Erwinia amylovora Expresses Fast and Simultaneously hrp/dsp Virulence Genes during Flower Infection on Apple Trees

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Abstract

Background: Pathogen entry through host blossoms is the predominant infection pathway of the Gram-negative bacterium Erwinia amylovora leading to manifestation of the disease fire blight. Like in other economically important plant pathogens, E. amylovora pathogenicity depends on a type III secretion system encoded by hrp genes. However, timing and transcriptional order of hrp gene expression during flower infections are unknown.

Methodology/Principal Findings: Using quantitative real-time PCR analyses, we addressed the questions of how fast, strong and uniform key hrp virulence genes and the effector dspA/E are expressed when bacteria enter flowers provided with the full defense mechanism of the apple plant. In non-invasive bacterial inoculations of apple flowers still attached to the tree, E. amylovora activated expression of key type III secretion genes in a narrow time window, mounting in a single expression peak of all investigated hrp/dspA/E genes around 24–48 h post inoculation (hpi). This single expression peak coincided with a single depression in the plant PR-1 expression at 24 hpi indicating transient manipulation of the salicylic acid pathway as one target of E. amylovora type III effectors. Expression of hrp/dspA/E genes was highly correlated to expression of the regulator hrpL and relative transcript abundances followed the ratio: hrpA>hrpN>hrpL>dspA/E. Acidic conditions (pH 4) in flower infections led to reduced virulence/effector gene expression without the typical expression peak observed under natural conditions (pH 7).

Conclusion/Significance: The simultaneous expression of hrpL, hrpA, hrpN, and the effector dspA/E during early floral infection indicates that speed and immediate effector transmission is important for successful plant invasion. When this delicate balance is disturbed, e.g., by acidic pH during infection, virulence gene expression is reduced, thus partly explaining the efficacy of acidification in fire blight control on a molecular level.

Introduction

The plant disease fire blight is caused by the Gram-negative bacterium Erwinia amylovora and is of recurring concern in pome fruit production. Economically relevant host plants include apple, pear and quince but many ornamentals of the Rosaceae family become infected as well [1]. Either blossoms, shoots or the rootstock can show blight symptoms leading to severe economic losses at varying extent. Large scale spreading of fire blight is ascribed to unintended trade with latent infected plants, whereas regional dissemination is due to pollinating insects, rain and wind [2]. Notably, new manifestations of the fire blight disease occur predominantly after blossoms of host plants were infected [2]. Thus, blossom infection plays an important role in gain of new geographical areas infested by the pathogen.

On host flowers, E. amylovora first multiplies on the stigmatic surface [3]. At high humidity, the bacteria enter the flower tissue through the nectarthodes located in the floral cup [4]. Although invasion of flowers is responsible for primary contamination with fire blight and substantially contributes to epidemics [2], molecular knowledge about the early infection process regarding bacterial virulence gene expression is absent. Regarding responsive plant gene expression, knowledge is limited to studies from leaves, stems, shoots, in vitro plantlets or immature fruits [5,6,7,8,9,10,11]. Only one study investigated the plant gene expression in detached flowers upon E. amylovora inoculation, but not bacterial gene expression [12]. The type III secretion system is an essential pathogenicity determinant during the early infection process in many phytopathogenic bacteria [13]. Plant pathogens such as Pseudomonas syringae and Xanthomonas campestris exploit natural openings on leaves, e.g., stomata or hydathodes for infection and manipulate the plant defense system with type III secreted effector proteins [14]. Also in E. amylovora the type III secretion system was shown to be essential for floral as well as shoot infections [8,15].
Main efforts to understand the early infection process by *E. amylovora* are based on studies in vegetative plant parts or immature pear, where infection has to be artificially assisted by wounding the plant [6,9,10,11]. However, the time point when *E. amylovora* genetically activates its type III secretion system, especially if not assisted by wounding and in the presence of the full plant defense such as in infections of flowers attached to the tree, still remains to be elucidated.

The type III secretion system consists of structural, regulatory and effector components and allows pathogenic bacteria to transmit effectors into the host cell [13]. In *E. amylovora*, the *hrp* genes (for hypersensitive reaction and pathogenicity) encode the components of the type III secretion system [15] and their expression is directly linked to virulence. The master switch for expression of this system is HrpL, an alternative sigma 70 factor, which can bind to the *hrp*-Box promoter elements present in all *hrp*- and *dsp*-genes [16,17]. The protein channel for effector transmission is composed of the pilin HrpA [18,19] and supports secretion of two functionally well characterized proteins, the harpin HrpN and the effector DspA/E. HrpN was initially isolated as elicitor of the hypersensitive reaction (HR) in non-host tobacco [20,21]. In host plants, HrpN was shown to be secreted along the pilus into the apoplast [18,22], where it probably forms pores in the plant plasma membrane and functions as the main translocator protein [23,24]. In support of this, HrpN proved to be necessary for efficient translocation of the effector DspA/E into plant cells [25]. DspA/E (for disease-specific) is absolutely required for *E. amylovora* virulence with mutants being apathogenic [26,27,28,29]. In the plant cell, DspA/E putatively interacts with specific host plant receptor-like serine/threonine kinases, thereby interfering with plant signaling [30]. These findings are in line with the inability of *dspA/E* mutants to effectively suppress salicylic-acid (SA)-activated plant immunity, such as callose deposition [31,32]. On the other hand, previous studies investigating the host transcriptional response upon *E. amylovora* inoculation did not find evidence for a differential expression of the pathogenesis-related protein 1 (*PR-1*), which would indicate an influence on the SA-mediated plant response [31,33,34]. This is astonishing, since the SA-mediated plant immunity is one of the major targets manipulated by type III effectors either directly or indirectly [31,35,36]. The *E. amylovora* DspA/E influences SA-dependent callose deposition [31,32] and thus would be a good candidate effector involved in manipulation of the plant SA-signaling. In this context, expression of *dspA/E* itself showed a transient peak in *E. amylovora* populations growing epiphytically on flowers [37] and a similar transcriptional induction upon inoculation on immature pear fruit [11]. Thus, one might expect a transient effect on the plant defense system as well. However, the timing of *dspA/E* expression relative to *hrp* gene expression during the development of infection remains to be determined [11,38].

