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Pseudomonas syringae enhances herbivory by suppressing the reactive oxygen burst in Arabidopsis

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ABSTRACT

Plant–herbivore interactions have evolved in the presence of plant-colonizing microbes. These microbes can have important third-party effects on herbivore ecology, as exemplified by drosophilid flies that evolved from ancestors feeding on plant-associated microbes. Leaf-mining flies in the genus *Scaptomyza*, which is nested within the paraphyletic genus *Drosophila*, show strong associations with bacteria in the genus *Pseudomonas*, including *Pseudomonas syringae*. Adult females are capable of vectoring these bacteria between plants and larvae show a preference for feeding on *P. syringae*-infected leaves. Here we show that *Scaptomyza flava* larvae can also vector *P. syringae* to and from feeding sites, and that they not only feed more, but also develop faster on plants previously infected with *P. syringae*. Our genetic and physiological data show that *P. syringae* enhances *S. flava* feeding on infected plants at least in part by suppressing anti-herbivore defenses mediated by reactive oxygen species.

1. Introduction

Herbivory has arisen in only a third of living insect orders, but is associated with high rates of diversification when it evolves: nearly 25% of all named species on Earth are herbivorous insects (Farrell, 1998; Mitter et al., 1988). These radiations may be promoted by the dynamics of plant–herbivore interactions, involving the emergence of novel defensive chemicals in plants and counter-adaptations to these defensive compounds in herbivores (Ehrlich and Raven, 1964; Dobler et al., 2012). Although insect-targeting defensive chemicals are often lineage-specific, their induction is regulated in large part by a common defense response pathway mediated by the phytohormone jasmonic acid (JA, see Glossary in Table 1) (Wasternack et al., 2013). Anti-herbivore chemicals are often augmented by generalized plant defense mechanisms, including a protective cuticle, trichomes (Traw and Bergelson, 2003), and the production of reactive oxygen species (ROS) such as oxygen ions and peroxides in a ROS burst induced by herbivore feeding (Collins et al., 2010; Summers and Felton, 1998; Wu et al., 2013). Microbial pathogens and commensals of plants must also overcome these plant defenses (Cao et al., 2012; Melotto et al., 2006; Torres et al., 2006), and the induction and manipulation of the plant immune system by microbial colonizers may have facilitated the evolution of herbivory (McFall-Ngai et al., 2013; Thaler et al., 2012).

The full extent of the influence of microbes on the ecology and evolution of herbivory in insects (excluding vertically transmitted symbionts of hemipterans) is largely unknown (Hansen and Moran, 2014; McFall-Ngai et al., 2013). Bacterial associations are likely to be involved in enhancing herbivore nutrition, but microbes may also be involved in making plants more palatable through detoxification of defensive chemicals (Freeland and Janzen, 1974), or though suppression of their production. In case of the latter, at least two factors are proposed to shape the likelihood and route by which microbes influence herbivory: (1) whether resource acquisition by microbes requires living plant tissue (biotrophy) or the killing of plant tissue (necrotrophy), and (2) whether biotrophic microbes can successfully colonize the host plant (i.e., the microbes are compatible with the host) or not (i.e.,

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Table 1
Glossary.

Acronyms	
COR	coronatine
DAB	3,3′-diaminobenzidine tetrahydrochloride
ETI	effector-triggered immunity
HR	hypersensitive response
ITC	isothiocyanate
JA	jasmonic acid
JA-Ile	jasmonic acid-isoleucine conjugate
LAM	leaf area mined
OPDA	12-oxophytodienoic acid (JA precursor)
PCD	programmed cell death
PTI	pattern-triggered immunity
ROS	reactive oxygen species
SA	salicylic acid
Arabidopsis thaliana	
aos (or *cyp74a/dde2*)	mutant deficient in accumulation of JA and OPDA
asFBP1.1 (or *H4*)	mutant with knocked-down expression of cell wall peroxidases
coi1-1	mutant insensitive to JA-Ile and COR
CYP81F2	PTI marker gene typically associated with ROS and callose accumulation
ein2-1	mutant insensitive to ethylene
myb51	mutant deficient in accumulation of indole glucosinolates
pad2-1	mutant deficient in accumulation of glutathione
pad4-1	mutant deficient in accumulation of salicylic acid and ethylene
pen4-1 (or *pcs1/cad1*)	mutant deficient in accumulation of phytochelatin
pmr4-1	mutant deficient in accumulation of callose
rbohD	NADPH oxidase mutant
rbohF	NADPH oxidase mutant
sid2-2	mutant deficient in accumulation of salicylic acid
Pseudomonas syringae	
Psm ES4326 *cfa6*	mutant deficient in production of COR
AvrPto, AvrPtoB, HopAI1	*P. syringae* effectors suppressing accumulation of ROS and callose
Scaptomyza flava	
Glutathione S-tranferase D1 (*GstD1*)	xenobiotic metabolism gene important in detoxification
Peritrophin A	integral to peritrophic membrane, physical antioxidant

the interaction is incompatible) (Lazebnik et al., 2014). Compatible biotrophic microbes tend to enhance feeding by chewing herbivores by suppressing the plant immune system with effectors or phytotoxins they deliver into plant cells (Cui et al., 2002; Groen et al., 2013; Lazebnik et al., 2014), whereas necrotrophic fungi and incompatible biotrophic microbes tend to reduce feeding (Cui et al., 2002; Lazebnik et al., 2014). In necrotrophic and incompatible biotrophic interactions, microbial phytotoxins or the recognition of effectors by the plant immune system trigger programmed cell death (PCD) in the plant, which is associated with a hypersensitive response (HR) and the release of ROS and plant defensive chemicals (Cui et al., 2002; Torres et al., 2006; Andersson et al., 2014). Such chemicals have broad-acting toxicity against microbes and animals in addition to their role as signaling molecules.

Herbivory has evolved several times independently in the family Drosophilidae from microbe-feeding ancestors (Lapoint et al., 2013). Herbivorous species in the genus *Scaptomyza*, which is nested within the paraphyletic genus *Drosophila*, generally specialize on plants from the order Brassicales. They overcome the physical barrier of the leaf cuticle by laying eggs in leaf punctures, which adult females create using a dentate ovipositor (Whiteman et al., 2011). The females also feed on sap from these punctures, and this behavior may play a role in oviposition preference. After the eggs hatch, the larvae develop inside the leaves as leafminers, consuming the mesophyll tissue. To some extent adult females and larvae detoxify the lineage-specific defensive chemicals in the Brassicales, the glucosinolate-derived isothiocyanates (ITCs), using the mercapturic acid pathway (Gloss et al., 2014). One key enzyme in this pathway that has been characterized in *Scaptomyza* spp. is Glutathione *S*-transferase D1 (GstD1) (Gloss et al., 2014). Less is known about how *Scaptomyza* spp. larvae respond to the toxic effects of ROS, which can severely damage the peritrophic membrane that lines the midgut and protects insects from microbial attack (Mittapalli et al., 2007c; Summers and Felton, 1998). Evidence from *Drosophila melanogaster*, the Hessian fly, and hemipteran and lepidopteran herbivores suggest that insect herbivores likely employ general xenobiotic metabolism pathways involving Glutathione *S*-transferases (including GstD1) to deactivate ROS species and downstream lipid peroxidation products (Landis et al., 2012; Lei and Zhu-Salzman, 2015; Luan et al., 2013; Mittapalli et al., 2007a,b; Sawicki et al., 2003; Sykiotis and Bohmann, 2008).

The ancestors of the herbivorous drosophilids in the *Scaptomyza* lineage were microbe-feeders, and it is possible that the evolutionary transition to herbivory in this group was influenced in part by bacterial plant colonizers (O'Connor et al., 2014). Indeed, bacterial colonizers of Brassicales inhibit the formation of or detoxify ROS and ITCs, and have evolved several mechanisms to do this (Abramovitch et al., 2003; Fan et al., 2011; Gimenez-Ibanez et al., 2009; Guo et al., 2012; Lewis et al., 2014; Tang et al., 1972; Xiang et al., 2008; Zhang et al., 2007).

