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The Oxylipin Signaling Pathway Is Required for Increased Aphid Attraction and Retention on Virus-Infected Plants

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Abstract

Many studies have shown that virus infection alters phytohormone signaling and insect vector contact with hosts. Increased vector contact and movement among plants should increase virus survival and host range. In this study we examine the role of virus-induced changes in phytohormone signaling in plant-aphid interactions, using *Pea enation mosaic virus* (PEMV), pea aphids (*Acyrthosiphon pisum*), and pea (*Pisum sativum*) as a model. We observed that feeding by aphids carrying PEMV increases salicylic acid and jasmonic acid accumulation in pea plants compared to feeding by virus-free aphids. To determine if induction of the oxylipin jasmonic acid is critical for aphid settling, attraction, and retention on PEMV-infected plants, we conducted insect bioassays using virus-induced gene silencing (VIGS), an oxylipin signaling inducer, methyl jasmonate (MeJA), and a chemical inhibitor of oxylipin signaling, phenidone. Surprisingly, there was no impact of phenidone treatment on jasmonic acid or salicylic acid levels in virus-infected plants, though aphid attraction and retention were altered. These results suggest that the observed impacts of phenidone on aphid attraction to and retention on PEMV-infected plants are independent of the jasmonic acid and salicylic acid pathway but may be mediated by another component of the oxylipin signaling pathway. These results shed light on the complexity of viral manipulation of phytohormone signaling and vector-plant interactions.

Keywords Luteovirus · Enamovirus · Lipoxygenase · Hyperoxide lyase · Hormone · Cross-talk · Phytobiome

Introduction

In a rapidly changing environment viruses are considered superior pathogens due to their high rates of mutation and capacity to evolve quickly (Fraile and García-Arenal 2018). This superiority is evident in the fact that most emerging infectious diseases are caused by viruses (Anderson et al. 2004). Over time and due to their ability to quickly evolve, viruses have developed myriad ways to change host physiology and ensure their own survival. This is evident when comparing changes in plant transcripts and metabolites in response to an evolved virus and an unevolved virus, where there are differential effects on stress-related transcripts, plant metabolism, growth, and development (Agudelo-Romero et al. 2008; Hillung et al. 2016; Cervera et al. 2018). Virus-induced changes in host physiology can also affect interactions with other organisms (Mauck et al. 2018). For example, more than 55% of all known plant viruses are dependent on aphid vectors for transmission (Hogenhout et al. 2008) and plant viruses are known to induce specific changes in plants which attract more aphids to infected plants in comparison to healthy plants (Eigenbrode et al. 2018). Some of the strategies exploited by viruses to increase vector attraction are to increase the plant's nutrient content (Casteel et al. 2014; Blanc and Michalakis 2016), to suppress the defense signaling pathway that target the vectors (Casteel et al. 2015; Wu et al. 2017), or to induce a blend of volatiles to attract more vectors (Claudel et al. 2018; Mwando et al. 2018; Safari et al. 2019). These changes in host physiology can make the infected plant more attractive to vectors and in some cases increase insect performance and the number of vectors in the environment. Increased rates of vector contact and increased vector populations should lead to more transmission of the virus and increased survival (Sisterson 2008).

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The oxylipin pathway has been reported to have an important role in attracting/repelling insect herbivores (Kessler et al. 2004; Schuman et al. 2018; Wei and Kang, 2011). A critical study by Kessler et al. (2004) demonstrated that plants deficient in the synthesis of jasmonic acid, one of the best-studied oxylipins, were more susceptible to insects feeding and more attractive to novel insects which were normally repelled from wild type plants. Numerous studies indicate that aphid feeding induces the production of jasmonic acid (Mai et al. 2014; Casteel et al. 2015; Sanchez-Arcos et al. 2016) and related defenses in several hosts (Morkunas et al. 2011) and, indeed, greater numbers of aphids settle and have higher fecundity on plants with lower jasmonic acid levels compared to controls (Morkunas et al. 2011; Zhang et al. 2018). Green leaf volatiles (GLVs) are another important oxylipin pathway derivative, which are produced from a separate branch of the oxylipin pathway. GLVs have been reported to have diverse effects on aphids. They can act as cues to attract aphids (Webster et al. 2008a, b) and also act as indirect defenses against aphids by attracting natural enemies to the aphid fed plants (Verheggen et al. 2008; Wei and Kang 2011).

Aphids are one of the most important vectors of plant viruses (Hogenhout et al. 2008) and viruses are able to manipulate plant signaling to increase host susceptibility to aphids. For example, *Barley yellow dwarf virus* and *Potato leaf roll virus* can induce GLVs to attract aphids to infected plants (dos Santos et al. 2016; Jiménez-Martínez et al. 2004; Rajabaskar et al. 2014; Werner et al. 2009). *Cucumber mosaic virus* was also reported to inhibit the jasmonic acid signaling pathway and induce host attractiveness to insect vectors (Wu et al. 2017). Often plants respond to virus infection by inducing the accumulation of salicylic acid, which upregulates defenses against viruses (Alazem and Lin 2015; Vlot et al. 2009; Wang et al. 2019). Virus induction of salicylic acid can benefit insect vectors through antagonistic impacts on jasmonic acid accumulation and defense induction (Abe et al. 2012; Li et al. 2018). Interestingly, a few studies have also demonstrated that vectors use this antagonistic phenomenon to make the plants more susceptible to vector feeding without the help from viruses (Zhang et al. 2018, 2019; Xu et al. 2019).

