTB4 restored the pathogenicity of the *dspA/E* mutants M52 in host plants.

Plant material and inoculum preparation.

Pathogenicity and electrolyte leakage assays. Apple seedlings were inoculated with strains CFBP1430, M52, and M52 (pTB4). Inoculum was prepared and inoculation was carried out as previously described (Perino et al. 1999). Appearance of fire blight symptoms was monitored over seven days. Electro-

lyte leakage was determined in apple and tobacco on leaf disks, as previously described (Gaudriault et al. 1998).

Transient expression experiments. Bacterial inocula were prepared as follows. *A. tumefaciens* EHA101 pFAJ3000 (*gus*) and EHA105 pAG1 (*dspA/E*) strains were resuspended in Murashige-Skoog broth and their optical density (OD) was adjusted to an OD₆₀₀ of 0.5.

Apple seedlings. Three-week-old 'Golden Delicious' apple seedlings were used. Prior to inoculation, seedlings were maintained in conditions of 100% humidity for 24 h to maximize stomatal opening. Each seedling was infiltrated with *A. tumefaciens* EHA101 pFAJ3000 (*gus*) or EHA105 pAG1 (*dspA/E*) under vacuum. Infiltrations were carried out on five of the uppermost leaves on the plant. The phenotype of the treated leaves was monitored for 7 days after inoculation.

Tobacco plants. Plants of *Nicotiana tabacum* cv. *xanthi* were grown in the greenhouse until they had 12 to 14 leaves. Leaves were perforated with a needle at the site of inoculation. Bacterial suspensions were then infiltrated into the leaf using a needleless syringe. After infiltration, we monitored the phenotype of the infected leaves for at least 48 h.

Determination of β -glucuronidase activity.

β -glucuronidase activity was detected by infiltrating leaves with 4 mM potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$), 0.05 mM potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3 \text{H}_2\text{O}$), 10 mM EDTA, 20 mM NaH_2PO_4 , 30 mM Na_2HPO_4 , 3 mM X-gluc. Leaves were then incubated overnight at 37°C. Leaf pigments were removed by immersing the leaves several times in 90% ethanol.

Microscopic observations.

Immunolocalization of DspA/E. Bacteria were grown in LB medium at 30°C up to an OD₆₀₀ of 2, prior to inoculation. In these growth conditions, the *E. amylovora* TTSS is not induced and DspA/E is not secreted (Gaudriault et al. 1997). Infected leaf tissues were harvested 4.5 h after inoculation—a time sufficient to allow induction of the TTSS (Boureau et al. 2002)—and were infiltrated with 3% paraformaldehyde, 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, under vacuum. Samples were then incubated at room temperature for 3 h, were washed four times in phosphate buffered saline (PBS; 1.9 mM KH_2PO_4 , 8.1 mM Na_2HPO_4 , and 150 mM NaCl at pH 7.4), were hand-sectioned with a razor blade, were incubated in permeabilization and blocking buffer (0.5% bovine serum albumin [BSA], 40% fish gelatin, 0.15% glycine, 1:1,000 (vol/vol) Tween 20, 3.3% goat serum in PBS) for 30 min, and then, were incubated overnight at 4°C with α -DspA/E rabbit antibody or α -LPS rabbit antibody, diluted 1:500 and 1:12,000, respectively, in PBS-T (PBS + 1:1,000 Tween-20 + 0.02% sodium azide). Samples were washed four times for 10 min each in PBS buffer and were incubated for 2 h at room temperature, with the secondary antibody diluted 1:20 in blocking buffer II (5 g of BSA per liter + 0.1% fish gelatin + 3.3% rabbit serum + 0.15% glycine + 1:12,000 Tween-20 in PBS). Samples were then washed twice for 10 min each in sterile distilled water.

For confocal microscopy, we used single labeling (DspA/E or LPS antibody) and double labeling (DspA/E and LPS antibodies). Secondary antibodies were conjugated to fluorescein isothiocyanate for DspA/E detection and to Texas Red for LPS detection, and samples were mounted in VectaShield for confocal microscopy.

For electron microscopy, only single labeling experiments were carried out, using a DspA/E polyclonal antibody. Secondary antibodies were conjugated to ultrasmall gold, and labeling was silver-enhanced using the Silver Enhancement kit (Amersham Biosciences Europe, Orsay, France). Samples were then washed twice for 10 min each in sterile distilled water.

Samples were contrast-stained by incubation in 1% uranyl acetate for 75 min. Samples were again washed twice for 10 min each in sterile distilled water, were dehydrated by incubation in 70% ethanol for two days, and were embedded in Epoxy-Araldite resin (FLUKA, Sigma-Aldrich, St. Quentin, Flavir, France). We observed 0.1-µm sections by TEM.

Topographic staining.

Tobacco leaf tissues were sampled from areas infiltrated with *A. tumefaciens* EHA105 pAG1 and EHA101 pFAJ3000 18 and 24 hpi, and were fixed, embedded in resin, sectioned, and stained with toluidine blue, as previously described (Perino et al. 1999). Observations were made on three independent samples. In each sample, 20 fields were observed and comparisons were made at each timepoint in blind tests for the 60 fields obtained in each set of conditions.

PCR.

Agroinfiltrated areas of tobacco leaves were crushed into fine powder in liquid nitrogen. Proteinase K lysis and total RNA extraction with phenol/chloroform were carried out using standard procedures (LiCl precipitation; Sambrook et al. 1989). Total RNA extracts were quantified by spectrophotometry, and their quality was checked on agarose gel. Isolation of mRNA was then carried out on 75 µg of total RNA, using the PolyATtract kit (Promega). Isolated mRNAs were resuspended in 50 µl of sterile distilled water and were stored at -80°C until further use. Isolated mRNAs (5 µl) were used for first-strand cDNA synthesis. Reverse transcription was performed with M-MLV reverse transcriptase (RNase H Minus, Point Mutant; Promega), using the protocols for cDNA synthesis described in the Universal RiboClone cDNA synthesis system (Promega). Second-strand cDNAs were obtained using conditions described in the Universal RiboClone cDNA synthesis system (Promega) and were resuspended in 30 µl of sterile distilled water.

PCR was carried out on double-stranded cDNAs generated from mRNA as described above. We used the following PCR amplification program: 94°C for 5 min, cycles of 94°C, 1 min; 52°C, 1 min; 72°C, 1 min, followed by a final incubation for 1 min at 72°C. *EF1* was amplified over 25 cycles, using primers based on the AF120093 sequence (forward: 5'-CCTTGTGGAAGTTTGAGACCACC-3' and reverse: 5'-CCAGTTTCCACACGACCAACAG-3'). *PR1* was amplified over 26 cycles, using primers based on the #X12737 sequence (forward: 5'-GTAGAACCTTTGACCTGGGACG-3' and reverse: 5'-GACCACTTGTCCTTTTATAGATCC-3').

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