A recent survey of *Scaptomyza flava* feeding on wild *Barbarea vulgaris* (Brassicaceae) in Flagstaff, Arizona, found that the fly's gut microbiota closely resemble the leaf microbiota of its host plants (O'Connor et al., 2014). This metamicrobiome is dominated by bacteria from the genus *Pseudomonas*, and encompasses the (hemi)biotrophic phytopathogen *Pseudomonas syringae*. Although *Scaptomyza* and *Pseudomonas* spp. do not strictly depend on one another to colonize plants, some findings suggest that they can engage in a mutually beneficial relationship. *P. syringae* grows epiphytically and apoplastically, and colonizes the leaf after entering through the stomata or wound sites, or through the vasculature after having colonized distal parts of the plant (Hirano and Upper, 2000; Misas-Villamil et al., 2011). Since both *Scaptomyza* spp. adult females and larvae create wound sites during feeding, insect feeding could promote the colonization of leaves by *P. syringae*. Indeed, adult female *S. flava* can act as vectors of *P. syringae* within and between plants (O'Connor et al., 2014). Additionally, adult *S. flava* females feed less and have a lower fecundity after treatment with antibiotics, suggesting that herbivory may be aided by the metabolic potential of gut-inhabiting microbes (O'Connor et al., 2014). These observations were paralleled by results from another field survey of herbivorous *Scaptomyza nigrita* feeding on *Cardamine cordifolia* (Brassicaceae) (Humphrey et al., 2014). The leaf microbiota were similarly dominated by *Pseudomonas* spp., and larvae showed a preference for feeding on plants infected with *P. syringae*. Moreover, in nature, bacterial titers were consistently higher in leaves that had damage from leafmining *S. nigrita*.

Combined with our earlier laboratory studies, where we identified some of the mechanisms by which *P. syringae* can disrupt plant defense against insect herbivores (Cui et al., 2002, 2005; Groen et al., 2013), these observations pose the intriguing possibility that adult and larval *Scaptomyza* spp. may either directly inoculate their host plants with bacteria that facilitate feeding, or indirectly benefit from the defense-suppressive effects of prior *Pseudomonas* spp. infections. This pattern has been observed in other plant–herbivore interactions, in which herbivores benefit from the defense-suppressive effects of the microbes they transmit. These "suppressive" microbes comprise a wide range of obligate pathogens including viruses, phytoplasmas and bacteria (Body et al., 2013; Casteel et al., 2014; Kaiser et al., 2010; Kazan and Lyons,

2014; Li et al., 2014; Sugio et al., 2011; Ziebell et al., 2011). One well-studied example is the Colorado potato beetle, which inoculates its host plants with *P. syringae* and other bacteria that in turn suppress plant anti-herbivore defenses (Chung et al., 2013).

How could *P. syringae* benefit insect herbivores? Several non-mutually exclusive mechanisms may be involved. Infections with many *P. syringae* strains, e.g. *P. syringae* pv. *maculicola* (*Psm*) ES4326, suppress JA-mediated insect defense signaling by activating plant immune signaling via salicylic acid (SA) (Chung et al., 2013; Groen et al., 2013; Wang et al., 2008). SA signaling generally has a suppressive effect on signaling by the major active JA molecule, the JA-isoleucine conjugate JA-Ile, by a process of crosstalk known as SA/JA antagonism (Thaler et al., 2012). Consistent with SA/JA antagonism, oviposition and larval feeding by *S. nigrita* in the field increased on leaves pre-treated with SA, and decreased on leaves pre-treated with JA (Humphrey et al., 2014). Furthermore, larvae of *S. flava* show enhanced feeding and develop more quickly on mutants of the reference plant, *Arabidopsis thaliana* (Arabidopsis; Brassicaceae), that are deficient in JA signaling (Whiteman et al., 2011). This relatively simple narrative of SA/JA antagonism is complicated however by the fact that although many strains of *P. syringae* activate SA-mediated defense responses, they also deploy a phytotoxin mimicking JA-Ile, coronatine (COR). COR counters SA-mediated suppression of JA signaling leading to induced susceptibility to bacteria and a neutralization of the SA-mediated susceptibility to herbivores (Cui et al., 2005).

A second mechanism by which microbes could suppress host insect defenses that are specific to plants in the family Brassicaceae is the detoxification of ITCs formed in response to herbivory. ITCs can have strong negative effects on *S. flava* and other herbivores (Agrawal and Kurashige, 2003; Jander et al., 2001; Whiteman et al., 2011, 2012). ITCs also have anti-bacterial effects and several strains of *P. syringae* including *Psm* ES4326 employ SAX enzymes to detoxify those (Fan et al., 2011). Although SAX-dependent detoxification of ITCs might benefit herbivores feeding on infected leaves, the SAX enzymes are not widely distributed among strains of *P. syringae* (Fan et al., 2011).

A third possible mechanism for microbe-mediated suppression of host defense is the detoxification of ROS or the inhibition of the ROS burst. *P. syringae* injects conserved effector proteins such as AvrPto, AvrPtoB and HopAI1 into plant cells, which suppress the formation of ROS (Abramovitch et al., 2003; Baltrus et al., 2011; Gimenez-Ibanez et al., 2009; Xiang et al., 2008; Zhang et al., 2007), and produces catalases to detoxify them (Guo et al., 2012). ROS such as hydrogen peroxide directly damage both bacterial pathogens and insect herbivores (Cao et al., 2012; Liu et al., 2010; Mittapalli et al., 2007a; Summers and Felton, 1998). Furthermore, ROS act not only in cross-linking plant cell walls, which could toughen leaf tissue to herbivores, but also play a key role in orchestrating the overall plant immune response (Levine et al., 1994; Mhamdi et al., 2010; Torres et al., 2006; Wu et al., 2013). *S. flava* larvae and adult females also induce production of hydrogen peroxide during feeding (Whiteman et al., 2011). Suppression of the ROS burst by *P. syringae* might thus protect *S. flava* from oxidative damage and plant defensive mechanisms regulated by ROS, and thereby enhance feeding.

In the experiments described here, we focus on the potential role of the ROS burst in Arabidopsis – *S. flava* – *P. syringae* interactions, and test the hypothesis that *P. syringae* associated with *S. flava* may make their shared host plant more palatable to the herbivore through suppressing the herbivore-induced ROS burst. After establishing that larvae, like adult flies, can transmit *P. syringae* within and between leaves, we found that infection of Arabidopsis with *P. syringae* enhances *S. flava* feeding and development. By testing a set of candidate defense-related mutants in Arabidopsis, we discovered that *S. flava* feeding was increased on Arabidopsis mutants that were deficient in producing wild-type (WT) ROS responses. Levels of feeding on these mutants were similar to those on WT plants that had been infected with *P. syringae*. On the other hand, larval feeding was decreased on mutants with a lowered threshold to initiate PCD. Cell death is an inevitable consequence when ROS levels reach a certain threshold and cause the release of additional ROS and ITCs (Andersson et al., 2014; Torres et al., 2006).

Congruent with a role for ROS in bacteria-induced susceptibility to herbivory, *P. syringae* suppressed the ROS burst generated in response to *S. flava* feeding, and *P. syringae* did not further promote larval feeding and development on intrinsically more palatable, engineered Arabidopsis plants, in which cell wall peroxidases are silenced and hydrogen peroxide production is disrupted. Furthermore, feeding by *S. flava* larvae on this ROS-deficient Arabidopsis line resulted in decreased expression of a xenobiotic detoxification gene in *S. flava*. Because pathogen- and herbivore-elicited ROS bursts form a widely conserved defense response among angiosperms (Torres et al., 2006), our findings are most likely relevant to other microbe-influenced interactions between plants and their attackers including insect herbivores and plant-parasitic nematodes (e.g. Luan et al., 2013; Vicente et al., 2013).

2. Materials and methods

2.1. Biological material and growth conditions

2.1.1. Plants

WT Arabidopsis accession Col-0 plants where grown as detailed in Whiteman et al. (2011). The loss-of-function Arabidopsis mutants *aos* (or *cyp74a*|*dde2*), *ein2-1*, *pad2-1*, *pad4-1*, *pen4-1*, *pmr4-1*, *pad4-1 pmr4-1*, *rbohD*, *rbohF*, and *sid2-2* have been described and are in the Col-0 background (Alonso et al., 1999; Clay et al., 2009; Dewdney et al., 2000; Glazebrook and Ausubel, 1994; Glazebrook et al., 1996; Guzmán and Ecker, 1990; Howden and Cobbett, 1992; Jirage et al., 1999; Nishimura et al., 2003; Torres et al., 2002, 2005; Vatamaniuk et al., 1999; Vogel and Somerville, 2000; Wildermuth et al., 2001; Zhou et al., 1998). The transgenic Arabidopsis reduction-of-function strain known as *asFBP1.1* (or *H4)*, in which expression of the cell wall-associated peroxidases *PRX33*, *PRX34*, and potentially other cell wall-associated peroxidases, is knocked down, has also been described and is in the Col-0 background (Bindschedler et al., 2006; Daudi et al., 2012; Mammarella et al., 2014). WT *B. vulgaris* plants were grown in the same conditions as described for Arabidopsis from seeds that descended from plants that were field-collected in New Hampshire.

