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Insect eggs induce a systemic acquired resistance in Arabidopsis

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SUMMARY

Although they constitute an inert stage of the insect's life, eggs trigger plant defences that lead to egg mortality or attraction of egg parasitoids. We recently found that SA accumulates in response to oviposition by the Large White butterfly *Pieris brassicae*, both in local and systemic leaves, and that plants activate a response that is similar to the recognition of pathogen-associated molecular patterns (PAMPs), which are involved in PAMP-triggered immunity (PTI). Here we discovered that natural oviposition by *P. brassicae* or treatment with egg extract inhibit growth of different *Pseudomonas syringae* strains in Arabidopsis through the activation of a systemic acquired resistance (SAR). This egg-induced SAR involves the metabolic SAR signal pipecolic acid, depends on ALD1 and FMO1, and is accompanied by a stronger induction of defence genes upon secondary infection. Although *P. brassicae* larvae showed a reduced performance when feeding on *P. syringae*-infected plants, this effect was less pronounced when infected plants had been previously oviposited. Altogether, our results indicate that egg-induced SAR might have evolved as a strategy to prevent the detrimental effect of bacterial pathogens on feeding larvae.

INTRODUCTION

Upon recognition of herbivory, plants deploy a variety of defences, including the production of poisonous metabolites, antinutritive proteins, and the emission of volatiles that attract predators (Howe and Jander, 2008). These responses are the result of massive transcriptional changes that are predominantly controlled by the jasmonic acid (JA) pathway (Reymond et al., 2004; de Vos et al., 2005; Kempema et al., 2007). Consequently, *Arabidopsis thaliana* mutants impaired in JA biosynthesis or signalling are more susceptible to insect feeding (McConn et al., 1997; Reymond et al., 2004; Bodenhausen and Reymond, 2007).

Even though insect eggs do not constitute a direct threat for plants they trigger host defences that lead to egg desiccation, drop-off, and mortality, or attraction of egg parasitoids (Hilker and Meiners, 2011; Reymond, 2013). However, the expression profile of Arabidopsis leaves after oviposition by the Large White butterfly *Pieris brassicae* is drastically distinct from the profile obtained after larval feeding (Little et al., 2007). Surprisingly, genes induced by oviposition include known targets of the salicylic acid (SA) signalling pathway (Little et al., 2007). Indeed, SA accumulates in Arabidopsis in response to oviposition by *P. brassicae*, both in local and systemic leaves (Bruessow et al., 2010), and major components of the SA pathway are required for egg extract-induced defence gene expression (Gouhier-Darimont et al., 2013). Also, egg-derived elicitors activate SA-dependent gene expression, a process that is similar to the recognition of pathogen-associated molecular patterns (PAMPs) during PAMP-triggered immunity (PTI) (Gouhier-Darimont et al., 2013). Unexpectedly, treatment with *P. brassicae* and *Spodoptera littoralis* egg extracts suppresses the induction of JA-dependent genes in a SA-dependent manner and leads to enhanced larval performance

of the generalist *S. littoralis* (Bruessow et al., 2010). Since SA- and JA-pathways are known to negatively impact each other (Pieterse et al., 2009), this finding suggests that insect eggs may highjack the SA pathway to down regulate defence against feeding larvae.

In addition to its negative impact on JA signalling, egg-triggered SA accumulation might prevent secondary infections, similarly to systemic acquired resistance (SAR). SAR is an inducible defence response that follows a primary infection by a pathogen and results in a systemic protection in the entire plant against a broad-spectrum range of pathogens (Sticher et al., 1997; Vlot et al., 2008; Shah and Zeier, 2013). SAR requires the activation of the SA pathway, involves the translocation of a mobile signal and primes systemic leaves for a stronger and prolonged expression of defences genes (Jung et al., 2009; Vlot et al., 2009; Conrath, 2011; Návarová et al., 2012; Shah and Zeier, 2013). SAR was initially associated with the perception of pathogen-derived effectors by plant-encoded resistance genes, a process called effector-triggered immunity (ETI) that is derived evolutionary from PTI, and with the development of a hypersensitive reaction (HR). However, it is now accepted that both PTI and ETI induce SAR (Mishina and Zeier, 2007; Dempsey and Klessig, 2012). Several metabolites have been identified that might play a role as SAR mobile signals. These include methyl salicylate, azelaic acid (AzA), glycerol-3-phosphate (G3P) and dihydroabietinal (DA) (for review, see Shah and Zeier, 2013; Fu and Dong, 2013). Genetic studies indicate that the transport of AzA, G3P and DA requires the putative lipid-transfer protein DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1) (Shah and Zeier, 2013; Fu and Dong, 2013). The recently discovered lysine catabolite pipecolic acid (Pip) plays also a crucial role in SAR. Upon pathogen infection, Pip accumulates in

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local and systemic leaves as well as in petiole exudate (Návarová et al., 2012). Elevated Pip is sufficient to enhance resistance to bacterial pathogens and prime plants for effective defence activation including SA accumulation, phytoalexin production, and defence gene expression (Návarová et al., 2012). Pip synthesis depends on the activity of the aminotransferase ALD1 (AGD2-LIKE DEFENCE RESPONSE PROTEIN1). Consequently, *ald1* mutant fails to accumulate SA in distal leaves and are compromised in SAR (Návarová et al., 2012). In addition to ALD1 (Song et al., 2004), FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE1) is required for systemic accumulation of SA, systemic defence gene expression, and SAR (Mishina and Zeier, 2006). FMO1 is also necessary for Pip-induced resistance and has therefore been proposed as a downstream component of Pip signalling (Mishina and Zeier, 2006; Zeier, 2013).

In this study, we show that natural oviposition or treatment with egg extract inhibit bacterial infection in Arabidopsis through the activation of SAR. This response is lost in *ald1* and *fmo1* mutants, implicating Pip as a systemic signal responsible for egg-induced SAR. Our results unravel a unique strategy potentially evolved by eggs to prevent the detrimental effect of bacterial pathogens on feeding larvae.

