The bacterial effector DspA/E is toxic in *Arabidopsis thaliana* and is required for multiplication and survival of fire blight pathogen

ALEXANDRE DEGRAVE^{1,2,3}, MANON MOREAU^{1,2,3}, ALBAN LAUNAY^{1,2,3}, MARIE-ANNE BARNY^{1,2,3}, MARIE-NOËLLE BRISSET⁴, ORIANE PATRIT^{1,2,3}, LUDIVINE TACONNAT⁵, REGINE VEDEL^{1,2,3} AND MATHILDE FAGARD^{1,2,3,*}†

¹INRA, Laboratoire des Interactions Plantes Pathogènes, UMR217 Paris, France

²UPMC, UMR217 Paris, France

³AgroParisTech, 16 rue Claude Bernard, Paris, France

⁴UMR 1345 IRHS (INRA, Université d'Angers, Agrocampus-Ouest) 42, rue George Morel, BP 60057, F-49071 Beaucouzé, France

⁵Unité de Recherche en Génomique Végétale, 91057 Evry cedex, France

SUMMARY

The type III effector DspA/E is an essential pathogenicity factor of the phytopathogenic bacterium *Erwinia amylovora*. We showed that DspA/E was required for transient bacterial growth in nonhost *Arabidopsis thaliana* leaves, as an *E. amylovora dspA/E* mutant was unable to grow. We expressed DspA/E in *A. thaliana* transgenic plants under the control of an oestradiol-inducible promoter, and found that DspA/E expressed *in planta* restored the growth of a *dspA/E* mutant. DspA/E expression in these transgenic plants led to the modulation by at least two-fold of the expression of 384 genes, mostly induced (324 genes). Both induced and repressed genes contained high proportions of defence genes. DspA/E expression ultimately resulted in plant cell death without requiring a functional salicylic acid signalling pathway. Analysis of *A. thaliana* transgenic seedlings expressing a green fluorescent protein (GFP):DspA/E fusion indicated that the fusion protein could only be detected in a few cells per seedling, suggesting the degradation or absence of accumulation of DspA/E in plant cells. Consistently, we found that DspA/E repressed plant protein synthesis when injected by *E. amylovora* or when expressed in transgenic plants. Thus, we conclude that DspA/E is toxic to *A. thaliana*: it promotes modifications, among which the repression of protein synthesis could be determinant in the facilitation of necrosis and bacterial growth.

INTRODUCTION

The type III secretion system (T3SS) is a major determinant required for the virulence of many Gram-negative bacterial pathogens on both animal and plant hosts. Bacteria use T3SS to inject type III effectors (T3Es) inside eukaryotic cells. For example, different strains of the phytopathogenic bacterium *Pseudomonas syringae* produce 15–35 effectors targeting a variety of cellular

compartments (Block and Alfano, 2011). Several of these T3Es have been characterized and many suppress eukaryotic cell defences using different strategies, such as interference with immune receptor signalling, blocking of RNA pathways and vesicle trafficking, and alteration of organelle function (Block and Alfano, 2011). T3Es can also interfere directly with plant gene expression: this is the case for *Xanthomonas* transcription activator-like (TAL) effectors which target different plant genes (Boch and Bonas, 2010), including genes encoding glucose export proteins, thereby inducing high glucose levels in the apoplast (Chen *et al.*, 2010). Although the cellular targets and/or protein activities of a number of T3Es of plant pathogenic bacteria have been identified (Alfano, 2009; Block and Alfano, 2011; Grant *et al.*, 2006), the majority remain unknown (Alfano, 2009). Although the primary function of T3Es is to promote disease, some effectors may be recognized by the corresponding plant disease resistance proteins in resistant plant cultivars, and trigger defence responses, including the hypersensitive response (HR), a very fast response of plant cells involving defence gene expression and cell death (Block *et al.*, 2008).

Erwinia amylovora is a Gram-negative bacterium responsible for fire blight disease affecting plants of the Pyreae tribe of Rosaceae, such as apple or pear. The main symptom of this disease is a progressive necrosis of actively growing aerial parts of susceptible hosts (Billing, 1983). The T3SS of *E. amylovora* is absolutely required for pathogenicity, as T3SS-deficient mutants are nonpathogenic and do not multiply in host leaves (Barny *et al.*, 1990). During disease, the T3SS of *E. amylovora* represses the flavonoid biosynthesis pathway, but induces the expression of three genes encoding pathogenesis-related (PR) proteins [*PR-5*, *chitinase* (*CHT*) and β -1,3-*glucanase* (*GLU*)] and a gene encoding an enzyme of the phenylpropanoid pathway (*phenylalanine ammonia lyase*, *PAL*) (Venisse *et al.*, 2002). The T3SS of *E. amylovora* has been shown to secrete at least 12 effectors (Nissinen *et al.*, 2007). Among them, DspA/E is primordial, as *E. amylovora* mutants defective for DspA/E are nonpathogenic and are unable to grow in host plants (Barny *et al.*, 1990; Gaudriault *et al.*, 1997) and in nonhost tobacco (Oh *et al.*, 2007).

*Correspondence: Email: mathilde.fagard@versailles.inra.fr

†Present address: IJPB UMR 1318 INRA Centre de Versailles-Grignon, Route de St-Cyr (RD10), 78026 Versailles cedex, France.

DspA/E belongs to a conserved family of effectors, present in most Gram-negative phytopathogenic bacteria (Kvitko *et al.*, 2009). Two of these related effectors have been demonstrated to be functional homologues of DspA/E: the AvrE effector of *P. syringae* pv. *tomato* (Bogdanove *et al.*, 1998) and the WtsE effector of *Pantoea stewartii* (Ham *et al.*, 2006). The virulence-promoting activity of this family of effectors is thought to depend on their ability to suppress the deployment of plant defences, such as callose deposition (DebRoy *et al.*, 2004; Kvitko *et al.*, 2009). However, to date, the specific cellular processes targeted by these effectors are unclear. These effectors have the particularity of triggering cell death in host plants (Badel *et al.*, 2006; Kvitko *et al.*, 2009), and a strong link seems to exist between their virulence-promoting activity and their ability to trigger cell death. For example, deletion derivatives of the WtsE protein of *P. stewartii* are affected in both bacterial growth promotion on host plants and the triggering of necrosis in nonhost plants (Ham *et al.*, 2008). Hence, several authors have suggested that the virulence-promoting activity of this family of effectors could be a result of their ability to cause plant cell death (Boureau *et al.*, 2006; Ham *et al.*, 2008). This hypothesis is supported by a recent report indicating that DspE from *Pectobacterium carotovorum* is not required to suppress callose deposition, but is required for the triggering of cell death and disease (Kim *et al.*, 2011).

We have demonstrated previously that DspA/E is required for the induction of necrosis by *E. amylovora* in nonhost *Arabidopsis thaliana* (Fig. 1A and Degraeve *et al.*, 2008). In this study, we use *A. thaliana* to unravel the early events involving DspA/E which enable *E. amylovora* to induce cell death and to survive in plant tissues during the first 48 h of the infection. We show that DspA/E is required for the growth and survival of *E. amylovora* in *A. thaliana*. We describe *A. thaliana* lines expressing the *dspA/E* gene under the control of an oestradiol-inducible promoter, which is able to rescue the *in planta* growth of a *dspA/E* mutant, but is toxic for *A. thaliana* plants. The expression of DspA/E leads to the induction of a large defence response at the transcriptional level, including the activation of the salicylic acid (SA) signalling pathway. Finally, we show that the toxicity of DspA/E is associated with an early repression of *de novo* protein synthesis. DspA/E also represses protein synthesis when injected by the bacterium into the plant cell, and the biochemical suppression of protein synthesis restores the *in planta* multiplication of a *dspA/E* mutant.