The de novo assembly of the type III secretion is energy consuming. This is why bacteria tightly restrict its expression until conditions arise which suggest host proximity [39]. In *E. amylovora*, these conditions include low nutrients, low temperature and low pH and generally resemble the plants apoplastic environment [10,29]. The inducing effect of acidic pH 5.5 on *hrp* gene expression [10] is particularly interesting regarding the use of acidifying products in fire blight control to prevent flower infections. Acidic stone meal or antagonistic yeast formulations with a pH around 4.0 were shown to inhibit pathogen growth [40]. On the other hand, *E. amylovora* strains which tolerate more acidity for growth are also described as more virulent (research report of project no 100448; www.dafne.at). It is currently not known if the inducing effect of acidic pH on *hrp* gene expression is balanced by the negative effect on pathogen growth at pH 4.0 and how this affects virulence.

We report here the temporal expression pattern of key genes for *E. amylovora* type III secretion for the first time during non-invasive bacterial inoculations on apple flowers still attached to the tree. The quantity and timing of *hrp* gene expression was determined by newly established quantitative real-time PCR analyses and compared to the expression of a virulence factor not involved in type III secretion, the amylovoran synthesis gene *amsG*. Parallel to *hrp* gene expression, expression of two host defense genes, *PR-1* and MalM/1, was monitored in the same flower tissues to assess plant defense response. Since acidification is relevant for fire blight control, the influence of acidic pH 4.0 on *hrp* expression was tested as well and compared to neutral pH.

**Materials and Methods**

**Apple flower-*E. amylovora* inoculations**

Freshly opened flowers of two-year-old potted *Malus domestica* ‘Golden Delicious’ were manually inoculated with *E. amylovora* 295/93 (deposited as CFBP 6449 in the French culture collection) by a non-invasive technique. For inoculation, liquid overnight cultures were resuspended in water buffered either with piperazine-1,4-bis-(2-ethanesulfonic acid) (Carl Roth, Karlsruhe, Germany) to pH 6.8 or with homopiperazine-1,4-bis-(2-ethanesulfonic acid) (Sigma-Aldrich, Vienna, Austria) to pH 4.0. The cell density was adjusted photometrically to 5×10⁸ cells ml⁻¹. On each single flower, two 10 μl droplets of bacterial suspension were placed, one to the stigmatic surface and one close to the hypanthium resulting in approximately 10⁷ bacterial cells per flower. Mock inoculations were performed with buffer only. Three replicate trees per treatment (pH 4.0 and pH 6.8 with and without *E. amylovora*) were inoculated in the greenhouse at 27/15°C day/night temperature and 80% relative humidity. Three single inoculated flowers per tree were sampled 6, 24, 48 and 72 hours post inoculation (hpi), immediately frozen in liquid nitrogen and kept at −80°C until further processing. Inoculation experiments for flower sampling were performed twice with new trees. For cDNA-synthesis, flowers were transcribed individually in the first (Set 1) and pooled per tree in the second independent experiment (Set 2).

To assess visual symptom development in flowers inoculated at pH 4.0 or pH 6.8, a modified standard test after Pusey, 1997 [41] with detached apple blossoms was applied. In a transparent box 15 detached apple flowers were placed in Eppendorf tubes filled with 1.5 ml 10% sucrose solution and inoculated on the stigmas with a 1 μl drop containing 10⁶ bacterial cells suspended in pH-adjusted water as described above. To attain high humidity 35 ml of 32% glycerine solution was added to each box and closed with a lid. 3 boxes per treatment and 3 boxes with buffer-only inoculated flowers were incubated at approximately 22°C and natural day/night light cycles. After 2 days the flowers were sprayed with pH-adjusted water containing a commercial fungicide. Visual symptom development was analyzed 8 days post inoculation. The detached apple blossom test was performed twice.

**RNA isolation**

Total RNA was isolated from sampled apple flowers with petal leaves removed according to the method of Chang et al., 1993 [42]. Isolated RNA was DNase-treated, checked for quality by gel electrophoresis and A₂₆₀/A₂₈₀ ratio determination, and quantified using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Vienna, Austria). Purified RNA extracts were checked at random for DNA contamination by using RNA extracts as templates in qPCR assays. High quality RNA (5.0 μg per sample...
in Set 1, 4.2 µg per sample in Set 2) was reverse transcribed with SuperScript VILO cDNA Synthesis Kit according to the manufacturer’s protocol (Invitrogen, Carlsbad, California, USA). Thereafter, the obtained cDNA was split for bacterial and plant gene expression analysis.

