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Root-Derived Oxylipins Promote Green Peach Aphid Performance on *Arabidopsis* Foliage [W]

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Oxylipins function as signaling molecules in plant growth and development and contribute to defense against stress. Here, we show that oxylipins also facilitate infestation of *Arabidopsis thaliana* shoots by the phloem sap–consuming green peach aphid (GPA; *Myzus persicae*), an agronomically important insect pest. GPAs had difficulty feeding from sieve elements and tapping into the xylem of *lipoxygenase5* (*lox5*) mutant plants defective in LOX activity. These defects in GPA performance in the *lox5* mutant were accompanied by reduced water content of GPAs and a smaller population size of GPAs in the mutant compared with the wild-type plant. *LOX5* expression was rapidly induced in roots in response to infestation of shoots by GPAs. In parallel, levels of *LOX5*-derived oxylipins increased in roots and in petiole exudates of GPA-colonized plants. Application of 9-hydroxyoctadecadienoic acid (an oxylipin produced by the LOX5 enzyme) to roots restored water content and GPA population size in *lox5* plants, thus confirming that a *LOX5*-derived oxylipin promotes infestation of the foliage by GPAs. Micrografting experiments demonstrated that GPA performance on foliage is influenced by the *LOX5* genotype in roots, thus demonstrating the importance of root-derived oxylipins in colonization of aboveground organs by an insect.

INTRODUCTION

Oxylipins, which encompass a large family of oxidized fatty acids, play a pivotal role as signaling molecules and protective compounds in plant response to biotic stress (Blée, 2002; Prost et al., 2005). Oxylipins are also implicated in cross-kingdom communication between plants and pathogenic fungi (Christensen and Kolomiets, 2011). The first step in the synthesis of oxylipins involves the formation of fatty acid hydroperoxides either by autooxidation or by the action of enzymes, such as lipoxygenases (LOXs) and α-dioxygenases (Feussner and Wasternack, 2002; Mosblech et al., 2009). Spontaneous or enzymatic modification of fatty acid hydroperoxides yields an additional array of oxylipins (Feussner and Wasternack, 2002; Mosblech et al., 2009).

The *Arabidopsis thaliana* genome contains six *LOX* genes, which encode proteins that are classified as 9- and 13-LOXs based on their ability to incorporate oxygen either at the C-9 or C-13 position of the fatty acid, yielding the 9- or 13-fatty acid hydroperoxides, respectively (Liavonchanka and Feussner, 2006). *Arabidopsis LOX1* and *LOX5* encode 9-LOXs, whereas *LOX2*, *LOX3*, *LOX4*, and *LOX6* encode 13-LOXs (Bannenberg et al., 2009). Jasmonic acid (JA), which is derived from the 13-LOX pathway, is one of the best-studied oxylipins that has a signaling function in plant growth and development and in stress response (Howe and Jander, 2008; Browse, 2009; Wu and Baldwin, 2010). 9-LOX–derived oxylipins are also involved in plant growth and development and in stress response. For example, 9-LOX–derived oxylipins are involved in lateral root development in *Arabidopsis* and maize (*Zea mays*) (Vellosillo et al., 2007; Gao et al., 2008) and in defense against bacterial pathogens in pepper (*Capsicum annuum*) and *Arabidopsis* (Vellosillo et al., 2007; Hwang and Hwang, 2010; López et al., 2011) and against fungal pathogens and nematodes in maize (Gao et al., 2007; Gao et al., 2008; Gao et al., 2009).

Insect infestation is a major factor that limits plant production (Ferry et al., 2006). Insect pests of plants can be classified into two broad groups, the chewing insects and the piercing/sucking insects. Chewing insects use their strong mandibles to chew on plant tissue, thus resulting in extensive physical damage and tissue loss (Karban and Baldwin, 1997; Kandoth et al., 2007). Among the piercing/sucking insects, thrips and spider mites are cell-content feeders that use their mouthparts, which are modified into stylets, to consume the content of mesophyll and/or epidermal cells (Walling, 2000). By contrast, aphids, whiteflies, and leafhoppers use their stylets to consume fluids from the vasculature. This feeding behavior of aphids and whiteflies results in minimal wounding to the plant (Walling, 2000).