RESULTS

DspA/E is required for multiplication and survival of *E. amylovora* in *A. thaliana*

To determine whether DspA/E is required for the transient growth of *E. amylovora* in *A. thaliana*, we inoculated wild-type *A. thaliana*

Fig. 1 DspA/E is required for *Erwinia amylovora* multiplication and survival in nonhost *Arabidopsis thaliana*. (A, B) Leaves of *A. thaliana* Col-0 5-week-old plants were inoculated with wild-type *E. amylovora* (*Ea*) or a *dspA/E* mutant (*dspA/E*) at 10^7 colony-forming units (CFU)/mL. (A) Representative photographs of symptoms at 6 days post-inoculation (dpi). (B) Bacteria were extracted and counted at the indicated time points. Asterisks indicate values significantly different from *Ea* at the corresponding time point according to the Mann–Whitney test ($P < 0.05$). hpi, hours post-inoculation; wt, wild-type.

plants of the Col-0 ecotype with a *dspA/E* mutant of *E. amylovora*. The *dspA/E* mutant showed an important reduction in bacterial population size at both 24 and 48 h post-inoculation (hpi) compared with wild-type *E. amylovora*, indicating that DspA/E is required for the transient multiplication of *E. amylovora* in *A. thaliana* (Fig. 1B). Furthermore, bacterial populations of the *dspA/E* mutant decreased immediately following inoculation, indicating that DspA/E is also required for bacterial survival *in planta*. Thus, DspA/E is required for the transient multiplication and survival of *E. amylovora*, as well as for the production of associated necrotic symptoms in nonhost *A. thaliana* plants.

DspA/E expressed in transgenic plants restores the multiplication of an *E. amylovora dspA/E* mutant

To identify the cellular modifications that could be attributed to the action of DspA/E alone, we wished to express the *dspA/E* gene in plant cells in the absence of bacteria. We generated, by *Agrobacterium*-mediated transformation, *A. thaliana* transgenic plants expressing a green fluorescent protein (GFP)::*dspA/E* fusion under the control of the oestradiol-inducible *lexA* promoter (Zuo *et al.*, 2000). We chose to construct an N-terminal GFP fusion as

the functional homologue of DspA/E in *P. stewartii*, WtsE, requires an intact C-terminus to be functional (Ham *et al.*, 2006). After the transformation of Col-0 plants, the selection of primary transformants led to the isolation of two independent lines carrying the *GFP::dspA/E* fusion (hereafter referred to as lines 13-1-1 and 13-1-2). As a control, we isolated a line carrying the *GFP* gene alone under the control of the oestradiol-inducible *lexA* promoter (hereafter referred to as line 7-2-1). Homozygous individuals for the T-DNA were selected in the progeny of each primary transformant, and further experiments were conducted on homozygous individuals. The overall results obtained with lines 13-1-1 and 13-1-2 were similar, and the results are therefore sometimes presented for one line only to simplify the presentation. Lines 13-1-1 and 13-1-2 showed a reduction in plant size in the absence of oestradiol treatment relative to control plants (Fig. 2A); however, they were able to bolt and were fertile. This phenotype is consistent with the fact that weak expression of the *dspA/E* gene could be detected by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the 13-1-1 and 13-1-2 lines in the absence of oestradiol treatment (Fig. 2B). The treatment of seedlings with oestradiol led to a 50-fold increase in the expression of DspA/E in lines 13-1-1 and 13-1-2 (Fig. 2B).

To determine whether DspA/E expressed in the transgenic plants was able to rescue the growth of a *dspA/E* mutant of *E. amylovora*, we co-inoculated leaves of transgenic plants with a *dspA/E* mutant and with oestradiol (Fig. 2C). Transient growth of the *dspA/E* mutant was observed in the transgenic plants expressing DspA/E (Fig. 2C). No bacterial growth-promoting effect of oestradiol treatment was obtained in the 7-2-1 control line inoculated with a *dspA/E* mutant (Fig. 2C). This result indicates that DspA/E produced in *A. thaliana* transgenic plants promotes the growth of *E. amylovora*, as also observed when it is injected directly by the bacterium through T3SS. DspA/E produced *in planta* was also able to increase the growth of wild-type *E. amylovora* (Fig. S1A, see Supporting Information).

DspA/E triggers electrolyte leakage and cell death in *A. thaliana*

DspA/E has been shown previously to induce electrolyte leakage and cell death when expressed transiently in tobacco and apple leaves (Boureau *et al.*, 2006). Electrolyte leakage from cells is commonly used to evaluate membrane damage caused by biotic or abiotic stresses. Electrolyte leakage was detected 1 h following oestradiol treatment of whole 13-1-2 seedlings and increased continuously thereafter (Fig. 3A). Interestingly, untreated 13-1-2 seedlings showed weak electrolyte leakage, which was not observed in 7-2-1 control seedlings (Fig. 3A), which is probably a consequence of the weak expression of DspA/E observed in untreated 13-1-2 seedlings. Expression of DspA/E in the transgenic

Fig. 2 Phenotype of DspA/E transgenic plants. (A) Four-week-old plants grown in 16 h day/8 h night. (B) Expression of the *dspA/E* gene in transgenic lines 24 h following treatment or not with oestradiol. (C) Bacterial counts of a *dspA/E* mutant of *Erwinia amylovora* in lines 7-2-1 and 13-1-2 (similar results were obtained with the 13-1-1 line). Bacteria were inoculated or not with 5 μ M oestradiol. Asterisks indicate values significantly different from the untreated 7-2-1 control line at the corresponding time point according to the Mann–Whitney test ($P < 0.05$).

lines ultimately led to the necrosis of treated leaf tissue: leaves started to wilt at 24 hpi with oestradiol and were completely necrotic at 5 days post-induction; the 7-2-1 control line showed no sign of necrosis whether or not it was treated with oestradiol (Fig. 3B). In order to determine the kinetics of induction of cell death, we stained the 13-1-2 transgenic seedlings with Evans blue and found that cell death could be detected at 16 h post-treatment (hpt) (Fig. S2, see Supporting Information).

Fig. 3 DspA/E triggers electrolyte leakage and cell death. (A) Electrolyte measurement in seedlings treated or not with oestradiol. Experiments were repeated twice with similar results. (B) The right halves of DspA/E transgenic leaves were infiltrated with dimethylsulphoxide (DMSO) or 20 μM oestradiol and photographs were taken 5 days after infiltration. (A, B) Similar results were obtained with the 13-1-1 line.