Analysis of *E. amylovora* gene expression

Quantitative real-time PCR (qPCR) assays for *E. amylovora* genes *hrpL*, *hrpA*, *hrpN*, *dspA/E*, *amsG*, *recA*, and *gyrA* were established and optimized. Primer sequences were derived from available sequence information in GenBank: *hrpL* (U36244), *hrpA* (U56662), *hrpN* (M92994.3), *dspA/E* (Y13831.1), *amsG* (Y77921.1), *recA* and *gyrA* (FN666575.1). To optimize primer concentrations, each primer was tested in 50 nM steps in a concentration range from 50 to 600 nM (M92994.3), qPCR-primers with 104 copies per reaction were used as templates. During the run, 72 h post inoculation (hpi), either stable or reduced *hrpL* transcript levels were observed in the first and second experiment, respectively. Parallel to the decline in *hrp* expression, the onset of flower aging was observed at 72 hpi. Petals fell off when touched and stigmas began to discolor (data not shown). The temporal expression pattern observed for *hrpL* was shown concomitantly by the structural gene *hrpA* as well as *hrpN* and *dspA/E* demonstrating a highly coordinated parallel expression during type III secretion (Figure 1). In contrast, expression of *amsG*, the first gene in the operon for amylovoran synthesis, remained for the first 72 h basally low without expression peak as observed for *hrp* genes. Control normalizations for all genes investigated against a second reference gene, *gyrA*, confirmed the observed transcriptional pattern (Figure S2).

To test acidification as one major parameter regulating the expression of the type III secretion system, flower inoculations on additional apple trees were performed in parallel. This time, the bacterial suspension was buffered to pH 4.0 prior to inoculation. As determined by qPCR in these samples, mean transcript levels of *hrpL*, *hrpA*, *hrpN* and *dspA/E* were diminished in comparison to expression levels at neutral pH and slowly increased linearly without peak expression (Figure 1). At the last sampling time point, 72 hpi, mean transcript abundances at pH 4.0 were always lower than values observed at peak expression under neutral conditions. Also at pH 4.0, the expression pattern of *hrpA*, *hrpN* and *dspA/E* followed closely *hrpL* expression with a minor deviation in *hrpN* expression indicating a slight peak expression. In order to test whether molecular expression patterns correlate with visual symptom development, we performed standard infection tests modified after Pusey, 1997 [41] by inoculating detached apple blossoms at the stigmas with 10^8 *E. amylovora* cells suspended in water buffered to pH 4 or pH 7. The inoculation density in this test system was lower than in greenhouse inoculations (10^8 cells), because the critical cell density necessary for infection in detached flowers is lower [46]. Evaluation at 8 dpi showed in two independent experiments significantly (T-test, p<0.05) less fire blight symptoms in flowers inoculated and wetted with acidic pH compared to neutral pH (Figure 2).

To determine the magnitude and relative quantities of *hrp* genes expressed during flower infection, transcript levels were analyzed separately for correlations between genes. After 24 hpi, *hrpA*, *hrpN*, and *dspA/E* transcript abundances were highly correlated with expression of *hrpL* with coefficients of determination being R²=0.7 and R²=0.9 for the first and second experiment, respectively (illustrated for the first experiment in Figure 3). In the early phase of induction (6 hpi) no such correlation with *hrpL* was observed (data not shown). In contrast to *hrp* genes, *amsG* expression showed little correlation with *hrpL* expression with R²=0.6 and R²=0.5 in the first and second experiment, respectively. To find out if *hrpL* expression itself is linearly dependent on the bacterial cell number, normalized *hrpL* expression was compared to the absolute copy

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**Results**

The transcriptional timing and coordination of the type III secretion system of *E. amylovora* was investigated for the first time at the site of primary infection, in flowers still attached to the tree. Without wounding, single flowers of the susceptible apple cv. Golden Delicious were manually inoculated with approximately 10^8 bacterial cells. Bacterial suspension was placed in two droplets at the stigmas and close to the hypophyllium (Figure S1).

Subsequently, the expression of selected genes essential for type III secretion and necessary for bacterial virulence was monitored. These genes comprised *hrpL* and *hrpA*, and two genes encoding for secreted proteins, *hrpN* and *dspA/E*. Transcript abundances of these genes were measured by newly developed qPCR protocols in reverse transcribed RNA-extracts of whole flowers. To account for pathogen abundance on the flower, expression was normalized against transcript abundance of the reference genes *recA* and *gyrA*.

No *hrp* expression was observed in non-inoculated flowers (not shown). In two independent inoculation experiments, the mean transcript level of *hrpL*, the main regulator gene of the type III secretion system, increased from low initial expression values 6 hours post inoculation (hpi) to peak expression between 24 to 48 hpi (Figure 1). At 72 hpi, either stable or reduced *hrpL* transcript levels were observed in the first and second experiment, respectively. Parallel to the decline in *hrp* expression, the onset of flower aging was observed at 72 hpi. Petals fell off when touched and stigmas began to discolor (data not shown). The temporal expression pattern observed for *hrpL* was shown concomitantly by the structural gene *hrpA* as well as *hrpN* and *dspA/E* demonstrating a highly coordinated parallel expression during type III secretion (Figure 1). In contrast, expression of *amsG*, the first gene in the operon for amylovoran synthesis, remained for the first 72 h basally low without expression peak as observed for *hrp* genes. Control normalizations for all genes investigated against a second reference gene, *gyrA*, confirmed the observed transcriptional pattern (Figure S2).

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Figure 1. Time-course of *E. amylovora* hrp virulence genes and *amsG* expression upon inoculation on apple flowers. Shown are two independent experiments, Set 1 (5–9 flowers per time point) and Set 2 (3–5 flowers per time point). Normalized expression values of indicated genes represent mean values of flower samples from three replicate trees. Expression is normalized to *recA*. Flowers were inoculated with bacterial suspension buffered to pH 7 (filled symbols) or buffered to pH 4 (open symbols). *hrpL* and *hrpL*-regulated genes are shown as circles whereas *hrp*-independent genes as squares.
doi:10.1371/journal.pone.0032583.g001
number of recA transcripts found on the respective flowers. The absence of correlation with recA with $R^2\leq0.3$ (both experiments) suggests that expression of hrpL does not directly depend on bacterial abundances at cell densities used for inoculation (illustrated for the first experiment in Figure S3). Comparing absolute transcript abundances during peak expression, \(\text{hrpA} : \text{hrpN} : \text{dspA} / \text{E} : \text{amsG} \) were expressed in a 567:7:4:1 (Set 1) and 131:9:4:1 (Set 2) ratio suggesting that the structural protein pilin is needed in highest abundance. At acidic pH 4.0 the relative proportions of hrp transcript abundances were similar to neutral conditions with \(\text{hrpA} : \text{hrpN} : \text{dspA} / \text{E} \) in a 049:10:14:1 ratio at 24 hpi (Set 1) and 106:3:4:1 ratio at 48 hpi (Set 2).