RESULTS

Oviposition and treatment with egg extract inhibit bacterial infection

We previously found that the SA pathway is activated in response to oviposition by *P. brassicae* (Bruessow et al., 2010; Gouhier-Darimont et al., 2013) and reasoned that this might enhance plant resistance against bacterial infection. *P. brassicae* butterflies were allowed to deposit egg batches on Arabidopsis plants and 4 to 5 days later eggs were gently removed from the plants, just before hatching of larvae. Local or distal leaves were then infected by dip inoculation with virulent *Pseudomonas syringae* pv. *tomato* DC3000 DB29 (*Pst* COR⁻) for 2 days. To avoid the antagonistic effect of the JA-mimic coronatine (COR) on the SA pathway, we initially tested a mutant strain of *Pst* that is unable to synthesize this virulence factor. Compared to control plants, oviposited plants showed a significantly reduced bacterial growth, both in local and distal leaves (Figure 1a). In a follow-up experiment, plants were pre-treated with *P. brassicae* egg extract for five days and further challenged with *Pst* COR⁻. The amount of egg extract applied to each leaf was equivalent to approximately one egg batch. A similar inhibition of bacterial growth was observed after treatment with by *P. brassicae* egg extract (Figure 1b), confirming earlier observations that application of egg extract mimics responses triggered by natural egg deposition (Little et al., 2007; Bruessow et al., 2010). Growth of wild-type *Pst* DC3000 was also inhibited by both oviposition or egg extract pre-treatment, suggesting that COR has no impact on this response (Figure 1c,d). Since oviposition by *P. brassicae* butterflies is more difficult to standardize, we thus carried-out all subsequent experiments with egg extract. Egg extract-induced resistance was observed with the avirulent strain *Pst* DC3000/AvrRPM1 (Figure 1e) and with the related pathogen *Pseudomonas syringae* pv. *maculicola* (Figure 1f). A resistance was

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also observed when leaves were infiltrated with *Pst* COR⁻, indicating that this response is robust and does not depend on the method of infection (Figure S1). Finally, a 3-days egg extract treatment already induced a significant inhibition of bacterial growth (Figure S2). Thus, leaves are resistant to infection even before hatching of the eggs.

Egg-induced inhibition of bacterial growth is similar to SAR

The observation that insect eggs enhanced resistance against bacterial infection in systemic leaves suggested that eggs might trigger SAR. To test this hypothesis, we first carried-out an experiment where the effect of primary infection or egg-extract treatment on secondary infection were compared simultaneously. Lower leaves were either treated with egg extract for five days or infiltrated with *Pst* COR⁻ for two days. After this initial treatment, upper leaves were challenged with *Pst* COR⁻ and bacterial growth was scored two days later. Both pre-treatments triggered a significant and similar reduction of bacterial growth in systemic leaves compared to control plants, consistent with the hypothesis that eggs induce a true SAR (Figure 2a).

Several chemical signals have been postulated to be mobile and mediate SAR in systemic leaves (Shah and Zeier, 2013; Fu and Dong, 2013). Among them, the recently discovered Pip is a Lys catabolite whose biosynthesis depends on ALD1 (Návarová et al., 2012). Having noticed that *ALD1* expression is induced by oviposition (Little et al., 2007) we thus tested the involvement of Pip in egg extract-induced SAR and analysed free amino acids in plants treated for five days with *P. brassicae* egg extract. A significant increase of several amino acids was observed, predominantly in treated leaves. Interestingly, Pip and alpha-aminoadipic acid (Aad), another Lys catabolite, showed a strong accumulation in both local and distal leaves (Figure 2b and Table S1).

Pip levels in distal leaves of egg extract-treated plants were in the same range as those reported for distal leaves of plants infected with *Psm* (Návarová et al., 2012). Consistent with our hypothesis, egg extract-induced SAR was lost in *ald1* and *fmo1* mutants (Figure 2c). Furthermore, egg extract-induced accumulation of Pip and SA in systemic leaves was abolished in *ald1* and *fmo1*, confirming that establishment of egg extract-induced SAR requires Pip and the regulator FMO1 (Figure 3a,b).

We further tested whether egg extract-induced inhibition of bacterial infection was dependent on SA signalling. We selected the double mutant *pad4* (*phytoalexin-deficient4*) x *enhanced disease susceptibility1* (*eds1*) and *npr1*, since PAD4 and EDS1 are central upstream regulators of the SA pathway (Wiermer et al., 2005; Wang et al., 2008; Fu and Dong, 2013) and NPR1 is a crucial component downstream of SA accumulation (Fu and Dong, 2013). Inhibition of *Pst* COR$^-$ growth by egg extract pre-treatment was abolished in *eds1-2 pad4-1* and *npr1* (Figure 2c), in accordance with the requirement of a fully functional SA pathway for egg extract-induced SAR.

Priming of defence gene expression

Establishment of SAR is accompanied by a stronger activation of defence upon secondary infection, a process called priming (Prime-A-Plant Group et al., 2006). In Arabidopsis, priming of defence metabolite accumulation and defence gene expression was observed after a secondary infection with *Psm* (Návarová et al., 2012). To investigate if egg extract-induced SAR was also conferring a primed state in distal leaves, we monitored the expression of *PR-1*, a known SA-associated marker gene. Plants were either treated with egg extract for five days or left untreated and, subsequently, distal leaves were infiltrated with *Pst* COR$^-$ for 24 h. Pre-treatment with

egg extract led to a significantly higher *PR-1* expression upon secondary infection (Figure 4a), suggesting that egg extract-induced SAR primes the plant for a more vigorous defence response and might explain reduced bacterial growth in egg-treated plants. A similar priming was observed for *NHL10* and *FRK1* (Figure 4b,c), which are two early PAMP-responsive genes (Boudsocq et al., 2010).

Reduced performance of *P. brassicae* larvae on infected plants

Since primary infection of plants with pathogens can either negatively or positively impact herbivore performance (reviewed Felton and Korth, 2000; Stout et al., 2006), we decided to test whether egg-induced inhibition of bacterial growth might benefit *P. brassicae* in Arabidopsis. First, leaves were inoculated with *Pst* and further challenged with first-instar *P. brassicae* larvae. After 6, 8, and 10 days of feeding, larvae were significantly smaller when fed with infected plants compared to mock inoculated plants, indicating that, in these conditions, infection of Arabidopsis leaves was detrimental to this lepidopteran species (Figure 5a). Then, to assess whether the presence of insect eggs may allow a better performance of *P. brassicae* on infected plants, we compared the weight of larvae after feeding on *Pst*-inoculated plants that were either exposed to prior oviposition or not. Strikingly, larvae gained significantly more weight on oviposited and infected plants than on plants that were only infected (Figure 5b). Thus, the bacterial growth inhibition that is observed after oviposition or egg treatment (Fig. 1) is strong enough to have a significant impact on larval development.

DISCUSSION

In nature, plants are generally exposed to simultaneous or sequential attacks by different enemies that compete for the same resources. Depending on the type of attacker, distinct signalling pathways are activated that lead to specific defence gene expression. In recent years, a growing body of literature showed that these pathways are intricately connected and their complex regulation is thought to allow the plant to optimize defences against various attackers (Pieterse et al., 2012). As a result of an on-going arms race, insects or pathogens have also evolved strategies to suppress or manipulate plant defences (Zhu-Salzman et al., 2005; Boller and He, 2009;). However, there is little information on direct interactions between enemies on the leaf surface and whether the plant contributes to these interactions. Here we demonstrate that both natural oviposition or egg extract treatment protect plants against bacterial infection for the benefit of larvae by activating SAR and unravels an unexpected aspect of the plant's response to oviposition. We also provide genetic and chemical evidence that egg extract-induced SAR is highly similar to a bacterial-induced SAR and that it requires the mobile signal Pip.

