

Signalling requirements for *Erwinia amylovora*-induced disease resistance, callose deposition and cell growth in the non-host *Arabidopsis thaliana*

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SUMMARY

Erwinia amylovora is the causal agent of the fire blight disease in some plants of the Rosaceae family. The non-host plant *Arabidopsis* serves as a powerful system for the dissection of mechanisms of resistance to *E. amylovora*. Although not yet known to mount gene-for-gene resistance to *E. amylovora*, we found that *Arabidopsis* activated strong defence signalling mediated by salicylic acid (SA), with kinetics and amplitude similar to that induced by the recognition of the bacterial effector *avrRpm1* by the resistance protein RPM1. Genetic analysis further revealed that SA signalling, but not signalling mediated by ethylene (ET) and jasmonic acid (JA), is required for *E. amylovora* resistance. *Erwinia amylovora* induces massive callose deposition on infected leaves, which is independent of SA, ET and JA signalling and is necessary for *E. amylovora* resistance in *Arabidopsis*. We also observed tumour-like growths on *E. amylovora*-infected *Arabidopsis* leaves, which contain enlarged mesophyll cells with increased DNA content and are probably a result of endoreplication. The formation of such growths is largely independent of SA signalling and some *E. amylovora* effectors. Together, our data reveal signalling requirements for *E. amylovora*-induced disease resistance, callose deposition and cell fate change in the non-host plant *Arabidopsis*. Knowledge from this study could facilitate a better understanding of the mechanisms of host defence against *E. amylovora* and eventually improve host resistance to the pathogen.

Keywords: callose, cell growth, defence signalling, effectors, endoreplication, fire blight, programmed cell death.

INTRODUCTION

The Gram-negative bacterium *Erwinia amylovora* belongs to the enterobacterial family, which includes human and animal pathogens, such as *Yersinia* spp., *Shigella* spp. and *Salmonella* spp. *Erwinia amylovora* is the causative agent of the devastating fire blight disease challenging certain rosaceous plants, including apples, pears and some ornamental plants (Eastgate, 2000; Vanneste and Eden-Green, 2001). Outbreaks of fire blight cause tremendous economic losses worldwide. The disease is usually managed through an integrated approach, consisting of horticultural practice, antibiotics and chemical applications, and cultivar breeding (Gusberti *et al.*, 2015). Genetic engineering provides a potentially powerful approach to enhance plant resistance to *E. amylovora*, the success of which relies on a thorough understanding of the mechanisms governing plant resistance to *E. amylovora*. Through whole-genome sequencing, transcriptomics and molecular studies, many genes with potential roles in the regulation of *E. amylovora* resistance have been identified in plants (Kamber *et al.*, 2016; Malnoy *et al.*, 2012). An understanding of the molecular mechanisms by which these genes act to enhance resistance against *E. amylovora* is important for the development of strategies to reduce and/or prevent fire blight and to mitigate losses in agriculture.

Erwinia amylovora employs various virulence factors to suppress plant innate immunity and promote bacterial virulence (Oh and Beer, 2005; Pique *et al.*, 2015; Vrancken *et al.*, 2013). One of the major virulence factors of *E. amylovora* is the type III secretion system (TTSS) that delivers effector proteins to plant cells to cause disease (Grant *et al.*, 2006; Hueck, 1998). *Erwinia amylovora* and *Pseudomonas syringae* share similar TTSS genes that are clustered in a large pathogenicity island on the bacterial chromosome, a region also called the hypersensitive response and pathogenicity (*hrp*) and disease-specific (*dsp*) gene cluster (Alfano and Collmer, 2004; Choi *et al.*, 2013). Among the effector proteins delivered by the TTSS of *E. amylovora*, DspA/E is a functional homologue of AvrE of *P. syringae* and plays a critical role in the promotion of *E. amylovora* virulence and cell death in both host and non-host plants (Bogdanove *et al.*, 1998a; Boureau *et al.*, 2006; Gaudriault *et al.*, 1997; Oh *et al.*, 2007). Either as a secreted effector of *E.*

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amylovora or as a cell-free elicitor, HrpN has been shown to induce a hypersensitive response (HR), callose deposition, broad-spectrum disease resistance and/or growth of treated plants (Boureau *et al.*, 2011; Dong *et al.*, 1999; Reboutier *et al.*, 2007; Wei *et al.*, 1992). *Erwinia amylovora* also produces and secretes two polysaccharides, amylovan and levan, which are important for biofilm formation of the bacterium and presumably also play a role in the survival of the bacterium in host tissue. These compounds could block host vascular tissue, thereby causing the wilting of vegetative shoots and promoting bacterial virulence (Eastgate, 2000; Pique *et al.*, 2015; Vrancken *et al.*, 2013).

The molecular mechanisms by which plants resist *E. amylovora* are not well understood. Such resistance is generally considered to be quantitative, possibly involving multiple genetic loci in host genomes (Khan *et al.*, 2013; Le Roux *et al.*, 2010; Malnoy *et al.*, 2012). No specific gene-for-gene interaction involving the recognition of *E. amylovora* effectors by specific plant resistance genes has been reported so far. In addition to host plants (e.g. apple and pear), non-host plants (e.g. Arabidopsis and tobacco) have been used to elucidate the plant mechanisms of resistance to *E. amylovora*. Although host plants show varying susceptibility to *E. amylovora*, non-host plants generally show durable resistance to the bacterium. Despite the differences in supporting the growth of the bacterium, both types of plant respond to *E. amylovora* infection by the activation of certain common defence pathways, including cell ion leakage, cell death, callose deposition, defence gene expression and defence signalling pathways (Bogdanove *et al.*, 1998a; Bourreau *et al.*, 2006, 2011; DebRoy *et al.*, 2004; Dong *et al.*, 1999; Gaudriault *et al.*, 1997; Launay *et al.*, 2016; Oh *et al.*, 2007; Reboutier *et al.*, 2007; Venisse *et al.*, 2001; Wei *et al.*, 1992). A study with Arabidopsis has also indicated that *de novo* protein synthesis is required for non-host resistance to *E. amylovora* (Moreau *et al.*, 2012). Thus, non-host plants provide a powerful system to elucidate the plant mechanisms of resistance to *E. amylovora*.

Although *E. amylovora* induces multifaceted defence responses in plants, whether some of these responses are required for plant resistance to the bacterium is not well understood. For instance, although exogenous jasmonic acid (JA) treatment protects apple plants against *E. amylovora*, some JA-related genes are induced and others are suppressed by *E. amylovora* infection (Degrave *et al.*, 2008; Duge De Bernonville *et al.*, 2012). Thus, it is unclear whether JA is required for *E. amylovora* resistance. Salicylic acid (SA) is another defence molecule linked to *E. amylovora* resistance. The treatment of apple and pear with SA analogues protects the plants from fire blight disease (Brisset *et al.*, 2000; Sparla *et al.*, 2004). The expression of many SA regulatory genes is induced by *E. amylovora* infection in apple and Arabidopsis (Bonasera *et al.*, 2006; Degrave *et al.*, 2008; Moreau *et al.*, 2012; Venisse *et al.*, 2002). Overexpression of the apple homolog of Arabidopsis *NONEXRESSOR OF PR GENES 1 (NPR1)*; a key SA

signalling gene), *MpNPR1*, confers increased disease resistance to *E. amylovora* in apple (Malnoy *et al.*, 2007). In addition, the Arabidopsis *NONEXRESSOR OF PR GENES 1 (NPR1)* gene is required for HrpN of *E. amylovora*-induced disease resistance in Arabidopsis (Dong *et al.*, 1999). Although these studies support the importance of SA in *E. amylovora* resistance, the lack of loss-of-function analysis of key SA genes, e.g. using mutants or gene silencing, makes it difficult to assess whether SA signalling is required for *E. amylovora* resistance in host plants. Indeed, some studies from the non-host Arabidopsis challenge this role of SA. Loss of function in the SA regulatory gene, *ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)*, compromises *E. amylovora* resistance in Arabidopsis (Aarts *et al.*, 1998; Degrave *et al.*, 2008; Moreau *et al.*, 2012). However, a mutant impaired in *ISOCHORISMATE SYNTHASE 1 (ICS1)* (also called *SA INDUCTION-DEFICIENT 2* and *EDS16*), encoding the major SA biosynthetic enzyme (Nawrath and Metraux, 1999; Ng *et al.*, 2011; Wildermuth *et al.*, 2001), did not alter *E. amylovora*-induced cell death, compared with the wild-type (Degrave *et al.*, 2008). Therefore, whether or not SA is required for *E. amylovora* resistance is still in question.