2.1.2. Insects

Information on the source population of *S. flava* and fly rearing and experimental conditions can be found in Whiteman et al. (2011). For all experiments, with the exception of testing Arabidopsis mutants described in Section 3.3, flies were reared on *B. vulgaris*. For these experiments, flies were reared on Arabidopsis.

2.1.3. Bacteria

We used a mutant of the laboratory strain *P. syringae* pv. *maculicola* (*Psm*) str. ES4326, which is closely related to *P. syringae* isolates associated with *Scaptomyza* spp. in the field (Humphrey et al., 2014). This mutant was the COR-deficient strain *Psm* ES4326 *cfa6*. We chose this COR-deficient mutant rather than the WT *Psm* ES4326 strain, because COR production is relatively rare

among *P. syringae* isolates that colonize Brassicaceae plants (Baltrus et al., 2011; Hwang et al., 2005; Sarkar et al., 2006). The COR-deficient mutant *Psm* ES4326 *cfa6* has been described by Cui et al. (2005).

2.2. Bacterial transmission experiment

Psm ES4326 *cfa6* bacteria were grown overnight in 2 ml liquid King's B supplemented with 50 μg ml^{-1} kanamycin (KB + K50) at 28 °C in a shaking incubator (225 rpm) for 18 hours prior to dilution and subsequent infection, as in Cui et al. (2005). The bacteria were then suspended in 10 mM $MgSO_4$ + 0.01% Tween20 at an optical density at 600 nm (OD_{600}) of 0.2. An OD_{600} of 0.2 corresponds to a density of *c.* 10^6 colony-forming units (cfu) cm^{-2} of leaf area (equivalent to an inoculum concentration of *c.* 10^8 cfu ml^{-1}). A titer of 10^6 cfu cm^{-2} of leaf area is regularly found for *P. syringae* in leaves damaged by *Scaptomyza* spp. larvae in the field (Humphrey et al., 2014), and this titer has previously been used in experiments in which a non-COR-producing *P. syringae* strain was inoculated epiphytically (see below) (Melotto et al., 2006).

Leaves of four 10-week-old *B. vulgaris* plants ($N = 2$ plants per treatment group) were either mock-inoculated with sterile 10 mM $MgSO_4$ + 0.01% Tween20 or inoculated with the *Psm* ES4326 *cfa6* suspension by gently painting the abaxial side of all the leaves in the *B. vulgaris* plants using sterile gloved fingers. We chose the abaxial side to mimic bacterial transmission by adult female flies as they mainly feed and oviposit on this side of the leaf (Whiteman et al., 2011).

Four days after inoculation, six two-day-old larvae per plant (total $N = 12$ per treatment group) were transferred from *B. vulgaris* nursing plants to the *B. vulgaris* source plants pre-inoculated with *Psm* ES4326 *cfa6*. Larvae were allowed to feed for 2 days on these infected source plants, after which three larvae per plant (total $N = 6$ per treatment group) were transferred again to non-inoculated *B. vulgaris* recipient plants to determine whether larvae can transfer *Psm* ES4326 *cfa6* from infected to healthy plants. After feeding for 4 days in recipient plants, when the larvae pupated, a sample from each of the six recipient leaves was collected for dilution plating to quantify the abundance of *Psm* ES4326 *cfa6* that had been transferred. For this and all subsequent assays, growth of *Psm* ES4326 *cfa6* was assessed on KB + K50 agar plates.

We enumerated bacterial growth within two of the 14 pre-inoculated source leaves at 4 days post inoculation just prior to the introduction of larvae from nursing plants (Fig. 1D, Source plant prior to larval feeding). We also measured bacterial growth within eight of these 12 pre-inoculated source leaves 2 days after larvae had been introduced from nursing plants at 6 days post inoculation (Fig. 1D, Source plant after larval feeding). Finally, we assessed bacterial growth within three of the six recipient leaves 4 days after larvae had been transferred from infected source plants (Fig. 1D, Recipient plant after larval feeding). Bacterial growth was determined with dilution plating. We also included mock-treated leaves to control for cross-contamination.

In parallel, we collected larvae from the pre-infected source plants and transferred them directly onto KB + K50 plates instead of transferring them into recipient plants. Two larvae per treatment group roamed freely on KB + K50 plates for 2 days, after which plates were visually inspected for growth. The cuticles of two additional larvae per treatment group were washed with sterile 10 mM $MgSO_4$ and then this wash was dilution-plated to see if bacteria were carried on the cuticle. Two final larvae per treatment

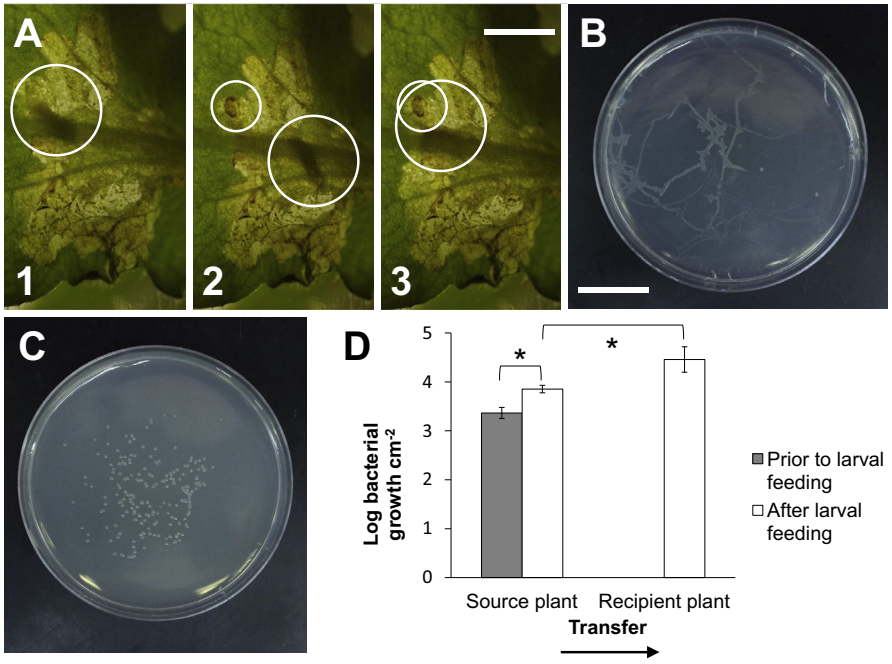

Fig. 1. *Scaptomyza flava* larvae can spread plant-colonizing bacteria within leaves. (A) A *S. flava* larva deposits frass ([2] small white circle) upon leaving the feeding site in the *B. vulgaris* leaf ([1 and 2] large white circles). After having fed elsewhere in the leaf, the larva later returns to continue feeding at this site ([3] overlapping large and small white circles). (B) *S. flava* larvae can spread plant-colonizing bacteria within leaves through the behavior described in (A). Bacteria attached to the larval cuticle can be deposited on other host plants, as shown here where a *S. flava* larva that fed on a leaf of a *Psm* ES4326 *cfa6*-infected *B. vulgaris* source plant was allowed to crawl on a selective King's B agar plate. (C) Cuticle wash from a larva that had fed on a *Psm* ES4326 *cfa6*-infected leaf spread on a selective King's B agar plate. (D) Mean accumulation of *Psm* ES4326 *cfa6* after pre-inoculation of *B. vulgaris* source leaves prior to and after 2 days of larval feeding, and accumulation of *Psm* ES4326 *cfa6* in recipient leaves on which larvae from infected source leaves were feeding. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences in bacterial accumulation in pair-wise comparisons (two-tailed Student's *t*-test, $P < 0.05$). Scale bars in (A) and (B) indicate 2 cm; (C) is depicted at the same scale as (B). Photo credit: Matthew Velazquez (A).

group were surface-sterilized using 70% EtOH, and then also allowed to roam freely on KB + K50 plates for 2 days.

