EDS1 Contributes to Nonhost Resistance of *Arabidopsis thaliana* Against *Erwinia amylovora*

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Erwinia amylovora causes fire blight in rosaceous plants. In nonhost *Arabidopsis thaliana*, *E. amylovora* triggers necrotic symptoms associated with transient bacterial multiplication, suggesting either that *A. thaliana* lacks a susceptibility factor or that it actively restricts *E. amylovora* growth. Inhibiting plant protein synthesis at the time of infection led to an increase in necrosis and bacterial multiplication and reduced callose deposition, indicating that *A. thaliana* requires active protein synthesis to restrict *E. amylovora* growth. Analysis of the callose synthase-deficient *pmr4-1* mutant indicated that lack of callose deposition alone did not lead to increased sensitivity to *E. amylovora*. Transcriptome analysis revealed that approximately 20% of the genes induced following *E. amylovora* infection are related to defense and signaling. Analysis of mutants affected in NDR1 and EDS1, two main components of the defense-gene activation observed, revealed that *E. amylovora* multiplied ten times more in the *eds1-2* mutant than in the wild type but not in the *ndr1-1* mutant. Analysis of mutants affected in three WRKY transcription factors showing EDS1-dependent activation identified WRKY46 and WRKY54 as positive regulators and WRKY70 as a negative regulator of defense against *E. amylovora*. Altogether, we show that EDS1 is a positive regulator of nonhost resistance against *E. amylovora* in *A. thaliana* and hypothesize that it controls the production of several effective defenses against *E. amylovora* through the action of WRKY46 and WRKY54, while WRKY70 acts as a negative regulator.

The gram-negative bacterium *Erwinia amylovora* is the causal agent of fire blight, a devastating disease of species of the *Pyraeae* tribe of *Rosaceae*, which includes apple, pear, and ornamentals (e.g., cotoneaster, pyracantha). Fire blight is a destructive disease affecting blossoms, shoots, and limbs and can sometimes kill entire trees. The main symptom of fire blight is progressive necrosis of aerial parts.

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E. amylovora depends on two main pathogenicity factors: i) the exopolysaccharide, required for biofilm formation, which is necessary for tissue colonization through the xylem and plays a role in bacterial survival (Koczan et al. 2009); ii) the type III secretion system (T3SS), which allows the injection of type III effectors (T3E) inside the plant cell. T3E are produced by many bacterial pathogens; it is generally accepted that their role inside the plant cell is to repress plant defense (Block et al. 2008), but they can also be involved in modifying plant metabolism for nutritional gain (Chen et al. 2010). Two T3E of *E. amylovora* have been shown to be involved in pathogenicity. DspA/E is known to be injected inside the plant cell and to repress callose deposition (DebRoy et al. 2004), a cell wall-associated plant defense. Harpin N (HrpN) is implicated in the translocation of T3E inside the plant cell (Bocsanczy et al. 2008; Boureau et al. 2011).

During an incompatible interaction, T3E can be detected by plant resistance (R) proteins, thereby triggering a hypersensitive response, a form of programmed cell death. At least three different signaling pathways leading to defense-gene expression in response to the activation of R proteins have been described in *Arabidopsis thaliana* (Glazebrook 2001). Two of these have been extensively studied, the EDS1- and the NDR1-dependent pathways. Resistance proteins known to require NDR1 (RPM1, RPS2, and RPS5) belong to the leucine-zipper subclass of R genes, while those known to require EDS1 (RPW8, RPP2, RPP4, RPP5, RPP10, RPP14, RPS4) belong to the subset of the R proteins with an amino-terminal domain similar to the *Drosophila* toll and mammalian interleukin 1 receptors (TIR). Other resistance genes (*RPP7*, *RPP8*, and *RPP13*) require neither EDS1 nor NDR1 (Eulgem et al. 2007).

During a compatible interaction, the absence of R-mediated recognition allows pathogen growth and disease. However, conserved molecules called pathogen associated molecular patterns (PAMPs) can trigger a defense response called PAMP-triggered immunity (PTI), which must be suppressed by pathogen effectors in order for disease to occur. Increasing evidence supports the existence of shared signaling pathways between PTI and R-mediated defense. One of these is the EDS1 pathway, which is necessary for both PTI and R gene-mediated signaling (Wiermer et al. 2005). Interestingly, EDS1 also plays a crucial role in transducing redox signals in response to biotic and abiotic stresses such as light (Mateo et al. 2004). Downstream responses controlled by EDS1 include the production of salicylic acid (SA), of reactive oxygen species (ROS) (Rustérucci et al. 2001) and of the secondary metabolite 2,3-dihydroxybenzoic acid (Bartsch et al. 2010) as well as the expression of both SA-dependent and -independent defense genes (Bartsch et al. 2006).

No gene-for-gene interaction has been described to date for the interaction between *E. amylovora* and its hosts. The molecular basis of the resistance against *E. amylovora* in resistant apple cultivars is thought to be of a quantitative nature (Pontais et al. 2008). These past few years, several resistance quantitative trait loci against fire blight have been identified in tolerant *Malus* cultivars (Calenge et al. 2005; Durel et al. 2009; Peil et al. 2007), suggesting that major resistance genes against fire blight could exist. *E. amylovora* infection of apple seedlings triggers several lines of defense, including expression of defense genes that are known to rely on the SA signaling pathway in *A. thaliana* (Venisse et al. 2002). However, no difference in the timing or level of expression of these SA-dependent genes was observed between a tolerant and a susceptible apple cultivar. This suggested that the SA pathway, although it is activated following infection, may not contribute to the re-

sistance of the tolerant cultivar. In *Nicotiana benthamiana*, the *NbSGT1* gene is required for nonhost resistance against *E. amylovora*, as plants in which *NbSGT1* expression was repressed by virus-induced gene silencing supported higher bacterial growth (Oh et al. 2007). *SGT1* being required for the stabilization of many resistance proteins suggested that R-mediated signaling is involved in the resistance of *N. benthamiana* to *E. amylovora*.