MeSA is another signal potentially involved in SAR. Experiments in tobacco showed that SA that accumulates in locally-infected leaves is converted to MeSA, which is mobile and can be converted back to SA in systemic tissues (Park et al., 2007). However, an Arabidopsis SA methyltransferase knockout mutant is still competent for bacterial-induced SAR (Attaran et al., 2009) and the corresponding *BSMT1* gene is not induced by oviposition (Little et al., 2007), nor SA treatment (Chen et al., 2003). Furthermore, MeSA emission could not be detected after oviposition by *P. brassicae* in Arabidopsis and *Brassica nigra* (Fatouros et al., 2012; Groux et al., 2014). Collectively,

these data do not support a role for MeSA in egg-induced SAR but future studies with

mutants should investigate the contribution of this metabolite. It will also be interesting

to test the role of DIR1-dependent mobile signals. However, our finding that egg extract

treatment failed to inhibit bacterial growth in the Pip biosynthesis mutant *ald1* clearly

indicates that this metabolite is critical for establishing a fully resistant state in both

local and distal leaves.

Strikingly, natural oviposition or egg extract treatment protected both local and

distal leaves against secondary infection. In bacteria-induced SAR, the development of

disease in local leaves after a primary infection prevents accurate measurement of a

secondary infection in the same leaf. Thus, the concept of SAR has always been

envisaged for distal leaves while it could already take place in the vicinity of a primary

infection. Indeed, in the case of a more localized stimulus triggered by egg deposition, a

protective effect could be observed in the treated leaf. This implies that a mobile signal

spread within the oviposited leaf where it triggered a local acquired resistance. Such

biological system could be helpful in the future to track the generation and early

movement of SAR signals.

Apparently, egg-induced SA accumulation may benefit larvae in two ways.

First, SA-dependent inhibition of the JA-pathway was shown to reduce the expression

of defence genes and to enhance larval performance of a generalist herbivore, although

this was not effective against the specialist *P. brassicae* (Bruessow et al., 2010).

Recently, two studies reported reduced larval performance of *P. brassicae* on plants that

were previously oviposited (Pashalidou et al., 2012; Geiselhardt et al., 2013). Thus, the

importance and specificity of SA-JA antagonism in response to oviposition might

depend on the species considered or on the feeding pressure. Second, we report here

that eggs induce SAR and that feeding on infected plants is detrimental to larvae. This SAR might protect larvae against a direct toxic effect of leaf pathogens and/or favour their development by inhibiting phytopathogens that compete for the same resources. Recently, root colonizing pseudomonads were shown to exhibit insecticidal activity (Ruffner et al., 2013). *Pseudomonas fluorescens* CHA0 contains a gene cluster encoding a Fit toxin that is conserved in other entomopathogenic bacteria and that kills several lepidopteran larvae via oral infection (Péchy-Tarr et al., 2008; Ruffner et al., 2013; Kupferschmied et al., 2014). The bean phytopathogen *Pseudomonas syringae* pv. *syringae* B728a was found to be toxic to the pea aphid, *Acyrthosiphon pisum* (Stavrinides et al., 2009). However, whether leaf bacterial pathogens are toxic to lepidopteran larvae is unknown and future studies should address this point. Assays that were developed to test the toxicity of fungal metabolites on *Spodoptera frugiperda* cell lines could be useful (Fornelli et al., 2004).

It is quite remarkable that Arabidopsis triggers an egg-induced SAR that is potentially beneficial to the attacker. One can envisage that during evolution *P. brassicae* eggs have hijacked the SA pathway for the success of their progeny. However, in response to oviposition the SA pathway controls the expression of hundreds of genes, some of which might be crucial for a direct defence against insect eggs (Little et al., 2007). For instance, an HR-like necrosis is observed underneath eggs in several plant species and has been associated with egg drop-off from the leaf (Reymond, 2013). Indeed, genes involved in programmed cell death are induced by *P. brassicae* oviposition (Little et al., 2007). Therefore, activation of the SA pathway may underlie a trade-off between a positive effect consisting of direct defences against insect eggs and a negative effect associated with enhanced larval survival and performance.

Obviously, the extent of this trade-off depends on the occurrence of insect eggs and leaf pathogens simultaneously. The discovery of egg-induced SAR is intriguing and its relevance will have to be further studied in natural environments. In addition, since oviposition has been shown to elicit the JA pathway in some plant/egg interactions (Meiners and Hilker, 2000; Büchel et al., 2012; Kim et al., 2012), whether SA-mediated responses to eggs are widespread will have to be studied.

An alternative explanation for the existence of egg-induced SAR is that larval feeding creates entries for pathogenic bacteria. In a recent field experiment with *Cardamine cordifolia* Humphrey et al. (Humphrey et al., 2014) found that plants damaged by chewing herbivores displayed a higher level of bacteria load. Infection might be more detrimental to plant development and reproductive success than herbivory. Detection of egg-associated elicitors provides an early "warning" signal that allows deployment of defences in anticipation of the attack (Beyaert et al., 2012; Geiselhardt et al., 2013; Lucas-Barbosa et al., 2013; Pashalidou et al., 2013). It will be interesting to compare the impact of herbivory and infection on plant fitness.

The observation that *P. brassicae* eggs induce SA accumulation in Arabidopsis (Bruessow et al., 2010), which in turn may lead to a better performance of feeding larvae by either reducing leaf pathogen growth (this study) or by inhibiting the JA pathway (Bruessow et al., 2010), raises the question whether strategies to control bacterial or fungal pathogens by spraying SA-analogues are optimal. For instance, application of the SAR activator benzothiadiazole (BTH) provides protection against a variety of leaf pathogens (Lawton et al., 1996; Görlach et al., 1996). It would be interesting to test whether BTH treatment in crop fields has an effect on the abundance and performance of insect herbivores.

Evidence that mutual interactions between plants, insects and bacteria are taking place during multiple attacks is accumulating. Local leaf infection by *P. syringae* was found to cause a systemic induced susceptibility (SIS) to larvae of the moth *Trichoplusia ni* in Arabidopsis (Cui et al., 2005). Induction of SIS is modulated by a combined action of PAMP-triggered SA signalling that inhibits JA-dependent defences and effector-induced ethylene signalling that blocks antiherbivore defence in a JA-independent way. In addition, SIS is counteracted by COR (Groen et al., 2013). Here, we showed that *P. brassicae* larval performance was reduced on infected plants. It this case larvae were feeding on leaves containing bacteria and this might have overridden a SIS effect. In another study, larvae of the Colorado potato beetle were found to suppress JA-dependent defences in tomato through the presence of bacteria in oral secretions (Chung et al., 2013). Recently, mutualistic ants associated with the acacia tree *Acacia hindsii* were found to protect their host against leaf pathogens, although this effect was attributed to the presence of competing bacteria on ant legs (González-Teuber et al., 2014). These fascinating examples and our discovery that insect eggs inhibit bacterial growth open the way for more studies on how plants respond to a combination of different attackers, how they affect each other, and what mechanism shape these complex interactions.