In the present study, we investigate the molecular and chemical interactions that occur between *Acrythosiphon pisum* (pea aphid), *Pea enation mosaic virus* (PEMV), and pea plants (*Pisum sativum*). Pea aphids are known to transmit PEMV in a persistent (circulative) and non-propagative manner (Demler et al. 1997). A review by Mauck et al. (2012) suggested that in persistent transmission, virus infection generally increases plant attractiveness for vectors and tends to increase the host quality for long-term feeding to ensure virus acquisition. Pea aphids show a strong preference for PEMV-infected peas and have a higher growth rate on PEMV-infected plants compared to controls (Hodge and Powell 2010; Wu et al. 2014). Wu et al. (2014) demonstrated that PEMV infection increased the ratio of GLVs-to-monoterpenes in plant volatile profiles and differences in aphid settling were attributed to these changes. PEMV-induced changes in plant visual cues have also been suggested to mediate differences in pea aphid settling between infected plants and controls (Hodge and Powell 2010). Recently it was reported that non-vector beetle herbivores prefer PEMV-infected plants compared to controls and that more beetle damage is observed on infected plants in the field (Chisholm et al. 2018, 2019). Increased plant susceptibility to beetles was attributed to phytohormone changes in PEMV-infected plants (Chisholm et al. 2018, 2019), though it is not known whether virus-induced changes in phytohormones may also mediate pea aphid attraction to PEMV-infected plants. To address this lack of knowledge, we first compared changes in salicylic acid, jasmonic acid and the abundance of related transcripts from plants infested with either *A. pisum* carrying PEMV (PEMV-aphids) or virus-free *A. pisum* (virus-free aphids). We hypothesized that aphids carrying PEMV will induce salicylic acid signaling more strongly and that jasmonic acid induction will be reduced compared to virus-free aphid feeding, which would benefit PEMV-aphids. Next, settling and migration assays were performed with pharmacological experiments and virus-induced gene silencing (VIGS) to study the role of changes in the oxylipin jasmonic acid in PEMV-aphid interactions. We expected that the migration assays performed after the pharmacological and VIGS perturbations in oxylipin signaling would disrupt aphid behaviors that are critical for virus transmission. Knowledge of the underlying molecular mechanism of this interaction might be useful for understanding vector-virus ecology in a broader perspective.

Material and Methods

Plants and Growth Conditions After sowing seeds, *Pisum sativum* cv. Banner and cv. Dark Skinned Perfection were grown in Conviron growth chambers under 25 °C/20 °C day/night temperature cycle with a photoperiod of 14/10 h day/night at a relative humidity of 50% and a light intensity of 200 mmol m^{-2} s^{-1}. Plants were grown for a week after sowing and were then immediately used in experiments, unless otherwise noted.

PEMV Infection Contrary to the previous published articles where field isolates of PEMV were used (Chisholm et al. 2018; Hodge and Powell 2010), we decided to use an infectious clone of PEMV. The advantage of using an infectious clone was to make sure of homogeneous populations of the virus leading to less variable response in the tritrophic interactions. Full-length infectious cDNA clones for PEMV1 and PEMV2 were obtained from Prof. Allen Miller (Doumayrou et al. 2016) and *in-vitro* transcription was performed

according to Demler et al. 1994 using mMESSAGE mMACHINE® T7 transcription kit (ThermoFisher, Carlsbad, CA, USA). *In-vitro* transcripts from each clone (1 μg of each transcript) were mixed equally and rub-inoculated on two *P. sativum* leaves previously dusted with carborundum. For control treatments, plants were rub-inoculated with buffer. To confirm PEMV infection, systemic leaves were collected around 7 days post-inoculation. Total plant RNA extraction and DNAse treatment were performed using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA), followed by Reverse Transcription with SMART ® MMLV (Takara Bio USA, Mountain view, CA, USA) and PCR. Ten days after inoculation, systemic leaves showed mosaic symptoms. For sap inoculation, two to three systemic leaves were collected from infected plants and crushed in an inoculation buffer consisting of 0.1 M sodium acetate (pH 6), 5% Sucrose, and 0.2% DIECA. This inoculation buffer/tissue mix was then rub-inoculated on two leaves of one-week-old *P. sativum* that had been previously dusted with carborundum.

Insects All experiments were conducted with *A. pisum* reared on a mixture of *Pisum sativum* cv. 'Dark Skinned Perfection' and *Vicia faba* under controlled conditions (28 °C/24 °C day/night with a photoperiod of 16/8 h day/night). PEMV-infected *A. pisum* were obtained by feeding virus-free adult *A. pisum* on PEMV-infected plants for 4 days before starting an experiment.

Kinetics Bioassay For the hormone and transcript kinetics experiment, 20 PEMV-aphids or 20 virus-free aphids were caged to individual one-week-old pea plants and allowed to feed and reproduce over time. At each time point, aphids were removed and leaves were collected separately from both treatments. The following time points were used for phytohormone quantification: 0, 4, 8, 24, 72 h, and 7 days. For relative transcript abundance 6, 24, 48, 72 h, and 7 days were selected (Fig. 1a & b). Leaves from each treatment were divided into two separate tubes with steel ball bearings in each, immediately flash frozen in liquid nitrogen, crushed using a Harbil 5-Gallon shaker (Fluid Management, Wheeling, IL, USA) and stored at −80 °C until processing. Frozen crushed leaf samples were used for quantifying transcripts and the phytohormones, jasmonic acid and salicylic acid (see methods below).