We described previously that *E. amylovora* is able to multiply and trigger necrotic lesions in leaves of nonhost *A. thaliana* plants; necrosis and bacterial growth both depend, as in the host plant, on the T3SS (Degrave et al. 2008). However, bacterial multiplication was weaker in *A. thaliana* leaves than in leaves of host plants. It was also transient, suggesting that *A. thaliana* lacked a susceptibility factor required for *E. amylovora* or that *A. thaliana* was able to mount an effective resistance against *E. amylovora* following infection (Degrave et al. 2008). We described previously that *A. thaliana* plants produce several lines of defense during the early phases of infection that are, for the most part, similar to those induced during disease on members of genus *Malus*, one of which is the SA pathway (Degrave et al. 2008). However, we determined that the SA pathway was not essential for this nonhost resistance, since the SA-deficient *sid2* mutant did not display any alteration in its response to *E. amylovora* (Degrave et al. 2008).

In this paper, we show that active plant protein synthesis is required for *A. thaliana* to restrict *E. amylovora* local growth and associated necrosis. We describe the global defense response triggered by *E. amylovora* in *A. thaliana* at the transcriptional level. Finally, we identify EDS1 as playing a significant role in *A. thaliana* resistance against *E. amylovora*, possibly through the action of two WRKY transcription factors, WRKY46 and WRKY54. In parallel, we identify WRKY70 as a negative regulator of defense against *E. amylovora*.

RESULTS

A. thaliana nonhost resistance to *E. amylovora* requires active protein synthesis.

In order to determine whether *A. thaliana* was actively restricting *E. amylovora* growth, we co-inoculated *A. thaliana*

Fig. 1. *Arabidopsis thaliana* requires active protein synthesis to restrict growth of the non adapted pathogen *Erwinia amylovora*. **A**, Symptom severity of wild-type (Col-0) *A. thaliana* leaves inoculated with 4 μ g per milliliter of eukaryotic protein synthesis inhibitor cycloheximide (CHX), *E. amylovora*, or both. Symptoms were observed 24 h postinoculation. Symptom severity was scored from 0 to 3, in which 0 corresponds to an absence of symptoms and 3 to a fully necrotic leaf, as described by Degrave and associates (2008). The symptom severity scale and representative symptoms are shown above the graph. The asterisk indicates a significant difference from leaves inoculated with *E. amylovora* alone (Fisher's exact test, $\alpha = 0.05$). **B**, Bacterial counts of *E. amylovora* cells in *A. thaliana* leaves inoculated with wild-type *E. amylovora* (*Ea*) or a type III secretion system mutant (*t3ss*) alone or together with 4 μ g of CHX per milliliter (T24-CHX). Bars represent standard errors. Columns connected with an asterisk are statistically different according to Mann and Whitney's test (P value < 0.05). **C**, Following treatment or not with CHX, leaf disks were labeled with ³⁵S methionine for 2 h. Total protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were stained with Coomassie blue to detect total protein (bottom). ³⁵S methionine incorporation was detected by autoradiography (top). Similar results were obtained for two biological replicates. **D**, Bacterial counts of *Escherichia coli* DH5 α cells in *A. thaliana* leaves inoculated alone or together with 4 μ g of CHX (T24-CHX) per milliliter. Bars represent standard errors. Bars with different letters are statistically different according to Kruskal and Wallis' test (P value < 0.05). Experiments were repeated three times with similar results.

leaves with *E. amylovora* cells and cycloheximide (CHX), which inhibits protein synthesis in eukaryotic cells. When plants were inoculated with CHX alone, plants displayed necrosis only rarely (Fig. 1A). Following inoculation with *E. amylovora* alone, approximately one third of the plants displayed necrosis 24 h postinoculation (hpi). However, when plants were co-inoculated with *E. amylovora* and CHX, all plants displayed necrosis at 24 hpi, and the proportion of plants displaying the strongest symptoms was higher. We then analyzed the proliferation of *E. amylovora* cells in planta when co-inoculated with CHX. In leaves co-inoculated with *E. amylovora* and CHX, the bacterial population was able to increase 10-fold more at 24 hpi than when leaves were inoculated with *E. amylovora* alone (Fig. 1B). We then analyzed the effect of CHX on in planta growth of an *E. amylovora* mutant lacking a functional T3SS. As described previously (Degraeve et al. 2008), the *t3ss* mutant alone could not multiply at all in *A. thaliana* leaves, and its bacterial population dropped dramatically at 24 hpi (Fig. 1B). However, when co-inoculated with CHX, the *t3ss* mutant was able to multiply at 24 hpi, but not to the same extent as wild-type bacteria treated with CHX. This result indicates that repressing plant de novo protein synthesis with CHX can partially restore the growth of a *t3ss* mutant of *E. amylovora* in nonhost *A. thaliana*. In order to quantify the effect of CHX on protein synthesis, we extracted total proteins from leaves treated or not with CHX and incubated in ^{35}S -radiolabeled methionine. De novo protein synthesis was reduced by approximately 75% in CHX-treated leaves (Fig. 1C). Finally, in order to determine the specificity of the effect of CHX, we inoculated *A. thaliana* leaves with *Escherichia coli* DH5 α cells with or without CHX. The treatment with CHX did not allow *Escherichia coli* cells to multiply in planta (Fig. 1D). However, the number of bacterial cells in planta did not decrease at 24 hpi in CHX-treated leaves while they dropped in untreated leaves, suggesting that *Escherichia coli* could be susceptible to active defenses in *A. thaliana*.