EXPERIMENTAL PROCEDURES

Plant material and insect growth conditions

Arabidopsis thaliana plants were sown in moist potting compost and vernalized for 2 days at 4°C. The *ald1* (*ald1*_T2; Song et al., 2004) mutant corresponds to the SALK T-DNA insertion line SALK_007673. Other lines used in this study are *fmo1* (Mishina and Zeier, 2006), *npr1-1* (Nottingham Arabidopsis Stock Centre) and *eds1-2 pad4-1* double mutant (*eds1pad4*; obtained from Jane Parker, MPI, Köln, Germany). All mutants are in the Col-0 background. Plants were grown in growth chambers as reported previously (Reymond et al., 2000) and were four-week-old at the time of treatment.

Pieris brassicae (large white butterfly) were reared on *Brassica oleracea* var. *gemmifera* as described previously (Schlaeppi et al., 2008).

Oviposition and treatment with egg extract

For experiments with natural oviposition, 15 plants were placed in a 60x60x60 cm tent containing ca. 30 *P. brassicae* butterflies. After 24 h, 4 plants containing one egg batch on two leaves were placed in a growth chamber for 4.5 days. Just before hatching, eggs were gently removed with a forceps and leaves were further infected with bacteria. Control plants were kept in the same conditions without butterflies.

For egg extract preparation*, Pieris brassicae* eggs collected from cabbage leaves were crushed with a pestle in Eppendorf tubes. After centrifugation (15'000 g for 3 min), the supernatant (egg extract) was stored at -20°C. For egg extract application, 2 x 2 µl of egg extract were spotted under the surface of each of two leaves of each treated

plant. Four plants were treated with egg extract 5 days before bacterial infection. Untreated plants were used as controls.

Cultivation of bacteria and plant infection

Bacterial strains *P. syringae* pv. *tomato* DC3000 (*Pst*), *P. syringae* pv. *tomato* DC3000 DB29 (*Pst* COR⁻), *P. syringae* pv. *tomato* DC3000/avrRpm1 (*Pst* AvrRpm1) and *P. syringae* pv. *maculicola* ES4326 (*Psm*) were streaked from a -80°C glycerol stock onto a low salt Luria Bertani (LB) medium (10 g/l BactoTryptone, 5 g/l Yeast Extract, 5 g/l NaCl and 14 g/l Bactoagar, pH=7.0). Antibiotics used for *P. syringae* strains was (in µg/ml): *Pst*: rif 50; *Pst* COR⁻: rif 50, kan 50 and spec 50; *Pst* avrRpm1: rif 50 and kan 50; *Psm*: strep 50. Bacteria were transferred into 6 ml of liquid culture in LB with antibiotic(s) and grown at 28°C O/N. 1 ml of the culture was then diluted in 5 ml of fresh LB for 4 hours to produce a bacterial culture in an exponential growth phase.

For infection by dipping, bacterial cultures were centrifuged at 7000 rpm for 2 min. The supernatant was discarded and the pellet diluted in 10 mM $MgCl_2$ and 0.02% (v/v) Silwet L-77 (Agridyne) to an OD600 of 0.05 for *Pst* COR⁻ and *Pst* avrRpm1, 0.02 for *Pst* and 0.1 for *Psm* in order to obtain an initial *in planta* titer of 50 to 500 cfu per 0.5 cm² leaf disc. Two leaves per plant were dipped into bacterial solution for 8 sec. Plants were watered and kept under high humidity under a cover for 24 h before infection. For infection by infiltration, bacterial solutions were prepared the same way except that Silwet L-77 was omitted and leaves were infiltrated using a 1 ml needleless syringe with a bacterial solution at an OD600 of 0.0005 to obtain a similar cfu/leaf disc than with dipping. For each treatment and for each time point, 8 leaves from 4 plants

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were used for bacterial titer determination. The experiment was replicated at least three times for each bacterial strain.

For priming experiments, local leaves were treated with egg extract for 5 days and then distal leaves were infiltrated with *Pst* COR⁻ for 24 h. Control plants were infiltrated with 10 mM MgCl$_2$. Distal leaves were collected for RNA extraction and gene expression analyses.

Bacterial growth determination

Leaves were collected 0 h and 48 h after infection and dipped in a bath of 70% ethanol to sterilize the leaf surface and then washed twice with water. For each leaf, a leaf disc of 0.5 cm^2 was excised and placed in a 2 ml Eppendorf tube with four glass beads. Leaf discs were grinded using a TissueLyser II (Qiagen) at 30'000 rpm for 1 min. 500 μl of 10 mM MgCl$_2$ was added in each tube and tubes were placed in the TissueLyser for 30 sec at 10'000 rpm to suspend bacteria. Each sample was then diluted in series of 1:10. 5 μl of each dilution were then spotted on LB plates with appropriate antibiotic(s). For t = 0 h, 100 μl of the non-diluted bacterial solution were directly spotted on LB plates with appropriate antibiotic(s). Plates were incubated at 28°C for 48 h and cfus were counted.

Free amino acid and SA analysis

Two leaves from three plants were pre-treated with 2 x 2 μl of *Pieris brassicae* egg extract. After 5 days, local and distal leaves were harvested, weighted, and frozen in liquid nitrogen in 2 ml Eppendorf tubes. Untreated plants were used as controls. Quantification of pipecolic acid, free amino acids, and salicylic acid was done as described previously (Návarová et al., 2012).

SUBMITTED MANUSCRIPT

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using an RNeasy® plant mini kit (Qiagen). DNase treatment was added to the protocol. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a final volume of 25 µl. Each cDNA sample was generated in triplicate and diluted eightfold with water. Gene-specific primers were designed to produce amplicons of 70 to 200 bp from the 3' end of the cDNA strand. At2g14610 (*PR-1*) forward: 5′-GTGGGTTAGCGAGAAGGCTA-3′, reverse: 5′-ACTTTGGCACATCCGAGTCT-3′; At2g19190 (*FRK1*) forward: 5'-TACGGCTCTTGTTGAACACT-3', reverse: 5'-TCACTATACGCGGTGTCCAT-3'; At2g35980 (*NHL10*) forward: 5'-GGATCGGACTCTACTACGAT-3', reverse: 5'-TAAAGTCCTAGACTGTCCGG-3'. Quantitative real-time PCR analysis was performed in a final volume of 20 µl containing 2 µl of cDNA, 0.2 µM of each primer, 0.03 µM of reference dye, and 10 µl of Brilliant III Ultra Fast SYBR® Green QPCR Master Mix (Agilent). Reactions were performed using an Mx3000P® real-time PCR machine (Agilent) with the following program: 95°C for 3 min, then 40 cycles of 10 sec at 95°C and 20 sec at 60°C. Relative mRNA abundance was normalized to the housekeeping gene At2g28390 (*SAND*), forward: 5'-AACTCTATGCAGCATTTGATCCACT -3', reverse: 5'-TGATTGCATATCTTTATCGCCATC -3'.