Construction of Virus Induced Gene Silencing (VIGS) Constructs The VIGS vector pCAPE1 and pCAPE-USER were obtained from Dr. Ida Elisabeth Johansen (University of Copenhagen) and was manipulated as per Constantin et al. (2004) to silence *Coronatine-insensitive 1*(*Coi1)* expression. Briefly, total plant RNA was extracted from *P. sativum* and cDNA was synthesized using Oligo dT, as above. A 459 bp fragment of *Coi1* was PCR-amplified using *Coi1* specific primers (FP: 5′- ggcaattuaattgcgttcccttatgcag and RP: 5′-gggtattuttcagcttttcgcaacctct). The *Coi1* fragment was cloned and subsequently inserted into linearized pCAPE-USER and was named pCAPE-Coi1. A control VIGS vector was made by PCR-amplifying a 529 bp fragment of eGFP using eGFP specific primers (FP: 5′-ggcaattugctgaccctgaagttcatctg and RP: 5′-gggtattugtgatcgcgcttctcgtt). The eGFP fragment was cloned and subsequently inserted into linearized pCAPE-USER and was named pCAPE-eGFPi.

Agrobacterium Infiltration for Silencing *Coronatine-insensitive 1* (*Coi1*) Agrobacterium infiltration was performed as per Constantin et al. (2004). Briefly, *Agrobacterium tumefaciens* containing either pCAPE1, pCAPE-eGFPi, or pCAPE-Coi1 were grown in 5 ml of Luria broth supplemented with 100 μg ml^{-1} kanamycin and 15 μg ml^{-1} rifampicin at 28 °C for 24 h with shaking. The bacteria were pelleted by centrifugation (4500 rpm, room temperature) and resuspended in infiltration media (10 mM $MgCl_2$ and 150 μM acetosyringone). OD_{600} was adjusted to 1.2–1.5 and incubated at room temperature for 90 mins without shaking. Agrobacterium cultures harbouring pCAPE1 and a pCAPE-USER derivative (pCAPE-eGFPi or pCAPE-Coi1) were mixed 1:1 prior to infiltration. The mixed culture was infiltrated to the abaxial side of the youngest pair of leaves on 10-day old *P. sativum* cv. 'Dark Skinned Perfection' using a 1-ml needleless syringe. Silencing of *Coi1* was confirmed through relative quantification of *Coi1* expression (explained below, Supplement Fig. 1). The entire process was also attempted with *P. sativum* cv. 'Banner' without success.

Relative Quantification of Transcripts To verify silencing of *Coi1* in VIGS experiments, systemic leaves were harvested from pea plants 21 days after infiltration. Total RNA was extracted from frozen tissue samples and treated with DNAse as described above. RNA integrity was verified using a 1% (*v*/v) agarose gel. After RNA extraction and DNAse treatment, 1 μg of total RNA was reverse transcribed as described above. Transcripts encoding ICS (ISOCHORISMATE SYNTHASE) and LOX (LIPOXYGENASE) were quantified relative to the *β-tubulin* transcripts using real-time quantitative reverse transcription (qRT)-PCR. Primers were designed following the recommendation of Primer 3 design: *β-tubulin* (FP: gtaacccaagctttggtgatc, RP: actgagagtcctgtactgct), *Ics* (FP: aatcttgcggtggaaacttg, RP: atatgcgcgaatcaaaggac), *Lox* (FP: catcacgcagcagtgaactt, RP: gggtcttctgttggcatgtt) and *Coi1* (FP: gcggcgatgtttaacttgat, RP: cgaaagtgcaacgacttcaa). qRT-PCR was performed using the Bio-Rad CFX384™ Real-Time System in a 10-μL mixture containing SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The thermocycling conditions were: 2 min polymerase activation at 50 °C followed by initial denaturation for 2 min at 95 °C and 40 cycles at 95 °C for 10 s, 55 °C for 30 s. Each sample was quantified in triplicates and included a no-

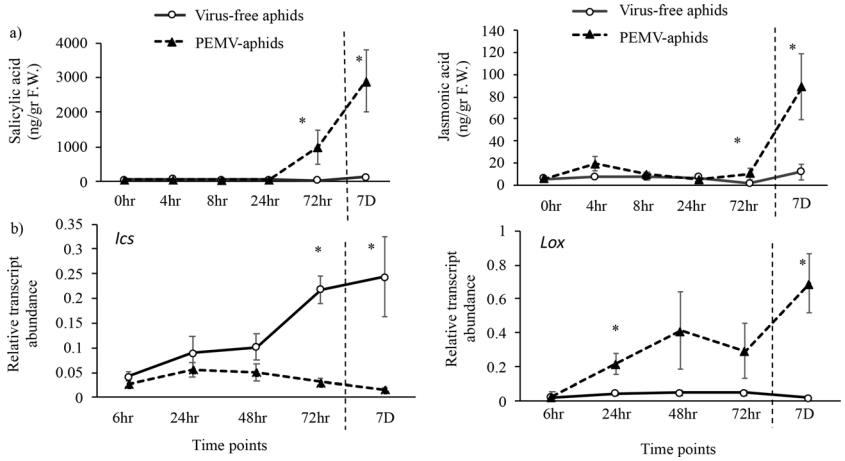

Fig. 1 Effect of virus-free aphids and PEMV-aphids on *Pisum sativum* phytohormones and associated transcripts. Twenty virus-free aphids or 20 PEMV-aphids were kept on each plant. (a) PEMV-aphids induced salicylic acid and jasmonic acid on *P. sativum* compared to virus-free aphids. (b) PEMV-aphids suppressed *Isochorismate synthase* [*Ics*] and induced *Lipoxygenase* [*Lox*] transcript abundance in *P. sativum* compared to virus-free aphids. Mean ± SE of $N = 6$ per treatment per time point. (* indicates significantly different as determined by *LSD* test, $P \leq 0.05$)