Altogether, these results indicate that active plant protein synthesis is required for *A. thaliana* to restrict *E. amylovora*-induced necrosis and bacterial growth.

Callose deposition in response to *E. amylovora* requires active protein synthesis.

Callose deposition is a wall-associated defense that is known to be repressed by *E. amylovora* during disease on host plants (DebRoy et al. 2004). In *A. thaliana*, on the contrary, callose deposition is induced following infection by the wild-type strain of *E. amylovora* (Degraeve et al. 2008), suggesting that callose deposition could play a role in the restriction of *E. amylovora* growth in *A. thaliana*. Analysis of callose deposition in plants co-inoculated with *E. amylovora* and CHX showed that CHX treatment inhibited the deposition of callose (Fig. 2A). In *A. thaliana*, callose deposition in response to pathogens depends on the *PMR4* gene (Nishimura et al. 2003). Indeed, the *pmr4-1* mutant inoculated with *E. amylovora* did not accumulate callose deposits, in contrast to wild-type plants (Fig. 2B). However, the bacterial growth and the intensity of necrotic symptoms induced by *E. amylovora* in the *pmr4-1* mutant was comparable to that in wild-type plants (Fig. 2C and D), indicating that callose deposition alone did not account for defense of *A. thaliana* against *E. amylovora*.

E. amylovora triggers important transcriptome modifications in *A. thaliana*.

We analyzed the transcriptome of *A. thaliana* in response to *E. amylovora*, in order to identify transcriptional changes that could be responsible for restricting *E. amylovora* proliferation. The transcriptome study was performed on *A. thaliana* leaves

6 and 24 h following inoculation with *E. amylovora*, using complete *A. thaliana* microarray (CATMA) chips (Crowe et al. 2003; Hilson et al. 2004). Control samples were prepared from mock-inoculated leaves. Two biological replicates and a dye-

Fig. 2. *Erwinia amylovora*-triggered callose deposition requires active protein synthesis and *PMR4*. **A** and **B**, Quantification of callose deposition of *Arabidopsis thaliana* leaf disks 8 h after infiltration with wild-type *E. amylovora* (*Ea*) or with water (mock). After staining with aniline blue, callose deposits appear as fluorescent dots. Bar length = 200 μm . **A**, Epifluorescence micrographs of *A. thaliana* leaf disks 8 h after infiltration. *E. amylovora* was co-infiltrated with 4 μg of cycloheximide (CHX) per milliliter (*Ea*+CHX) or not (*Ea*) in wild-type Col-0 plants. The mean number of callose deposits per square millimeter is indicated for each treatment in the bar graph. **B**, *E. amylovora* was infiltrated in the callose synthase-deficient *pmr4-1* mutant and wild-type Col-0 plants. **C**, Symptom severity of wild-type (Col-0) and *pmr4-1* mutant leaves inoculated with *E. amylovora*. Symptoms were observed 24 h postinoculation and were scored according to our rating scale (shown on the right). No statistical difference was found between wild type and *pmr4-1*, according to Fisher's exact test, $\alpha = 0.05$. **D**, Bacterial counts of wild-type *E. amylovora* cells in leaves of wild-type Col-0 and *pmr4-1* mutant plants. No statistical difference was found between wild type and *pmr4-1*, according to Student's *t* tests (*P* value < 0.05). Experiments were repeated twice with similar results.

swap were performed for each set of pairwise comparison. Statistical analysis (discussed below) revealed that, altogether, 15.3% (3,762) of the 24,576 probes represented on the CATMA array showed at least a twofold modulation (P value ≤ 0.05) of their transcript accumulation, following *E. amylovora* infection at 6 or 24 hpi. This indicated that infection with *E. amylovora* led to a very broad remodeling of the transcriptome during the first 24 hours following infection (Supplementary Table 1). In order to reduce the number of false positives, we arbitrarily selected for further analysis the genes showing at least a 2.5-fold induction or repression of their expression level at 6 or 24 hpi following inoculation with *E. amylovora*. Totals of 1,185 induced genes and 1,073 repressed genes corresponded to this criterion (Fig. 3A).

We analyzed the putative functions of these genes using the Munich Information Center for Protein Sequences database (Ruepp et al. 2004). More than 80% of these genes were annotated as encoding proteins of known or predicted function, distributed among seven main categories (Fig. 3B). All functional categories were represented in both induced and repressed genes, except for the “protein synthesis” category, which corresponded almost exclusively to repressed genes (7.9% of repressed genes) and contained mainly ribosomal proteins (Supplementary Fig. 1). The largest category in both induced (29.8%) and repressed genes (23.2%) corresponded to metabolism-related genes, suggesting that the metabolism of infected plant cells was deeply modified; this was already true at the

early 6 hpi timepoint (not shown). The “defense” and “signal transduction” categories together represented an important proportion (approximately 20%) of induced genes. We confirmed, by reverse transcription-polymerase chain reaction (RT-PCR), the modulation of a subset of these genes (Supplementary Fig. 2). Altogether, our data point to a very large transcriptional reprogramming in *A. thaliana* leaves infected with *E. amylovora*, in which defense-gene regulation plays an important part.