Insect performance assays

All leaves of four-week-old Col-0 plants were infiltrated using a needleless syringe with *Pst* diluted to an OD600 of 0.005. Control plants were infiltrated with a 10 mM MgCl$_2$

mock solution. Plants were incubated for two days in a growth chamber in order to let

bacteria proliferate. After incubation, five freshly hatched *P. brassicae* larvae were

placed on each of 4 infected plants. Plants were placed in a transparent plastic box and

kept in a growth room during the whole experiment. After six days of feeding, larvae

were weighed on a precision balance (Mettler-Toledo, Switzerland) and placed back on

a new set of infected plants as described above. Larvae were allowed to feed another

two or four days before their final weight was measured.

To test the effect of oviposition, four-week-old Col-0 plants were placed in

insect tents in presence of approximately 30 *P. brassicae* adults for 2-3 days allowing

females to lay eggs. Control plants were placed in empty tents. Four days later, eggs

were gently removed and all leaves from oviposited or control plants were infiltrated

using a needleless syringe with *Pst* diluted to an OD600 of 0.0005 or with a 10 mM

MgCl2 solution. Plants were incubated for 24 hours in a growth chamber in order to let

bacteria proliferate. After incubation, five freshly hatched *P. brassicae* larvae were

placed on each of 8 plants from each treatment. Plants were placed in a transparent

plastic box and kept in a growth room. After six days of feeding, larvae were weighed

on a precision balance (Mettler-Toledo, Switzerland). This experiment was repeated

twice.

Statistical analyses

For experiments on bacterial growth, values were log-transformed to meet the normality

assumption and analysed with a linear mixed model fit by REML (package "lme4" in R,

http://www.R-project.org). Insect performance assays were analysed with a linear

mixed model fit by REML or by two-way ANOVA. Analyses on gene expression data

were performed using one-way ANOVA and Tukey's test for post hoc comparisons.

Data from metabolite quantifications were analysed by Student's t test.

SUPPORTING INFORMATION

Figure S1. Egg extract-induced inhibition of bacterial growth after *Pst*COR⁻ infiltration.

Figure S2. Inhibition of bacterial growth in plants treated for 3 days with *P. brassicae* egg extract.

Table S1. Free amino acid levels in plants treated for 5 days with *P. brassicae* egg extract.

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FIGURE LEGENDS

Figure 1. Oviposition or treatment with egg extract protects Arabidopsis against bacterial infection.

Growth of *Pseudomonas syringae* strains (see Materials and Methods) was monitored in local (grey bars) or distal (white bars) leaves after oviposition (ovi) by *P. brassicae* (a,c) or application of *P. brassicae* egg extract (b, d-f) on local leaves for 5 days. Infection was done by dipping. Control plants (black bars) were only infected with bacteria. Means ± SE (n = 8) of three biological replicates are shown, except (d) with two replicates. Significant differences between control and treated plants are indicated (Linear mixed model, *P <0.05, **P <0.01, ***P <0.001).

Figure 2. Egg extract-induced inhibition of bacterial growth is similar to SAR.

(a) Growth of *Pst* COR⁻ in distal leaves (2°) after treatment of local leaves (1°) by either *P. brassicae* egg extract (EE) for 5 days or infiltration with *Pst* COR⁻ for 48 h (white bars), or no treatment (black bars). Secondary infection was done by infiltration. Means ± SE (n = 8) of three biological replicates are shown. Significant differences between control and treated plants are indicated (Linear mixed model, ***P <0.001). (b) Pipecolic acid levels in plants treated with egg extract for 5 days. Untreated plants were used as controls (CTL). Means ± SE (n = 6) of three biological replicates are shown. Significant differences between control and treated plants are indicated (Student's t test, **P <0.01). (c) Growth of *Pst* COR⁻ was monitored in local (grey bars) and distal (white bars) leaves of Col-0 and mutant plants 48 h after infection with *Pst* COR⁻ by dipping. Local leaves were pre-treated for 5 days with egg extract. Control plants (black

bars) were only infected with bacteria. Means ± SE (n = 8) of at least three biological replicates are shown. Significant differences between control and treated plants are indicated (Linear mixed model, **P <0.01, ***P <0.001).

Figure 3. Egg extract-induced Pip and SA accumulation is abolished in *ald1* and *fmo1* mutants.

Pipecolic acid (a) and salicylic acid (b) levels in distal leaves of plants treated with egg extract for 5 days (white bars). Untreated plants were used as controls (black bars). Means ± SE (n = 6) of four (a) and six (b) biological replicates are shown. Significant differences between control and treated plants are indicated (Student's t test *P <0.05, ***P <0.001).

Figure 4. Egg extract-induced priming of defence genes in distal leaves.

Local (1°) leaves were either treated with *P. brassicae* egg extract (EE) for 5 days or not treated (-). Distal (2°) leaves were then infiltrated with *Pst* COR⁻ or MgCl$_2$ (mock) for 24 h. Expression of defence genes *PR-1* (a), *NHL10* (b), and *FRK1* (c) was monitored in distal leaves. Means ± SE of three technical replicates are shown. Different letters indicate significant differences at P <0.05 (ANOVA followed by Tukey's honest significant difference test, (a) $F_{(3,7)}$ = 78.7, P <0.001; (b) $F_{(3,8)}$ = 42.6, P <0.001; (c) $F_{(3.8)}$ = 1720, P <0.001). This experiment was repeated twice with similar results.

Figure 5. *P. brassicae* larval development on *Pst*-infected plants.

(a) Plants were infiltrated with *Pst* (white bars) or $MgCl_2$ (black bars) for 2 days. Then, neonate *P. brassicae* larvae were placed on infected plants for 6 days, weighed and further placed on newly infected plants, after which the final weight was recorded at 8 or 10 days. Means ± SE (n ≥ 10) from six (day 6), two (day 8) and four (day 10) biological replicates are shown. Asterisks indicate significant differences (Linear mixed model, ***P <0.001). (b) *P. brassicae* butterflies were allowed to lay eggs on 4-week-old plants (E). Control (C) plants were kept in empty tents. Four days later plants were infiltrated with *Pst* or $MgCl_2$ for 24 hours. Then, neonate *P. brassicae* larvae were placed on treated plants for 6 days and weighed. Means ± SE (n ≥ 25) of three biological replicates are shown. Different letters indicate significant differences at P <0.05 (two-way ANOVA followed by Tukey's honest significant difference test, treatment: $F_{(3,388)}$ = 109.81, P <0.001).

Figure S1. Egg extract-induced inhibition of bacterial growth is independent on the mode of infection.