In this report, we examined the defence signalling pathways required for resistance to *E. amylovora*. We confirmed that *E. amylovora* infection activates SA production in both the host plant apple and the non-host plant Arabidopsis. Although not yet known to mount gene-for-gene resistance to *E. amylovora* in plants (Malnoy *et al.*, 2012), Arabidopsis in the presence of *E. amylovora* activates SA signalling with a similar amplitude and kinetics to that induced by recognition of the *P. syringae* effector *avrRpm1* by the Arabidopsis resistance protein *RPM1*. Genetic analysis revealed that *E. amylovora* resistance in Arabidopsis is dependent on SA, but independent of JA and ethylene (ET) pathways. We further showed that SA, ET and JA pathways are not required for callose synthesis induced by *E. amylovora*. In addition, we observed that *E. amylovora* induced tumour-like growths in Arabidopsis, which contain enlarged cells with increased nuclear DNA content. The formation of such abnormal growths is largely independent of SA and *E. amylovora* effectors. Together, this study reveals signalling requirements for *E. amylovora*-induced disease resistance, callose deposition and cell fate change in the non-host plant Arabidopsis. Information obtained from this study could be used to better understand the defence mechanisms of host plants in response to *E. amylovora*, and subsequently aid in the development of strategies to improve plant resistance to the destructive fire blight disease.

RESULTS

Arabidopsis activates strong SA-mediated defence in response to *E. amylovora*

The model plant Arabidopsis shows non-host resistance to *E. amylovora* and has been used as a powerful system to study the plant

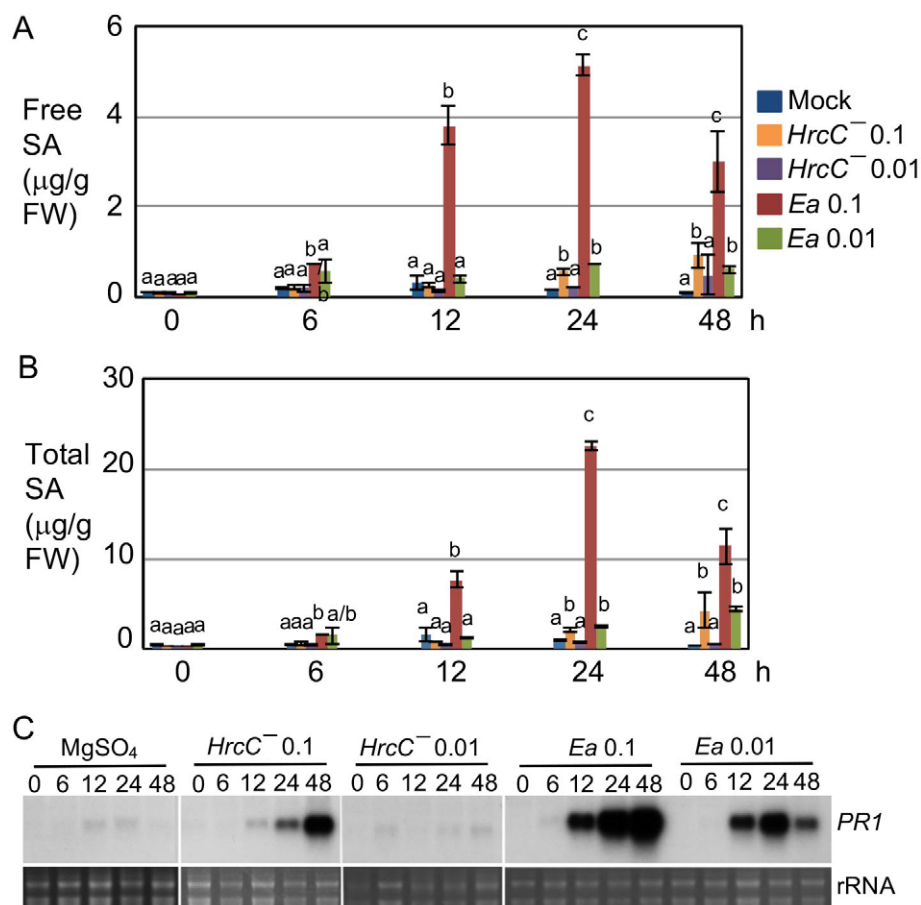


Fig. 1 *Erwinia amylovora* induces dynamic changes in salicylic acid (SA) accumulation and *PR1* expression in Arabidopsis. The fourth to sixth leaves of Col-0 plants were infiltrated with *E. amylovora* (*Ea*) at an optical density at 600 nm (OD_{600}) of 0.1 or 0.01, *Pseudomonas syringae* pv. *maculicola* strain *HrcC*⁻ at OD_{600} = 0.1 or 0.01 or 10 mM MgSO₄. The infected leaves were collected at 0, 6, 12, 24 and 48 h post-inoculation (hpi) for SA and RNA analyses. (A) Free SA measurement. (B) Total SA measurement. (C) Northern blotting analysis of *PR1* expression. Images of *rRNA* were used as loading controls. Statistical analysis was performed with one-way analysis of variance (ANOVA) Fisher's partial least-squares difference (PLSD) tests (StatView 5.0.1). Different letters in (A) and (B) indicate significant difference among the samples at each time point ($P < 0.05$). These experiments were repeated twice with similar results. FW, fresh weight.

mechanisms of *E. amylovora* resistance. In order to further elucidate how Arabidopsis activates defence against *E. amylovora*, we compared the kinetics of SA accumulation and SA signalling activation (using the expression of the SA marker gene *PR1*) induced by *E. amylovora* with those induced by some *P. syringae* strains. We used two doses {optical density at 600 nm (OD_{600}) = 0.1 [10^8 colony-forming units (cfu)/mL] and 0.01 (10^7 cfu/mL)} of *E. amylovora* and collected the inoculated leaves at 0, 6, 12, 24 and 48 h post-inoculation (hpi). In parallel, we also included three *P. syringae* pv. *maculicola* (*Pma*) strains. *Pma HrcC*⁻ lacks the T3SS to deliver effectors to plant cells, and thus only activates basal defence in the host (Hauck *et al.*, 2003; Tsuda *et al.*, 2008a; Wang *et al.*, 2009). *Pma DG3* is a virulent strain that induces effector-triggered susceptibility and *Pma avrRpm1* (also called *Pma DG34*) expresses the avirulent effector *avrRpm1*, recognized by the R protein RPM1, and can induce effector-triggered immunity (Alfano *et al.*, 2000; Guttman *et al.*, 2002). The data for the *P. syringae* strains have been reported previously (Hamdoun *et al.*, 2013). Here, we only included the data for *Pma HrcC*⁻ in Fig. 1 for a comparison. We found that SA accumulation induced by *E. amylovora* (0.1) was observed as early as 6 hpi and was much higher than that induced by *Pma HrcC*⁻ (0.1) (Fig. 1A,B). Consistent with SA

accumulation, *PR1* transcripts were also much higher in plants infected with *E. amylovora* (0.1) than those infected with *Pma HrcC*⁻ (0.1) (Fig. 1C). The degree of SA and *PR1* induction by *E. amylovora* was dosage dependent, as much lower accumulation of SA and *PR1* transcripts was observed with the infection at the lower dose of *E. amylovora* (0.01) (Fig. 1). Interestingly, the kinetics and amplitude of SA and *PR1* transcripts induced by *E. amylovora* (0.1) were actually comparable with those induced by the avirulent strain *Pma avrRpm1*, and faster and higher than those induced by the virulent strain *Pma DG3* (Fig. 1 and Hamdoun *et al.*, 2013). *Erwinia amylovora*-induced SA accumulation occurs largely through the major SA biosynthetic pathway catalysed by ICS1, because a loss-of-function mutation in *ICS1*, *ics1-1*, abolished SA accumulation on *E. amylovora* infection (Fig. S1, see Supporting Information).