In parallel, the plant defense response was monitored by expression profiling of the pathogen related protein-1 (PR-1) (GenBank: AF507974.1) and the malus miraculin 1 (MalMir1) (GenBank: FK938848.1) in the same cDNA samples as used for bacterial expression analysis. Whereas normalized MalMir1 expression revealed no consistent pattern related to \(E.\ amylovora\) inoculation, expression of PR-1 was transiently downregulated in flowers 24 hpi compared to uninoculated flowers (Figure 4). At 24 hpi, PR-1 was significantly 6.5-fold less ($p<0.05$) expressed in the first experiment and 5.5-fold less in the second experiment at neutral pH. At acidic pH, transient downregulation of PR-1 was less pronounced but in contrast to neutral pH, PR-1 expression was upregulated 48 hpi (Figure 4).

**Discussion**

Type III secretion is essential for \(E.\ amylovora\) infection of host vegetative tissue or flowers [17,47,48]. However, no expression studies revealed timing of hrp gene expression in its entity during infection.
Transcript values for PR-1 and MalMir1 are normalized to actin and GAPDH expression. Flowers were inoculated with bacterial suspension buffered to pH 7 (filled symbols) or buffered to pH 4 (open symbols) and compared to un inoculated (only buffer treated) flowers. The asterisk indicates significant differential expression (p < 0.05, REST 2008 analysis).

doi:10.1371/journal.pone.0032583.g004

Figure 4. Fold change of PR-1 and MalMir1 gene expression in E. amylovora inoculated flowers relative to uninoculated flowers.

The infection process or addressed expression order. Thus, we investigated virulence gene expression for the first time during the major infection mode of E. amylovora: floral infection. Because in this case bacteria can invade the healthy flower without the need for artificial wounding, we adopted a non-invasive inoculation method: we placed two droplets of bacterial suspension at flower parts of the susceptible apple ‘Golden Delicious’ where this pathogen can also naturally be detected, the stigmas and close to the hypanthium [4]. This method allows controlling the applied bacterial number per flower and normally yields in our routine greenhouse inoculations 25–48% blighted blossoms in this cultivar.

A characteristic temporal hrp expression profile during flower infection included an induction time of approximately 6 hours. This time lies in between time points found for hrp inducing medium (3–6 h) and wound-inoculated immature pear (24 h) and comprises time for hrp de novo RNA and protein synthesis [10,29]. For comparison, in the Pseudomonas syringae-plant interaction expression of hrp genes is induced not until bacteria have reached the intercellular space where direct contact with plant cells is possible [49,50]. A similar local dependence for full hrp expression can be assumed in E. amylovora flowers infections because bacteria have to migrate first towards nectaries. There, the rising hrpL expression would have downregulated flagellar synthesis thereby opposing motility [51]. The early occurrence of peak expression of hrp genes and dspA/E in all flowers was intriguing, because it indicates a fast bacterial infection effort rather than sequential attacking. Similarly, a single peak expression of the effector dspA/E at 48 hpi was found in E. amylovora populations growing epiphytically on apple stigmas [37]. Fast infections might be advantageous, since plant defense is encountered in an initially uninoculated state (Figure 4). Notably, even the presence of an avirulent E. amylovora strain is detected by the plant and triggers a rapid defense response [52]. Also important in this context is flower age. E. amylovora infects successfully flowers 1–3 days old but susceptibility drastically decreases with flower age [2,53]. This is in good accordance with what we have observed in our greenhouse experiments visually and in gene expression. Petal leaf fall and necrosis of the stigmas were observed from the third day on and concomitantly also expression of the type III secretion system declined 72 h post inoculation. This emphasizes the importance of a fast expression of the type III secretion system especially during early plant invasion by E. amylovora. In contrast to the expression profile of the type III secretion system, expression of amsG, the first gene in the amylovoran synthesis operon [54], was only basally expressed during early infection. This is in accordance with previous observations that this virulence factor is important later in pathogenicity [55].

When comparing the E. amylovora hrp/dspA/E gene expression profile with the leaf pathogen Pseudomonas syringae pv. phaseolicola, a similar peak expression curve is evident, however, the period of hrp expression was shortened to 24 h upon P. syringae infiltration into host leaves [56]. Characteristic for both pathogens seems to be that strong hrp expression is followed by a decline in expression during the infection. P. syringae cells turned their type III secretion off as soon as they had established inside the plant [56] which might be similar for E. amylovora but has not been investigated yet. For other plant pathogens with similar type III secretion systems such as Erisia herbicola or Pantoea stewartii information about expression profiles during plant invasion are currently not available but could reveal if this expression pattern is common to plant pathogens.

Accumulation of hrp transcripts differed between single inoculated flowers indicative for induction to various degrees (Figure 3). These variable transcript levels did not correlate with the bacterial cell number as estimated by abundance of two independent reference gene transcripts (Figure S3), but correlated significantly with hrpL transcript abundance. The close co-expression of hrpA, hrpN, and dspA/E with hrpL demonstrated by correlation coefficients above 0.7 is consistent with a direct genetic induction of this system through hrpL [17]. hrpL transcript...
accumulation itself varied up to 5-fold during peak expression between individual flowers, which might be explained by a different amount of bacteria that reached the hypanthium. We hypothesize that highest expression could be expected in bacterial populations staying at the nectaries or inside the plant whereas lower expression could be expected in epiphytically growing populations. In conclusion, the variable overall expression would explain why even in artificially inoculated flowers, only a portion become infected and shows symptoms later on.