Growth of *Pseudomonas syringae* COR$^-$ was monitored in local (grey bars) or distal (white bars) leaves after application of *P. brassicae* egg extract on local leaves for 5 days. Leaf infection was done by dipping in a bacterial solution at an OD600 of 0.05 (a) or by infiltration using a 1 ml needleless syringe with a bacterial solution at an OD600 of 0.0005 (b). Control plants (black bars) were only infected with bacteria. Means ± SE (n = 8) of three biological replicates are shown. Significant differences between control and treated plants are indicated (Linear mixed model, **P <0.01, ***P <0.001).

Figure S2. Egg extract-induced inhibition of bacterial growth after 3 days of treatment. Growth of *Pseudomonas syringae* COR$^-$ was monitored in local (grey bars) or distal (white bars) leaves after application of *P. brassicae* egg extract on local leaves for 3 days. Leaf infection was done by dipping. Control plants (black bars) were only infected with bacteria. Means ± SE (n = 8) of three biological replicates are shown. Significant differences between control and treated plants are indicated (Linear mixed model, ***P* <0.01, ****P* <0.001).

Figure 1
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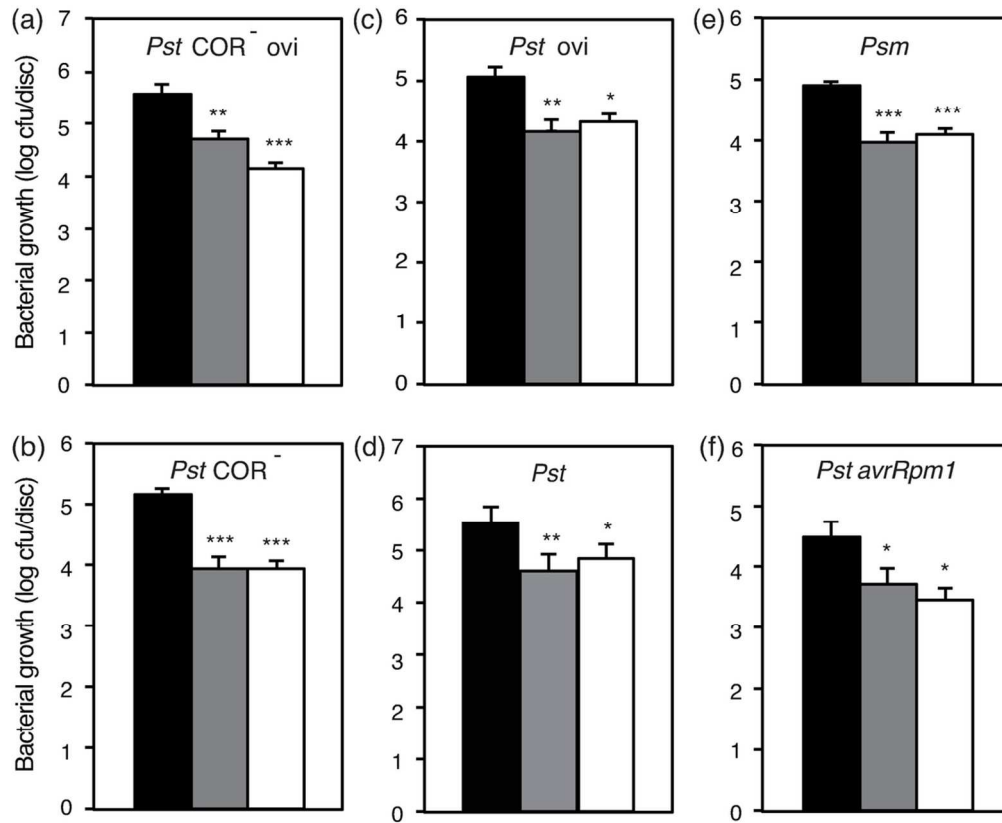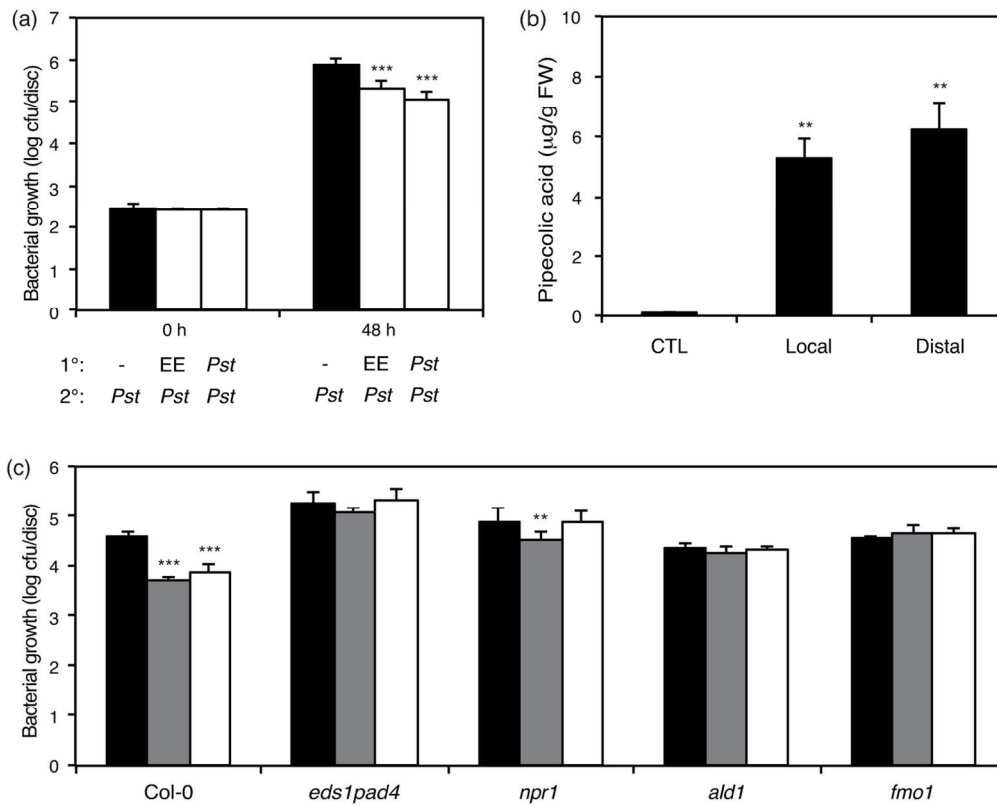129x103mm (300 x 300 DPI)