Compared with the rapid and strong activation of the SA pathway in Arabidopsis on *E. amylovora* infection (Fig. 1), the host plant apple (*Malus × domestica* cv. 'Royal Gala') only produced modest levels of SA when challenged with *E. amylovora* (10^8 cfu/mL) at 48 hpi (Fig. S2, see Supporting Information). Such a modest activation of the SA pathway has also been reported previously (Milcevicová *et al.*, 2010). The difference in SA pathway activation

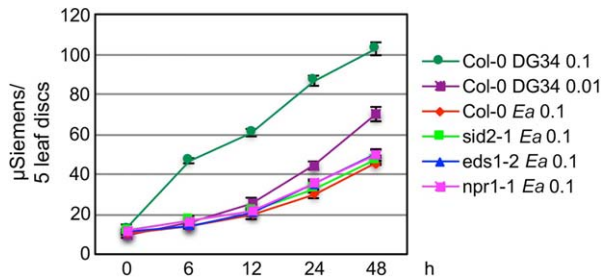


Fig. 2 *Erwinia amylovora*-induced ion leakage in Arabidopsis is independent of salicylic acid (SA) and less than that induced by RPM1-avrRpm1 recognition. Arabidopsis leaves were infiltrated with *E. amylovora* (*Ea*) or *Pseudomonas syringae* pv. *maculicola* (*Pma* DG34) avrRpm1 and collected after infection for ion leakage measurement. The data represent the average of triplicate samples \pm standard deviation. These experiments were repeated twice with similar results.

between the host plant apple and the non-host plant Arabidopsis underscores the importance of SA signalling in non-host resistance against *E. amylovora*.

Arabidopsis responds to *Pma* avrRpm1 via typical gene-for-gene interaction, using the plant R protein RPM1 to recognize the bacterial avirulence protein AvrRpm1. This recognition could result in an HR within hours of infection. Although *E. amylovora*-induced SA signalling activation showed similar kinetics and amplitude to that induced by *Pma* avrRpm1 (Fig. 1 and Hamdoun *et al.*, 2013), *E. amylovora* is not known to activate a rapid HR in Arabidopsis as shown with *Pma* avrRpm1 infection. Only limited leaf necrosis was observed in *E. amylovora*-infected leaves several days post-infection (Degrave *et al.*, 2008 and our observation). The HR is usually correlated with massive ion leakage in the dying cells. Consistent with the lack of acute HR at the early infection stage, we found that *E. amylovora* induced much less ion leakage in Col-0, compared with *Pma* avrRpm1 (Fig. 2). Thus, unlike *Pma* avrRpm1, *E. amylovora*-induced SA signal activation is uncoupled from massive cell death.

Non-host resistance to *E. amylovora* in Arabidopsis is SA dependent, but independent of ET- and JA-mediated signalling

Although SA and some SA pathway-related genes are known to be induced by *E. amylovora* in Arabidopsis and apple, whether or not SA is required for *E. amylovora* resistance has not been firmly established. The Arabidopsis mutant defective in the SA regulatory gene *EDS1*, but not in the major SA synthesis gene *ICS1*, showed greater cell death than wild-type plants on *E. amylovora* infection (Degrave *et al.*, 2008; Moreau *et al.*, 2012). These observations suggest that *EDS1*, but not SA in general, is required for *E. amylovora* resistance in Arabidopsis.

To further clarify the role of SA in defence against *E. amylovora* in Arabidopsis, we infiltrated additional SA mutants (*eds5-3*,

pad4-1, *npr1-1* and *nahG*, in addition to *ics1-1* and *eds1-2*) with *E. amylovora*. *EDS5* encodes a chloroplast protein proposed to transport SA from the chloroplast to the cytoplasm in a cell (Nawrath *et al.*, 2002; Serrano *et al.*, 2013; Yamasaki *et al.*, 2013). PHYTOALEXIN DEFICIENT 4 (*PAD4*) interacts with *EDS1* and probably acts in the same pathway as *EDS1* for defence regulation based on biochemical, genetic and microarray studies (Bartsch *et al.*, 2006; Feys *et al.*, 2001; Jirage *et al.*, 1999; Ng *et al.*, 2011; Sato *et al.*, 2007). Both *eds5-3* and *pad4-1* mutants accumulate much reduced SA levels under defence conditions (Feys *et al.*, 2001; Nawrath *et al.*, 2002; Ng *et al.*, 2011). *NPR1* is a master regulator of SA signalling (Seyfferth and Tsuda, 2014; Yan and Dong, 2014). The *nahG* plant overexpresses the bacterial SA hydroxylase which metabolizes SA to catechol, leading to a greater susceptibility of plants to a range of pathogens (Delaney *et al.*, 1994; Friedrich *et al.*, 1995; Gaffney *et al.*, 1993; Kachroo *et al.*, 2000; Lu *et al.*, 2001; Mur *et al.*, 1997; van Wees and Glazebrook, 2003). To allow better growth of *E. amylovora* in Arabidopsis plants, we covered the infiltrated plants with a dome to maintain high humidity. In addition, we used an *E. amylovora* strain with streptomycin resistance for a more accurate quantification of *E. amylovora* replication on Arabidopsis leaves based on antibiotic selection. Under these conditions, we found that all SA mutants tested were able to support greater *E. amylovora* growth than Col-0, and the *nahG* plant showed the highest bacterial population (Fig. 3). These data establish that SA is required for non-host resistance to *E. amylovora* in Arabidopsis.

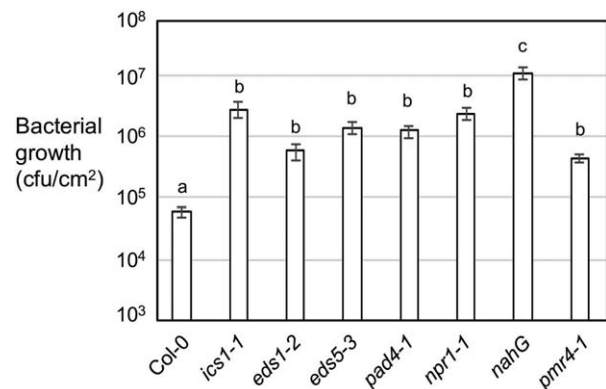


Fig. 3 Salicylic acid (SA)-defective plants and the *pmr4-1* mutant disrupted in callose synthesis are more susceptible than Col-0 to *Erwinia amylovora*. The fourth to sixth leaves of 30-day-old plants were infiltrated with *E. amylovora* at an optical density at 600 nm (OD_{600}) of 0.1. Infected leaf discs (38 mm^2) were taken for the measurement of bacterial growth at 3 days post-inoculation (dpi). The initial inoculum (0 dpi) was about 8×10^3 colony-forming units (cfu)/leaf disc for these plants. Each data point represents the average of 20–24 samples from four independent experiments. The error bars represent the standard error of the mean. Statistical analysis was performed with Student's *t*-test (StatView 5.0.1). Different letters indicate significant difference among the samples ($P < 0.05$).

To further test how the SA pathway is related to cell death formation during the Arabidopsis–*E. amylovora* interaction, we measured ion leakage in several SA mutants. We found that the SA mutants showed a similar level of ion leakage to Col-0 on *E. amylovora* infection (Fig. 2). These data further support that SA-mediated defence is uncoupled from *E. amylovora*-induced cell death.

To investigate whether additional defence signalling pathways are involved in *E. amylovora* resistance, we infected mutants impaired in ET or JA signalling. *ein2-1* is an ET-insensitive mutant (Alonso *et al.*, 1999), whereas *jar1-1* and *jin1-7* are JA-insensitive mutants (Lorenzo *et al.*, 2004; Staswick *et al.*, 1992). We found that these ET and JA mutants did not differ from Col-0 in their ability to support *E. amylovora* growth (Fig. S3, see Supporting Information). Therefore, we conclude that ET and JA pathways are dispensable for resistance to *E. amylovora* in Arabidopsis.