2.3. Larval feeding and development assays

Larval feeding on WT and mutant Arabidopsis was measured using the leaf area-mined (LAM) assay as described in Whiteman et al. (2011) in which larvae were allowed to feed for 4 days after being transferred from nursing plants.

In a variant of this experiment, we measured larval feeding and development on WT and mutant Arabidopsis plants that were either pre-inoculated with *Psm* ES4326 *cfa6* or mock-inoculated. Notably, bacterial infections and herbivory took place in the same leaves (co-infections). Prior to the start of an experiment, bacteria were prepared and inoculated as described in Section 2.2 above. On the day of the inoculation of experimental plants, adult flies were allowed to mate and oviposit on non-inoculated WT Arabidopsis nursing plants for 24 hours. This time point was chosen to mimic the timing of events in nature, where adult females likely transmit *P. syringae* during feeding and oviposition on leaves that form the substrate for subsequent larval feeding (Humphrey et al., 2014; O'Connor et al., 2014). After 4 days, late first instar larvae were transferred to the experimental plants as described in Whiteman et al. (2011). Larval feeding was then measured after 2 days using the LAM assay.

After measuring LAM, larvae were allowed to complete their development in the experimental plants as described by Whiteman et al. (2011). Adult eclosion from pupae was assessed 20 days after the parental generation was allowed to oviposit on the nursing plants (Fig. 2B), or was assessed every day until all pupae had eclosed (Fig. 4B). A subset of leaves was harvested to verify *Psm* ES4326 *cfa6* infection as described above.

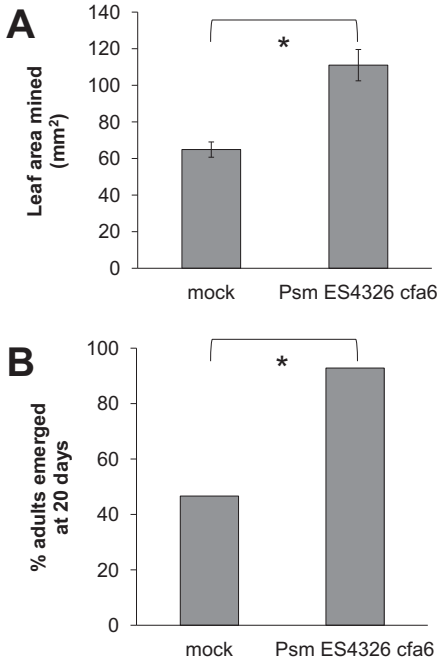

Fig. 2. *Scaptomyza flava* larvae consume more and develop faster in *P. syringae*-infected leaves. (A) Mean leaf area mined (LAM) by *S. flava* larvae reared in wild-type (WT; Col-0) Arabidopsis leaves pre-infected with *Psm* ES4326 *cfa6* compared to LAM by larvae reared in mock-inoculated leaves. Larvae were transferred from WT nursing plants in the second instar into the experimental plants and allowed to feed for 48 hours. (B) Percentage of *S. flava* pupae having eclosed as adults at 20 days after the day of oviposition in the nurse plants. Error bars represent standard error of the mean. Asterisks indicate a statistically significant difference (two-tailed Student's *t*-test, $P < 0.05$ in (A); χ^2 test, $P < 0.05$ in (B)) between treatments ($N = 15–20$ plants per treatment).

2.4. Detection of reactive oxygen species in Arabidopsis

We measured the production of hydrogen peroxide in Arabidopsis after feeding by *S. flava* adult females. Experimental plants were either mock-inoculated or inoculated with *Psm* ES4326 *cfa6* as described in Section 2.2 4 days prior to exposing them to adult *S. flava*. Adult *S. flava* were allowed to mate, feed and oviposit for 24 hours. Thereafter, three leaves per plant that received similar levels of damage and were matched developmentally ($N = 3$ plants per treatment group) were harvested for analysis. *In situ* detection of hydrogen peroxide production in Arabidopsis leaves using 3,3′-diaminobenzidine tetrahydrochloride (DAB) was carried out following the methods described in Whiteman et al. (2011) and Daudi et al. (2012). We subsequently quantified the level of increase in DAB staining intensity around sites of feeding damage relative to the background levels of DAB staining in undamaged surrounding tissue in the same leaves ($N = 9$ leaves per treatment sourced from 3 independently treated and processed plants) using ImageJ (Abràmoff et al., 2004).

2.5. Expression of xenobiotic metabolism genes in S. flava

Expression of the xenobiotic metabolism genes *Glutathione S-transferase D1* (*GstD1*) and *Peritrophin A* in *S. flava* larvae feeding on Arabidopsis WT and mutant plant lines was measured using real-time quantitative PCR (RT-qPCR). The materials, methods and primers used have been described in Whiteman et al. (2011). For each Arabidopsis line, RNA was isolated from whole bodies of 5–7 late second instar larvae feeding on these plant lines, which were divided over each of three biological replicates for each line. One exception was the *asFBP1.1* line for which only two biological replicates of larvae feeding on this line were performed.

2.6. Statistical analysis

Statistical analysis was performed using R version 3.0.2 (R Core Development Team, 2012), and the R Commander package version 2.1-7 (Fox, 2005).

3. Results

3.1. S. flava larvae can promote the spread and growth of P. syringae within leaves

To determine whether *S. flava* larval movement facilitates the spread of *P. syringae* within leaves, we first characterized larval behavior. Although the larvae are less mobile than adult females, they still move frequently within leaves and occasionally between leaves of the same plant. *S. flava* larvae typically deposit their frass at the edges of the mines they create (Fig. 1A1 and A2). Using the deposition of frass as evidence that an area was previously visited by larvae, we observed that larvae often leave the area of a mine where they have deposited frass for a few hours, but return later to resume feeding at these sites (Fig. 1A3). This behavior is consistent with that described in the literature (Hendel, 1928; Hering, 1951a). We hypothesized that this larval movement behavior would promote the spread of *P. syringae* if it was already present inside the leaf, whether it had been deposited there by ovipositing and feeding adult females or through other mechanisms of colonization (Hirano and Upper, 2000; Humphrey et al., 2014; Morris et al., 2008; O'Connor et al., 2014).

To determine if larvae mechanically vectored viable *P. syringae*, we released larvae that had been feeding on *B. vulgaris* leaves infected with *P. syringae* onto selective KB + K50 plates. For these experiments and others described in this paper, we used a coronatine (COR)-deficient, kanamycin resistant mutant of *P. syringae* pv. *maculicola* strain ES4326 (*Psm* ES4326 *cfa6*). We chose this strain because it closely resembles *P. syringae* strains associated with *S. flava* in the wild (Humphrey et al., 2014) and because COR production is relatively rare among *P. syringae* isolates that colonize Brassicaceae plants (Baltrus et al., 2011; Hwang et al., 2005; Sarkar et al., 2006). Three days later, we observed that fluorescent *Psm* ES4326 *cfa6* bacterial colonies developed wherever larvae traversed the agar surface (Fig. 1B). We then investigated whether bacteria were transferred via the cuticle of the larvae (Hirano and Upper, 2000). To do this, we rinsed larvae that had been feeding on *Psm* ES4326 *cfa6*-infected *B. vulgaris* leaves with sterile water and plated the washes. Three days after plating, colonies of *Psm* ES4326 *cfa6* developed on plates that had been streaked with washes (Fig. 1C), showing that larvae can mechanically spread bacteria. This experiment suggested that the larval behavior shown in Fig. 1A is conducive to the dissemination of bacterial colonizers within leaves through mechanical transfer via the cuticle.

We next performed a larval transfer experiment in which larvae that were feeding on *B. vulgaris* source leaves that had previously been inoculated epiphytically with *Psm* ES4326 *cfa6* (Melotto et al., 2006) were transferred to non-inoculated recipient leaves of separate plants. Larvae successfully transmitted *Psm* ES4326 *cfa6* in three out of six transfers. The transmissions of bacteria to recipient leaves show that in these cases *Psm* ES4326 *cfa6* had entered the apoplastic space of the source leaves through the stomata as they could otherwise not have been picked up by larvae (Melotto et al., 2006). Moreover, when bacteria successfully established in the recipient leaves, they grew to titers that were the same or higher than those in the source leaves (Fig. 1D). This is in line with results obtained in similar experiments involving three-way interactions between *P. syringae*, chewing herbivores and Brassicaceae host plants (Appel et al., 2014; Humphrey et al., 2014). Our results in the between-leaf transfer experiments further establish that larval foraging can promote the colonization of leaves by *P. syringae*.