More than 250 species of aphids are pests of plants (Dixon, 1998; Blackman and Eastop, 2000). Aphids affect plant productivity by removal of photoassimilates and by altering source-sink patterns (Dixon, 1998; Blackman and Eastop, 2000; Goggin, 2007). In addition, some aphids are also vectors for plant viruses (Kennedy et al., 1962; Matthews, 1991; Guerrieri and Digilio, 2008). Although the aphid stylet penetrates the host tissue primarily intercellularly, occasionally it punctures cells, allowing the insect to sample cell contents (Pollard, 1973). Two types of saliva are released by aphids into the plant. The gelling saliva, which is secreted when the stylet is penetrating host tissue, insulates the stylet from plant tissue and forms a tight seal around the penetrated site. By contrast, the watery saliva, which contains several hydrolytic enzymes and is intermittently released into the sieve elements during insect feeding from the phloem (Miles, 1999; Tjallingii, 2006), likely aids in reversal of phloem

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occlusion (Will et al., 2007), thus facilitating continued feeding from the sieve elements. The sugar-rich phloem sap that is consumed by aphids has an osmotic potential that is considerably more negative than the aphid hemolymph, and the resulting gradient could result in the dehydration of the aphid (Douglas, 2006). Occasionally, aphids also ingest xylem sap (Spiller et al., 1990; Tjallingii and Hogen Esch, 1993; Powell and Hardie, 2002; Douglas, 2006). The lower solute concentration in xylem sap and hence a higher osmotic potential as compared with phloem sap (Mattson, 1980; Taiz and Zeiger, 2002) is suggested to enable aphids to osmoregulate their hemolymph and prevent dehydration (Pompon et al., 2010, 2011).

Some aphids have a restricted host range and are considered as specialists. For example, the host range of cabbage aphid (*Brevicoryne brassicae*) and mustard aphid (*Lipaphis erysimi*) is limited to related cruciferous species (Blackman and Eastop, 2000). By contrast, a generalist aphid like *Myzus persicae* Sülzer, more commonly known as the green peach aphid (GPA), is polyphagous and has a wide host range that includes more than 50 plant families (Blackman and Eastop, 2000). It has been suggested that generalist aphids use nutritional cues to make their host selections (Powell et al., 2006), whereas specialist aphids utilize plant secondary metabolites as cues for host recognition, feeding, and oviposition (Raybould and Moyes, 2001; Macel and Vrieling, 2003).

The coevolution of plants and their insect pests has resulted in the development of complex interactions between them. Whereas plants have evolved a variety of defense and tolerance mechanisms against insect herbivory (Howe and Jander, 2008), insects have evolved countering strategies to bypass and/or overcome the barriers used by plants (Hogenhout and Bos, 2011). Much of our current understanding of molecular mechanisms that influence plant interaction with insects arises from studies dealing with response of the aboveground shoot foliage to insect attack (Howe and Jander, 2008). However, although less well studied, physiological changes in the roots also affect the severity of infestation in the shoots (Erb et al., 2009). For example, a variety of secondary metabolites (e.g., nicotine) that are involved in protecting aboveground tissues are synthesized in roots, from which they are transported to shoots in response to insect infestation (Baldwin et al., 1994; Erb et al., 2009; Morita et al., 2009). Here we show that in *Arabidopsis*, the *LOX5*-encoded 9-LOX–derived oxylipin(s) functions as a susceptibility factor in plant interaction with GPA. *LOX5* facilitates insect feeding from sieve elements and water consumption from xylem. We provide genetic and biochemical evidence that roots are the source of the *LOX5*-derived oxylipin(s) that facilitates colonization of *Arabidopsis* shoots by GPA, thus highlighting the dependence of GPA on root-derived oxylipins for colonizing *Arabidopsis*.