***Erwinia amylovora*-induced callose deposition is independent of SA, ET and JA pathways**

Erwinia amylovora is known to induce massive callose deposition in apple and Arabidopsis plants (Boureau *et al.*, 2011; DebRoy *et al.*, 2004; Degraeve *et al.*, 2008). To better understand the signalling pathways required for *E. amylovora*-induced cell wall defence in plants, we examined callose deposition on *E. amylovora* infection in Arabidopsis mutants disrupted in SA, ET and/or JA-mediated defence. We found that, on *E. amylovora* infection, single mutants disrupted in SA, ET or JA pathways showed similar levels of callose deposition to Col-0 (Figs S4 and S5, see Supporting Information). Further, disruption of two or more pathways mediated by SA, ET and/or JA did not affect *E. amylovora*-induced callose deposition (Fig. 4). Thus, we conclude that *E. amylovora*-induced callose deposition is independent of SA, ET and JA pathways. However, callose deposition was completely abolished in the powdery mildew resistant 4–1 (*pmr4-1*) mutant, which shows impaired callose synthesis (Fig. 4 and Moreau *et al.*, 2012; Nishimura *et al.*, 2003). Interestingly, we found that *pmr4-1* supported greater *E. amylovora* growth (Fig. 3). Because the SA mutants were compromised in *E. amylovora* resistance, but not in callose deposition, and the callose-deficient mutant *pmr4-1* was compromised in *E. amylovora* resistance, we conclude that callose deposition is necessary, but not sufficient, for *E. amylovora* resistance.

***Erwinia amylovora* infection induces cell growth in Arabidopsis**

Bacteria, fungi and nematodes are known to induce abnormal cell growth in addition to promoting cell death in plants (de Almeida Engler *et al.*, 1999; Chalupowicz *et al.*, 2006; Chandran *et al.*, 2009; Hamdoun *et al.*, 2013; Hansen and Meins, 1986; Marois *et al.*, 2002). *Erwinia amylovora* is known to induce cell death in

both host and non-host plants, but whether it affects cell growth has not been reported. We observed tumour-like growths on *E. amylovora*-infiltrated Arabidopsis leaves at 4 days post-inoculation (dpi), which appeared to be transparent protrusions that varied in size (Fig. S6, see Supporting Information). For a closer observation of the abnormal growths, we fixed the growth regions and embedded them in LR white resin. With thin sections (1 μ m), we found that the growth regions contained mostly enlarged mesophyll cells and the number of enlarged cells varied in different tumours (Fig. 5A). Quantification of abnormal growths by counting the clear protrusions on *E. amylovora*-infiltrated leaves revealed that abnormal growths were induced in an *E. amylovora* dosage-dependent manner (Fig. 5B).

Enlarged cells are usually associated with increased nuclear content, which probably results from endoreplication, a process involving multiple rounds of DNA synthesis without cell mitosis (Bramsiepe *et al.*, 2010; De Veylder *et al.*, 2011). To quantify the nuclear content of the enlarged cells, we fixed the abnormal growth regions and embedded them in paraffin. Leaf cross-sections, 15 μ m thick, were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Consistent with their increased cell size, the enlarged cells had much larger nuclei (Fig. 6A–F). The stained nuclei were measured for relative fluorescence intensity using ImageJ (Version 1.45s). We arbitrarily set the average fluorescence value measured for guard cells as 2C. The relative nuclear DNA content of other cell types was calculated on the basis of that of the guard cells. We found that normal mesophyll cells had an average of about 20C nuclear DNA content. The enlarged cells, however, had an average nuclear DNA content of 60C, suggesting an activation of endoreplication in these cells (Fig. 6G).

To elucidate the factors from Arabidopsis which affect *E. amylovora*-induced cell growth, we examined several SA mutants. We found that *ics1-1*, *pad4-1* and *npr1-1* mutants had a similar number of tumour-like growths to Col-0, whereas *nahG* plants showed far fewer growths than Col-0 (Fig. 7). It is worth noting that *nahG* plants were hypersusceptible to *E. amylovora* (Fig. 3) and most leaves had wilted by the time tumour growths were counted, preventing the assessment of cell growth (data not shown). Like the SA mutants, *pmr4-1* had a similar number of tumour-like growths to Col-0 in the presence of *E. amylovora*. These results suggest that *E. amylovora*-induced cell growth in Arabidopsis is largely independent of the SA pathway and callose formation.

We further tested whether *E. amylovora* effectors are required for cell growth control in Arabidopsis. Both *dspE/A* and *hrpN* are *E. amylovora* effectors delivered to the host via the TTSS, and these proteins play crucial roles in *E. amylovora* virulence (Bogdanove *et al.*, 1998a; Boureau *et al.*, 2006, 2011; Dong *et al.*, 1999; Gaudriault *et al.*, 1997; Oh *et al.*, 2007; Wei *et al.*, 1992).

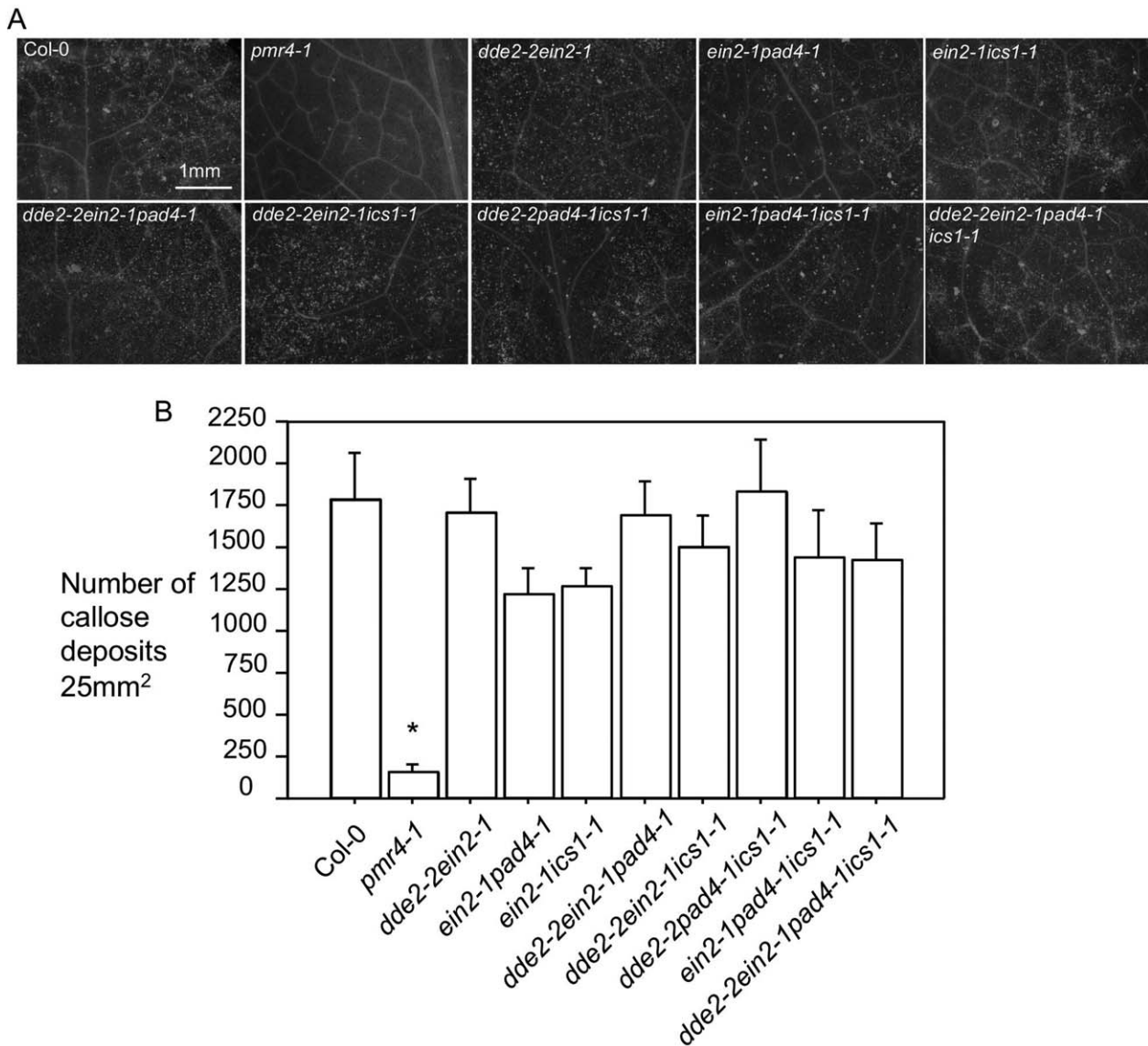


Fig. 4 *Erwinia amylovora*-induced callose deposition in Arabidopsis is independent of salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) signalling. *Erwinia amylovora* [optical density at 600 nm (OD_{600}) = 0.01]-infiltrated leaves of each genotype were harvested for fixation and staining with aniline blue. (A) Images of callose deposits. Callose deposits appeared as fluorescent dots and were photographed with an AxioCam MRc5 camera connected to a fluorescence stereoscope microscope. (B) Quantification of callose deposits. The number of callose deposits of each genotype was quantified using ImageJ (Version 1.45s). Each data point was an average of at least six images from four different leaves \pm standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA) Fisher's partial least-squares difference (PLSD) tests (StatView 5.0.1). The asterisk indicates significant difference of *pmr4-1* from other genotypes ($P < 0.05$). These experiments were repeated twice with similar results.