3.2. S. flava larvae perform better on leaves infected with P. syringae

We previously observed that *Scaptomyza* spp. can promote leaf colonization by *P. syringae* (Humphrey et al., 2014). This led us to test the hypothesis that inoculation of feeding sites with *P. syringae* might facilitate herbivory by *S. flava*. We addressed this by pre-inoculating leaves with *Psm* ES4326 *cfa6*, and assessing larval feeding and development.

Pre-inoculation of leaves with *Psm* ES4326 *cfa6* led to enhanced feeding by *S. flava* larvae as determined by the leaf area-mined (LAM) assay (Fig. 2A), which resulted in faster development into adults (Fig. 2B). These findings are consistent with the enhanced feeding by *S. nigrita* observed on plants infected with a *P. syringae* isolate from the field (Humphrey et al., 2014).

3.3. S. flava second- and third-instar larval feeding rates correlate with plant capacity for ROS formation

Given that *Psm* ES4326 *cfa6* infection enhanced feeding by *S. flava*, we tested a set of Arabidopsis mutants corresponding to candidate genes potentially involved in defense against *S. flava* larvae. We previously reported that larvae fed more on the JA signaling-deficient *aos* (or *cyp74a*/*dde2*) and *coi1-1* mutants, and the glucosinolate-deficient *myb51* mutant than on WT plants (Whiteman et al., 2011). This is consistent with findings that JA signaling- and glucosinolate-deficient mutants show enhanced susceptibility to a variety of chewing insect herbivores (Bodenhausen and Reymond, 2007; Gigolashvili et al., 2007; Groen et al., 2013; Müller et al., 2010).

We focused on testing mutants implicated in ROS-mediated immune responses. Our rationale for this was that ROS are generally produced upon the initiation of pattern-triggered immunity (PTI). This is the first layer of the plant immune system activated after recognition of conserved pathogen and/or insect elicitors such as chitin (Miya et al., 2007). ROS can have direct negative effects on insects and initiate downstream anti-herbivore defense responses that are regulated via JA, SA, ethylene and other hormones (Kim et al., 2014; Tsuda et al., 2009). The presence of crosstalk and feedback mechanisms within this regulatory network means that mutants with altered ROS production often have pleiotropic effects on JA signaling, regulation of PCD, and the production of defensive metabolites such as glucosinolates and callose.

The *ein2-1*, *sid2-2* and *pad4-1* mutants are affected in ROS production upon PTI initiation, but also show enhanced JA signaling due to a decrease in SA/JA antagonism (Cui et al., 2002; Groen et al., 2013; Kim et al., 2014; Mühlenbock et al., 2008; Tsuda et al., 2009; Wildermuth et al., 2001; Yi et al., 2014). It was therefore not unexpected that larvae consumed either equal amounts of or less leaf material on these mutants than on WT plants (Fig. 3A). In contrast to the *pad4-1* mutant, the callose-deficient mutant *pmr4-1* has constitutively suppressed JA signaling due to high levels of SA (Nishimura et al., 2003), but a strongly enhanced capacity for producing ROS and for initiating PCD (Nishimura et al., 2003). In other words, the *pmr4-1* and *pad4-1* mutants have opposite phenotypes. The *pmr4-1* mutant was not statistically different from WT plants with respect to levels of larval feeding (Fig. 3B), consistent with the conclusion that enhanced susceptibility to feeding due to reduced JA signaling is balanced by enhanced resistance through a strongly increased capacity for ROS production, which has previously been observed for another mutant with similar characteristics, *acd2* (Cui et al., 2002). A *pad4-1 pmr4-1* double mutant exhibited enhanced larval feeding compared to the *pad4-1* and *pmr4-1* single mutants (Fig. 3B), consistent with the hypothesis that, in the double mutant, the opposite effects of the mutant alleles on JA signaling, ROS formation, and PCD initiation negate one another (Nishimura et al., 2003).

The two main types of enzymes responsible for the ROS burst associated with defense-related responses are the NADPH oxidases D and F (RbohD and RbohF) (Torres et al., 2002, 2005), and cell wall-associated peroxidases including PRX33 and PRX34 (Bindschedler et al., 2006; Daudi et al., 2012; O'Brien et al., 2012b; Mammarella et al., 2014). The cell wall peroxidases form a major source of ROS during PTI (Daudi et al., 2012; O'Brien et al., 2012a,b), whereas the ROS produced by NADPH oxidases are involved in the suppression of HR-related PCD in addition to contributing to the ROS burst during both pattern- (PTI) and effector-triggered immunity (ETI) (Daudi et al., 2012; O'Brien et al., 2012a,b; Torres et al., 2002, 2005). Interestingly, we found that *S. flava* larvae consumed *c.* 30% more leaf tissue on the cell wall peroxidase-deficient anti-sense transgenic line *asFBP1.1* (Bindschedler et al., 2006; Daudi et al., 2012), which suggests that cell wall peroxidases are a major source of ROS during herbivore attack as they are during pathogen attack. Although the NADPH oxidases also produce ROS during herbivore and pathogen attack, and contribute to plant defense against aphids (Jaouannet et al., 2015; Miller et al., 2009; Torres et al., 2002, 2005), and caterpillars (Wu et al., 2013), *S. flava* larvae consumed equal amounts of leaf tissue on the *rbohD* and *rbohF* mutants vs. WT plants (Fig. 3C). In

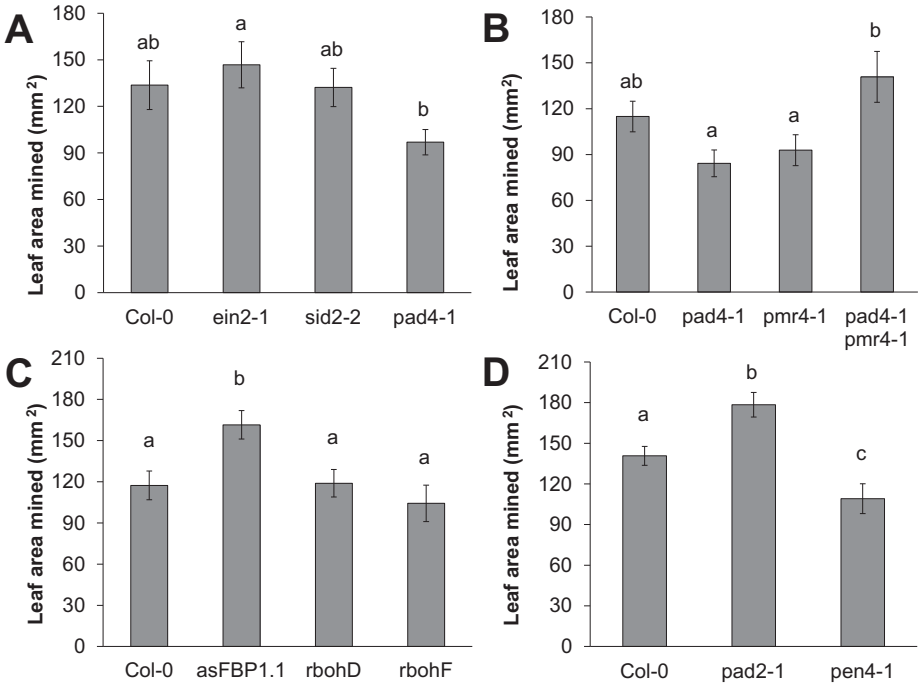

Fig. 3. *Scaptomyza flava* larval leaf consumption in Arabidopsis mutants varies with the plants' defensive potential. (A–D) Mean leaf area mined (LAM) by *S. flava* larvae reared in Arabidopsis mutants relative to LAM by larvae reared in wild-type (WT; Col-0) plants. Larvae that had just reached the second instar were transferred from WT nursing plants to experimental and control plants and allowed to feed for 96 hours. Error bars represent standard error of the mean. Letters indicate a statistically significant difference (one-way ANOVA with *post hoc* Tukey's HSD tests; $P < 0.05$) between treatments ($N = 15–20$ plants per treatment).

these latter mutants, any reduction in ROS-mediated resistance to *S. flava* might be offset by the enhanced propensity for initiating PCD as is the case in the *pmr4-1* mutant.