The relative transcript abundances between genes were found in both independent inoculation experiments in the order hrpA > hrpN > hrpL > dspA/E. The exceptionally low but efficient amount of dspA/E was also previously recognized in transient expression experiments of dspA/E in apple leaves, where neither the mRNA nor protein were detectable despite necrosis was elicited [57]. Our results support this conclusion since even with the highly sensitive qPCR method dspA/E transcripts were detectable in lowest abundances only. Consistent with the here observed relative transcript abundances, the encoded proteins are secreted in similar proportions into inducing medium [58]. Congruently, expression of the homologous hgp genes in P. syringae followed closely hgpL expression over time and in similar relative quantities with hgpL > hgpN > hgpE [56]. Together with our data, this suggests that strong upregulation of structurally important transcripts as hgpN and hgpL are necessary to provide efficient effector placement into flower tissue.

One important question for understanding host susceptibility is how plant defense systems are manipulated by E. amylovora during floral infection. We addressed this question by analyzing the expression of two plant genes possibly involved in host defense: a gene encoding for the putative proteinase inhibitor Miraculin (MalMir1), which was highly upregulated upon E. amylovora shoot infections [59] and the pathogenesis-related protein 1 (PR-1), which is a well known indicator for salicylic acid (SA) signaling. The flowers in our experiments were still attached to the living tree to ensure a natural plant defense reaction. For the putative proteinase inhibitor Miraculin encoded by MalMir1 no consistent expression pattern was observed, which indicates no role in defense against E. amylovora in flowers. Contrary, expression of PR-1 was lowered at 24 hpi in both experiments suggesting a transient suppression caused by E. amylovora since no such expression change was observed in mock-inoculated flowers. In Malus domestica several PR-genes were identified with three different PR-1-like genes PR-1a, PR-1b and PR-1c [33]. None of these PR-1-like genes were upregulated due to E. amylovora inoculation in apple shoots [33] or detached flowers [54]. Our PR-1 real-time primers specifically target PR-1a and we found not only absence of induction but a transient suppression in PR-1 expression upon E. amylovora infection in flowers. Several previous studies presumed manipulation of the SA pathway by this pathogen, however, could not find transcriptional evidence probably due to the temporal limited and transient nature of expression or methodical sensitivity [31,33]. Also, a recent microarray study did not detect differential expression of Pr-1 [34], which might have been missed, because the plant response was investigated in flowers which were detached from the plant. However, manipulation of the SA pathway either directly or indirectly, e.g., via the antagonistic jasmonic acid pathway would be a critical function of certain type III effectors for successful host infection [31,35]. Therefore, we speculate that in our experiments DspA/E caused the observed PR-1 suppression, since this effector was suggested to modulate basal, probably SA-dependent host plant defense such as callose deposition [31,32]. We suggest that expression of dspA/E had reached already at 24 hpi a threshold level that caused the observed PR-1 suppression, even though in the second experiment maximal hgp expression was only reached at 48 hpi (Figure 1). Further indirect evidence for involvement of DspA/E in SA-defense manipulation is given by delayed PRI-expression, when dspA/E is transiently expressed in non-host tobacco leaves [57].

Acidification as potential mechanism to prevent new fire blight infections in flowering orchards is discussed since a long time. Several commercially available products are either based solely on this mode of action such as acidic stone meal or are formulated in acidic buffers including disinfectants [60] or acid-tolerant antagonistic yeasts [40]. Recently, natural acidification by the antagonist P. agglomerans was suggested as potential additional antagonistic mechanism against E. amylovora [61]. The common understanding how acidic pH prevents fire blight infections is mainly derived from the growth-inhibiting effect of pH<5 on E. amylovora [40,62,63] thereby preventing multiplication on the flower, which is necessary to reach a critical cell density for infection [2]. Also in our experiments E. amylovora did not grow below pH 5 (Figure S4). The critical cell density necessary for infection is estimated in fire blight forecasting models to be at least 10^7 colony-forming units (CFU) per flower [64,65] which is close to the naturally observed E. amylovora population densities of 10^6–10^7 CFU per flower [2]. Less is known how acidic pH affects the further development of infection when the critical cell density is reached. To investigate hgp gene expression at pH 4 and pH 7 without the effect of growth retardation by acidic pH, we applied E. amylovora inoculum densities (10^8 cells/flower) above the threshold density necessary for infection. Interestingly, Wei et al. [10] demonstrated that acidic pH 5.5 is inducing type III secretion in E. amylovora in liquid culture, which might hypothetically increase virulence and in consequence would be unwanted in fire blight control. Thus, we addressed the question if a more acidic pH 4 still increases expression of the type III secretion system during flower infection. On a molecular level, expression of hgp genes at acidic pH 4 was reduced without a typical peak expression curve as compared to bacterial suspension buffered to pH 7 on flowers (Figure 1). Therefore, in contrast to pH 5.5 the more acidic pH 4 does not induce hgp expression anymore. The non-inducing effect of pH 4 is meaningful considering the pH range naturally encountered by plant pathogenic bacteria during infection, which is usually between 5.0–6.5 in the apoplast [66]. However, over time expression of hgp genes at acidic pH increased slowly but linearly but never reached peak expression levels observed at neutral pH. On some of the flowers, a minor expression peak for hgpN was observed indicating few successful infection events. Thus, under field conditions where flowering time may be prolonged compared to the greenhouse, the pathogen might still be able to infect albeit slower and at lower frequency. Interestingly, in flowers inoculated at acidic pH the plant defensive gene PR-1 was less suppressed at 24 hpi compared to neutral inoculations (Figure 4). Moreover, a clear upregulation of PR-1 at 48 hpi indicated that acidification disturbs the bacterial infection progress leading to activation of the plant defense. Regarding visible symptoms on inoculated blossoms, acidification of the bacterial suspension with pH 4 buffered water could reduce (significant at p<0.05; T-test) the number of flowers showing symptoms typically for fire blight (Figure 2). Together with our gene expression data, this indicates that acidification leads to slower and reduced infection rates and might well be an effective measure to reduce fire blight.