Identification of defense genes modulated at the transcriptional level.

Defense genes activated following infection are obvious candidates for restricting *E. amylovora* growth in planta. Therefore, we analyzed in more detail the defense and signaling categories of genes modulated following infection with *E. amylovora*. As expected because of our previous results (Degraeve et al. 2008), markers of the SA pathway as well as markers of oxidative stress were induced (Table 1). Concerning genes responsive to jasmonic acid (JA) and ethylene (ET), our data indicate that the *CHI-B*, *ETRI*, and *ERF1* genes were induced; however, most other JA-responsive genes were either repressed during the infection or not induced (Table 1). Flavonoid biosynthesis genes were repressed as is described on host plants (Venisse et al. 2002), which could be confirmed by RT-PCR. The expression of 10 putative *R* genes (Lewis et al. 2010) was affected following infection with *E. amylovora*, only one of which was repressed (Table 1). The majority of *R* genes induced by *E. amylovora* belong to the TIR-NBS (nucleotide binding site) and TIR-NBS-LRR (leucine-rich repeat) category and only one to the coiled coil (CC)-NBS-LRR category (Meyers et al. 2003). The expression of 40 putative LRR receptor-like kinase (RLK)-encoding genes was modulated, with 30 being induced and 10 repressed (Supplementary Fig. 3). The expression of a large number (47) of transcription factor-encoding genes was affected by *E. amylovora* infection: 23 genes encoding transcription factors of the WRKY family were induced, 12 genes encoding transcription factors of the MYB family were repressed or induced, eight genes encoding transcription factors of the bZIP family were induced (Supplementary Fig. 4).

In *A. thaliana*, several pathways of *R*-gene signaling exist, two of which have been well described, i.e., the NDR1 pathway involved in the signaling in response to activation of CC-NBS-LRR-type *R* genes (Meyers et al. 2003), and the EDS1 pathway involved in the signaling of *R* genes mainly of the TIR-NBS-LRR type (Wiermer et al. 2005). Of nine *R* genes induced in response to *E. amylovora*, five contained a TIR domain. Furthermore, the *EDS1* gene was strongly induced as early as 6 hpi, and this induction occurred mainly through the perception of *E. amylovora* PAMPs, since a *t3ss* mutant of *E. amylovora* also induced *EDS1*. Finally, most of the *EDS1*-dependent genes, as defined by Bartsch and associates (2006), were strongly induced in response to *E. amylovora* as early as 6 hpi (Supplementary Fig. 5), among which were three WRKY transcription factors (*WRKY46*, *WRKY54* and *WRKY70*), *FMO1*, and *NUDT7*, characterized as actors of the EDS1 pathway (Bartsch et al. 2006), and the SA-dependent gene *PRI*, all of which we confirmed to show at least partial EDS1-dependent activation in response to *E. amylovora*, since quantitative (q)RT-PCR experiments showed that their expression was lower in response to *E. amylovora* in an *eds1-2* mutant (Fig. 4). Altogether, these data led us to hypothesize a role for *EDS1* in the defense of *A. thaliana* against *E. amylovora*.

The EDS1 pathway contributes to *A. thaliana* nonhost resistance against *E. amylovora*.

To assess whether *EDS1*-dependent defense induction played a role during the interaction between *E. amylovora* and

Fig. 3. *Erwinia amylovora* causes deep modifications of the *Arabidopsis thaliana* transcriptome. **A**, Venn diagrams of the number of *A. thaliana* genes showing at least a 2.5-fold modification of their expression 6 and 24 h following inoculation with *E. amylovora* compared with mock-inoculated leaves. **B**, Functional distribution of the 1,185 genes induced (left) and 1,073 genes repressed (right) 6 and 24 h following inoculation with *E. amylovora*. The genes were classified according to the functional organization of the Munich Information Center for Protein Sequences functional catalog (Ruepp et al. 2004).

A. thaliana, we used the *eds1-2* mutant (Glazebrook 2001; Wiermer et al. 2005). For comparison, we used the *ndr1-1* mutant, affected in the signaling pathway in response to a different set of *R* genes than EDS1 (Century et al. 1995; Glazebrook 2001). The mutants were inoculated with the wild-type strain of *E. amylovora*, and necrotic symptoms were scored (Fig. 5A). In the *ndr1-1* mutant, the number of necrotic leaves was slightly higher than in wild-type plants. However, this difference was not associated with a modification of in planta bacterial growth (Fig. 5B). In contrast, the *eds1-2* mutant showed significantly more symptomatic leaves and their symptoms were stronger than in the wild-type plants (Fig. 5A). These stronger symptoms were associated with a tenfold increase in bacterial growth of wild-type *E. amylovora* in the *eds1-2* mutant compared with growth in wild-type plants (Fig. 5B), while the *t3ss* mutant remained unable to multiply in the *eds1-2* mutant (Supplementary Fig. 6).

WRKY transcription factors are known to play an important role in defense-gene activation in plants. Among the EDS1-dependent genes, we found three WRKY transcription factors (*WRKY46*, *WRKY54*, and *WRKY70*) that were, therefore, good candidate regulators of effective defense against *E. amylovora*. We analyzed the response to *E. amylovora* of mutants corresponding to each of these three genes. The *wrky46-3* and *wrky54-2* mutants both showed a significant increase in the number of necrotic leaves and in the intensity of necrosis (Fig. 5C), indicating that these transcription factors are positive regulators of defense against *E. amylovora*. On the contrary,

the *wrky70-1* mutant showed almost no symptoms in response to *E. amylovora* (Fig. 5C), indicating that *WRKY70* is a negative regulator of defense against *E. amylovora*.