RESULTS

The GPA Population Size Is Smaller in *lox5* Mutant Plants Than in Wild-Type *Arabidopsis* Plants

Accumulation of 9-LOX–synthesized oxylipins and volatile compounds derived from these oxylipins was previously shown to be stimulated in leaves of plants infested with aphids (Gosset et al., 2009). Some 9-LOX–derived volatiles induce oviposition in aphid predators (Verheggen et al., 2008), thus implicating a potential role for 9-LOXs in indirect defense against aphids. 9-LOX–synthesized oxylipins also accumulate both in phloem sap and in aphids feeding on plants (Harmel et al., 2007), suggesting that these oxylipins may also have a more direct role in plant–aphid interaction. *Arabidopsis* contains two 9-LOX–encoding genes, *LOX1* (At1g55020) and *LOX5* (At3g22400) (Bannenberg et al., 2009). Whereas LOX1 is localized to the plastids (see Supplemental Figures 1C, 1G, and 1K online), LOX5 exhibits extraplastidic localization (see Supplemental Figures 1D, 1H, and 1L online).

To determine whether either *LOX1* or *LOX5* has a role in plant–aphid interaction, a no-choice bioassay was conducted in which 20 adult apterous (wingless) GPAs were released both on wild-type *Arabidopsis* plants (accession Columbia-0 [Col-0]) and on *lox1* or *lox5* mutant plants that lack *LOX1* or *LOX5* function, respectively. Two d after inoculation, the size of the insect population on each plant was determined. As shown in Figure 1A, GPA population size on the *Arabidopsis lox5-1* and *lox5-3* mutants, which contain T-DNA insertions within the *LOX5* coding sequence (see Supplemental Figures 2A to 2C online), was significantly smaller than in wild-type plants. Ectopic expression of LOX5 or a LOX5-green fluorescent protein (GFP) protein fusion from the *Cauliflower mosaic virus 35S* promoter attenuated the *lox5-1*– and *lox5-3*–conferred enhanced resistance to GPA (Figure 1B), thus confirming that some *LOX5*-dependent activity is a susceptibility factor that predisposes *Arabidopsis* to infestation by GPA. In comparison with the *lox5* mutants, insect numbers in the *lox1-1* and *lox1-3* mutants were greater and comparable with those in the wild type (Figure 1A). Furthermore, in comparison with the wild type and *lox1-1* single mutant, insect numbers were comparably low on the *lox1-1 lox5-1* double mutant and the *lox5-1* single mutant plant (Figure 1C), thus indicating that unlike *LOX5*, *LOX1* does not have a discernible role in the *Arabidopsis*–GPA interaction.

Vascular Sap from GPA-Infested *lox5* Mutant Plants Lacks an Activity That Facilitates GPA Infestation

Vascular sap-enriched petiole exudates of uninfested *Arabidopsis* contain an inhibitory activity that limits the size of the GPA population on an artificial diet (Figure 2) (Pegadaraju et al., 2007; Louis et al., 2010a; Louis et al., 2010b). However, by contrast with petiole exudates from leaves of uninfested plants, petiole exudates from leaves of GPA-infested wild-type plants increased the insect population size on an artificial diet (Figure 2), thus suggesting that GPA infestation manipulates the host to make vascular sap more suitable for the insect. Although *LOX5* did not affect the accumulation of this infestation-promoting activity in petiole exudates from uninfested leaves (Figure 2), petiole exudates collected from GPA-infested leaves of the *lox5-1* and *lox5-3* mutants were less effective in promoting GPA population than comparable petiole exudates from wild-type leaves (Figure 2). We thus conclude that *LOX5* is required for the GPA-infestation–associated alterations of vascular sap that make the sap more palatable to the insect.