In summary, a non-invasive inoculation technique allowed us to study the virulent behaviour of the E. amylovora pathogen on the flower and, in parallel, to observe the plant defense reaction in flowers still attached to the living tree. E. amylovora expressed key
genes for type III secretion, namely the pilin hpaA, the putative translocator hpaV and the effector dfrA/E in a narrow time frame of 24–48 hpi and in well-defined ratios under the control of the regulator hpaL. No hierarchy for the expression of these genes (this study) or for the encoded secreted proteins was found [50]. This leads to a model where simultaneous expression of the type III components is required for successful infection. The bacterial presence as well as secreted HrpN is recognized by host cells [32,52,67], thus concomitant injection of effectors is necessary to counteract elicitation of defense responses. Interestingly, main expression of hpa genes coincided with a transient suppression in plant PR-1 expression at 24 hpi suggesting that E. amylovora quickly impacts the major SA-dependent plant defense pathway. This implies that co-transcription of E. amylovora structural genes with effectors of the type III secretion system is necessary to outrun plant defense.

Supporting Information

Figure S1 Non-invasive inoculation of an apple flower with E. amylovora cell suspension. One droplet was applied to the stigmatic surface, one close to the hypanthium. (TIF)

Figure S2 E. amylovora hpa virulence genes and amsG expression upon apple flower inoculation. Shown are expression profiles of indicated genes for two independent experiments, Set 1 (5–9 flowers per time point) and Set 2 (3–5 flowers per time point). Expression values of indicated genes were normalized to gusA expression and represent mean values of flower samples from three replicate trees. Flowers were inoculated with bacterial suspension buffered to pH 7 (filled symbols) or buffered to pH 4 (open symbols). hpaL and hpaL–regulated genes are shown as circles whereas hpa-independent genes as squares. (EPS)

Figure S3 Transcript abundances of reference genes as estimate for bacterial abundance compared to relative hpaL expression. A, nea transcript numbers on single flowers (as estimate for E. amylovora cell numbers) plotted against relative expression of hpaL (hpaL/nea) at indicated time points post inoculation. B, The same transcript values are plotted against gus transcript numbers and gave virtually identical results. (EPS)

Figure S4 Growth curves of E. amylovora 295/93 in minimal medium at different pH. Growth at 26°C was determined as increase in optical density at 630 nm in minimal medium modified after Pusey [68]: K2HPO4 0.7 g/l, KH2PO4 0.2 g/l, 10% sucrose, buffered with homopiperazine-1,4-bis(2-ethanesulfonic acid) to pH 4.8, with 2-(N-morpholino)ethanesulfonic acid to pH 5.8, with piperazin-N,N′-bis(2-ethanesulfonic acid) to pH 6.8, and with N-Tris(hydroxymethyl)methyl-3-amino propanesulfonic acid to pH 7.8 or pH 8.8. Values shown represent the mean of at least 3 independent trials with 5 replicates and standard deviations (not visible for pH 4.8). (EPS)

Table S1 Primer sequences and PCR conditions used in qPCR analyses and standard PCR. (DOC)

Acknowledgments

We are grateful for the technical assistance and support provided in verification of plant expression results by Dr. F. Trognitz, Austrian Institute of Technology, Vienna, Austria. We also want to thank two anonymous reviewers for their valuable comments, which helped to improve this manuscript.

Author Contributions

Conceived and designed the experiments: DP RM EW. Performed the experiments: DP RM JS. Analyzed the data: DP RM EW. Contributed reagents/materials/analysis tools: DP RM EW SB. Wrote the paper: DP RM JS. Contributed analysis tools: DP RM EW. Performed the experiments: DP RM EW. Contributed analysis tools: DP RM EW SB. Wrote the paper: DP RM JS. Contributed reagents/materials/analysis tools: DP RM EW SB. Wrote the paper: DP RM EW SB. Critical revision of article: EW.