template control. Cycle time values were automatically determined for all plates and genes using the Bio-Rad CFX384™ Real-Time System software. Analysis of qRT-PCR fluorescence data was performed and expressed in fold change relative to *β-tubulin* using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Phytohormone Analysis One Milliliter of Extraction Buffer (isopropanol:water:HCl [2:1:0.005, *v*/v]) was added to each frozen sample, internal standards (d_4- salicylic acid and d_5-jasmonic acid; CDN Isotopes) were added, and then samples were homogenized in a paint shaker for 45 s. Samples were extracted with dichloromethane, evaporated, redissolved in 100 μL of methanol, and 10 μL was analyzed using an Agilent Technologies 6420 triple quad liquid chromatography-tandem mass spectrometry instrument (Agilent, Santa Clara, CA, USA) as in (Casteel et al. 2015). For separation, a Zorbax Extend-C18 column 3.0×150mm (Agilent, Santa Clara, CA, USA) was used with 0.01% formic acid in water (A) and 0.01% (*v*/v) formic acid in acetonitrile (B) at a flow rate of 600 mL min^{-1}. The gradient was 0–1 min, 20% B; 1–10 min, linear gradient to 100% B; 10–13 min, 100% A).

Placeholder Text

Choice Bioassay Choice assays with chemical inhibitors and inducers were conducted using *P. sativum* cv. Banner (14 days after sowing). To assess the effect of the inducer, 0.45 mM MeJA +0.01% TWEEN solution, and the inhibitor, 2 mM phenidone +0.01% TWEEN solution, plants were given ten 'mists' of one of the chemicals 2 h before initiating an experiment. A mist represented one spray or ~0.5 ml. For controls, plants were treated with ten mists of 0.01% TWEEN solution in water. For each choice assay, ten adult aphids were placed in the center of a 150 mm petri dish, equidistance from the youngest, non-excised true leaf of a treated and a control plant, as in (Bak et al. 2019; Patton et al. 2019). Aphid choice was recorded 2 h after aphid release. The experiment was performed in dark conditions to remove visual cues.

Choice assays with *Coi1* silenced and control plants (eGFPi) were conducted 21 days after *Agrobacterium* infiltration. This time point was determined earlier to have the maximum silencing of *Coi1* expression. Choice assays were performed as above but using the youngest, non-excised true leaf of a *Coi1* silenced plant and control plant.

Field Experiments An open field experiment was conducted at the Spillman Agronomy Farm in Pullman, WA, USA in 2016 to study the effect of exogenous application of MeJA acid to *P. sativum* plants on *A. pisum* abundance. We established 12 5 × 5 m plots on 27 April, with each plot receiving 72 uniformly transplanted 2-wk old *P. sativum* plants (cv. Banner); plots were separated by a 5 m buffer of open space. After two weeks for plants to establish (11 May), all plants in plots were exposed to one of three randomly selected treatments: (i) control – 0.01% TWEEN solution (a surfactant) mixed with water, (ii) 0.01% TWEEN plus 0.45 mM MeJA, mixed in water, and (iii) insecticide – 0.01% TWEEN plus a 5% formulation of imidacloprid in water, which kills *A. pisum* (Chisholm et al. 2019). Four replicated plots were used for each treatment, and each plant in a plot was sprayed with the specific solution using a water spray bottle to coat the entire plant surface. As effects of treatments were expected to decay over time in the field, treatments were re-applied to each plot on 25 May and 8 June. In each plot, we counted the

total number of *A. pisum* nymphs and adults on 12 randomly selected plants on 18 May, 1 June, and 15 June, approximately one week after each treatment application.

Migration Assays Emigration and immigration assays were adapted with modifications from Chesnais et al. (2019) and performed on *P. sativum* cv. Banner. To estimate emigration, two plants were planted in a single pot 5 cm apart. One week after sowing, 20 PEMV-aphids were caged on one of the plants in each pot and allowed to feed and reproduce for 6 days. All aphids were removed on the last day (i.e., 6th day) and the aphid-inoculated plant was sprayed with either 2 mM pheniodone +0.01% TWEEN or water +0.01% TWEEN until run-off. The other plant in the pot was left untreated. Twenty-four hours after treatment, ten virus-free adult aphids were placed on the chemical sprayed+aphid inoculated PEMV plant in each pot. Immediately after adding aphids to plants a piece of black plastic was placed over the cages to remove all visual cues. The experiment was kept under intermittent observation over a 24-h period and the number of aphids that moved to the healthy untreated plant was counted at the end of 24-h. To estimate immigration, the same procedure was used except that the bioassay was initiated by placing ten aphids on the virus-free untreated plant in each pot and the number of aphids moving to the treated plant was counted.

Statistical Analysis All experiments were repeated at least 5 times on two separate occasions. The specific number of repetitions for presented data is provided in the figure legends. The distribution of the values of all variables analyzed was tested for normality using the Shapiro-Wilk test (Sokal and Rohlf 1995) and were also tested for homogeneity of variances using the Levene test (Sokal and Rohlf, 1995). To determine if PEMV-aphid feeding had impacts on transcripts encoding ICS or LOX, and salicylic acid or jasmonic acid levels across different time points, the data (Fig. 1) were analyzed by generalized linear models (GLM) with a normal distribution which fitted the observed data. The model included aphids, hormones/transcripts, and time points as fixed factor in a full factorial model. GLM was also used to analyze migration assay (Fig. 3) and phytohormones quantification data (Fig. 4) with a normal distribution which fitted the observed data and included phenidone treatment as a fixed factor. The GLM analysis was selected because it is a robust method with respect to the distribution of the data and allows contrasting both balanced and non-balanced models. To determine if the observed differences between classes of the same factor were significant, least significant difference (LSD) analysis were performed. A binomial distribution was used to analyze the choice assays data (Fig. 2). The statistical analyses were performed using the SPSS v.24.0 program (SPSS Inc., IL, USA).