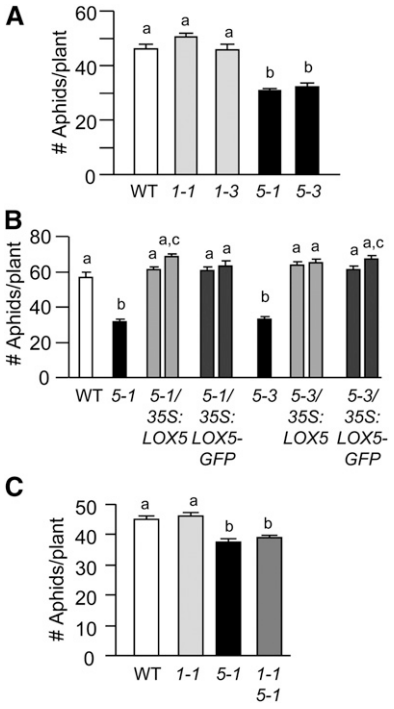

Figure 1. GPA Population Size Is Smaller on the *lox5* Mutants Than the Wild-Type Plant.

(A) No-choice test. Comparison of total numbers of GPAs (adults + nymphs) on the wild type (WT) and 9-LOX mutants, *lox1-1*, *lox1-3*, *lox5-1*, and *lox5-3*.

(B) No-choice test. Comparison of total number of GPAs on the wild type, *lox5-1*, *lox5-3*, and two independent *35S:LOX5* and *35S:LOX5-GFP* transgenic lines each in the *lox5-1* and *lox5-3* backgrounds.

(C) No-choice test. Comparison of GPA numbers on the *lox1-1* and *lox5-1* single mutants and the *lox1-1 lox5-1* double mutant.

In **(A)** to **(C)**, all values are means from 12 plants ± SE. Different letters above the bars indicate values that are significantly different ($P < 0.05$) from each other by GLM/ANOVA.

GPA Is Unable to Tap into Xylem and Spends Less Time in Sieve Elements of *Arabidopsis lox5* Mutant Plants

Electrical penetration graph (EPG) analysis provides a sensitive technique to study insect feeding behavior in plants (Walker, 2000). Previously, EPG has been used to compare the effect of wild-type and mutant *Arabidopsis* genotypes on GPA feeding behavior (Pegadaraju et al., 2007; Louis et al., 2010a; Louis et al., 2010b; Singh et al., 2011). In EPG, a wired insect that is part of a low-voltage circuit is released on a plant, and its activity is electrically monitored. The pattern of different waveforms generated provides insights into the time spent by the insect in different activities, including the nonprobing phase (NP), when the insect stylet is not inserted into the plant, the pathway phase, when the insect stylet, although inserted in the plant tissue, is outside the phloem and xylem, the xylem phase (XP), when the insect is drinking from the xylem, and the sieve-element phase (SEP), when the stylet is in the sieve element.

Comparisons of GPA feeding behavior in the wild-type and *lox5-1* mutant plants revealed differences in the time spent by GPAs in SEP. As shown in Table 1, GPAs spent less time in SEP in *lox5-1* mutant plants than in wild-type plants. GPAs also had difficulty tapping into the xylem of the *lox5-1* mutant plant compared with the wild-type plant. Although 50% of the insects feeding on wild-type *Arabidopsis* exhibited one or more XP, no XP was observed in more than 100 h of EPG recording involving 16 insects on the *lox5-1* mutant plant (Table 1). The reduced time spent by GPAs in SEP and XP on the *lox5-1* mutant was paralleled by an increase in NP on the *lox5-1* mutant compared with the wild-type plant. These results suggest that a product of LOX5 activity facilitates GPA feeding from sieve elements and water consumption from xylem.