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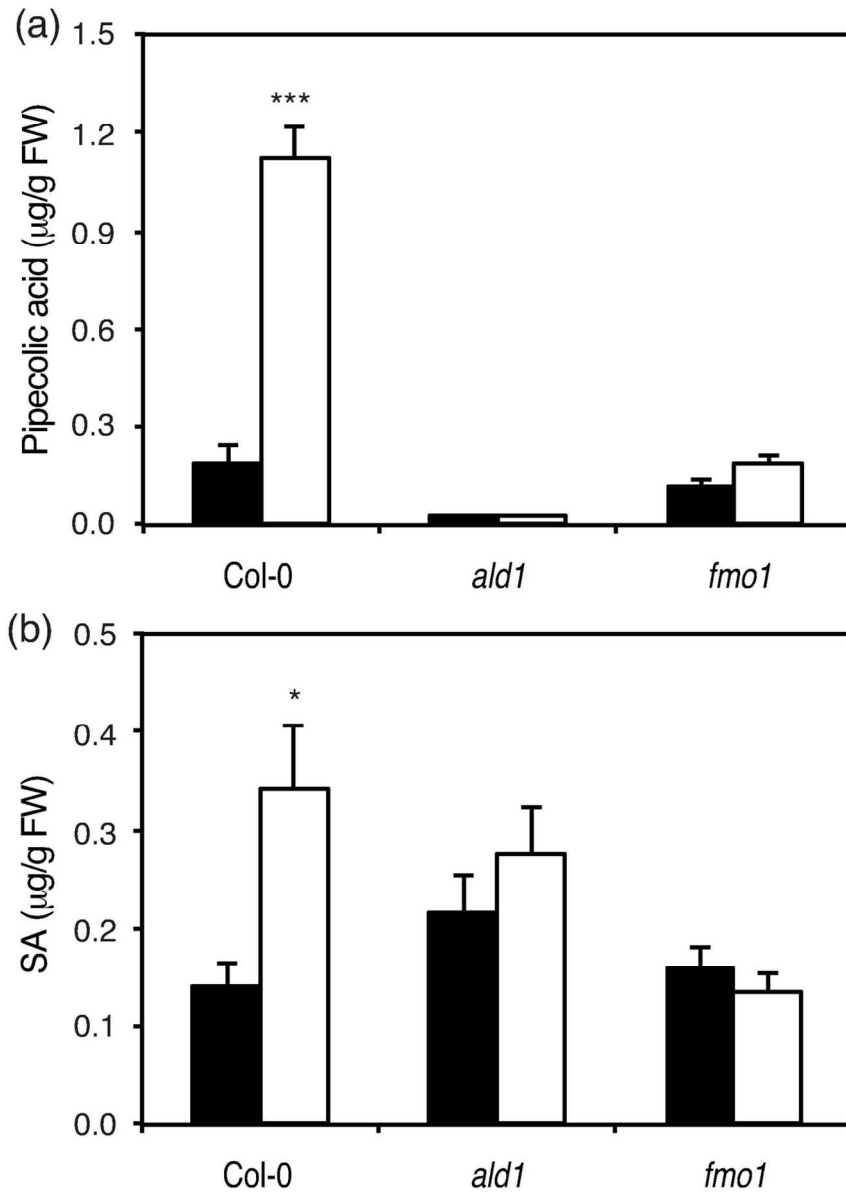41
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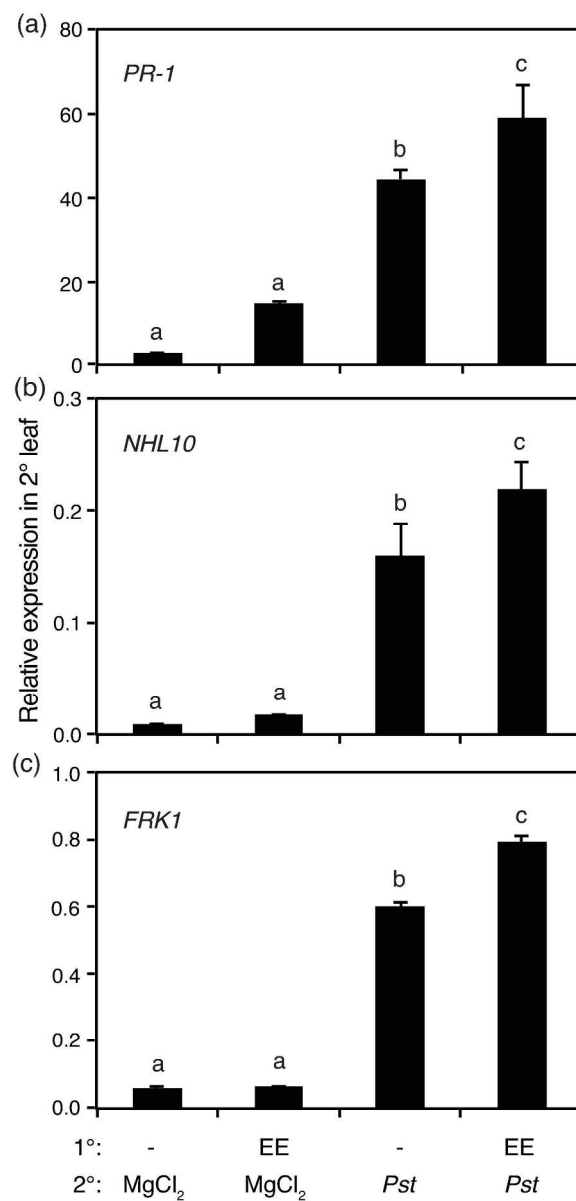51
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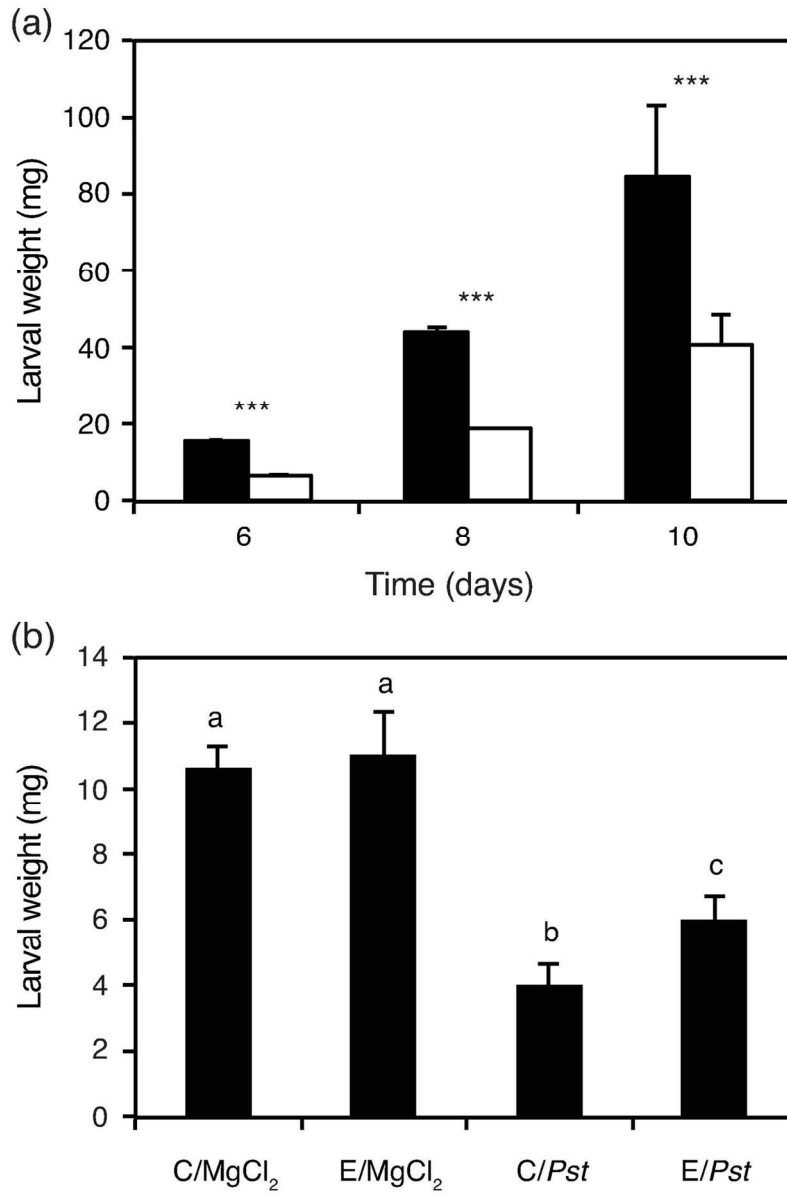57
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Figure 4
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Figure 5
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Figure S1