DspA/E inhibits the growth of leaf and root in *A. thaliana*

When oestradiol treatment of transgenic plants was performed by spraying instead of infiltrating, we found that the youngest leaves of the rosette did not undergo necrosis, but were severely reduced in size (Fig. 4A). This sublethal effect of DspA/E could be a result of an inefficient penetration of oestradiol into the leaves using this method, and suggests that low concentrations of oestradiol induce a reduction in plant growth, which would be consistent with the dwarf phenotype of the 13-1-1 and 13-1-2 transgenic plants. In order to test this hypothesis, we analysed the primary root growth of 2-week-old transgenic plants treated with a range of oestradiol concentrations. As shown in Fig. 4B, treatment of transgenic seedlings with the lowest concentrations (10 and 20 nM) reduced root growth relative to mock-treated seedlings, whereas treatment with the highest concentration of oestradiol (100 nM) completely inhibited root growth. Oestradiol treatment had no effect on the primary root growth of the 7-2-1 control line (Fig. S1B). Staining of root cells with fluorescein diacetate (FDA) and propidium iodide (PI) indicated that, at the lowest concentrations used (10 nM), we could not detect cell death and root cells

Fig. 4 DspA/E inhibits plant growth. (A) Five-week-old 13-1-2 transgenic plants were sprayed each day with 5 μM oestradiol or dimethylsulphoxide (DMSO) for 3 weeks. The photograph shows representative plants for each treatment. The control 7-2-1 plants were unaffected by oestradiol treatment (data not shown). (B) 13-1-2 seedlings were immersed for 10 min in oestradiol-containing solutions and cleared. Each bar represents the mean growth in millimetres per day for 15 seedlings ± standard error (SE). Results are shown for the 13-1-2 line. Root length in the 7-2-1 control line was unaffected (Fig. S1B). (C) Two days after treatment, the root tips of 13-1-2 transgenic seedlings were coloured for 5 min in a fluorescein diacetate–propidium iodide (FDA–PI) staining solution, rinsed and observed under a fluorescence microscope using the appropriate filter. Living tissues are stained green by FDA; dead tissues are stained red by PI. Photographs show one representative root tip for each treatment. No significant PI staining was found in 7-2-1 control seedlings (Fig. S3).

were still alive (Fig. 4C), whereas treatment with the highest concentration (100 nM) induced cell death. At the intermediate concentration (20 nM), both PI- and FDA-stained patches could be found, indicating the presence of both dead and live cells. The 7-2-1 control line did not show significant staining with PI following oestradiol treatment (Fig. S3, see Supporting Information). Collectively, these data show that DspA/E has a progressive inhibitory effect on primary root growth, and that low expression of DspA/E is sufficient to repress root growth without causing apparent cell death.

To determine whether DspA/E could also reduce plant growth when injected by the bacterium, we infected *in vitro*-grown *A. thaliana* seedlings with wild-type *E. amylovora* or a *dspA/E* mutant. Six days after infection, we found no apparent necrosis, but the seedlings infected with wild-type *E. amylovora* showed

a reduction in growth compared with the *dspA/E* mutant- or mock-treated seedlings (Fig. S4, see Supporting Information).

DspA/E induces the expression of an array of *A. thaliana* defence genes

To determine the impact of DspA/E alone on gene expression in *A. thaliana*, we analysed the transcriptome of 13-1-2 transgenic plants using complete *A. thaliana* microarray (CATMA) chips (Crowe *et al.*, 2003; Hilson *et al.*, 2004). We used a low concentration of oestradiol (30 nM), which does not suppress plant growth completely (Fig. 4B), and chose the 6-h time point (before cell death could be detected using Evans blue; Fig. S2), in order to identify early gene expression changes associated with DspA/E expression: 2-week-old seedlings of the 13-1-2 line were treated with 30 nM oestradiol or mock-treated, and RNA was extracted 6 h following treatment. The 7-2-1 transgenic line expressing GFP was used as a control. Two biological replicates and a dye swap were performed for each set of pairwise comparisons.

Statistical analysis (see Experimental details) showed that oestradiol treatment of the control line (7-2-1) led to the

modulation of 22 genes, all of which were affected by less than two-fold in their expression. These 22 genes were removed from our analysis (data not shown). Eighty-one genes were expressed more than two-fold in the untreated 13-1-2 line relative to the untreated control line (Table S1, see Supporting Information). Several enhanced disease susceptibility 1 (EDS1)- and/or SA-dependent genes, such as *phytoalexin deficient4* (*PAD4*), *PR1* and *WRKY70*, were induced significantly in the untreated 13-1-2 line. This suggested that the low expression of DspA/E in line 13-1-2 was sufficient to induce, at least partly, these signalling pathways. Oestradiol-induced DspA/E expression in the 13-1-2 line led to the induction of 321 genes and the repression of 60 genes by at least two-fold (Table S2, see Supporting Information). Figure 5A shows the functional distribution of these genes. Most functional categories were affected by DspA/E; however, the defence category was very highly represented in both induced and repressed genes. Among the induced genes, we found genes modulated by the SA pathway and many WRKY transcription factors (Fig. S5, see Supporting Information). Genes encoding jasmonic acid (JA) biosynthesis or signalling were, for the most part, not induced (Fig. S5). We analysed by qRT-PCR a subset of the

Fig. 5 Identification of gene expression modifications induced by DspA/E in *Arabidopsis thaliana*. (A) Functional distribution of genes modulated in the 13-1-2 line 6 h following oestradiol treatment according to the MIPS Functional Catalogue (Ruepp *et al.*, 2004). (B) Gene expression levels in seedlings 6 h following mock or oestradiol treatment by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). FMO, flavin-containing monooxygenase; ICS, isochlorismate synthase; PAD, phytoalexin deficient; PR, pathogenesis-related.

most strongly induced genes, with a focus on SA signalling and WRKY transcription factors, in the two transgenic lines bearing the *dspA/E* gene (Fig. 5B). These genes showed higher levels of expression in untreated 13-1-1 and 13-1-2 lines relative to the control line (7-2-1). However, most of these genes were induced following oestradiol-induced expression of DspA/E in 13-1-1 and 13-1-2 lines, but not in the 7-2-1 control line. Only *WRKY46* was not induced by oestradiol treatment in the 13-1-1 line, suggesting that the high level of expression of *WRKY46* in untreated plants of this line was at its maximum. We compared the list of genes modulated by DspA/E alone with the genes identified previously as modulated by *E. amylovora* infection (Moreau *et al.*, 2012). Among the 1121 genes induced by *E. amylovora* infection, 366 were induced significantly (among which 174 were induced by at least two-fold) in the 13-1-2 transgenic plants following oestradiol treatment. Among the 1035 genes repressed by *E. amylovora*, 97 were repressed significantly (among which 12 were repressed by at least two-fold) in the 13-1-2 transgenic plants following oestradiol treatment (Table S3, see Supporting Information). In addition, only 12 genes showed opposite modulation by *E. amylovora* and DspA/E (Table S3). Most defence genes and WRKY genes were modulated in a similar manner by *E. amylovora* and DspA/E (Fig. S5). Furthermore, we confirmed the involvement of DspA/E in the induction of the SA pathway and WRKY transcription factors, as these genes were less induced at 6 or 24 hpi by a *dspA/E* mutant than by the wild-type strain of *E. amylovora* (Fig. S6, see Supporting Information).