The *eds1-2* mutation and CHX treatment have overlapping effects.

In order to determine whether the *eds1-2* mutation and CHX treatment affected the same plant response to *E. amylovora* infection, we analyzed the multiplication of *E. amylovora* co-inoculated with CHX in the *eds1-2* mutant background. The number of *E. amylovora* cells 24 hpi was higher in wild-type plants treated with CHX than in the *eds1-2* mutant plants not treated with CHX (Fig. 6), suggesting that the effect of CHX was stronger than that of the *eds1-2* mutation. Furthermore, *E. amylovora* multiplication was higher in the *eds1-2* mutant when it was co-inoculated with CHX, but bacterial cells did not multiply more than in wild-type plants treated with CHX (Fig. 6). Altogether, our data indicate that the CHX treatment bypasses the effect of the *eds1-2* mutation on *E. amylovora* in planta proliferation. One possibility to explain these results could be that CHX inhibits EDS1-triggered defenses as well as other defenses triggered by a separate pathway that remains to be identified.

DISCUSSION

E. amylovora, the fire blight pathogen, is able to induce necrotic symptoms and to multiply transiently in leaves of the

Table 1. Regulation of selected defense genes in response to *Erwinia amylovora*

AGI number	Gene description	6 hpi ^a	24 hpi ^a
Response to salicylic acid			
AT3G52430	Phytoalexin-deficient 4 protein (<i>PAD4</i>)	3.23	3.25
AT1G74710	Isochorismate synthase 1 (<i>ICS1</i>)	3.48	2.66
AT4G39030	Enhanced disease susceptibility 5 (<i>EDS5</i>)	2.22	2.62
AT3G48090	Disease resistance protein (<i>EDS1</i>)	1.94	1.20
AT1G21270	Wall-associated kinase 2 (<i>WAK2</i>)	1.46	1.52
AT1G64280	Regulatory protein (<i>NPR1</i>)	1.03	1.09
Response to jasmonic acid and ethylene			
AT4G17500	Ethylene-responsive element-binding protein 1 (<i>ERF1</i>)	0.62*	1.32
AT3G12500	Basic endochitinase (<i>CHI-B</i>)	0.22*	0.79
AT1G66340	Ethylene receptor 1 (<i>ETR1</i>)	0.82*	0.77
AT1G75830	Plant defensin-fusion protein, putative (<i>PDF1.1</i>)	-1.25	-0.71*
AT5G42650	Allene oxide synthase (<i>AOS</i>)	-1.28	-0.39*
AT3G16470	Jacalin lectin family protein	-0.95	0.53*
AT1G13280	Allene oxide cyclase family protein	-0.95	-0.96
AT1G19640	S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (<i>JMT</i>)	-0.02*	-0.77
AT2G43710	Stearoyl-ACP desaturase (<i>SSI2</i>)	0.01*	-1.19
AT2G06050	12-oxophytodienoate reductase (<i>OPR3</i>)	0.14*	0.58*
AT2G39940	Coronatine-insensitive 1 (<i>COI1</i>)	0.81*	0.32*
Response to oxidative stress			
AT3G22370	Alternative oxidase 1a, mitochondrial (<i>AOX1A</i>)	2.01	1.20
AT1G64060	Respiratory burst oxidase protein F (<i>RbohF</i>)	1.69	0.90
Flavonoid biosynthesis			
AT5G13930	Chalcone synthase (<i>CHS</i>)	-0.81*	-2.88
AT5G08640	Flavonol synthase (<i>FLS</i>)	-1.34	-2.99
Resistance genes			
AT1G66090	Disease resistance protein family (TIR-NBS class) ^b	4.63	3.67
AT3G14470	Disease resistance protein family (NBS-LRR class)	2.02	1.92
AT4G11170	Disease resistance protein family (TIR-NBS-LRR class)	1.54	2.55
AT5G51630	Disease resistance protein family (TIR-NBS-LRR class)	0.96	2.08
AT4G14370	Disease resistance protein family (TIR-NBS-LRR class)	1.39	1.08
AT4G33300	ADR1-like1, disease resistance protein family (CC-NBS-LRR class)	1.92	1.55
AT5G45510	LRR family protein	1.17	1.63
AT1G17615	Disease resistance protein family (TIR-NBS class)	0.19*	1.38
AT5G38350	Disease resistance protein family (NBS-LRR class)	0.26*	1.44
AT4G19530	Disease resistance protein (TIR-NBS-LRR class), putative	-1.25	-1.53

^a Values are log₂ signal ratios between infected and water-treated control plants. Positive and negative values correspond to genes up- and down-regulated in response to *E. amylovora*, respectively. Asterisks (*) indicate that gene expression was not significantly changed. hpi = hours postinoculation.

^b TIR = toll and mammalian interleukin 1 receptors, NBS = nucleotide binding site, LRR = leucine-rich repeat, and CC = coiled coil.

Fig. 4. Modulation of EDS1-dependent genes in response to *Erwinia amylovora*. Analysis of gene expression levels by quantitative real-time reverse transcription-polymerase chain reaction. Arbitrary units are calculated relatively to the EF1 α gene expression level. Analysis of gene expression was performed in Ler (Landsberg erecta) and *eds1-2* plants harvested 6 h after mock (m) or *E. amylovora* (Ea) inoculation.

model plant *A. thaliana* (Degraeve et al. 2008). Both transient bacterial growth and necrotic symptoms triggered by *E. amylovora* require a functional T3SS, as does the pathogenicity of *E. amylovora* on host plants. However, in nonhost *A. thaliana*, *E. amylovora* does not grow as much as in host plants. The aim of this work was to identify plant factors possibly involved in restricting the growth of *E. amylovora* in *A. thaliana*.