The data from the open field experiment were analyzed using a *generalized linear mixed model* with a negative binomial distribution, which fit the observed count data, to explore effects of treatments on abundance of *A. pisum* (total nymphs + adults). The model included fixed effects of treatment, date, and the date × treatment interaction; the model included a random effect of plot to account for the repeated measures. Analyses were conducted in R (R Core Team 2017) using the lme4 package (Bates et al. 2015).

Results

PEMV-Aphid Feeding Increases Salicylic Acid and Jasmonic Acid in Pea Plants PEMV-aphid feeding significantly increased salicylic acid and jasmonic acid levels in pea plants relative to feeding by virus-free aphids ($P \leq 0.042$, Fig. 1a). Although there was not a significant difference in salicylic acid and jasmonic acid levels at early time points (4, 8, & 24 h), salicylic acid and jasmonic acid levels were significantly higher after 72 h and 7 days of PEMV-aphid feeding compared to virus-free aphid feeding ($P \leq 0.02$, Fig. 1a). Despite higher salicylic acid levels, PEMV-aphid feeding did not increase abundance of transcripts encoding ISOCHORISMATE SYNTHASE (ICS), a critical enzyme for the synthesis of salicylic acid (Wildermuth et al. 2001) (Fig. 1b). Rather, PEMV-aphids suppressed the ICS transcript accumulation after 72 h and 7 days relative to virus-free aphids ($P \leq 0.004$, Fig. 1b). Abundance of the transcript encoding LIPOXYGENASE (LOX), which is responsible for jasmonic acid synthesis (Wasternack and Hause 2013), was also quantified. Consistent with changes in jasmonic acid accumulation, there was a slight increase in transcripts that encode LOX after 72 h and significant increase after 7 days of PEMV-aphid feeding compared to virus-free aphid feeding ($P < 0.001$, Fig. 1b). These data suggest aphid induction of salicylic acid and jasmonic acid are altered when aphids are carrying PEMV.

PEMV-Aphids and Virus-Free Aphids Respond Differently to the Oxylipin Pathway To investigate the importance of oxylipin signaling in *A. pisum*-pea interactions, we next conducted field and laboratory experiments with MeJA, a known inducer of the oxylipin jasmonic acid and downstream defenses (Fig. 2a; Adio et al. 2011). In open-air field experiments the abundance of *A. pisum* decreased over time ($\chi^2 = 6.50$, df = 1, $P = 0.011$) and was affected by the plot treatments ($\chi^2 = 34.1$, df = 2, $P = 0.011$). The total number of *A. pisum* was highest in the control plots, followed by the MeJA plots, followed by the insecticide plots; significant differences were

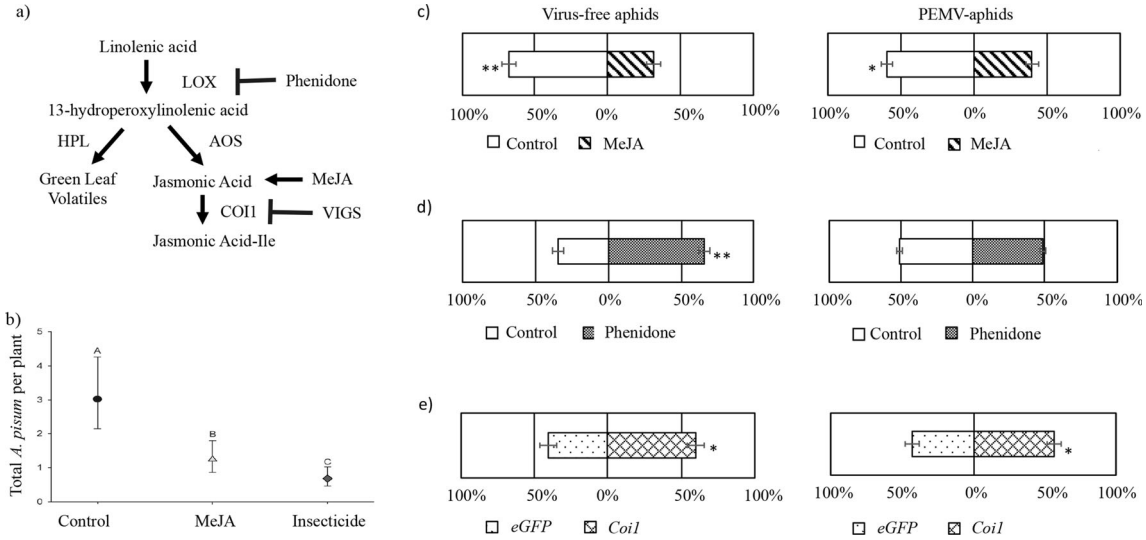

Fig. 2 Manipulation of the oxylipin pathway differentially affects virus-free aphids and PEMV-aphids. (a) A generalized overview of the oxylipin pathway and points of manipulation. (b) The number of aphids per plant in open *Pisum sativum* fields treated with either control (0.01% TWEEN), methyl jasmonate (MeJA) or, insecticide (5% imidacloprid). For each choice, ten adult aphids were placed in the center of a petri dish equidistance from the youngest true leaf of two plants. (c) Upon MeJA treatment, greater numbers of virus-free and PEMV-aphids settled on the control plants. (d) Upon phenidone treatment, greater numbers of virus-free aphids settled on the phenidone treated plants, while PEMV-aphids showed no difference in their settling preferences. (e) Upon VIGS treatment, greater numbers of virus-free and PEMV-aphids settled on plants with *Coi1* silenced. Mean ± SE of (b) $N = 48$ and, (c, d, e) $N = 15–18$ * represent significant differences at $P < 0.05$, *Binomial* test. Abbreviations: LIPOXYGENASE (LOX), CORONATINE-INSENSITIVE 1 (COI1), ALLENE OXIDE SYNTHASE(AOS), HYDROPEROXIDE LYASE (HPL), virus-induced gene silencing (VIGS)

detected between each of these three treatments (Fig. 2b). These results were consistent over time (date x treatment effect: $\chi^2 = 2.18$, df = 2, $P = 0.34$). In laboratory experiments, fewer PEMV-aphids and virus-free aphids settled on plants treated with MeJA compared to untreated plants ($P = 0.0001$, Fig. 2c).