To determine whether the absence of XP in GPAs reared on the *lox5* mutant affects the hydration status of insects, water content was evaluated in aphids collected from the wild-type and *lox5-1* and *lox5-3* mutant plants. As shown in Figure 3, the percentage water content of GPAs reared on wild-type plants was significantly higher than in GPAs collected from *lox5-1* and *lox5-3* mutant plants. By contrast with insects reared on the *lox5-1* and *lox5-3* mutant, water content in GPAs reared on the *lox5-1* and *lox5-3* plants expressing either the *35S:LOX5* or *35S:LOX5-GFP* transgenes was comparable with that in insects reared on the wild-type plant (Figure 3). These results indicate that the inability of GPA to tap into the xylem of the *lox5* mutants is associated with lower water content in the insect. We therefore conclude that a *LOX5*-dependent factor(s) facilitates the insect's ability to tap into xylem and phloem.

LOX5 Function in *Arabidopsis* Roots Promotes Insect Performance in Leaves

A survey of publicly available microarray data sets (http://www.genevestigator.com/) and quantitative RT-PCR experiments (see Supplemental Figure 3 online) revealed that *LOX5* expression in *Arabidopsis* leaves was very poor. Furthermore, *LOX5* expression was not significantly altered in GPA-infested leaves (see Supplemental Figure 3 online). Considering that GPA is unable to tap into xylem of the *lox5* mutant and that the general flow of xylem contents is from roots to shoots, we hypothesized

Table 1. EPG: Time Spent by GPAs for Various Activities on *Arabidopsis* Wild-Type and *lox5-1* Plants

Insect Activity	Wild Type	*lox5-1*	P Values
Time spent in NP[a]	1.89 ± 0.39	3.08 ± 0.44	0.055[b]
Time spent in pathway phase[a]	4.63 ± 0.44	4.26 ± 0.48	0.678
Time spent in SEP[a]	1.29 ± 0.29	0.66 ± 0.12	0.056[b]
Time spent in XP[a]	0.19 ± 0.06	n.d.	
Time to first probe[a]	0.19 ± 0.05	0.16 ± 0.04	0.910
Time to first SEP[a]	2.78 ± 0.52	1.93 ± 0.38	0.122
Time to first XP[a]	4.58 ± 0.84	n.d.	

EPG recording was conducted on 16 aphids on plants of each genotype. Recording with each insect was conducted for 8 h. n.d., none detected.

[a]Each value in hours is the average ± SE of 16 replications.

[b]Parameters that are significantly different (Kruskal-Wallis test) between GPAs reared on the wild type versus *lox5-1* mutant.

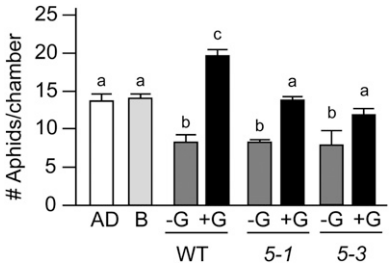

Figure 2. *lox5* Petiole Exudates Lack an Insect-Fecundity–Promoting Activity.

Artificial diet assay showing a comparison of GPA numbers on petiole exudates collected from uninfested (−G) and GPA-infested (+G) wild-type (WT) and *lox5-1* and *lox5-3* plants. Controls were an artificial diet alone (AD) and a diet supplemented with the buffer (B) used to collect petiole exudates. Values are means (±SE) of six replicates for each treatment. Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; GLM/ANOVA).

that *LOX5* function might be required in roots of GPA-infested plants. Indeed, *LOX5* expression was rapidly induced in the roots within 3 h of release of GPA on the leaves (Figure 4A). *LOX5* expression was sustained at elevated levels through the 48-h duration of the experiment.