Another important prerequisite for ROS formation is the availability of glutathione (Dubreuil-Maurizi et al., 2011). Indeed, *S. flava* larvae consumed more leaf tissue in the glutathione-deficient mutant *pad2-1* (Fig. 3D), which produces lower levels of hydrogen peroxide after PTI initiation (Dubreuil-Maurizi et al., 2011; Parisy et al., 2007; Schlaeppi et al., 2008). Furthermore, larval feeding was diminished in the phytochelatin synthase-deficient *pen4-1* mutant (Fig. 3D). Phytochelatin-deficient mutants contain stabilized glutathione levels, and accumulate ROS and glucosinolates after PTI initiation (Clay et al., 2009; Dubreuil-Maurizi et al., 2011; Grill et al., 1989; Parisy et al., 2007). The JA produced after herbivore attack likely enhances glutathione production through induction of the expression of glutathione biosynthetic enzymes (Schlaeppi et al., 2008; Xiang and Oliver, 1998).

Despite the pleiotropic effects on JA signaling, regulation of PCD, and biosynthesis of callose and glucosinolates in some of the mutants analyzed here, when viewed together, these new data strongly suggest a role for ROS, in particular hydrogen peroxide produced by cell wall-associated peroxidases, in defense against *S. flava*.

3.4. *P. syringae*-induced susceptibility to herbivory depends on hydrogen peroxide production

S. flava feeding activates the production of hydrogen peroxide (Whiteman et al., 2011), which plays important direct and indirect (regulatory) roles in anti-herbivore defense in Arabidopsis and other plant species (Collins et al., 2010; Summers and Felton, 1998; Wu et al., 2013). Several widely conserved *P. syringae* effectors prevent the formation of ROS by blocking upstream positive regulatory mechanisms (Abramovitch et al., 2003; Baltrus et al., 2011; Gimenez-Ibanez et al., 2009; Xiang et al., 2008; Zhang et al., 2007). In addition, *P. syringae* produces catalases that actively break down ROS (Guo et al., 2012). We therefore reasoned that the action of these bacterial enzymes and effectors might promote insect feeding by suppressing the accumulation of cell wall peroxidase-produced hydrogen peroxide or detoxifying it after herbivore attack.

We tested the hypothesis that *P. syringae* suppresses the generation or activity of ROS after herbivory by pre-inoculating WT and *asFBP1.1* mutant plants with *Psm* ES4326 *cfa6* or sterile 10 mM $MgSO_4$ as mock control and measuring LAM and development time. The *asFBP1.1* mutant has no strong pleiotropic effects on JA signaling, callose deposition, or PCD initiation after infection with a diverse group of *P. syringae* isolates (Mammarella et al., 2014). In contrast to WT plants (see Fig. 2), if the primary reason that *Psm* ES4326 *cfa6* causes enhanced feeding is because it suppresses ROS generation or activity, we predicted that *Psm* ES4326 *cfa6* would not enhance the susceptibility of *asFBP1.1* plants, which generate reduced amounts of ROS. To control for the possibility that the mock-inoculated *asFBP1.1* would already be maximally susceptible to *S. flava*, we included mock- and *Psm* ES4326 *cfa6*-inoculated *aos* mutant plants. We previously found that the *aos* mutant, which is deficient in the biosynthesis of JA, is highly susceptible to *S. flava* attack (Whiteman et al., 2011).

As hypothesized, pre-inoculation of *Psm* ES4326 *cfa6* did not enhance *S. flava* feeding on the *asFBP1.1* mutant (Fig. 4A). Similarly, the rate of larval development was not significantly enhanced in the *asFBP1.1* line following inoculation with *Psm* ES4326 *cfa6* (Fig. 4B). As expected, the *aos* mutant allowed even more feeding than the *asFBP1.1* mutant (Fig. 4A), allowing us to rule out the possibility that the *asFBP1.1* line is maximally susceptible to *S. flava*, and therefore conclude that hydrogen peroxide plays a key role in Arabidopsis defense against *S. flava*. However, because *Psm* ES4326 *cfa6* did not enhance susceptibility of the *aos* mutant either, it is also possible that *P. syringae* suppresses

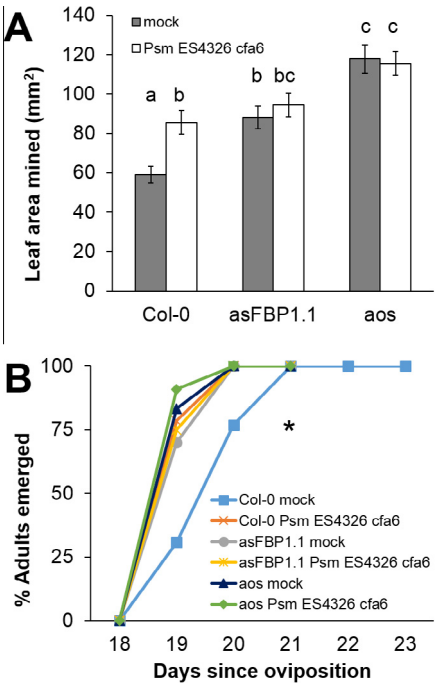

Fig. 4. Enhanced consumption of leaf material and accelerated development of *S. flava* larvae in *P. syringae*-infected leaves are not found in the *asFBP1.1* and *aos* mutants of Arabidopsis. (A) Mean leaf area mined (LAM) by *S. flava* larvae reared in wild-type (WT; Col-0) and mutant Arabidopsis leaves pre-infected with *Psm* ES4326 *cfa6* compared to LAM by larvae reared in mock-inoculated leaves. Larvae were transferred from WT nursing plants in the second instar into the experimental plants and allowed to feed for 48 hours. Error bars represent standard error of the mean. (B) Percentage of *S. flava* pupae having eclosed as adults over time shown in days after the day of oviposition in the nursing plants. Treatment groups: Col-0 mock, light blue squares; Col-0 *Psm* ES4326 *cfa6*, orange crosses; *asFBP1.1* mock, grey circles; *asFBP1.1Psm* ES4326 *cfa6*, yellow stars; *aos* mock, dark blue triangles; *aosPsm* ES4326 *cfa6*, green lozenges. Letters in (A) indicate statistically significant differences (two-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). The asterisk in (B) indicates a statistically significant difference between mock-inoculated Col-0 WT plants and the other treatment groups (χ^2 tests, $P < 0.05$). $N = 15–20$ plants per treatment group.

the JA signaling pathway by activating SA-mediated defense responses, i.e., by classic SA/JA antagonism. This is discussed in more detail below.

3.5. P. syringae suppresses hydrogen peroxide levels after herbivore damage

To further determine whether bacterial suppression of herbivore-induced ROS accumulation underlies the beneficial effect of *P. syringae* on *S. flava* feeding, we tested whether *Psm* ES4326 *cfa6* suppresses the ROS burst after herbivore damage. To do this, we visualized hydrogen peroxide production at *S. flava* feeding sites in plants that had either been pre-infected with *Psm* ES4326 *cfa6* for 4 days as in the LAM and development time assays (Fig. 4A and B), or in plants that had been mock-inoculated. We focused on feeding damage caused by adult females, since these feeding sites are more amenable to quantifying ROS production using light microscopy and DAB staining than larval mines. Adult females create open wounds for feeding, whereas the larval mines are enclosed in the leaf cuticle and various mesophyll cell layers. This means that it is difficult to accurately estimate hydrogen peroxide production in and around larval mines, because results can be confounded by the amount of tissue consumed by the larvae, which reduces the thickness of the leaf. This issue notwithstanding, adult females and larvae both induce the production of hydrogen peroxide upon feeding (Whiteman et al., 2011).

Plants pre-infected with *Psm* ES4326 *cfa6* produced significantly less hydrogen peroxide after subsequent feeding by *S. flava* than mock-inoculated plants (Fig. 5A and B). This was quantified by measuring the area of positive DAB staining around sites of feeding damage (Fig. 5E), and the DAB staining intensity around sites of feeding damage relative to the background levels of staining in undamaged surrounding tissue in the same leaves (Fig. 5F). The same pattern was visible in the *asFBP1.1* mutant (Fig. 5C–F), albeit this mutant produces significantly less hydrogen peroxide than WT plants in response *S. flava* feeding without the presence of *Psm* ES4326 *cfa6* (Fig. 5E–F). The observation that prior infection with *P. syringae* also reduced the levels of DAB staining in the *asFBP1.1* line is consistent with previous reports showing that *asFBP1.1* plants are not completely deficient in the production hydrogen peroxide (Bindschedler et al., 2006; Daudi et al., 2012; Mammarella et al., 2014). Although we did not sample enough eggs to quantify the effects of the presence of egg elicitors on ROS accumulation, the staining around sites of oviposition did not appear to differ visibly from staining around feeding sites without eggs (black arrows in Fig. 5A–D). These results suggest that *P. syringae* promotes *S. flava* feeding by suppressing the ROS burst or by detoxification of ROS.