Erwinia amylovora strains lacking these effectors or the functional TTSS compromised the virulence of the bacterium (Fig. 8A and Boureau *et al.*, 2006; Wei *et al.*, 1992). We found that these three strains did not affect the number of abnormal growths in Arabidopsis, compared with the wild-type *E. amylovora* strain (Fig. 8B). These data indicate that *E. amylovora* effectors are not necessary for cell growth in Arabidopsis.

DISCUSSION

Although not a natural host to *E. amylovora*, Arabidopsis activates strong defence against the bacterium and thus has been used as a powerful system for the dissection of the mechanisms of resistance to *E. amylovora*. Here, we report a study to elucidate the signalling requirements for some *E. amylovora*-induced phenotypes in order to gain a better understanding of the non-host resistance

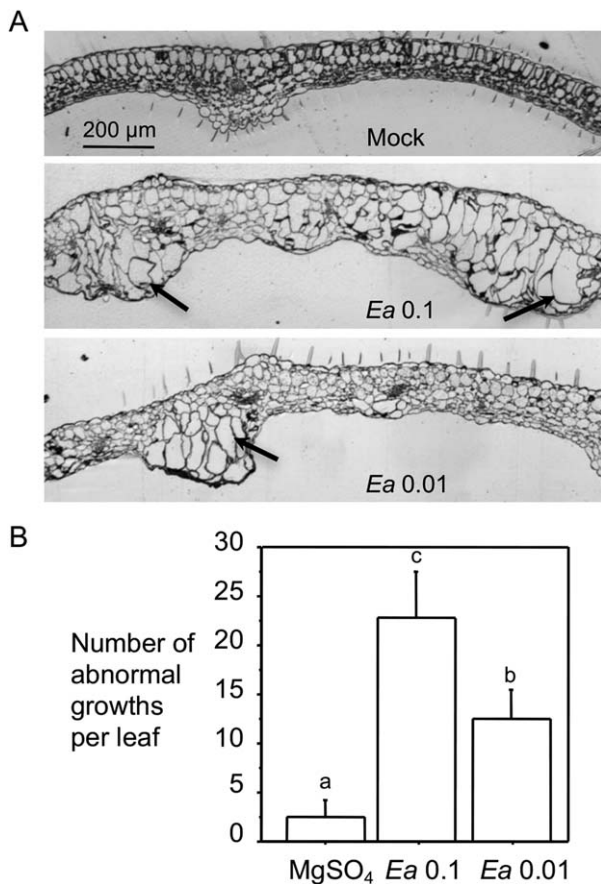


Fig. 5 *Erwinia amylovora* induces abnormal cell growths in Arabidopsis. *Erwinia amylovora*-infiltrated (Ea) and 10 mM MgSO₄-treated (Mock) leaves were observed for leaf morphology. (A) Leaf cross-sections. The treated leaves were fixed, embedded in LR white resin and cut with an ultramicrotome into 1-µm sections. Leaf cross-sections were stained with 0.1% toluidine blue O and photographed using an AxioCam MRc5 camera (Zeiss, Inc., Göttingen, Germany) connected to a dissecting microscope. Arrows indicate enlarged cells in abnormal growth regions in leaves. The size bar represents 200 µm and applies to all images. (B) Quantification of the tumour-like growths. The abnormal growths appeared to be transparent protrusions on the infected leaves and were counted at 5 days post-inoculation (dpi) with the assistance of a dissecting microscope. At least 25 leaves were used for counting. Error bars represent standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA) Fisher's partial least-squares difference (PLSD) tests (StatView 5.0.1). Different letters indicate significant difference amongst the samples ($P < 0.05$).

displayed by Arabidopsis to the bacterium. Our data showed that *E. amylovora* infection activates strong SA production and signalling in Arabidopsis with kinetics and amplitude similar to that induced by RPM1-avrRpm1 recognition during Arabidopsis-*P. syringae* interaction. An intact SA pathway, but not JA and ET pathways, is required for *E. amylovora* resistance in Arabidopsis. Callose deposition activated by *E. amylovora* can be uncoupled from defence signalling mediated by SA, JA and ET. *Erwinia*

amylovora also induces cell growth in Arabidopsis, largely independent of plant SA signalling or effectors from the pathogen. Overall, this study has revealed the molecular components involved in the interaction between the non-host plant Arabidopsis and *E. amylovora*. The knowledge obtained from this study could be employed to better understand the resistance mechanisms used by host plants, such as apple and pear, to defend against *E. amylovora*.

Non-host resistance is the most common resistance against a broad range of pathogens in plants and can be grouped into two types (Gill *et al.*, 2015; Senthil-Kumar and Mysore, 2013; Thordal-Christensen, 2003). Type I non-host resistance induces no symptoms because the pathogens cannot overcome pre-existing obstacles in plants, such as physical and chemical barriers. Type II non-host resistance is associated with the development of cell death at the infection site. *Erwinia amylovora* activates type II non-host resistance in Arabidopsis. However, we did not observe the typical HR symptoms at early infection stages as shown with *Pma avrRpm1*. Limited necrotic cell death was only observed at later stages of *E. amylovora* infection (Degraeve *et al.*, 2008 and data not shown). Consistent with these observations, *E. amylovora* elicited much less ion leakage than *Pma avrRpm1* in Arabidopsis (Fig. 2). Nevertheless, *E. amylovora* induces rapid and strong accumulation of SA and *PR1* transcripts in Arabidopsis, the kinetics and amplitude of which are similar to those induced by *RPM1-avrRpm1* recognition (Fig. 1 and Hamdoun *et al.*, 2013). Thus, non-host resistance to *E. amylovora* observed with Arabidopsis shares similarities to, but is also different from, the resistance triggered by certain gene-for-gene interactions. The similarities could be explained by the activation of certain common downstream defence signalling pathways in plants. Indeed, both *E. amylovora* and *P. syringae* share the conserved TTSS and some effector genes (Alfano and Collmer, 2004; Choi *et al.*, 2013; Oh and Beer, 2005), which could induce these downstream defence pathways in plants. However, Arabidopsis-*P. syringae* interaction involves classical gene-for-gene resistance, whereas it is unknown whether plants can use their specific resistance genes to recognize any cognate effectors of *E. amylovora* (Khan *et al.*, 2013; Le Roux *et al.*, 2010; Malnoy *et al.*, 2012). In general, resistance to *E. amylovora* is considered as a quantitative trait which may involve multiple gene loci. Such differences in pathogen recognition by plants could explain the lack of acute HR in *E. amylovora*-infected plants. However, we cannot rule out the possibility that Arabidopsis has evolved R proteins to recognize specifically certain *E. amylovora* effectors without invoking any HR.