To test whether *LOX5* activity in roots facilitates infestation of *Arabidopsis* shoots by GPA, reciprocal micrografting of seedlings was used to generate chimeric *LOX5*/*lox5-1* plants that contained wild-type *LOX5* scions (shoots) and *lox5-1* mutant rootstock and *lox5-1*/*LOX5* chimeric plants that contained *lox5-1* scion and *LOX5* rootstock. *LOX5*/*LOX5* and *lox5-1*/*lox5-1* self-grafted plants were used as the wild-type and *lox5-1* mutant controls. Allele-specific PCR was used to confirm the genotype of the scion and rootstock (see Supplemental Figure 4 online). These plants were used to determine the effect of root genotype at the *LOX5* locus on aphid performance in the shoots. It was anticipated that, if *LOX5* function in the roots facilitates GPA infestation in the shoots, then the GPA population would be smaller on chimeric plants that have a *lox5-1* mutant rootstock compared with plants with a wild-type *LOX5* rootstock. As expected, in a no-choice assay, GPA population in the control *lox5-1*/*lox5-1* self-graft was smaller than in the *LOX5*/*LOX5* plant (Figure 4B). The GPA population was also significantly smaller in the *LOX5*/*lox5-1* chimera, which contains a wild-type *LOX5* scion and a *lox5-1* mutant rootstock, than in the *LOX5*/*LOX5* plant. By contrast, the GPA population in the *lox5-1*/*LOX5* chimera, which contains a *lox5-1* mutant scion and a wild-type *LOX5* rootstock, was comparable with that in the *LOX5*/*LOX5* plant and significantly higher than in the *LOX5*/*lox5-1* and *lox5-1*/*lox5-1* plants. These results, taken along with the upregulation of *LOX5* in roots of GPA-infested plants, confirm that *LOX5* activity in the roots facilitates colonization of the shoots by GPA.

9-LOX Products Accumulate at Elevated Levels in Roots and Petiole Exudates of GPA-infested Plants

Because *LOX5* encodes a 9-LOX (Bannenberg et al., 2009), we monitored levels of the following 9-LOX synthesized 9-hydroperoxy- and 9-hydroxy-fatty acids (9-HPs) in roots of wild-type and *lox5-1* mutant plants in which shoots were colonized by GPA: 9-hydroxyoctadecadienoic acid (9-HOD), 9-hydroperoxyoctadecadienoic acid (9-HPOD), 9-hydroxyoctadecatrienoic acid (9-HOT), and 9-hydroperoxyoctadecatrienoic acid (9-HPOT). As shown in Figure 4C, levels of all four 9-HPs were higher in roots of GPA-infested wild-type plants compared with roots of uninfested wild-type plants. By contrast, in roots of GPA-infested *lox5-1* mutant, this increase in 9-HPs was significantly lower, confirming that *LOX5* contributes to the increase in 9-HPs in roots of GPA-infested plants.

Because the *lox5-1* allele affected the amount of time spent in SEP and XP by GPAs and also the accumulation of an activity in petiole exudates that is associated with an increase in GPA population size (Table 1, Figure 2), 9-HP levels were also determined in petiole exudates collected from leaves of GPA-infested plants, and as controls, uninfested wild-type and *lox5-1* mutant plants. As shown in Figure 5A, levels of these 9-HPs were elevated in petiole exudates collected from GPA-infested leaves of wild-type plants. However, the accumulation of these 9-HPs was attenuated in petiole exudates collected from GPA-infested leaves of the *lox5-1* mutant. To determine whether one or more of these 9-HPs were capable of promoting GPA fecundity, GPA was cultivated on an artificial diet containing either 9-HOD, 9-HPOD, 9-HOT, or 9-HPOT. As shown in Figure 5B, in comparison with control diets, a diet containing ethanol (0.1%) that was used to dissolve the 9-HPs, insect numbers were higher on 9-HOD– and 9-HPOD–supplemented diets, confirming that some 9-LOX products enhance GPA population size. 9-HOT and 9-HPOT did not enhance insect numbers at the concentrations used. Quite the contrary, 9-HOT adversely affected insect numbers in this assay. Irrigation of the *lox5-1* plants with 9-HOD

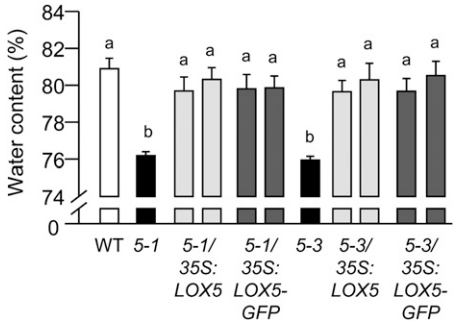

Figure 3. GPAs Reared on the *lox5* Mutant have Reduced Water Content.