The SA pathway is involved in the toxicity of DspA/E in *A. thaliana* only at low levels of DspA/E expression

The SA signalling pathway has been shown previously to be involved in the induction of cell death in plants. Indeed, several lesion mimic mutants have been shown to require the SA pathway for the triggering of necrotic lesions (Lorrain *et al.*, 2003; Meng *et al.*, 2009). We therefore crossed the 13-1-2 transgenic line to the NahG transgenic plants in which SA cannot accumulate (Lawton *et al.*, 1995). In the F2 progeny, we selected plants homozygous for the *GFP::dspA/E* transgene and for the *NahG* transgene. As observed for the 13-1-2 plants, the 13-1-2 NahG plants showed a reduction in plant size relative to the wild-type (Fig. 6A). As expected, the SA-dependent marker gene *PR1* was not activated in these double homozygous plants (Fig. 6B). We analysed the induction of membrane damage provoked by DspA/E in these plants by measuring electrolyte leakage: electrolyte leakage was weaker in untreated 13-1-2 NahG plants than in untreated 13-1-2 plants. However, when seedlings were treated with 5 μ M oestradiol, DspA/E was still able to induce electrolyte leakage in the presence of the NahG transgene (Fig. 6C). We also checked whether the inhibition of root growth was affected in the double homozygous plants. We found that DspA/E was still able to

Fig. 6 Role of the salicylic acid (SA) pathway in the toxicity of DspA/E. (A) Two-week-old *in vitro*-grown seedlings. Both 13-1-2 and 13-1-2 NahG plants show a reduction in plant size compared with the wild-type and NahG seedlings. (B) Top two panels: *pathogenesis-related 1* (*PR1*) and *elongation factor 1 α* (*EF1 α*) expression detected by end-point reverse transcription-polymerase chain reaction (RT-PCR). The *EF1 α* gene is used as a constitutive control. Bottom panel: intensity of *PR1* signal relative to the *EF1 α* signal measured using ImageJ software. No expression was found in 13-1-2 NahG plants whether they were treated or not with oestradiol. (C) Electrolyte leakage of mock or oestradiol-treated leaves measured at 24 h post-treatment (hpt). Control plants did not display significant electrolyte leakage. (D) Root growth 24 h following treatment with different concentrations of oestradiol. Asterisks indicate a significant difference from the control line at the same oestradiol concentration (Mann–Whitney test; $P < 0.05$). We also found a significant difference between electrolyte leakage of 13-1-2 NahG in oestradiol- and mock-treated seedlings.

inhibit root growth at the highest concentration of oestradiol used (5 μ M), but that root growth was slightly less inhibited by DspA/E in the presence of the NahG transgene at lower concentrations (10 and 30 nM; Fig. 6D).

Altogether, these data indicate that the SA signalling pathway contributes to the toxicity of DspA/E when DspA/E is not highly expressed (in mock-treated seedlings or those treated with 10 or 30 nM oestradiol), but that the SA pathway is not required for DspA/E toxicity in *A. thaliana* when it is highly expressed (in seedlings treated with 5 μ M oestradiol).

DspA/E represses pathogen-associated molecular pattern (PAMP)-induced callose deposition in *A. thaliana* in response to *P. syringae*

During the infection of host plants, such as apple, by *E. amylovora*, no callose deposition occurs (DebRoy *et al.*, 2004). However, callose deposition occurs in *A. thaliana* infected by *E. amylovora* (Degrave *et al.*, 2008). It has been shown that, in host plants, DspA/E is required to repress callose deposition triggered by HrpN, as a *dspA/E* mutant triggers callose deposition (Boureau *et al.*, 2011; DebRoy *et al.*, 2004). We wondered whether the accumulation of callose in *A. thaliana* following *E. amylovora* infection was a result of the inability of DspA/E to suppress callose deposition in this species. We therefore analysed callose deposition in leaves of 13-1-2 and control 7-2-1 plants following inoculation with a *t3ss* mutant of *P. syringae* pv. *tomato* and wild-type *E. amylovora*, which both induce callose deposition in *A. thaliana*. Both bacterial strains induced the accumulation of callose deposits in leaves of the 7-2-1 control line, whether treated or not with oestradiol (data not shown). We found that, when DspA/E expression was induced by oestradiol treatment in the 13-1-2 line, leaves infected with the *t3ss* mutant of *P. syringae* accumulated significantly less callose than did untreated leaves, indicating that DspA/E was able to repress PAMP-induced callose deposition in *A. thaliana* (Fig. 7). However, leaves of 13-1-2 plants infected with *E. amylovora* accumulated as much callose whether or not DspA/E expression was induced (Fig. 7). This indicates that DspA/E does not suppress callose deposition induced by *E. amylovora* in *A. thaliana* leaves.

The GFP::DspA/E fusion protein is transiently detected in the cytoplasm and the nucleolus

To determine the intracellular localization of DspA/E, we analysed the fluorescence of roots in 13-1-2 transgenic seedlings following oestradiol treatment. In transgenic 7-2-1 control seedlings, the fluorescent signal was detected in the cytoplasm and in the nucleus at 2 hpt (Fig. S7A, see Supporting Information) and was still visible at 24 hpt. In 13-1-2 transgenic seedlings, a transient fluorescent signal appeared at 2 hpt in isolated cells of the root (Fig. S7A), but could not be detected in any cell at 3 or 24 hpt.

Fig. 7 DspA/E represses pathogen-associated molecular pattern (PAMP)-induced callose, but not *Erwinia amylovora* (Ea)-induced callose, in *Arabidopsis thaliana*. Epifluorescence micrographs of leaf discs 8 h after infiltration with *Pseudomonas syringae* pv. *tomato* hrp mutant with (+) or without (–) 5 μ M oestradiol. Callose deposits appear as fluorescent dots.

In 13-1-2 seedlings, the fluorescence signal at 2 hpt was not similar to that of control seedlings: it was weak in the cytoplasm and the nucleoplasm, but strong in a circular structure that resembled the nucleolus. 4',6-Diamidino-2-phenylindole (DAPI) staining of nuclei confirmed that the fluorescence signal in the 13-1-2 transgenic line was mainly detected in a region of the nucleus that excludes DAPI stain and is reminiscent of the nucleolus (Fig. S7B). In order to confirm that this specific subcellular localization was caused by an intact GFP::DspA/E fusion protein, we attempted to detect it by Western blotting using both an anti-DspA/E antibody and an anti-GFP antibody. We could not detect any signal in the 13-1-2 seedlings at any of the time points tested, whereas GFP alone was detected in control seedlings (data not shown), suggesting that the fusion protein is present either transiently or that the quantity of protein does not reach the sensitivity threshold for detection by Western blot. The absence of detection of DspA/E in transgenic plants by Western blotting is in agreement with the transient nature of the fluorescent signal detected in GFP::DspA/E seedlings (Fig. S7A).

DspA/E represses protein synthesis

As the GFP::DspA/E fusion could only be observed transiently, and as it was detected in the nucleolus, which is the site of ribosomal RNA (rRNA) transcription, pre-rRNA processing and ribosome subunit assembly (Lam *et al.*, 2005), we wondered whether DspA/E expression *in planta* was correlated with impaired *de novo* protein synthesis. We analysed the incorporation of 35 S-labelled methionine into newly synthesized proteins of *A. thaliana* seedlings expressing DspA/E. In *A. thaliana* seedlings expressing DspA/E, almost no 35 S-labelled methionine was incorporated into

the newly synthesized proteins (Fig. 8A), indicating a rapid decrease in protein synthesis when DspA/E was expressed in the plant cell. We then analysed the incorporation of ^{35}S -labelled methionine in proteins of 13-1-2 seedlings treated with a range of oestradiol concentrations, and found that the inhibition of *de novo* protein synthesis was dependent on the oestradiol concentration (Fig. 8A). No obvious modification of total protein content was visible at this stage in DspA/E-expressing seedlings.