Amongst the known defence signalling pathways, SA, JA and ET pathways have been linked to plant responses to *E. amylovora*. The data reported here demonstrate that SA signalling is required for *E. amylovora* resistance in Arabidopsis, whereas JA and ET pathways are dispensable. This role of SA in *E. amylovora*

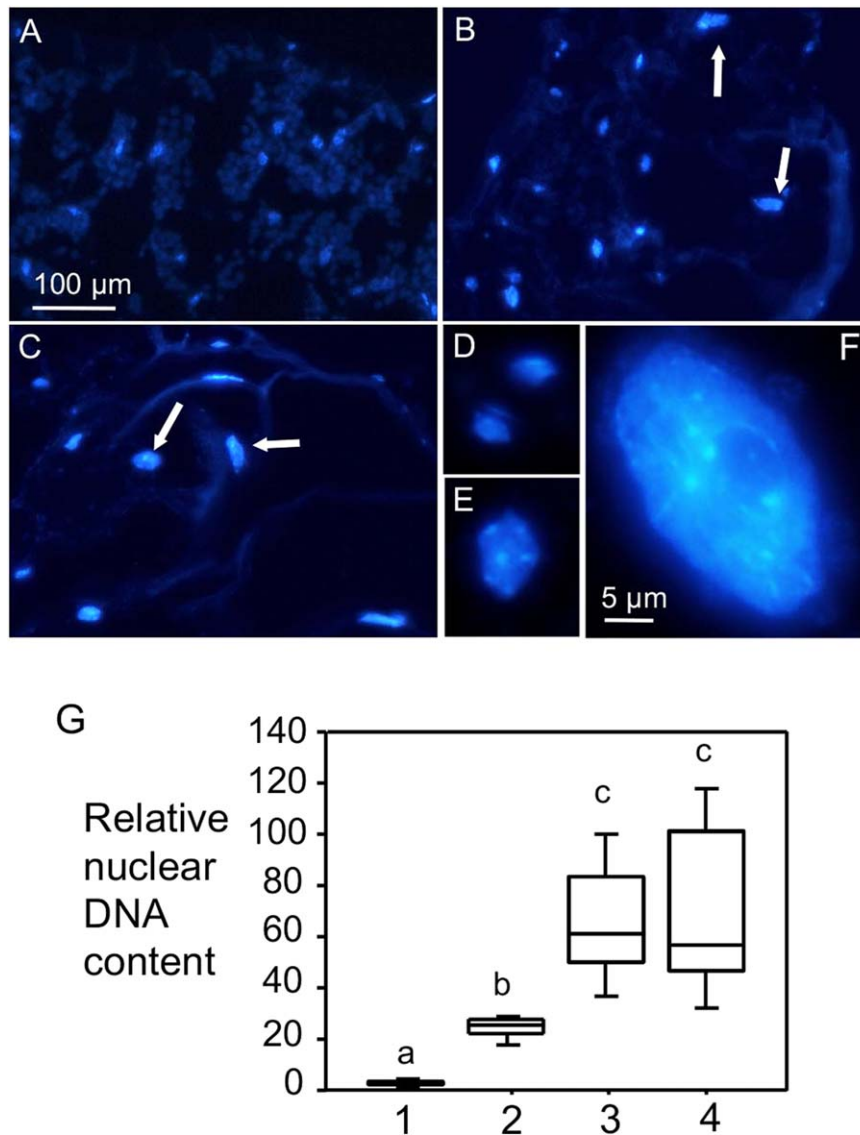


Fig. 6 *Erwinia amylovora* infection induces the formation of enlarged mesophyll cells with increased nuclear content. *Erwinia amylovora*-infiltrated or mock-treated leaves were harvested at 5 days post-inoculation (dpi), fixed and embedded in paraplast, and sectioned for 15-μm slices with a microtome. Leaf cross-sections were stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Nuclei images (A–F) were captured using a Nikon DS cooled camera attached to a compound fluorescence microscope. The images are from the following samples: (A) 10 mM MgSO₄; (B) *E. amylovora* [optical density at 600 nm (OD₆₀₀) = 0.1]; (C) *E. amylovora* (OD₆₀₀ = 0.01); (D) typical nuclei of guard cells from (A); (E) a typical nucleus of a normal mesophyll cell from (A); (F) a typical nucleus of an enlarged mesophyll cell induced by *E. amylovora*. Arrows indicate large nuclei (B, C). The size bar in (A) represents 100 μm and applies to (A–C), whereas the size bar in (F) represents 5 μm and applies to (D–F). (G) Quantification of relative nuclear DNA content. The numbers represent the relative nuclear DNA contents of the following types of cell: 1, guard cell nuclei from mock-treated leaves; the value was set to 2C for the calculation of the relative nuclear contents of the other cell types; 2, normal mesophyll cells from mock-treated leaves; 3, enlarged mesophyll cells induced by *E. amylovora* (OD₆₀₀ = 0.1); 4, enlarged mesophyll cells induced by *E. amylovora* (OD₆₀₀ = 0.01). At least 60 nuclei were used for each data point. Error bars represent standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA) Fisher's partial least-squares difference (PLSD) tests (StatView 5.0.1). Different letters indicate significant difference amongst the samples ($P < 0.05$). The nuclear contents of guard cells and normal mesophyll cells from *E. amylovora*-infected leaves were comparable with those from the corresponding cells in mock-treated leaves (data not shown).

resistance is consistent with the known function of SA in regulating both host and non-host resistances in several other plant pathosystems (Kachroo *et al.*, 2000; Lu *et al.*, 2001; Tsuda *et al.*, 2008b; van Wees and Glazebrook, 2003), and could be applied to

E. amylovora host plants. Indeed, SA has been shown to be important for *E. amylovora* in apple. SA accumulation and the expression of several SA regulatory genes were induced by *E. amylovora* infection in apple (Fig. 1; Bonasera *et al.*, 2006; Moreau *et al.*,

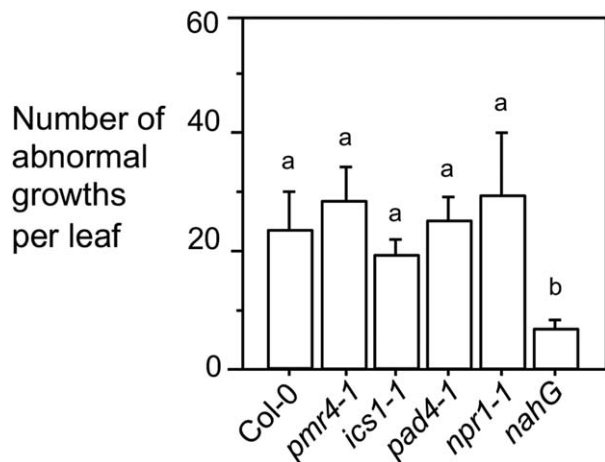


Fig. 7 *Erwinia amylovora*-induced tumour growths are largely independent of salicylic acid (SA). Plants were infiltrated with *E. amylovora* [optical density at 600 nm (OD_{600}) = 0.1] or 10 mM $MgSO_4$ as a control and observed for leaf morphology. The number of abnormal growths was counted at 5 days post-inoculation (dpi) on the infected leaves with the assistance of a dissecting microscope. At least 25 leaves per genotype were used for counting. Error bars represent standard deviation. Different letters indicate significant difference amongst the samples ($P < 0.05$). These experiments were repeated twice with similar results.

2012; Venisse *et al.*, 2002). In addition, manipulation of the SA pathway in apple plants, either by exogenous SA analogue application or overexpression of the *NPR1* gene, conferred increased disease resistance to *E. amylovora* (Brisset *et al.*, 2000; Malnoy *et al.*, 2007; Sparla *et al.*, 2004). Interestingly, we only observed a modest increase in SA in apple plants susceptible to *E. amylovora*. On the contrary, the non-host plant *Arabidopsis* activates quick and strong SA signalling in the presence of *E. amylovora*. It is possible that such a difference in SA signal activation can account for the different resistance to *E. amylovora* displayed by apple and *Arabidopsis*. Thus, increasing the SA pathway could be a powerful approach to enhance the resistance to *E. amylovora* in host plants such as apple and pear.