We used CHX, an inhibitor of eukaryotic protein synthesis, which inhibited de novo protein synthesis by approximately 75% in *A. thaliana* leaves. Inhibiting protein synthesis in plant cells at the time of infection allowed *E. amylovora* cells to multiply 10-fold more than in untreated leaves. This clearly indicates that *A. thaliana* plants are actively restricting the multiplication of *E. amylovora* cells in the apoplast and limiting the associated necrotic symptoms. However, although we show that de novo protein synthesis is reduced by treatment with CHX, we cannot exclude the possibility that the effect of CHX

Fig. 5. The *eds1-2* mutant displays increased sensitivity to *Erwinia amylovora*. **A**, Symptom severity on wild type and mutants as indicated at 24 h postinoculation (hpi). The symptom severity scale is shown on the right. The *ndr1-1* mutant is in the Col-0 ecotype and the *eds1-2* mutant is in the Ler ecotype. Columns with different letters are statistically different according to Fisher's exact test ($\alpha = 0.05$). **B**, Bacterial count of *E. amylovora* in *eds1-2* and *ndr1-1* mutants. The number of CFU present in leaf extracts was counted following inoculation (T0) and 24 hpi (T24). The asterisk indicates a significant difference from leaves of corresponding wild-type ecotype inoculated with *E. amylovora* (Student's *t* tests, *P* value < 0.05). **C**, Symptom severity on wild type and mutants as indicated at 48 hpi. The symptom severity scale is shown on the right. Columns with different letters are statistically different according to Fisher's exact test ($\alpha = 0.05$). Experiments were repeated three times with similar results.

was due to the triggering of nuclear signals such as the phosphorylation of nuclear proteins, as was shown previously in animal cells (Edwards and Mahadevan 1992). Two days following inoculation, the population of bacterial cells dropped slightly in CHX-treated plants (not shown), suggesting that the effect of CHX was only transient. Furthermore, in contrast to what is observed in host plants, no sign of bacterial propagation along the veins was detected in plants treated or not with CHX (not shown). Propagation of *E. amylovora* in host plants has been shown to occur in the xylem of leaves in which bacterial numbers were high (Koczan et al. 2009). The lack of propagation of *E. amylovora* in *A. thaliana* leaves could be due to the lack of a susceptibility factor allowing progression through the xylem.

Interestingly, CHX-treated plants also supported higher bacterial growth of a *t3ss* mutant of *E. amylovora*. This suggested that CHX treatment suppressed a plant response that was triggered, at least partly, following the perception of the bacterial PAMPs harbored by the *t3ss* mutant. This is consistent with the idea that PAMPs play an important role in triggering non-host resistance (Navarro et al. 2004; Schulze-Lefert and Panstruga 2011). However, we previously showed that a *t3ss* mutant of *E. amylovora* only weakly induced the well-described defense signaling pathways dependent on JA, ET, and SA (Degraeve et al. 2008), suggesting that one or more other defense pathways are activated by a *t3ss* mutant or that the weak induction observed is sufficient to restrict growth of the *t3ss* mutant. In addition, CHX treatment did not fully rescue the *t3ss* mutation, since the growth of a *t3ss* mutant in the presence of CHX remained lower than that of wild-type *E. amylovora* in the presence of CHX. This suggests either that CHX does not inhibit the expression of all the plant reactions triggered by PAMPs or that the T3E of *E. amylovora* are important for bacterial growth. Indeed, it was shown recently that the T3SS of *Pseudomonas syringae* and of *Xanthomonas oryzae* pv. *oryzae* were able to induce the expression of several sugar transporter-encoding genes termed *SWEET* in *A. thaliana* and rice (Chen et al. 2010). Furthermore, the authors showed that RNA interference of *OsSWEET11* led to the resistance to *X. oryzae* pv. *oryzae*, indicating that the induction of the expression of this transporter by *X. oryzae* pv. *oryzae* T3E in rice was required for the bacteria, probably through their ability to transport glucose into the apoplast.

We found that, following infection with *E. amylovora*, the transcriptome of *A. thaliana* was widely remodeled. A large portion of these genes belong to the “metabolism” category, i.e., 23% of repressed genes and 29% of induced genes. It was shown recently that bacterial infection leads to important metabolic changes as early as 8 hpi and that part of this response could be attributed to transcriptional reprogramming (Ward et al. 2010). Thus, it is likely that *E. amylovora* provokes important metabolic changes in *A. thaliana* leaves, as does infection with *P. syringae*. However, the precise metabolic changes induced by *E. amylovora* remain to be characterized.

WRKY transcription factors constitute one of the largest families of transcriptional factors in plants. They are known to work both as positive and negative regulators of specific processes (Rushton 2010). Interestingly, of the 23 WRKY transcription factors induced by *E. amylovora*, nine were previously described as being induced by PAMPs of *P. syringae* (Navarro et al. 2004; Thilmony et al. 2006) or the conserved peptide flg22 (*WRKY6* and *WRKY15*) (Navarro et al. 2004), suggesting that the response of *A. thaliana* to *E. amylovora* is, in part, due to the recognition of its PAMPs; six were described as being induced by other pathogen-related stimuli such as infection by an avirulent pathogen, a necrotrophic pathogen, or a toxin (AbuQamar et al. 2006; Wang et al. 2006), and eight

were described as being induced following oxidative stress or other abiotic stresses (Balazadeh et al. 2008; Ma et al. 2006; Scarpeci et al. 2008; Tosti et al. 2006), suggesting that another part of the response of *A. thaliana* could be due to the induction of the oxidative stress, which is known to occur in response to *E. amylovora* during disease on host (Venisse et al. 2003) as well as during the nonhost interaction with *A. thaliana* (Degraeve et al. 2008).