To determine whether DspA/E also reduced *de novo* protein synthesis during infection, we analysed the incorporation of ^{35}S -labelled methionine in *A. thaliana* and apple leaves infected with

wild-type *E. amylovora* or a *dspA/E* mutant. When *A. thaliana* and apple leaves were infected with *E. amylovora*, *de novo* protein synthesis was reduced in a DspA/E-dependent manner (Fig. 8B,C). Finally, we determined that repression of protein synthesis using cycloheximide (CHX) partially rescued the growth of a *dspA/E* mutant in *A. thaliana* plants (Fig. 8D).

DISCUSSION

DspA/E is a T3E that is essential for the virulence of the phytopathogenic bacterium *E. amylovora* on host plants. DspA/E

Fig. 8 DspA/E inhibits *de novo* protein synthesis. (A) Ten-day-old transgenic seedlings were treated with dimethylsulphoxide (DMSO) or oestradiol (5 μM) for 3 h and labelled with ^{35}S methionine. (B) Leaves of 5-week-old Col-0 plants were syringe infiltrated with water (mock) or with bacterial suspensions as indicated. (C) Leaf discs of apple seedlings were vacuum infiltrated with water (mock) or with different strains of *Erwinia amylovora* as indicated. (A–C) Total protein extracts were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue; ^{35}S methionine incorporation was detected by autoradiography. Similar results were obtained for two biological replicates. (D) Bacterial counts of *E. amylovora* in *Arabidopsis thaliana* leaves inoculated with wild-type *E. amylovora* (*Ea* wt) or a *dspA/E* mutant (*Ea dspA/E*), alone or together with 4 $\mu\text{g}/\text{mL}$ of cycloheximide (CHX). Bars represent standard errors. hpi, hours post-inoculation.

belongs to a family of effectors conserved in most plant pathogenic bacteria, corresponding to large proteins with no homology to any protein of known biochemical function. Depending on the bacterial species, DspA/E homologues are either required for virulence or for fitness (Boureau *et al.*, 2006). However, the precise role of these effectors during infection has not been established clearly. Two DspA/E homologues, *P. syringae* AvrE and *P. stewartii* WtsE, have been shown to suppress SA-dependent callose deposition and/or expression of the *PR1* defence gene, and the virulence-promoting activity of this family of effectors is thus generally thought to be linked to their ability to suppress deployment of plant defence (DebRoy *et al.*, 2004; Ham *et al.*, 2008). However, several authors have suggested that the virulence-promoting activity of this family of effectors could be a result of their ability to cause plant cell death (Boureau *et al.*, 2006; Ham *et al.*, 2008; Kim *et al.*, 2011).

We have shown previously that *E. amylovora* multiplies transiently in nonhost *A. thaliana* (Degrave *et al.*, 2008). We have shown in this article that DspA/E promotes bacterial multiplication and bacterial survival of *E. amylovora* in *A. thaliana*, as a *dspA/E* mutant was unable to multiply in *A. thaliana* leaves and bacterial numbers decreased rapidly after inoculation. The fact that DspA/E can promote bacterial growth in nonhost plants has been observed previously: indeed, DspA/E is required for early growth of *E. amylovora* in leaves of nonhost *Nicotiana benthamiana* (Oh *et al.*, 2007). In order to analyse the modifications in the plant cell caused by DspA/E alone, we transformed *A. thaliana* with constructs allowing the inducible expression of DspA/E in a translational fusion with GFP. When DspA/E was expressed directly inside the plant cell in the transgenic plants, it was able to restore the growth of a *dspA/E* mutant of *E. amylovora*. This is consistent with the fact that DspA/E is known to be injected inside plant cells by *E. amylovora* and is thought to function inside plant cells (Bocsanczy *et al.*, 2008). DspA/E has been shown previously to repress callose deposition, a cell wall-based plant defence, and it has been suggested that the bacterial growth-promoting activity of DspA/E is linked to its ability to repress plant defence (DebRoy *et al.*, 2004). In this article, we have shown that DspA/E can indeed repress callose deposition induced by *P. syringae* PAMPs in *A. thaliana*, but that it does not repress callose induced by *E. amylovora*, which is in agreement with the fact that *A. thaliana* leaves accumulate callose deposits following *E. amylovora* infection (Degrave *et al.*, 2008). Furthermore, this suggests that *P. syringae*- and *E. amylovora*-induced callose deposition require independent pathways, consistent with previous reports indicating that callose deposition in *A. thaliana* involves more than one pathway (Luna *et al.*, 2011). As DspA/E promotes bacterial multiplication in *A. thaliana*, but does not repress callose deposition, this suggests that repression of callose deposition by DspA/E is not critical to its bacterial growth-promoting role. Indeed, we have reported previously that a *pmr4-1* callose-deficient mutant of *A. thaliana* does

not show increased sensitivity to *E. amylovora* (Moreau *et al.*, 2012).

The two transgenic lines transformed with the *dspA/E* gene (13-1-1 and 13-1-2) show low levels of electrolyte leakage and a growth delay in the absence of oestradiol treatment. This indicates that the oestradiol-inducible system is leaky and confirms previous data suggesting that very low levels of DspA/E are toxic for plant cells (Boureau *et al.*, 2006; Oh *et al.*, 2007). Indeed, agrobacteria bearing a dexamethasone-inducible *dspA/E* gene construct can induce cell death in the absence of dexamethasone, indicating that the dexamethasone-inducible system is leaky and that very low expression levels of DspA/E are sufficient to induce cell death in *N. benthamiana* (Oh *et al.*, 2007). This high toxicity of DspA/E could explain the relatively small number of transgenic lines isolated that were able to express DspA/E after oestradiol treatment. When the expression of DspA/E was induced by low oestradiol concentrations, this led to root and leaf growth reduction. *Erwinia amylovora* infection of seedlings also led to a DspA/E-dependent reduction in growth. Other effectors are known to affect plant growth; for example, the *E. amylovora* harpin HrpN and the *P. syringae* T3E AvrRpt2 both promote root growth in *A. thaliana* (Chen *et al.*, 2007; Ren *et al.*, 2008). The induction of DspA/E expression using high concentrations of oestradiol led to growth arrest, as well as electrolyte leakage and necrosis, as described previously in tobacco and apple (Boureau *et al.*, 2006).