Next, we conduced bioassays with phenidone, an inhibitor of lipoxygenase enzymes, and *Coi1* silenced pea plants using VIGS (Fig. 2a, d, e). When plants were treated with phenidone greater numbers of virus-free aphids settled on treated plants compared to controls ($P = 0.0007$, Fig. 2d), however, there was no impact of phenidone treatment on PEMV-aphid settling ($P = 0.09$, Fig. 2d). In VIGS experiments, greater numbers of PEMV-aphids and virus-free aphids settled on *Coi1* silenced plants compared to VIGS controls ($P \leq 0.047$, Fig. 2e). These results suggest inhibition of a component of the oxylipin signaling pathway downstream from jasmonic acid affects PEMV-aphids and virus-free aphids similarly, but a component upstream from the jasmonic acid branch had a differential effect on PEMV-aphids and virus-free aphids.

PEMV Induction of Oxylipin Signaling is Critical for Aphid Attraction and Retention As phenidone treatment had a differential effect on PEMV-aphid and virus-free aphid settling, it was used further to investigate the role of oxylipin pathway on aphid emigration to, and immigration from, virus-infected plants (Fig. 3). In the emigration assay, significantly higher numbers of pea aphids moved off PEMV-infected plants after the phenidone treatment compared to untreated PEMV-infected plants ($P = 0.059$, Fig. 3a,b). In the immigration assay, significantly fewer aphids migrated from healthy plants to PEMV-infected plants treated with phenidone, compared to untreated PEMV-infected plants ($P = 0.063$, Fig. 3c,d). To determine if there is a direct impact of phenidone on pea aphid settling we conducted a control experiment. There was no difference in aphid settling between phenidone treated and untreated healthy plants without virus ($P = 1$; Supplemental Fig. 2), suggesting phenidone had no direct effect on aphid migration.

As it is known that jasmonic acid and salicylic acid can have an inhibitory impact on each other (Abe et al. 2012; Li et al. 2018), we next wanted to determine the impact of phenidone treatment on jasmonic acid and salicylic acid levels in PEMV-infected plants. Surprisingly, we did not observe any significant difference in jasmonic acid levels between phenidone-treated and untreated PEMV-infected plants ($P = 0.402$, Fig. 4a). Moreover, there was no effect of phenidone on salicylic acid levels in PEMV-infected plants compared to untreated PEMV-infected plants ($P = 0.709$, Fig. 4b). To verify our methods were working properly, we conducted a control experiment. We observed MeJA treatment significantly increased jasmonic acid content in pea plants and treatment with phenidone inhibited the MeJA induction of jasmonic acid ($P = 0.006$, Supplemental Fig. 3). These results together suggest the impact of phenidone on aphid attraction to, and