Comparison of our transcriptome data with published data indicated that the expression of genes described as *EDS1*-dependent was strongly induced in response to *E. amylovora*, which we could confirm by showing that their expression was less induced by *E. amylovora* infection in the *eds1-2* mutant. The *eds1-2* mutant was more sensitive to *E. amylovora* than wild-type plants, indicating that *EDS1*-dependent defenses were effective against *E. amylovora*. The *EDS1* pathway is known to be triggered during both PTI and effector-triggered immunity. Our data indicate that *EDS1* was mainly induced through the PAMPs of *E. amylovora*, thus, mainly through the activation of PTI. This is, again, consistent with the idea that PTI plays a major role in nonhost resistance (Navarro et al. 2004; Schulze-Lefert and Panstruga 2011). *EDS1* was shown to regulate several defense-related pathways, one of which is the SA pathway (Wiermer 2005). However, we previously showed that the SA-deficient *sid2* mutant was not more sensitive to *E. amylovora* (Degraeve et al. 2008), indicating that the defenses effective against *E. amylovora* are *EDS1* dependent but SA independent. This is consistent with the fact that *EDS1* was shown to play a complex role in regulating the plant's response to both biotic and abiotic stress by regulating the expression of SA-dependent and SA-independent genes, cell death, ROS production, as well as plant growth (Bartsch et al. 2006; Ochsenbein et al. 2006; Straus et al. 2010). We found that the growth of *E. amylovora* in the different accessions used in this study was not always equivalent (Fig. 5). We did not detect, overall, a significant and reproducible difference in the growth of *E. amylovora* between the Ler and Col-0 accessions. However, we cannot exclude the existence of a quantitative difference in the response between these two accessions to *E. amylovora*.

Among the *EDS1*-dependent genes, we found three WRKY transcription factors (*WRKY46*, *WRKY54*, and *WRKY70*), which were, therefore, good candidate regulators of effective defense against *E. amylovora*. Analysis of the corresponding mutants

Fig. 6. Cycloheximide (CHX) increases *Erwinia amylovora* proliferation in the *eds1-2* mutant. Bacterial count of *E. amylovora* in *eds1-2* and Ler plants treated or not with 4 µg of CHX per milliliter at the time of inoculation. The number of CFU present in leaf extracts was counted 24 h postinoculation. Columns with different letters are statistically different according to Kruskal and Wallis's test (P value < 0.05). Experiments were repeated twice with similar results.

revealed that *wrky46-3* and *wrky54-2* mutants are more sensitive to infection by *E. amylovora*, while the *wrky70-1* mutant was less sensitive compared with wild type. This indicates that *WRKY46* and *WRKY54* are positive regulators of defense against *E. amylovora*, while *WRKY70* is a negative regulator of defense against *E. amylovora*. A *wrky46* knock-out mutant (*wrky46-1*) was previously shown to be unaffected in its response to *Botrytis cinerea* and to *P. syringae* pv. *tomato* (Gao et al. 2011), and a *wrky54* knock-out mutant (*wrky54-1*) was shown to be unaffected in its response to *P. syringae* pv. *maculicola* (Wang et al. 2006). To our knowledge, the present report, therefore, constitutes the first indication that *WRKY46* and *WRKY54* are involved in the response of *A. thaliana* to pathogen infection. *WRKY70* has the capability to suppress JA-mediated defenses and the *wrky70-1* mutant is more resistant to the necrotroph *Alternaria brassicicola*, which is sensitive to JA-mediated defenses (Li et al. 2006). Therefore, one possibility, although this remains to be determined, is that the decreased sensitivity of the *wrky70-1* mutant to *E. amylovora* is linked to an increase in JA-mediated defense. It is unlikely, however, that the increased sensitivity of the *eds1-2* mutant to *E. amylovora* is linked to JA-mediated defenses, since JA-responsive genes are only weakly or not induced in response to *E. amylovora* in wild-type plants and since the lack of SA signaling in the *eds1-2* mutant is expected to lead to an increase in JA-mediated defense (Wiermer et al. 2005).

Altogether, we show that *A. thaliana* nonhost resistance against *E. amylovora* involves both an EDS1-dependent plant response, suppressed in the *eds1-2* mutant, and an EDS1-independent plant response, suppressed by CHX treatment. The precise nature of the effective defenses against *E. amylovora* remains, however, to be identified.

MATERIALS AND METHODS

Bacterial strains and plant lines.

The wild-type strain of *E. amylovora* (CFBP1430) and the *t3ss* mutant strain PMV6023 hrcV::MudIIPR13 (Barney et al. 1990) used in this study are described by Degraeve and associates (2008).

Seeds of the *A. thaliana* Col-0 and Ler ecotypes were obtained from the Institut National de la Recherche Agronomique Versailles collection; seeds of the *eds1-2* (Ler) mutant were provided by P. Schulze-Lefert; seeds of the *ndrl-1* (Col-0) and *pmr4-1* (Col-0) mutants were obtained from the European *A. thaliana* Stock Centre. The *wrky70-1* (Ulker et al. 2007) and *wrky46-3* (GABI-Kat 038C07) insertion mutants were provided by I. Somsich. The *wrky54-2* mutant analyzed in this study corresponds to line SAIL_319_E03. Homozygous individuals were identified by PCR, and absence of expression of *WRKY54* was confirmed by qRT-PCR.