3.6. Expression of S. flava xenobiotic metabolism genes negatively correlates with levels of plant defense

Finally, we tested whether the altered feeding of *S. flava* on Arabidopsis ROS mutants was reflected in the expression of xenobiotic metabolism genes in the larvae. We previously identified two candidate xenobiotic metabolism genes, *Glutathione S-tranferase D1* (*GstD1*) and *Peritrophin A*. GstD1 is an important detoxification enzyme in *Drosophila* species, involved in xenobiotic and pesticide resistance, including resistance to ROS and downstream lipid peroxidation products (Landis et al., 2012; Mittapalli et al., 2007b; Sawicki et al., 2003), and to ITCs (Gloss et al., 2014). *Peritrophin A* is integral to the peritrophic membrane, which lines the midgut of most insects, and serves as a physical antioxidant in herbivorous insects protecting the epithelium from ROS (Summers and Felton, 1998). The expression of glutathione *S*-transferases and peritrophins changed in another herbivorous dipteran, the Hessian fly, in relation to levels of ROS produced by their wheat host plants (Mittapalli et al., 2007a,b,c). Furthermore, the expression of *GstD1* and *Peritrophin A* is upregulated in *S. flava* larvae in response to chitin pretreatment of Arabidopsis plants, which is known to lead to an ROS burst (Miya et al., 2007; Whiteman et al., 2011). The expression of these two xenobiotic metabolism genes is therefore consistent with the hypothesis that the insect midgut responds transcriptionally to differing levels of plant defensive chemicals such as ROS.

We found that expression of *Peritrophin A* and *GstD1* was downregulated in larvae reared on the cell wall-associated peroxidase-deficient line *asFBP1.1* relative to those reared on the NADPH oxidase mutant *rbohD* (Fig. 6). Conversely, expression of *Peritrophin A* was upregulated in larvae feeding on the NADPH oxidase mutants *rbohD* and *rbohF* compared to those consuming WT plant leaves (Fig. 6). In all three mutants, the expression of *GstD1* changed in the same direction as *Peritrophin A*, but the changes in *GstD1* expression were not significant relative to larvae feeding on WT plants (Fig. 6).

4. Discussion

We found that *P. syringae* enhances *Scaptomyza flava* herbivory by suppressing ROS-based plant defenses. However, the nature of the association between *P. syringae* and *Scaptomyza* spp. remains

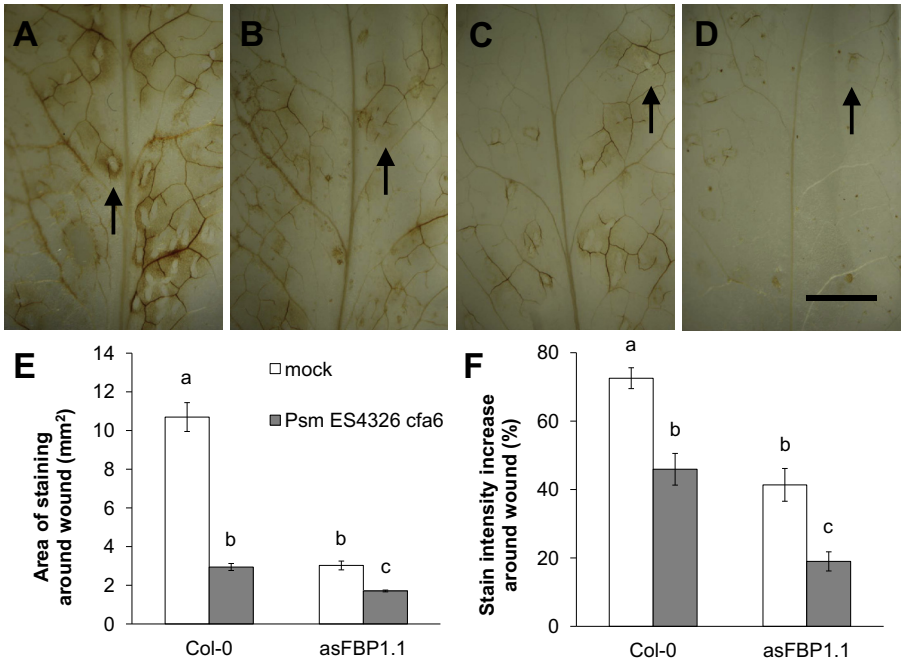

Fig. 5. Infection with *P. syringae* reduces plant hydrogen peroxide levels upon *S. flava* feeding. (A–D) Staining of hydrogen peroxide production (brown coloration) using 3,3′-diaminobenzidine tetrahydrochloride (DAB) in leaves of wild-type (WT; Col-0) (A–B) and *asFBP1.1* mutant (C–D) Arabidopsis plants that were either pre-infected with *Psm* ES4326 *cfa6* 4 days prior to insect feeding (B and D) or mock-inoculated (A and C), left with adult female *S. flava* flies for 24 hours. Note the difference in the relative contrast in staining around the feeding sites (“wounds”) compared to the undamaged surrounding tissue between leaves pre-infected with *Psm* ES4326 *cfa6* and mock-inoculated leaves, and the area of staining around individual feeding sites. Black arrows indicate sites of oviposition in which an egg was laid in the feeding site. (E–F) Leaves representative for each treatment group, such as the leaves of which a close up is shown in (A–D), were selected for quantitative analysis. Letters in (E–F) indicate statistically significant differences (two-way ANOVA with *post hoc* Tukey’s HSD tests (E), χ^2 tests (F), $P < 0.05$). Error bars represent standard error of the mean. Scale bar in (D) for (A–D) indicates 5 mm.

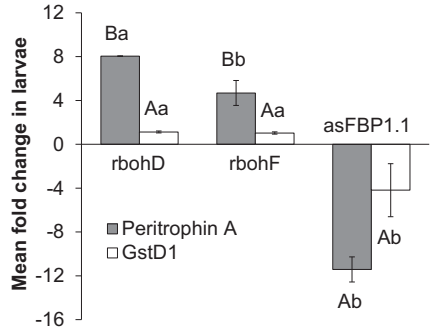

Fig. 6. Expression of two xenobiotic metabolism genes in *S. flava* negatively correlates with the defensive potential of their host plants. The xenobiotic metabolism genes *Peritrophin A* and *Glutathione S-transferase D1* (*GstD1*) are expressed at higher levels in *S. flava* larvae feeding on the *rbohD* and *rbohF* Arabidopsis mutants and at lower levels in larvae feeding on the *asFBP1.1* mutant. This correlates with the lower potential for reactive oxygen species (ROS) formation in *asFBP1.1*, and with the enhanced propensity for programmed cell death and concomitant release of ROS and other defensive chemicals in *rbohD* and *rbohF*. The figure shows the expression of *Peritrophin A* and *GstD1* relative to the control housekeeping gene *RpL32* in larvae reared on Arabidopsis mutants compared to wild-type (WT; Col-0) plants. Error bars represent standard error of the mean. Capital letters indicate statistically significant differences in gene expression between larvae feeding on mutant plants compared to those feeding on WT plants; lowercase letters indicate statistically significant differences in gene expression between larvae feeding on the *asFBP1.1* mutant and larvae feeding on the *rbohD* and *-F* mutants (one-way ANOVA with *post hoc* Tukey’s HSD tests; $P < 0.05$).

an open question: a key consideration is whether *P. syringae* can be vertically transmitted by *Scaptomyza* spp. *Pseudomonas* spp. are the dominant group of bacteria in the gut microbiota of *S. flava*. This is also the case in the Hessian fly (Bansal et al., 2011, 2014), an independently evolved dipteran herbivore. In the Hessian fly, vertical transmission of *Pseudomonas* spp. has been established (Bansal et al., 2011, 2014). Given that *P. syringae* has been found to die in the digestive tracts of Lepidoptera, and that ice-nucleating bacteria such as *P. syringae* might harm the insect in sub-zero temperatures (Mignon et al., 1998), it is unclear whether the association between *Scaptomyza* spp. and *P. syringae* could be maintained through vertical transmission. Nevertheless, *Pseudomonas* spp., including *P. syringae* isolates, form the dominant group of bacteria associated with *Scaptomyza* spp., and the leaves they parasitize, in nature. Our results show that *P. syringae* can be vectored by adult females within and between plants (O’Connor et al., 2014), and by larvae at least within and between leaves (this study).