We found that the induction of the expression of DspA/E by oestradiol treatment modulated the expression of 388 *A. thaliana* genes 6 h after treatment. A very high proportion of the modulated genes were induced (326 genes). Approximately 25% of the genes that were induced following the expression of DspA/E showed a higher level of expression in untreated 13-1-2 transgenic plants bearing the *dspA/E* transgene than in the 7-2-1 control line, indicating that the low level of DspA/E expression in 13-1-2 plants was sufficient to induce a transcriptional response. The fact that these genes were further induced when DspA/E expression was increased by oestradiol treatment indicates that at least part of the transcriptional response is dose dependent, which is in agreement with the dose dependence of the repression of root growth by DspA/E expression. Analysis of the functional categories of the modulated genes using the FunCat database (Ruepp *et al.*, 2004) showed that DspA/E triggered a strong defence reaction at the transcriptional level. Indeed, the SA pathway was strongly induced by DspA/E expression, consistent with our previous observations that the SA pathway is induced in a DspA/E-dependent manner following infection by *E. amylovora* (Degrave *et al.*, 2008). SA is known to be involved in some forms of cell death in plants, and many of the mutants in which SA signalling is constitutively active develop necrotic lesions spontaneously (Lorrain *et al.*, 2003; Meng *et al.*, 2009). Therefore, it is possible that DspA/E induces cell death in *A. thaliana* via the induction of the SA pathway. However, we showed in this article that activation

of the SA pathway is not required for the toxicity of DspA/E in *A. thaliana*. It has been shown recently that the DspE homologue of *P. carotovorum* has no effect on plant gene expression, although it is able to induce cell death and promote disease (Kim *et al.*, 2011). Thus, the action of DspA/E and its functional homologues on the SA pathway seems to be variable from plant to plant (Boureau *et al.*, 2006; Pester *et al.*, 2012), but does not seem to have an impact on the ability of these effectors to promote bacterial growth or plant cell death. We also found that the EDS1 signalling pathway is strongly induced by DspA/E at the transcriptional level. We have shown previously that the EDS1 pathway is involved in *A. thaliana* nonhost resistance to *E. amylovora* (Moreau *et al.*, 2012). Thus, it seems that, although DspA/E is required for *E. amylovora* growth in *A. thaliana*, it also contributes to the detection of *E. amylovora* by *A. thaliana* cells and to the triggering of the resistance response. This situation is similar to the observations in nonhost tobacco, in which DspA/E is required for both bacterial growth and the resistance response (Oh *et al.*, 2007).

We have shown in this study that the expression of DspA/E leads to a rapid repression of *de novo* protein synthesis. This could explain why we were unable to detect the DspA/E protein *in planta* by Western blot and why the GFP::DspA/E fusion protein was only transiently detected. Previous experiments on the *Agrobacterium*-mediated transient expression of DspA/E or WtsE in *N. benthamiana* also led to plant cell death, but the authors were unable to detect the DspA/E or WtsE protein in plant tissues (Ham *et al.*, 2009; Oh *et al.*, 2007). However, the lack of accumulation of DspA/E in plant cells could also be a result of an inherent instability of the DspA/E protein. In animal bacterial pathogens, it has been shown that effectors can repress host translation and that this repression is required for pathogenesis. The intracellular bacterial pathogen *Legionella pneumophila* secretes inside the host cell five effectors which are required for bacterial growth and virulence and which repress host cell translation (Fontana *et al.*, 2011). The authors suggest that the inhibition of host translation could benefit the pathogen by increasing the availability of amino acids or by dampening the host's response. However, they also demonstrate that host cells are able to detect the arrest of translation, which triggers a transcriptional response and could counterbalance the positive effects of the effectors (Fontana *et al.*, 2011). To our knowledge, the present work is the first demonstration of the repression of protein synthesis by a plant pathogen T3E. This led us to investigate *de novo* protein synthesis in infected plants, and we found that it was rapidly inhibited in both *A. thaliana* and apple leaves following infection by *E. amylovora* in a DspA/E-dependent manner. Furthermore, we found that repression of host protein synthesis with CHX partially restored the multiplication of a *dspA/E* mutant, in agreement with our previous observations that CHX can promote *E. amylovora* multiplication and associated necrosis (Moreau *et al.*, 2012). The fact that the growth

of a *dspA/E* mutant could only partially be restored by CHX indicates that the function of DspA/E in the plant cell cannot be fully complemented by the biochemical repression of protein synthesis.

Altogether, we have shown that DspA/E is required for early bacterial growth in the nonhost plant *A. thaliana* and that DspA/E represses protein synthesis and triggers a strong defence response. At this point, the causal relationships, if they exist, between cell death, defence triggering, repression of protein synthesis and the bacterial growth-promoting effect of DspA/E, are unknown. However, previous data concerning WtsE deletion mutants have suggested that at least the cell death-inducing and bacterial growth-promoting activities of WtsE are strongly linked (Ham *et al.*, 2008). Although this remains to be proven, our data argue in favour of the induction of cell death by DspA/E being responsible for its bacterial growth-promoting activity, possibly linked to its protein synthesis-suppressing activity.

EXPERIMENTAL PROCEDURES

Bacterial strains, yeast strains and plant lines

The *E. amylovora* strains used in this study are described in Degraeve *et al.* (2008), except for the *dspA/E* mutant (M81). To construct the M81 *dspA/E* mutant, plasmid pSL4 containing the mutagenized insert (Appendix S1) was introduced into *E. amylovora* by electroporation. Isolated colonies were screened for streptomycin resistance and ampicillin sensitivity. The recombination event was checked by Southern blotting. A secretion test was performed which confirmed that DspA/E was not secreted by the M81 strain. The *tts* mutant of *P. syringae* pv. *tomato* was obtained from the Collection Française des Bactéries Phytopathogènes (Angers, France). Apple seedlings (*Malus × domestica*) were generated from seeds obtained from open-pollinated 'Golden Delicious', which is susceptible to fire blight disease.

Generation of DspA/E transformation constructs and plant transformation

The GFP::*dspA/E* construct was obtained by cloning the *eGFP* coding region in frame with the *dspA/E* coding region under the control of the inducible *lexA* promoter in the pER8 transformation vector (Zuo *et al.*, 2000) (see Appendix S1 for details). *Arabidopsis thaliana* Col-0 plants were transformed by the *Agrobacterium* sp. strain C58C1 using the floral dipping procedure (Clough and Bent, 1998).

Plant culture, oestradiol treatment and inoculation methods

To measure *in planta* bacterial multiplication, *A. thaliana* plants were grown in soil for 5 weeks and leaves were inoculated as described in Degraeve *et al.* (2008). *In vitro*-grown seedlings were sown on solid 1 × Murashige and Skoog (MS) medium containing 1% sucrose after surface sterilization and vernalized by incubation at 4 °C for 2 days. Seedlings were subjected to a cycle of 16 and 8 h of light and darkness,

respectively, at 25 °C (day) and 20 °C (night). To measure seedling root length, the plates were oriented vertically so that the roots grew along the surface of the medium. Oestradiol treatment was conducted by immersion of 10-day-old seedlings in 17 β -oestradiol (Sigma, St. Louis, OH, USA) for 10 min. For the infection of *in vitro*-grown *A. thaliana*, 2-week-old seedlings were submerged with a 10⁷ colony-forming unit (CFU)/mL bacterial suspension for 10 min, and the phenotype of the plants was assessed 6 days after infection. For ³⁵S-labelled methionine assays, leaf discs of 3-week-old apple seedlings were vacuum infiltrated with bacterial suspensions at 5 $\times$ 10⁸ CFU/mL and kept in a wet Petri dish for 8 h at 22 °C; 5-week-old *A. thaliana* Col-0 plants were inoculated with bacterial suspensions at 10⁷ CFU/mL and leaf discs were taken at 14 hpi.