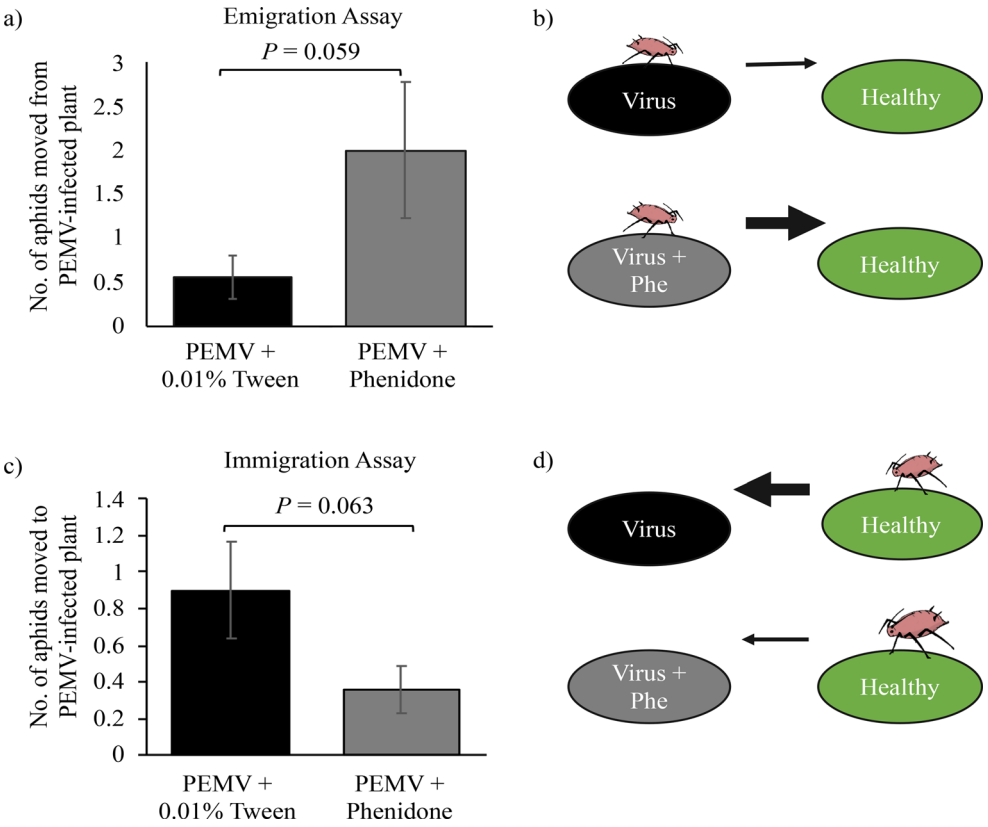

Fig. 3 Inhibition of the lipoxygenase affects the migration of virus-free aphids from, or to, PEMV-infected *Pisum sativum* plants. (a, b) Ten virus-free aphids were kept on phenidone treated or untreated PEMV-infected plants with access to a healthy plant in the same pot for 24 h. (a) More aphids moved from the PEMV-infected plants treated with phenidone compared to untreated infected plants, (b) as illustrated in the cartoon. (c, d) Ten virus-free aphids were kept on a healthy plant with access to a phenidone treated or untreated PEMV-infected plant in the same pot for 24 h. (c) Fewer aphids moved to PEMV-infected plants treated with phenidone compared to untreated infected plants, (d) as illustrated in the cartoon. Mean ± SE of $N = 10–14$; P determined from a *LSD* Test

retention on, PEMV-infected plants is dependent on the oxylipin pathway but independent of the jasmonic acid and salicylic acid.

Discussion

Our results demonstrate that PEMV increases *A. pisum*-mediated induction of jasmonic acid and salicylic acid in *P. sativum* plants and indicates a complex role for the oxylipin pathway in *P. sativum*-*A. pisum*-PEMV interactions. It had been previously shown that PEMV infection induces salicylic acid accumulation in pea plants compared to uninfected controls (Chisholm et al. 2018, 2019) and we hypothesized that this would inhibit the induction of jasmonic acid by aphids. We observed elevated levels of salicylic acid after 7 days of PEMV-aphid feeding compared to virus-free aphid feeding (Fig. 1). However, we did not observe inhibition of jasmonic acid, but instead observed elevation of jasmonic acid content in plants with PEMV-aphids compared to virus-free aphids (Fig. 1). These results suggest that the increases in *A. pisum* attraction and performance on PEMV-infected plants (Wu et al. 2014; Hodge and Powell, 2010) were not due to cross-talk between salicylic acid and jasmonic acid. Chisholm et al. (2018) also observed a small increase in jasmonic acid in PEMV-aphid treatments compared to virus-free aphid treatments, although the increase was not significant ("Sham" in Chisholm et al. 2018). The lack of significance in the previous experiment may be due to differences in experimental methods. In our study PEMV-aphids were allowed to feed

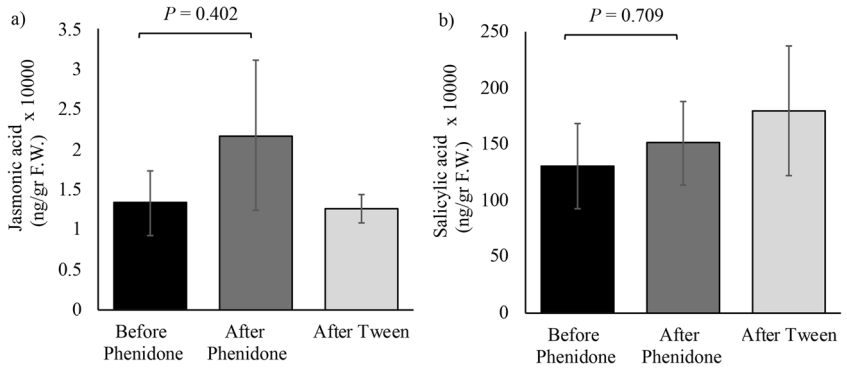

Fig. 4 Quantification of phytohormones in PEMV-infected plants before and after phenidone treatment. (a) Jasmonic acid and (b) salicylic acid content in 14-day-old *Pisum sativum* previously fed with PEMV-aphids for 6 days followed by treatment with either phenidone or 0.01% TWEEN as a control. Mean ± SE of $N = 5$; P determined from a *LSD* Test

for 7 days and then tissue was collected, while in Chisholm et al. (2018) PEMV-aphids were allowed to feed for two days and then removed. Ten days after aphid removal tissue was collected for phytohormone analysis. These differences add to the growing literatures showing that virus-induced changes in plant physiology differ when their insect vectors are present (Bak et al. 2017).

Although we did not observe evidence of antagonistic cross-talk between jasmonic acid and salicylic acid at the metabolite level as has been shown previously (Vlot et al. 2009; Alazem and Lin 2015; Wang et al. 2019), we did at the transcriptional level (Fig. 1b). The ICS protein catalyzes an important step in salicylic acid synthesis (Wildermuth et al. 2001). Surprisingly, aphids induced *Ics* transcription relative to PEMV-aphids feeding on plants, but salicylic acid accumulation was not higher in these plants (Fig. 1a,b). This result indicates that the aphids carrying PEMV might be inducing salicylic acid in an ICS-independent manner. The ICS pathway is thought to be the primary producer of salicylic acid in *Arabidopsis thaliana*, though salicylic acid can also be produced through PHENYLALANINE AMMONIA LYASE (PAL) (Chen et al. 2009). Recently it was demonstrated that both the ICS and PAL pathways are equally important for pathogen-induced biosynthesis of salicylic acid in soybean (*Glycine max*), another legume like *P. sativum* (Shine et al. 2016). LOX is a critical enzyme in the early steps of oxylipin and jasmonic acid biosynthesis (Wasternack and Hause 2013). PEMV-aphids induced both LOX transcripts and jasmonic acid metabolite levels relative to virus-free aphid feeding on pea plants (Fig. 1a,b), showing a direct correlation between LOX transcript level and jasmonic acid. Chisholm et al. (2018), (2019) showed that non-vector beetle (*Sitona lineatus*) feeding also induced jasmonic acid in healthy pea plants relative to controls and greater numbers of virus-free aphids settled on these plants compared to undamaged controls. Considering our data along with the studies of Hodge and Powell (2010), and Chisholm et al. (2018) and (2019), it is interesting to see that when there is jasmonic acid induction (beetle-damaged plants or PEMV-aphid-induced plants) virus-free aphids prefer to settle on these plants and perform better.