Although in our larval performance assays we could only directly study the benefits of plant pre-infection with *P. syringae* on the feeding and development of second and third instar *S. flava* larvae, *P. syringae* may also suppress anti-herbivore defenses during the egg and first instar stages. Eggs from Lepidoptera and Diptera (including *D. melanogaster*) can activate PTI and induce ROS accumulation and callose deposition at the site of oviposition (Bruessow et al., 2010; Gouhier-Darimont et al., 2013; Little et al., 2007), leading to reduced larval performance (Geiselhardt et al., 2013). We have here and previously observed the accumulation of ROS at the sites of feeding and oviposition by adult female *S. flava*, and found that egg deposition activated expression of the PTI marker gene *CYP81F2* (Whiteman et al., 2011), which is typically associated with ROS accumulation (Daudi et al., 2012). Since the process of oviposition increases the amount of ROS produced, this will likely make the ROS burst-suppressive effect of the bacteria on *S. flava* larval performance even more important.

The expression of *CYP81F2* is also typically associated with callose deposition (Clay et al., 2009). We have incidentally observed desiccated first-instar larvae and eggs encased by callose in *B.*

vulgaris in nature (Groen and Whiteman, personal observation), and this has been reported in the literature as well (Hering, 1951b). In our larval performance assays we could not assess the potential suppression of callose deposition by *P. syringae* in response to *S. flava* eggs or first-instar larvae. However, ROS accumulation around adult female feeding sites, as well as around sites of egg deposition, was effectively suppressed by infection with *Psm* ES4326 *cfa6* (Fig. 5). Furthermore, a number of *P. syringae* effectors that suppress ROS accumulation, including AvrPto, AvrPtoB, and HopAI1, also suppress callose deposition (Gimenez-Ibanez et al., 2009; Xiang et al., 2008; Zhang et al., 2007). This suggests that *P. syringae* can suppress defenses induced by oviposition, and might thereby facilitate first instar as well as late instar larval feeding. Our data are in striking contrast to results obtained by studying three-way interactions between Arabidopsis, *P. syringae* pv. *tomato* DC3000, and the chewing lepidopteran herbivore *Pieris brassicae*, in which pre-infection with *P. syringae* induced resistance to herbivory (Hilfiker et al., 2014). It will be interesting to disentangle which mechanisms govern the variety of outcomes of three-way plant-microbe-herbivore interactions.

Given the lack of *P. syringae*-induced susceptibility to *S. flava* feeding in both the *asFBP1.1* and the *aos* mutants, it is possible that *P. syringae*-induced susceptibility works at least in part through SA/JA antagonism. That is, infection by *P. syringae* elicits a strong SA-mediated defense response, which in turn antagonizes JA signaling, thereby phenocopying the *aos* mutant. According to this model, ROS and JA-mediated signaling function independently and additively to confer resistance to *S. flava,* and infection with *P. syringae* suppresses both ROS production and JA signaling. This seems unlikely, however, for the following reason: infection of *asFBP1.1* knockdown plants with *Psm* ES4326 *cfa6* does not lead to more *S. flava* feeding than the mock-infected *asFBP1.1* controls. If *Psm* ES4326 *cfa6* independently suppressed both hydrogen peroxide production and JA signaling, we would predict that the level of feeding on infected *asFBP1.1* plants (and on infected WT plants) would have been similar to the higher levels observed on the *aos* mutant, but this was not the case (Fig. 4A).

Furthermore, the *aos* mutant is not only deficient in JA production, which at least partially accounts for the enhanced susceptibility to *S. flava* (Fig. 4), but is also deficient in the biosynthesis of the JA precursor 12-oxophytodienoic acid (OPDA), which is an anti-herbivore defense compound and a regulator of anti-herbivore defense in its own right (Dabrowska et al., 2009; Park et al., 2013; Stintzi et al., 2001; Taki et al., 2005). OPDA signaling is necessary for glutathione homeostasis and, as in the case of the *pad2-1* mutant, glutathione levels are diminished in the *aos* (or *cyp74a*|*dde2-2*) mutant, leading to reduced capacity for class III peroxidase expression (the class that includes cell wall peroxidases) and lower levels of ROS accumulation after attack (Dubreuil-Maurizi et al., 2011; Park et al., 2013; Taki et al., 2005). Thus, both the *aos* mutant and the *asFBP1.1* knockdown line may be defective in ROS production, and the lack of *P. syringae*-induced susceptibility to *S. flava* in *asFBP1.1* and *aos* plants may be a consequence of the diminished potential for ROS accumulation and defensive compounds that depend on ROS signaling for production. OPDA signaling was not the focus of the current study, but future work could elucidate its potential role in three-way plant–microb e–insect interactions.

In the case of the Arabidopsis NADPH oxidase mutants *rbohD* and *rbohF*, enhanced expression of the *Peritrophin A* gene in *S. flava* larvae feeding on these mutants (Fig. 6) contrasted with the data on larval leaf consumption on these mutants, which was unchanged (Fig. 3C), and suggests that activity of the NADPH oxidases do have an effect on *S. flava* physiology. The *rbohD* and *rbohF* mutants have less control over limiting the spread of PCD, which we have previously observed around *S. flava* feeding sites (Whiteman et al., 2011). The release of ROS and other defensive compounds, such as ITCs in the case of Arabidopsis (Andersson et al., 2014), from cells undergoing PCD around feeding sites could exert physiological costs on the herbivore (Agrawal and Kurashige, 2003; Summers and Felton, 1998; Whiteman et al., 2012). However, in this case these costs may fall within the limits of what the larvae can tolerate, so that larval feeding rates do not change. The two opposing roles of the NADPH oxidases in suppressing HR-related PCD and promoting PTI may explain the lack of a difference in the amount of leaf tissue consumed by *S. flava* larvae between the *rboh* mutants and WT plants. Overall, these data are consistent with previous reports showing that *asFBP1.1* plants, but not *rbohD* and *rbohF* mutants, are significantly more susceptible to infection by a variety of bacterial and fungal pathogens (Bindschedler et al., 2006; Galletti et al., 2008; Pogány et al., 2009; Daudi et al., 2012; Mammarella et al., 2014).

5. Conclusions

We found that *S. flava* larvae can vector *P. syringae* within and between leaves, as was previously shown for adults (O'Connor et al., 2014), and that larval performance is enhanced when plants are infected with these bacteria. Enhanced larval feeding and more rapid larval development correlated with bacterial suppression of the plant ROS burst elicited by *S. flava* attack. A causal mechanism for this correlation was suggested by our finding that larval feeding was significantly enhanced in transgenic Arabidopsis *asFBP1.1* plants engineered to produce low levels of hydrogen peroxide, but that pre-infection with *P. syringae* did not increase larval feeding on *asFBP1.1* plants. The increased larval feeding on *asFBP1.1* plants was reflected in the decreased expression of a key gene involved in the insect's xenobiotic response. However, more work is needed to assess the relative importance of suppression of the ROS burst by *P. syringae* in facilitating herbivory compared to other possible mechanisms for the bacterial suppression of anti-herbivore defenses such as the suppression of JA signaling and callose deposition, and the detoxification of ITCs.

Given the apparently close association of *P. syringae* and *S. flava* on their shared host plants and their potentially mutually beneficial relationship, there is a clear potential for either partner to use the other to manipulate and colonize new host plants, as has been described for numerous viral and bacterial plant pathogens and their insect vectors (Casteel et al., 2014; Chung et al., 2013; Kazan and Lyons, 2014; Mauck et al., 2012; Palukaitis et al., 2013; Sugio et al., 2011; Ziebell et al., 2011). In this way, *Scaptomyza* spp. might complement their own offensive mechanisms (e.g. proteinaceous effectors, phytohormones or phytohormone analogs secreted with the saliva) with those offered by microbes, as has been observed in other plant–herbivore and plant–plant parasitic nematode interactions (e.g. Favery et al., 2016; Guiguet et al., 2016; Harris et al., 2015; Kazan and Lyons, 2014; Zhang et al., 2016; Zhao et al., 2016). Future field-based experiments such as those described by Humphrey et al. (2014) and O'Connor et al. (2014) will allow us to gauge the extent and relevance of these associations.

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