Plant inoculations and bacterial counting.

Plants were grown for 5 weeks in soil and were subjected to an 8-h-light and 16-h-dark cycle at 20°C, with 70% relative humidity. Leaves were infiltrated with bacterial suspensions at a concentration of 10⁷ CFU per milliliter in sterile water or with sterile water (mock-inoculation), using a needleless syringe. Symptom severity was scaled as described by Degraeve and associates (2008). For symptom rating, at least 12 rosette leaves were used per condition for each biological replicate. Maximal symptoms appeared at 24 or 48 hpi, depending on biological replicates. Therefore, representative experiments are presented either at 24 or 48 hpi. Bacterial counting was performed by grinding infected leaves in sterile water using glass beads in a TissueLyser (Qiagen/Retsch, Hilden, Germany). The resulting suspension was used for serial dilutions, fol-

lowed by plating on LB medium and counting of colonies. Bacterial counting was performed at 24 hpi.

Staining of callose deposits.

Leaves of 5-week-old *A. thaliana* plants were syringe-infiltrated with bacterial suspensions in water with an optical density at 600 nm of 0.1. Callose was stained with aniline blue, as previously described (Degraeve et al. 2008), for 30 min in 0.01% aniline blue in 150 mM K₂HPO₄ (pH 9.5) buffer. Leaf disks were examined by epifluorescence microscopy. Experiments were repeated twice with similar results. Representative pictures are shown. The number of callose deposits per picture was determined using ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.) and were compared using Fisher's exact test ($\alpha = 0.05$). We analyzed 25 to 30 pictures corresponding to more than five independent leaves for each treatment.

RNA extraction and RT-PCR analysis.

For RNA extraction, infiltrated leaves were cut off at the indicated times, were immediately frozen in liquid nitrogen, and were then stored at -80°C. Total RNA was prepared by phenol-chloroform extraction followed by LiCl precipitation, as described by Verwoerd and associates (1989). For RT-PCR analysis, first-strand cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) from 1 µg of DNase-treated (Invitrogen) total RNA in a 20-µl reaction volume. The cDNA were precipitated, were washed in 70% ethanol, and were resuspended in 50 µl of water. For semiquantitative RT-PCR, 1 µl of the cDNA was subjected to PCR in a 20-µl reaction volume. PCR runs were of 25 to 30 cycles, each cycle consisting of 94°C for 30 s, 52 to 55°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 2 min to complete polymerization. qRT-PCR reactions were achieved using Maxima SYBR Green/Rox qPCR MasterMix 2× (Fermentas, Vilnius, Lithuania), following the manufacturer's protocols. The expression of the *EF1α* gene was used as a constitutive reference. The gene-specific primers used in this analysis are indicated in Supplementary Table 2.

³⁵S-labeled methionine incorporation.

Arabidopsis leaf disks treated or not with 4 µg of CHX per milliliter were transferred 16 h post-treatment to 1 ml of liquid 1× Murashige Skoog (MS) solution containing 50 µCi of ³⁵S-labeled methionine (PerkinElmer, Waltham, MA, U.S.A.). The leaf disks were incubated in ³⁵S-labeled methionine for 2 h. Leaf disks were rinsed twice for 5 min in 1× MS. Total proteins were extracted in 1 mM Na-EDTA, 1 mM MgCl₂, 25 mM Tris-HCl (pH 7.6), 13.3 µM β-mercaptoethanol, and antiprotease cocktail at 1 µg/ml (Roche), were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the methionine incorporation rate was revealed by autoradiography.

Transcriptome studies.

Microarray analysis was performed with the Complete Arabidopsis Transcriptome MicroArray containing 24,576 gene-specific tags corresponding to 22,089 genes from *A. thaliana* (Crowe et al. 2003; Hilson et al. 2004). Two independent biological replicates were produced. Leaves of 5-week-old Col-0 plants were inoculated with bacterial suspensions of wild-type *E. amylovora* or sterile water. Leaves were collected 6 or 24 h after inoculation. Total RNA was extracted using the Qiagen RNeasy kit according to the supplier's instructions. RNA integrity, cDNA synthesis, hybridization, and array scanning were performed as described by Lurin and associates (2004). cDNA from leaves inoculated with *E. amylovora* were hybridized against cDNA of leaves inoculated with water collected at the same timepoint.

Statistical analysis of microarray data.

Statistical analysis was based on two dye swaps, as described by Gagnot and associates (2008). To determine differentially expressed genes, we performed a paired *t*-test on the log ratios. Spots displaying extreme variance (too small or too large) were excluded. The raw *P* values were adjusted by the Bonferroni method, which controls the family-wise error rate. We considered as being differentially expressed the genes with a Bonferroni *P* value ≤ 0.05 , as described by Gagnot and associates (2008).

Data deposition.

Microarray data from this article were deposited at Gene Expression Omnibus database (accession number GSE8701) and at CATdb (project RA05-02_Erwinia) according to the "Minimum Information About a Microarray Experiment" standards.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

European *A. thaliana* Stock Centre (NASC); arabidopsis.info
 The Gene Expression Omnibus database: www.ncbi.nlm.nih.gov/geo
 CATdb, a complete *Arabidopsis* transcriptome database:
urgv.evry.inra.fr/CATdb