References

1. Momol MT, Aldwinckle HS (2000) Genetic Diversity and Host Range of Erwinia amylovora. In: Vanneste JL, ed. Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora. London: CAB, International. pp 55–72.
2. Thomson SV (2000) Epidemiology of Fire Blight. In: Vanneste JL, ed. Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora. London: CAB International. pp 9–36.
3. Thomson SV (1996) The Role of the Sigma in Fire Blight infections. Phytopathology 76: 476–482.
4. Bubin T, Orosz-Kovács Z, Farkas A (2003) The nectary as the primary site of infection by Erwinia amylovora (Burr). Winslow et al.: a mini review. Plant Syst Evol 230: 183–194.
5. Baldo A, Norell JL, Farrell RE, Jr., Bassett CL, Aldwinckle HS, et al. (2010) Identification of genes differentially expressed during interaction of resistant and susceptible apple cultivars (Malus x domestica) with Erwinia amylovora. BMC Plant Biol 10: 1.
6. Blachinsky D, Stienenberg D, Zarnoki E, Weinhal D, Manulis S (2006) Effects of pear tree physiology on fire blight progression in perennial branches and on expression of pathogenicity genes in Erwinia amylovora. Eur J Plant Pathol 116: 313–324.
7. Norrell J, Farrell R, Bassett C, Baldo A, Lalli D, et al. (2009) Rapid transcriptional response of apple to fire blight disease revealed by cDNA suppression subtractive hybridization analysis. Tree Genetics & Genomes 5: 27–40.
8. Venise JS, Maloy M, Faire M, Paulin JP, Brisset MN (2002) Modulation of defense responses of Malus spp. during compatible and incompatible interactions with Erwinia amylovora. Mol Plant Microbe Interact 15: 1204–1212.
9. Wang D, Korhan SS, Zhao Y (2010) Molecular signature of differential virulence in natural isolates of Erwinia amylovora. Phytopathology 100: 192–196.
10. Wei ZM, Snedden BJ, Beer SV (1992) Expression of Erwinia amylovora hpa genes in response to environmental stimuli. J Bacteriol 174: 1873–1882.
11. Zhao Y, Blumer SE, Sundin GW (2005) Identification of Erwinia amylovora genes induced during infection of immature pear tissue. J Bacteriol 187: 8008–8103.
12. Sarowar S, Zhao Y, Soria-Guerra RE, Ali S, Zheng D, et al. (2011) Expression profiles of differentially regulated genes during the early stages of apple flower infection with Erwinia amylovora. J Exp Bot 62: 4951–4961.
13. Buttner D, He SY (2009) Type III protein secretion in plant pathogenic bacteria. Plant Physiol 150: 1656–1664.
14. Melotto M, Underwood W, He SY (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. Annu Rev Phytopathol 46: 101–122.
15. Oh CS, Beer SV (2005) Molecular genetics of Erwinia amylovora involved in the development of fire blight. FEMS Microbiol Lett 253: 185–192.
16. Oh CS, Kim JF, Beer SV (2005) The Hrp pathogenicity island of Erwinia amylovora and identification of three novel genes required for systemic infection. Mol Plant Pathol 6: 125–138.
17. Wei ZM, Beer SV (1995) hpaL activates Erwinia amylovora hpa gene transcription and is a member of the ECF subfamily of sigma factors. J Bacteriol 177: 6201–6210.
18. Jin Q, Hu W, Brown I, McGhee G, Hart P, et al. (2001) Visualization of secreted Hrp and Aer proteins along the Hrp pilus during type III secretion in Erwinia amylovora and Pseudomonas syringae. Mol Microbiol 40: 1129–1139.
19. Kim JF, Wei ZM, Beer SV (1997) The hpaL and hpaO genes encode components of a type III pathway that secretes harpin. J Bacteriol 179: 1690–1697.
20. Barny MA (1995) Erwinia amylovora hpaV mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. Eur J Plant Pathol 101: 335–340.
21. Wei ZM, Laby RJ, Zumoff CH, Bauer DW, He SY, et al. (1992) Harpin, elicitor of the hypersensitive response produced by the plant pathogen Erwinia amylovora. Science 257: 85–88.
22. Perino C, Gaudriault S, Vian B, Barny MA (1999) Visualization of harpin secretion in planta during infection of apple seedlings by Erwinia amylovora. Cell Microbiol 1: 131–141.
23. Engelhardt S, Lee J, Gabler Y, Kemmerling B, Haapalainen ML, et al. (2009) Separable roles of the Pseudomonas syringae pv. phaseolicola accessory protein HrpZ1.
in ion-conducting pore formation and activation of plant immunity. Plant J 57: 706–717.

24. Oh, J, Kim, JG, Jeon, E, Yoo, CH, Moon, JS, et al. (2007) Amyloidogenesis of type III-dependent harpins from plant pathogenic bacteria. J. Biol Chem 282: 15601–15609.

25. Bosanczy AM, Niissen RM, Oh CS, Beer SV (2008) HrpN of Erwinia amylovora functions in the translocation of DspA/E into plant cells. Mol Plant Pathol 9: 423–434.

26. Barro MA, Guinebertiere MH, Marcian R, Couissac E, Paulin JP, et al. (1990) Cloning of a large gene cluster involved in Erwinia amylovora CFBP1430 virulence. Mol Microbiol 4: 777–786.

27. Bogdanove AJ, Bauer DW, Beer SV (1998) Erwinia amylovora secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. J Bacteriol 180: 2244–2247.

28. Bogdanove AJ, Kim JF, Wei Z, Kolchinsky P, Charkowski AO, et al. (1998) Homology and functional similarity of an hrp-linked pathogenicity locus, dseEF, of Erwinia amylovora and the avirulence locus arcA of Pseudomonas syringae pathovar tomato. Proc Natl Acad Sci U S A 95: 1932–1937.

29. Gauthriault S, Malandrin L, Paulin JP, Barny MA (1997) DspA, an essential pathogenicity factor of Erwinia amylovora showing homology with AvrE of Pseudomonas syringae, is secreted via the Hrp secretion pathway in a DspH-dependent way. Mol Microbiol 26: 1057–1069.

30. Meng X, Bonasera JM, Kim JF, Niissen RM, Beer SV (2006) Apple proteins that interact with DspA/E, a pathogenicity effector of Erwinia amylovora, the fire blight pathogen. Mol Plant Microbe Interact 19: 53–61.

31. DebRoy S, Thilmony R, Kwack YB, Nomura K, He SY (2004) A family of Erwinia amylovora HrpN(ea) harpin from E. amylovora hrpA mutant of Pseudomonas syringae pv. tomato DC3000 in infected tomato leaves. Mol Plant Microbe Interact 17: 1250–1258.