One striking feature of the plant response to *E. amylovora* infection is the formation of massive callose deposits in the infected area. The major callose synthase gene *PMR4* is required for callose deposition in *Arabidopsis* (Moreau *et al.*, 2012). However, it is unclear whether callose deposition is related to *E. amylovora* resistance and how the main defence signalling pathways affect callose deposition. In particular, the role of SA in *E. amylovora*-induced callose deposition in *Arabidopsis* has not been completely defined. On the one hand, a study by Degraeve *et al.* (2008) showed SA-independent callose deposition induced by *E. amylovora*. On the other, another study by DebRoy *et al.* (2004) showed that the *avrE* effector expressed by *P. syringae* could suppress callose deposition in an SA-dependent manner. They suggested that the *E. amylovora* effector *dspA/E*, a homologue of *P. syringae* *avrE*

that functionally cross-complements with *dspA/E*, could similarly target SA-dependent cell wall defence. Here, using single or combinations of two or more mutants disrupting SA, JA and/or ET pathways, our data conclusively showed that callose deposition is independent of these three main defence signalling pathways in *Arabidopsis*. As the SA mutants were more susceptible to *E. amylovora*, yet accumulated wild-type levels of callose, and the callose-deficient mutant *pmr4-1* was more susceptible to *E. amylovora*, we conclude that callose deposition is necessary, but not sufficient, for *E. amylovora* resistance in *Arabidopsis*. We also recognized the discrepancy in our *E. amylovora* growth data for the *ics1* and *pmr4-1* mutants (Fig. 3), compared with the observations reported earlier (Moreau *et al.*, 2012). Such a difference could be explained by many factors, for instance, the different *E. amylovora* strains, sampling times and plant growth conditions used in the two laboratories. Nevertheless, our data suggest that the cell wall-based resistance to *E. amylovora* is different from defence signalling mediated by SA, JA and ET in non-host *Arabidopsis*. It would be interesting to further investigate whether cell wall modification induced by *E. amylovora* is important for resistance in the host plants. If so, manipulation of the cell wall could provide an additional strategy to SA signalling modification to enhance host resistance to fire blight disease.

We further reported here that *E. amylovora* infection activated tumour-like growths in *Arabidopsis*. Many pathogens are known to induce cell fate change in the infected plants, including the activation of cell death, cell division and cell enlargement in the infected area. Although cell death has been widely recorded as a readout of disease symptoms (Greenberg and Yao, 2004), cell division and cell enlargement have been largely overlooked in

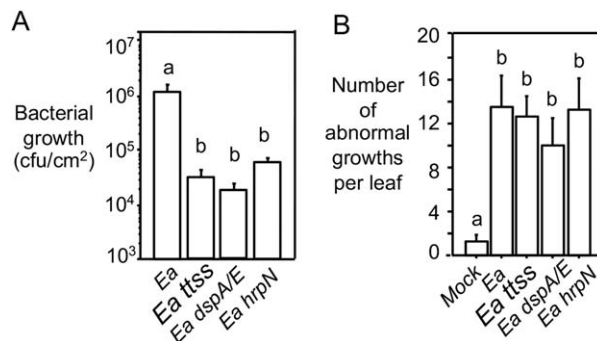


Fig. 8 *Erwinia amylovora* effectors are not required for tumour growth in *Arabidopsis*. Plants were infiltrated with different *E. amylovora* strains [optical density at 600 nm (OD_{600}) = 0.1] (*Ea*) or with 10 mM $MgSO_4$ as a control (Mock). (A) Quantification of bacterial growth (cfu, colony-forming unit). Each data point represents the average of 12 samples from two independent experiments \pm standard error of the mean. (B) Quantification of abnormal growth. At least 25 leaves per genotype were used for each counting. Error bars represent standard deviation. Different letters indicate significant difference amongst the samples ($P < 0.05$). This experiment was repeated twice with similar results.

studies of plant–pathogen interactions. Several pathogens have been reported to induce cell division and/or cell enlargement, in some cases leading to tumour-like growths in plants (de Almeida Engler *et al.*, 1999; Braun, 1956; Chalupowicz *et al.*, 2006; Chandran *et al.*, 2009; Hamdoun *et al.*, 2013; Marois *et al.*, 2002). We found that *E. amylovora*-induced cell growth in Arabidopsis was independent of some effectors of the bacterium and SA signalling of Arabidopsis. Additional experiments are needed to identify molecular factors from *E. amylovora* and Arabidopsis that are important for abnormal growths in plants.

We speculate that such tumour-like growths resemble cankers found in *E. amylovora*-infected apple. It has been proposed that, in order to limit the spread of *E. amylovora*, apple plants produce extra cell layers to seal off diseased tissue, thus forming a canker. In turn, the canker provides an important structure *in planta* for *E. amylovora* to survive the winter, from which the bacterium emerges as ooze in the next spring for further infection (Eastgate, 2000; Malloy *et al.*, 2012). However, how *E. amylovora* induces canker formation in apple and the morphology of these cankers are not well understood. Further investigation of bacterial and/or plant factors contributing to tumour-like growths in Arabidopsis could help to reveal the molecular mechanisms underlying canker formation in *E. amylovora*-infected apples, leading to a better control of fire blight.

Erwinia amylovora is one of the top 10 plant-pathogenic bacteria identified by the international scientific community. It causes the devastating fire blight disease in economically important pome fruit-producing plants and some ornamental plants (Mansfield *et al.*, 2012). The mechanisms of plant resistance to *E. amylovora* are not well understood. Here, we provide evidence to show that SA signalling and callose deposition are required for *E. amylovora* resistance in the non-host plant Arabidopsis. Although it is difficult to directly test the requirements of SA signalling and callose deposition in host resistance to *E. amylovora* because of the inherent experimental restrictions with the host genetic system, the conservation of the genes involved in SA signalling and callose formation in Arabidopsis and host plants strongly suggests a similar requirement of these two processes for *E. amylovora* resistance in host plants. Thus, genes important for SA- and cell wall-mediated defence provide potentially powerful molecular tools for genetic modification in order to improve the resistance of economically important crops to the destructive fire blight disease.

EXPERIMENTAL PROCEDURES

Plant materials

The Arabidopsis plants used in this work were all in the Columbia-0 background and were grown in growth chambers with a 12-h light/12-h dark cycle, light intensity of 200 $\mu\text{mol}/\text{m}^2/\text{s}$, 60% humidity and 22 °C. The mutants *ics1-1*, *eds1-2*, *eds5-3*, *pad4-1*, *npr1-1*, *jar1-1* and *nahG* have been described previously (Lu *et al.*, 2009; Ng *et al.*, 2011; Staswick *et al.*,

1992; Wang *et al.*, 2011). *ein2-1* was provided by Caren Chang at University of Maryland College Park, *jin1-7* by Barbara Kunkel at Washington University and *pmr4-1* by Shunyuan Xiao at University of Maryland College Park. The multiple SA, ET and/or JA gene knockout mutants (*dde2-2ein2-1*, *ein2-1pad4-1*, *ein2-1sid2-2*, *dde2-2ein2-1pad4-1*, *dde2-2ein2-1sid2-2*, *dde2-2pad4-1sid2-2*, *ein2-1pad4-1sid2-2* and *dde2-2ein2-1pad4-1sid2-2*) have been described previously (Tsuda *et al.*, 2009) and were obtained from the Arabidopsis Biological Resource Center.

Malus × domestica cv. 'Royal Gala' was propagated *in vitro* as described previously (Ko *et al.*, 2002; Norelli *et al.*, 1988) and grown in a glasshouse under supplemental lighting to maintain a 16-h day length and average temperature of 28 °C. Plants were watered daily and treated weekly with nutrient solution (MiracleGro) and Osmocote (Scott's Miracle-Gro Products, Marysville, Ohio, USA).

Bacterial culture and infection assays

Erwinia amylovora strain Ea273 (*Ea*) is a wild-type strain isolated from apple in New York, and the *E. amylovora* strain with streptomycin resistance was isolated from pear in Washington State. *Ea dspA/E* is a *dspA/E* deletion mutant and *Ea hrpN* is an *hrpN* deletion mutant. The *Ea ttss* mutant cannot export effectors to the host cell. These *Ea* mutants are in the Ea273 background and were created by homologous recombination (Bogdanove *et al.*, 1998b; Wei and Beer, 1993). *Erwinia amylovora* strains were kindly provided by Steven V. Beer at Cornell University. All *E. amylovora* strains were grown at 30 °C in Lysogeny broth (LB) medium with appropriate antibiotics for selection. For apple infection, actively growing shoots were sprayed with *E. amylovora* (10^8 cfu/mL) or the mock solution containing 10 mM phosphate buffer, pH 6.5, to run off using a hand-pumped spray bottle, and then immediately wounded with a sterile florist frog as described previously (Bonasera *et al.*, 2006). Treated leaves were harvested at 48 hpi for SA analysis.