³⁵S-labelled methionine incorporation

Seedlings treated with dimethylsulphoxide (DMSO) or oestradiol for 3 h and inoculated *A. thaliana* and apple leaf discs were transferred to 1 mL of liquid 1 $\times$ MS containing 50 μ Ci of ³⁵S-labelled methionine (Perkin-Elmer, Waltham, MA, USA), and incubated for 2 h, except for the seedlings treated with a range of oestradiol concentrations (30 min of incubation). Seedlings or leaf discs were rinsed twice for 5 min in 1 $\times$ MS. Total proteins were extracted in 1 mM sodium ethylenediaminetetraacetate (Na-EDTA), 1 mM MgCl₂, 25 mM Tris-HCl (pH 7.6), 13.3 μ M β -mercaptoethanol and 1 μ g/mL antiprotease cocktail (Roche), subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the methionine incorporation rate was revealed by autoradiography.

Microscopy and 2,7-dichlorofluorescein diacetate (DCFH-DA), callose, DAPI and FDA-PI staining

Callose detection is described in Degrave *et al.* (2008). For DAPI staining, seedlings were immersed in 5 μ g/mL DAPI (Sigma) and 10 mM phosphate-buffered saline (PBS), and rinsed twice in 10 mM PBS. For FDA-PI analysis, seedlings were immersed in 5 μ g/mL FDA (Sigma) and 5 μ g/mL PI (BD Pharmingen, San Diego, CA, USA), and rinsed twice with sterile water. Seedlings were mounted in 50% glycerol or water (for FDA-PI) and observed with a Zeiss Axiophot light microscope (Zeiss, Oberkochen, Germany) (eGFP and FDA: excitation filter, 470 nm; bandpass filter, 525 nm; PI: excitation filter, 546 nm; barrier filter, 590 nm; DAPI: excitation filter, 365 nm; barrier filter, 420 nm). H₂O₂ detection using 300 μ M DCFH-DA was performed as described by Degrave *et al.* (2008).

RNA extraction and qRT-PCR

RNA extraction and cDNA synthesis are described in Degrave *et al.* (2008). cDNAs were amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's license in an Applied Biosystems 7300 Real Time PCR System. Results were analysed with Applied Biosystems Sequence Detection Software v1.3.1. Gene expression was normalized using the constitutive *elongation factor 1 α* (*EF1 α*) gene. Primers are described in Fagard *et al.* (2007) and in Appendix S1.

ACKNOWLEDGEMENTS

We thank B. Szurek for *Agrobacterium tumefaciens* C58C1 and C. Manceau for the *Pseudomonas syringae* *tts* mutant. We thank J. P. Renou,

L. Deslandes, Y. Marco, L. Rajjou and V. Gaudin for fruitful discussions and D. Expert for critical reading of the manuscript. We thank A. Christ, J. Fromentin and Q. Clément for technical help. This work was supported by the French National Research Agency (ANR-07-JCJC-0132) and the French Ministry for Higher Education and Research to AD and AL.

REFERENCES

- Alfano, J.R. (2009) Roadmap for future research on plant pathogen effectors. *Mol. Plant Pathol.* **10**, 805–813.
- Badel, J.L., Shimizu, R., Oh, H.S. and Collmer, A. (2006) A *Pseudomonas syringae* pv. *tomato* *avrE1/hopM1* mutant is severely reduced in growth and lesion formation in tomato. *Mol. Plant-Microbe Interact.* **19**, 99–111.
- Barry, M.A., Guinebretiere, M.H., Marçais, B., Coissac, E., Paulin, J.P. and Laurent, J. (1990) Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. *Mol. Microbiol.* **4**, 777–786.
- Billing (1983) Fire blight. *J. R. Hortic. Soc.* **55**, 206–210.
- Block, A. and Alfano, J.R. (2011) Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr. Opin. Microbiol.* **14**, 39–46.
- Block, A., Li, G.Y., Fu, Z.Q. and Alfano, J.R. (2008) Phytopathogen type III effector weaponry and their plant targets. *Curr. Opin. Plant Biol.* **11**, 396–403.
- Boch, J. and Bonas, U. (2010) *Xanthomonas* AvrBs3 family-type III effectors: discovery and function. *Annu. Rev. Phytopathol.* **48**, 419–436.
- Bocsanczy, A., Nissinen, R., Oh, C. and Beer, S. (2008) HrpN of *Erwinia amylovora* functions in the translocation of DspA/E into plant cells. *Mol. Plant Pathol.* **9**, 425–434.
- Bogdanove, A., Bauer, D. and Beer, S. (1998) *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the hrp (type III secretion) pathway. *J. Bacteriol.* **180**, 2244–2247.
- Boureau, T., ElMaarouf-Bouteau, H., Garnier, A., Brisset, M., Perino, C., Pucheu, I. and Barny, M. (2006) 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. *Mol. Plant-Microbe Interact.* **19**, 16–24.
- Boureau, T., Siemer, S., Perino, C., Gaubert, S., Patrit, O., Degrave, A., Fagard, M., Chevreau, E. and Barny, M.A. (2011) The HrpN effector of *Erwinia amylovora*, which is involved in type III translocation, contributes directly or indirectly to callose elicitation on apple leaves. *Mol. Plant-Microbe Interact.* **24**, 577–584.
- Chen, L.Q., Hou, B.H., Lalonde, S., Takanaga, H., Hartung, M.L., Qu, X.Q., Guo, W.J., Kim, J.G., Underwood, W., Chaudhuri, B., Chermak, D., Antony, G., White, F.F., Somerville, S.C., Mudgett, M.B. and Frommer, W.B. (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. *Nature*, **468**, 527–532.
- Chen, Z., Agnew, J., Cohen, J., He, P., Shan, L., Sheen, J. and Kunkel, B. (2007) *Pseudomonas syringae* type III effector AvrP2 alters *Arabidopsis thaliana* auxin physiology. *Proc. Natl. Acad. Sci. USA*, **104**, 20 131–20 136.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Crowe, M., Serizet, C., Thareau, V., Aubourg, S., Rouzé, P., Hilson, P., Beynon, J., Weisbeek, P., van Hummelen, P., Reymond, P., Paz-Ares, J., Nietfeld, W. and Trick, M. (2003) CATMA: a complete *Arabidopsis* GST database. *Nucleic Acids Res.* **31**, 156–158.
- DeRoy, S., Thilmony, R., Kwack, Y., Nomura, K. and He, S. (2004) A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. USA*, **101**, 9927–9932.
- Degrave, A., Fagard, M., Perino, C., Brisset, M.N., Gaubert, S., Laroche, S., Patrit, O. and Barny, M.A. (2008) *Erwinia amylovora* type three-secreted proteins trigger cell death and defense responses in *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* **21**, 1076–1086.
- Fagard, M., Dellagi, A., Roux, C., Perino, C., Rigault, M., Boucher, V., Shevchik, V.E. and Expert, D. (2007) *Arabidopsis thaliana* expresses multiple lines of defense to counterattack *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* **20**, 794–805.
- Fontana, M.F., Banga, S., Barry, K.C., Shen, X., Tan, Y., Luo, Z.Q. and Vance, R.E. (2011) Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent *Legionella pneumophila*. *Plos Pathog.* **7**, e1001289.
- Gaudriault, S., Malandrin, L., Paulin, J.P. and Barny, M.A. (1997) DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* **26**, 1057–1069.