32. Bouroue T, Rombaut J, Rouine E, Taira S, Romantochuk M (2002) Localization of hrp-induced Pseudomonas syringae pv. tomato DC3000 in infected tomato leaves. Mol Plant Pathol 3: 451–460.

33. Jin Q, Thilmory R, Zwieслer-Völlick J, He SY (2003) Type III protein secretion in Pseudomonas syringae. Microbes Infect 5: 301–310.

34. Gesbron S, Paulin JP, Thraourd M, Barny MA, Brisset MN (2006) The alternative sigma factor HrpL negatively modulates the flagellar system in the phytopathogenic bacterium Erwinia amylovora under hrp-inducing conditions. FEMS Microbiol Lett 257: 221–227.

35. Barro MA, Brisset M, Perino C, Vian B, Barny M, et al. (2006) Protection of apple against fire blight induced by an hrpB mutant of Erwinia amylovora. Biologia Plantarum 50: 667–674.

36. Thomson SV, Gouk SC (2003) Influence of Age of Flowering Plants on Erwinia amylovora and Biological Control Agents. Plant Disease 87: 562–569.

37. Bugert P, Gräder K (1995) Molecular analysis of the ams operon required for exopolysaccharide synthesis of Erwinia amylovora. Mol Microbiol 15: 917–933.

38. Verisse JS, Barny MA, Paulin JP, Brisset MN (2003) Involvement of three pathogenicity factors of Erwinia amylovora in the oxidative stress associated with compatible interaction in pear. FEBS Lett 537: 198–202.

39. Thaïsères R, Sampa PD, Panopoulos NJ, Stevens C, Mansfield JW (2004) Transcriptional regulation of components of the type III secretion system and effectors in Pseudomonas syringae pv. phaseolicola. Mol Plant Microbe Interact 17: 1259–1265.

40. Bouroue T, Dizakrouz-Bouteau H, Garnier A, Brisset MN, Perino C, et al. (2006) DspA/E, a type III effector essential for Erwinia amylovora pathogenicity and growth in plants, induces cell death in host apple and nonhost tobacco plants. Mol Plant Microbe Interact 19: 16–24.

41. Niissen RM, Zierberb AJ, Bogdanove AJ, van Wijk KJ, Beer SV (2007) Analyses of the secretomes of Erwinia amylovora and selected hrp mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. Mol Plant Pathol 8: 55–67.

42. Milevičová R (2009) Different aspects of plant responses to fire blight in apple [PhD thesis]. Vienna, Austria: University of Natural Resources and Life Sciences. 109 p.

43. Pauliñas PG, Tisantos J (2000) Epidemiology of Fire Blight. In: Vanneste JL, ed. Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora. London: CAB International. pp 199–234.

44. Bugert P, Gräder K (1995) Molecular analysis of the ams operon required for exopolysaccharide synthesis of Erwinia amylovora. Mol Microbiol 15: 917–933.

45. Pusey PL, Stockwell VO, Rudell DR (2008) Antibiosis and acetylation by Pantoea agglomerans strain EI25 may contribute to suppression of Erwinia amylovora. Phytopathology 98: 1136–1143.

46. Nakka S, Qi M, Zhao Y (2010) The Erwinia amylovora PhoPQ system is involved in resistance to antimicrobial peptide and suppresses gene expression of two novel type III secretion systems. Microbes Res 165: 665–673.

47. van der Zet T, Keil HL (1979) Fire blight: A bacterial disease of rosaceous plants. Washington: USDA, Washington, DC. 200 p.

48. Chauvet A, Fu ZQ, Alfano JR (2008) Phytopathogen type III effector

49. Milcevicova´ R (2009) Different aspects of plant responses to fire blight in apple [PhD thesis]. Vienna, Austria: University of Natural Resources and Life Sciences. 109 p.

50. Pauliñas PG, Tisantos J (2000) Epidemiology of Fire Blight. In: Vanneste JL, ed. Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora. London: CAB International. pp 199–234.

51. Pusey PL, Rudell DR (2008) Antibiosis and acetylation by Pantoea agglomerans strain EI25 may contribute to suppression of Erwinia amylovora. Phytopathology 98: 1136–1143.

52. Chauvet A, Fu ZQ, Alfano JR (2008) Phytopathogen type III effector

53. Milcevicova´ R (2009) Different aspects of plant responses to fire blight in apple [PhD thesis]. Vienna, Austria: University of Natural Resources and Life Sciences. 109 p.

54. Bogdanove AJ, Kim JF, Beer SV (2000) Disease-specific Genes of Erwinia amylovora: Keys to Understanding Pathogenesis and Potential Targets for Disease Control. In: Vanneste JL, ed. Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora. London: CAB International. pp 163–177.

55. He SY, Nomura K, Whitman TS (2004) Type III protein secretion mechanism in mammalian and plant pathogens. Biotechnol Bioprospecta 1694: 101–206.

56. Kuenen S (2003) Fire blight control in organic fruit growing – systematic investigation of the mode of action of potential control agents. 1st Symposium on Biocontrol of Bacterial Plant Diseases; 2005 October, 23–26; University of Konstanz, Germany. Mitt. Biol. Bundesanst. Land-Forstwirtschaft 408, pp 249–253.

57. Pusey PL (1997) Crab apple blossoms as a model for research on biological control of fire blight. Phytopathology 87: 1096–1102.

58. Chang S, Puryear J, Caimrey J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11: 133–136.

59. Takeda GW, Toth IK, Burkhart MB (2007) Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen Pellicbacter atrocipticum. BMC Plant Biol 7: 50.

60. Milevičová R, Gusch C, Halbshwirth H, Stich K, Hanke M-V, et al. (2010) Erwinia amylovora-induced defense mechanisms of two apple species that differ in susceptibility to fire blight. Plant Pathol 179: 60–67.

61. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.