For Arabidopsis infection, the fourth to sixth leaves of 30-day-old plants were infiltrated with *E. amylovora* strains at the indicated concentrations, using a 1-mL needleless syringe. The 10 mM MgSO_4 solution was used as mock treatment. All bacterial cultures and plant material inoculated with *E. amylovora* strains were maintained in isolation and autoclaved at the end of the experiment to prevent bacterial escape from the laboratory. The infected plants were covered with a clear dome to maintain 100% humidity. At 3 dpi, leaf discs of 7 mm in diameter from the infected leaves were excised using a core borer and ground in 10 mM MgSO_4 . The surface area of each leaf disc is 38 mm². Serial dilutions of the ground mixture were made and plated on LB plates containing appropriate antibiotics. Each bacterial data point was an average of samples taken from at least eight different plants from two or more independent experiments \pm standard error of the mean. Except for the bacterial growth assay, all other infection assays were performed with plants uncovered.

The culture of *Pma* ES4326 strains and the inoculation of Arabidopsis have been described previously (Wang *et al.*, 2011).

Ion leakage assay

Arabidopsis leaves infected with *E. amylovora* or *Pma* avrRpm1 were collected immediately after inoculation and cut with a 7-mm core borer. Five

leaf discs from five different plants were washed in de-ionized water twice and placed in 5 mL of de-ionized water. Each time point had triplicate samples for each genotype and each treatment. Solution conductivity was measured using an EC meter (The London Company, Welwyn International Inc., Cleveland, OH, USA) at the indicated times after leaf collection.

SA measurement

Bacteria-infected and mock (10 mM MgSO₄)-treated leaves from Arabidopsis or apple were extracted for free and total SA (glucosylated SA), and quantified with a high-performance liquid chromatograph as described previously (Ng *et al.*, 2011; Wang *et al.*, 2011).

RNA analysis

Bacteria-infected and mock-treated leaves from 30-day-old Arabidopsis plants were collected for RNA extraction and northern blotting, as described previously (Lu *et al.*, 2009). Radioactive probes were prepared by PCR, using an antisense primer specific for a gene fragment in the presence of [³²P]dCTP. Primers for *PR1* have been described previously (Lu *et al.*, 2009).

Analysis of leaf morphology using light microscopy

Bacteria-infected and mock-treated leaves from 30-day-old Arabidopsis plants were examined for tumour-like growths, which appeared to be transparent protrusions on leaves and could be observed with the assistance of a dissection microscope. At least 25 leaves from 12–15 plants were used for the quantification of tumour-like growths for each treatment. To observe more detailed leaf morphology, the treated leaves were collected at 4 dpi and cut into 2 × 4-mm² sections. The sections were fixed, embedded in LR white resin and further sliced with an ultramicrotome into 1-μm sections. Leaf cross-sections were stained with 0.1% toluidine blue O and photographed using an AxioCam MRC5 camera (Zeiss, Inc., Göttingen, Germany) connected to a dissection microscope.

Nuclear DNA quantification by DAPI staining

Leaves of 30-day-old Arabidopsis plants treated with *E. amylovora* or 10 mM MgSO₄ were collected and cut into 3 × 6-mm² sections, using at least six sections from six plants for each treatment. The sections were fixed, embedded in paraplast, cut into 15-μm slices with a microtome, and stained with DAPI to visualize nuclei. Relative nuclear DNA quantification was analysed as described previously (Hamdoun *et al.*, 2013).

Callose staining

Leaves of 30-day-old Arabidopsis plants treated with *E. amylovora* (0.01) or 10 mM MgSO₄ were boiled in alcoholic lactophenol (phenol–glycerol–lactic acid–water: 1 : 1 : 1 : 1, v/v) for 2 min and rinsed with 50% ethanol. The transparent leaves were incubated for 1 h in 0.15 M phosphate buffer (pH 9.5) containing 0.01% aniline blue (Lu *et al.*, 2003), prior to microscopic analysis. The stained leaves were visualized and photographed with a fluorescence dissection microscope (Leica M80, Leica Microsystems, Wetzlar, Germany) connected to a CCD camera (Leica IC80 HD). Callose deposition was quantified by measurement of the relative

fluorescence intensity emitted by aniline blue stain using ImageJ (Version 1.45s).

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

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

Fig. S1 *Erwinia amylovora*-induced salicylic acid (SA) accumulation is *ISOCHORISMATE SYNTHASE 1 (ICS1)* dependent in Arabidopsis. The fourth to sixth leaves of Col-0 and *ics1-1* plants were infiltrated with *E. amylovora* at an optical density at 600 nm (OD_{600}) of 0.1 and collected at 24 h post-inoculation (hpi) for SA analyses. (A) Free SA measurement. (B) Total SA measurement. Statistical analysis was performed with one-way analysis of variance (ANOVA) Fisher's partial least-squares difference (PLSD) tests (StatView 5.0.1). Asterisks indicate significant difference between *E. amylovora*-inoculated Col-0 and *ics1-1* plants at the same time point ($P < 0.05$). These experiments were repeated twice with similar results. FW, fresh weight.

Fig. S2 *Erwinia amylovora* induces salicylic acid (SA) accumulation in apple. *Erwinia amylovora* (*Ea*; 0.1)-inoculated and mock-inoculated apple leaves were harvested at 48 h post-inoculation (hpi) and extracted for total and free SA followed by high-performance liquid chromatography (HPLC) measurement. Asterisks indicate significant difference between mock- and *E. amylovora*-inoculated samples for free or total SA level ($P < 0.05$). These experiments were repeated twice with similar results. FW, fresh weight.

Fig. S3 Arabidopsis mutants disrupted in ethylene (ET) and jasmonic acid (JA) signalling are not compromised in resistance to *Erwinia amylovora*. The fourth to sixth leaves of 30-day-old plants were inoculated with *E. amylovora* at an optical density at 600 nm (OD_{600}) = 0.1 [10^8 colony-forming units (cfu)/mL]. Inoculated leaf discs (each 38 mm²) were taken for the measurement of bacterial growth at 3 days post-inoculation (dpi). Each data point represents the average of 12 samples from two independent experiments \pm standard error of the mean. Note that there is no difference between the samples. These experiments were repeated twice with similar results.

Fig. S4 *Erwinia amylovora*-induced callose deposition in Arabidopsis is independent of salicylic acid (SA) signalling. *Erwinia amylovora*-infected leaves of each genotype were harvested for fixation and staining with aniline blue. (A) Images of callose deposits. (B) Quantification of callose deposits. Each data point was an average of at least six images from four different leaves. The asterisk indicates significant difference of *pmr4-1* from the other genotypes ($P < 0.05$). These experiments were repeated twice with similar results.

Fig. S5 *Erwinia amylovora*-induced callose deposition in Arabidopsis is independent of ethylene (ET) and jasmonic acid (JA) signalling. *Erwinia amylovora*-inoculated leaves of each genotype were harvested for fixation and staining with aniline blue. (A) Images of callose deposits. Callose deposits appeared as fluorescent dots and were photographed with an AxioCam MRc5 camera connected to a fluorescence stereoscope microscope. (B) Quantification of callose deposits. The number of

callose deposits of each genotype was quantified using ImageJ (Version 1.45s). Each data point was an average of at least six images from four different leaves. Note that there is no difference between the samples. These experiments were repeated twice with similar results.

Fig. S6 *Erwinia amylovora* induces tumour-like growths in Arabidopsis leaves. Plants were infiltrated with *E. amylovora* at an optical density at 600 nm (OD_{600}) of 0.1 [10^8 colony-forming units (cfu)/mL] (*Ea*) or mock solution (10 mM $MgSO_4$) (Mock). The abnormal growth phenotype usually appeared at about 4 days post-inoculation (dpi). (A) Images of abnormal growths on the leaf abaxial side. (B) Leaf cross-section. Leaves were sectioned into thin slices by hand sectioning and imaged with a camera connected to a dissection microscope. Arrows indicate abnormal growths as transparent protrusions on the infected leaves.