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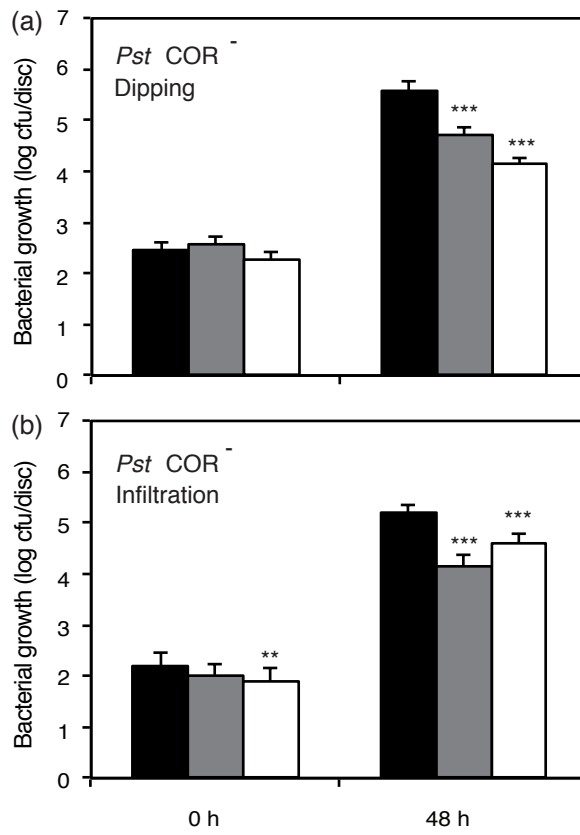53
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Figure S2

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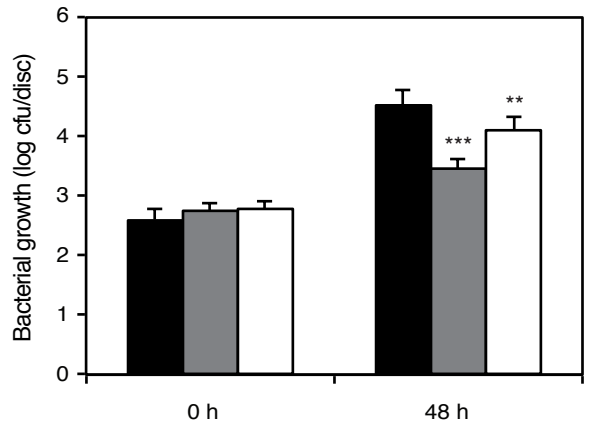51
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Table S1. Free amino acid levels in plants treated for 5 days with *P. brassicae* egg extract.

Amino acid	Control	Local	Distal
Gly	6.69 ± 1.01	23.03 ± 4.47	7.91 ± 0.59
Ala	18.42 ± 3.7	95.53 ± 12.17	27.20 ± 5.06
Val	5.58 ± 0.88	21.19 ± 2.04	6.52 ± 1.14
β-Ala	0.48 ± 0.13	1.66 ± 0.23	0.61 ± 0.19
β-Aib	0.03 ± 0.01	0.94 ± 0.23	0.04 ± 0.00
Leu	2.28 ± 0.52	14.42 ± 1.44	2.63 ± 0.69
Ile	2.19 ± 0.41	9.23 ± 0.94	2.50 ± 0.53
GABA	4.32 ± 1.91	45.60 ± 10.88	4.89 ± 1.48
Thr	59.04 ± 4.51	77.14 ± 0.53	82.82 ± 17.26
Ser	98.58 ± 28.32	179.29 ± 11.58	106.35 ± 20.28
Pro	25.91 ± 3.57	133.27 ± 21.96	24.00 ± 2.46
4HYP	0.30 ± 0.03	1.27 ± 0.07	0.32 ± 0.03
Met	0.22 ± 0.04	2.11 ± 0.40	0.27 ± 0.05
Cys	0.20 ± 0.01	0.28 ± 0.03	0.30 ± 0.04
Asp	560.07 ± 48.48	641.17 ± 49.27	682.44 ± 29.75
Asn	16.02 ± 1.43	23.65 ± 1.69	20.35 ± 2.96
Glu	335.14 ± 16.78	440.35 ± 8.51	387.19 ± 32.53
Gln	46.04 ± 5.36	72.92 ± 11.98	83.46 ± 17.62
Orn	0.34 ± 0.06	0.99 ± 0.14	0.30 ± 0.05
Lys	4.16 ± 0.26	26.91 ± 3.48	4.34 ± 0.40
Pip	0.11 ± 0.00	5.28 ± 0.67	6.21 ± 0.93
Aad	0.12 ± 0.02	0.84 ± 0.11	0.99 ± 0.23
Phe	2.91 ± 0.15	12.24 ± 1.27	3.92 ± 0.15
Tyr	0.86 ± 0.20	5.90 ± 0.49	0.95 ± 0.18
Trp	0.14 ± 0.03	0.55 ± 0.06	0.14 ± 0.01
CTH	0.01 ± 0.00	0.38 ± 0.06	0.02 ± 0.00
DOPA	0.00 ± 0.00	0.56 ± 0.04	0.00 ± 0.00

Mean values (±SE) of three independent replicates are given in μg/g fresh weight. Untreated plants were used as controls. β-Aib: beta-aminoisobutyric acid; GABA: gamma-aminobutyric acid; 4HYP: 4-hydroxyproline; Orn: ornithine; Pip: pipecolic acid; Aad: alpha-aminoaminoadipic acid; CTH: cystathionine; DOPA: 3,4-dihydroxyphenylalanine.