- Grant, S.R., Fisher, E.J., Chang, J.H., Mole, B.M. and Dangl, J.L. (2006) Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annu. Rev. Microbiol.* **60**, 425–449.
- Ham, J.H., Majerczak, D.R., Arroyo-Rodriguez, A.S., Mackey, D.M. and Coplin, D.L. (2006) WtsE, an AvrE-family effector protein from *Pantoea stewartii* subsp. *stewartii*, causes disease-associated cell death in corn and requires a chaperone protein for stability. *Mol. Plant–Microbe Interact.* **19**, 1092–1102.
- Ham, J., Majerczak, D., Ewert, S., Sreerekha, M., Mackey, D. and Coplin, D. (2008) WtsE, an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants. *Mol. Plant Pathol.* **9**, 633–643.
- Ham, J., Majerczak, D., Nomura, K., Mecey, C., Uribe, F., He, S., Mackey, D. and Coplin, D. (2009) Multiple activities of the plant pathogen type III effector proteins WtsE and AvrE require WxxxE motifs. *Mol. Plant–Microbe Interact.* **22**, 703–712.
- Hilson, P., Allemeersch, J., Altmann, T., Aubourg, S., Avon, A., Beynon, J., Bhalerao, R., Bitton, F., Caboche, M., Cannoot, B., Chardakov, V., Cognet-Holliger, C., Colot, V., Crowe, M., Darimont, C., Durinck, S., Eickhoff, H., de Longevialle, A., Farmer, E., Grant, M., Kuiper, M., Lehrach, H., Léon, C., Leyva, A., Lundeberg, J., Lurin, C., Moreau, Y., Nietfeld, W., Paz-Ares, J., Reymond, P., Rouzé, P., Sandberg, G., Segura, M., Serizet, C., Tabrett, A., Taconnat, L., Thareau, V., Van Hummelen, P., Vercruyse, S., Vuylsteke, M., Weingartner, M., Weisbeek, P., Wirta, V., Wittink, F., Zabeau, M. and Small, I. (2004) Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. *Genome Res.* **14**, 2176–2189.
- Kim, H.S., Thammarat, P., Lommel, S.A., Hogan, C.S. and Charkowski, A.O. (2011) *Pectobacterium carotovorum* elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant–microbe interactions. *Mol. Plant–Microbe Interact.* **24**, 773–786.
- Kvitko, B.H., Park, D.H., Velasquez, A.C., Wei, C.F., Russell, A.B., Martin, G.B., Schneider, D.J. and Collmer, A. (2009) Deletions in the repertoire of *Pseudomonas syringae* pv. *tomato* DC3000 type III secretion effector genes reveal functional overlap among effectors. *PLoS Pathog.* **5**, e1000388.
- Lam, Y.W., Trinkle-Mulcahy, L. and Lamond, A.I. (2005) The nucleolus. *J. Cell Sci.* **118**, 1335–1337.
- Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. and Ryals, J. (1995) Systemic acquired resistance in Arabidopsis requires salicylic acid but not ethylene. *Mol. Plant–Microbe Interact.* **8**, 863–870.
- Lorrain, S., Vailleau, F., Balaque, C. and Roby, D. (2003) Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends Plant Sci.* **8**, 263–271.
- Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B. and Ton, J. (2011) Callose deposition: a multifaceted plant defense response. *Mol. Plant–Microbe Interact.* **24**, 183–193.
- Meng, P., Raynaud, C., Tcherkez, G., Blanchet, S., Massoud, K., Domenichini, S., Henry, Y., Soubigou-Taconnat, L., Lelarge-Trouverie, C., Saindrenan, P., Renou, J. and Bergounioux, C. (2009) Crosstalks between myo-inositol metabolism, programmed cell death and basal immunity in Arabidopsis. *PLoS ONE* **4**, e7364.
- Moreau, M., Degrave, A., Vedel, R., Bitton, F., Patrit, O., Renou, J.P., Barny, M.A. and Fagard, M. (2012) EDS1 contributes to nonhost resistance of *Arabidopsis thaliana* against *Erwinia amylovora*. *Mol. Plant–Microbe Interact.* **25**, 421–430.
- Nissinen, R.M., Ytterberg, A.J., Bogdanove, A.J., Van Wijk, K.J. and Beer, S.V. (2007) Analyses of the secretomes of *Erwinia amylovora* and selected hrp mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. *Mol. Plant Pathol.* **8**, 55–67.
- Oh, C.S., Martin, G.B. and Beer, S.V. (2007) DspA/E, a type III effector of *Erwinia amylovora*, is required for early rapid growth in *Nicotiana benthamiana* and causes NbSGT1-dependent cell death. *Mol. Plant Pathol.* **8**, 255–265.
- Pester, D., Milčevićová, R., Schaffer, J., Wilhelm, E. and Blümel, S. (2012) *Erwinia amylovora* expresses fast and simultaneously hrp/dsp virulence genes during flower infection on apple trees. *PLoS ONE* **7**, e32583.
- Ren, X., Liu, F., Bao, Z., Zhang, C., Wu, X., Chen, L., Liu, R. and Dong, H. (2008) Root growth of *Arabidopsis thaliana* is regulated by ethylene and abscisic acid signaling interaction in response to HrpN(Ea), a Bacterial Protein of Harpin Group. *Plant Mol. Biol. Rep.* **26**, 225–240.
- Ruepp, A., Zollner, A., Maier, D., Albermann, K., Hani, J., Mokrejs, M., Tetko, I., Güldener, U., Mannhaupt, G., Münsterkötter, M. and Mewes, H. (2004) The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res.* **32**, 5539–5545.
- Venisse, J., Malnoy, M., Faize, M., Paulin, J. and Brisset, M. (2002) Modulation of defense responses of *Malus* spp. during compatible and incompatible interactions with *Erwinia amylovora*. *Mol. Plant–Microbe Interact.* **15**, 1204–1212.
- Zuo, J., Niu, Q.W. and Chua, N.H. (2000) Technical advance: an estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J.* **24**, 265–273.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 DspA/E expressed *in planta* increases the growth and survival of wild-type *Erwinia amylovora* in *Arabidopsis thaliana*. Root growth of the 7-2-1 control line is not affected by oestradiol treatment.

Fig. S2 Kinetics of DspA/E-induced cell death in *Arabidopsis thaliana* transgenic plants.

Fig. S3 Detection of cell death in transgenic seedlings using propidium iodide (PI) staining.

Fig. S4 *Erwinia amylovora* reduces plant growth in a DspA/E-dependent manner.

Fig. S5 Modulation of selected *Arabidopsis thaliana* genes by *Erwinia amylovora* and DspA/E.

Fig. S6 DspA/E is required for full induction of defence genes in *Arabidopsis thaliana* by *Erwinia amylovora*.

Fig. S7 DspA/E does not accumulate in transgenic plants.

Table S1 List of 81 *Arabidopsis thaliana* genes modulated in the untreated 13-1-2 line.

Table S2 *Arabidopsis thaliana* genes modulated by at least two-fold following induction of *dspA/E* expression.

Table S3 Comparison of genes modulated by *Erwinia amylovora* and by DspA/E alone in 13-1-2 transgenic plants.

Appendix S1