Our choice assays demonstrate that the oxylipin pathway is important for aphid settling preferences and has differential effects on PEMV-aphids compared to virus-free aphids (Fig. 2). When components of jasmonic acid signaling were manipulated using MeJA or VIGS (Fig. 2a), virus-free and PEMV-aphids preferred to settle on leaves with lower jasmonic acid levels, irrespective of virus status (Fig. 2b,c,e). In contrast, when upstream components of oxylipin signaling where manipulated using phenidone, virus-free and PEMV-aphids responded differentially (Fig. 2d). While greater numbers of virus-free aphids settled on phenidone-treated leaves compared to untreated, PEMV-aphids did not show any settling preferences (Fig. 2d). This may suggest that aphids are not able to respond to changes in volatile profiles related to the oxylipin signaling pathway after PEMV acquisition. Conditional host cue responses after virus acquisition by aphids have been demonstrated in other systems (Ingwell et al. 2012), however, this is the first time it has been demonstrated for the pea aphid/PEMV pathosystem, and the first time it was specifically linked to the oxylipin signalling pathway. In an ecological framework our data may support a role of oxylipin signaling in increasing virus transmission or accumulation in the environment. Chisholm et al. (2018) reported that non-vector beetle feeding induces jasmonic acid and positively affects virus titer and aphid performance. Thus, in nature non-vector-fed plants with induced jasmonic acid will repel virus-free aphids whereas PEMV-aphids, which show no preference, can settle on the damaged plants, which are also better hosts for the virus.

The increase in aphid retention and attraction induced by PEMV infection was dependent on components of the oxylipin signaling pathway (Fig. 3), possibly oxylipin-dependent host volatiles. We demonstrated that PEMV-aphid feeding induces jasmonic acid accumulation in pea plants over time (Fig. 1a) and that fewer aphids were attracted to PEMV-infected plants treated with phenidone compared to untreated infected plants (Fig. 3c,d). As we did not observe a decrease in jasmonic acid levels in PEMV-infected plants treated with phenidone compared to untreated PEMV-infected plants (Fig. 4a), this suggests that another component of the oxylipin pathway is mediating the interaction. Jasmonic acid is the final product of the ALLENE OXIDE SYNTHASE (AOS) branch of the oxylipin pathway. Another branch of the oxylipin pathway is catalyzed by HYDROPEROXIDE LYASE (HPL) and is responsible for the synthesis of many GLVs (Scala et al. 2013). Several viruses have been implicated in regulating volatiles derived from the oxylipin pathway to attract aphids (Eigenbrode et al. 2002; Jiménez-Martínez et al. 2004; Sharifi et al. 2017), including PEMV (Wu et al. 2014). As both branches are downstream from lipoxygenase (Fig. 2a), manipulating the oxylipin pathway using the phenidone in PEMV-infected plants may disrupt one or both branches of the pathway. Although significant, induction of jasmonic acid by PEMV-aphids was very low in our study, compared to induction of jasmonic acid levels in pea after MeJA treatment (Supplemental Fig. 3) and beetle damage (Chisholm et al. 2018). Thus, inhibition of lipoxygenases by phenidone may primarily impact PEMV-induced GLVs production and the HPL branch of oxylipin signaling if the AOS branch is not induced as high in comparison. The increase of jasmonic acid and related transcripts in PEMV-aphid treatments might also be a side effect of virus-induced increases in oxylipin-dependent GLV production, as suggested in Scala et al. (2013). Additional downstream components of the oxylipin pathway or intermediates may be involved, as well as other

plant hormones. In addition to changes in plant defense and signaling, virus infection is known to alter primary metabolites such as amino acids (Patton et al. 2019, Casteel et al. 2014). Many primary metabolites can act as phagostimulants and important components of host nutritional quality, thus changes in these compounds may be important for viruses that are transmitted in a persistent manner, where longer feeding is required. It has been suggested that aphids vectors will use volatiles initially for host selection, while host nutritional quality should play a more important role on aphid retention (Mauck et al. 2012). Additional experiments will be required to determine the specific role of oxylipin signaling pathway in virus transmission, though the current findings open the door for more molecular studies in *A. pisum-P. sativum*-PEMV interactions.

In a span of 80 to 100 years, viruses can evolve rapidly to adapt themselves to new hosts and in the process they may lose the capacity to infect old hosts (Fraile et al. 1997; Bera et al. 2018). PEMV-1 (*Luteoviridae*) and PEMV-2 (*Tombusviridae*) contain RNA genomes, and cause huge losses in production for legume producers (Chisholm et al. 2019; Clement et al. 2010). RNA viruses are prone to mutations, which are considered the raw agents driving evolution. High mutation rates allow viruses to evolve rapidly in agricultural and natural ecosystems, where plants, vectors, and non-vectors co-exist (Chisholm et al. 2019; Clark et al. 2019). A relevant study by Pagan and Holmes (2010) on long-term evolution of different virus species from the *Luteoviridae* family showed that most of the new species originated in the last few hundred years, during the rise of agriculture (Pagan and Holmes 2010). Thus, understanding evolution and ecological interactions at the molecular level has the potential to help in developing better disease management tools.

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