Percentage water content in GPAs reared on wild-type (WT) and *lox5-1* and *lox5-3* mutant plants and *35S*:*LOX5* and 35*S*:*LOX5-GFP* complemented lines. Insects were starved for 12 h prior to release of 20 adult GPAs on each wild-type, *lox5-1*, and *lox5-3* mutant and the *35S*:*LOX5* and 35*S*:*LOX5-GFP* complemented lines. After 12 h, 30 to 40 insects from two plants were collected and pooled. The fresh weight and dry weight of the pooled insects were measured to calculate the percentage water content. Each bar represents the mean of percentage water content ± SE (n = six pools of 30 to 40 insects). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; GLM/ANOVA).

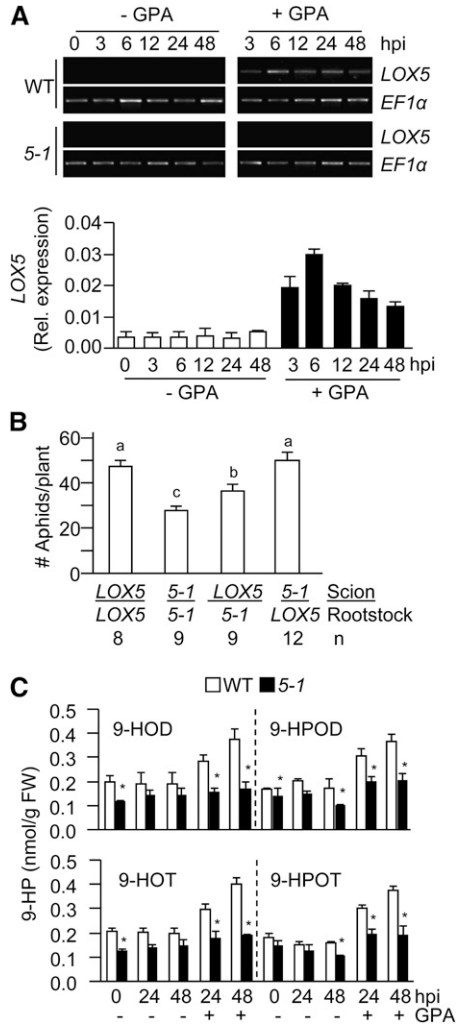

Figure 4. *LOX5* Activity in Roots Promotes GPA Infestation on Shoots.

(A) *LOX5* expression in roots of uninfested (−GPA) and GPA-infested (+GPA) wild-type (WT) and *lox5-1* plants. RT-PCR (top) and quantitative real-time PCR (bottom) were performed on RNA from roots of uninfested (white bars) and GPA-infested (black bars) plants harvested at 0, 3, 6, 12, 24, and 48 h postinfestation (hpi). The *EF1α* gene provided the control for RT-PCR. For quantitative real-time PCR, the expression level of *LOX5* relative to expression level of *EF1α* at each time point is presented. Values are means ± SE ($n = 3$).

(B) *LOX5* function in roots facilitates GPA infestation on shoots. No-choice assay comparison of GPA numbers on self- and reciprocal-grafts between wild-type and *lox5-1* plants. The genotype of the scion/root-stock (shoot/root) for each graft combination is indicated below the bars. Data points indicate means (±SE). Different letters above the bars indicate values that are significantly different from each other ($P < 0.05$; GLM/ANOVA). The numbers (n) of plants used for each graft combination are indicated below each bar.

(C) 9-HP concentrations in roots of uninfested (−GPA) and GPA-infested (+GPA) wild-type and *lox5-1* plants. Root tissues were collected at 0, 24, and 48 h after GPA-infestation. Each bar represents the mean (±SE) concentration of 9-HOD, 9-HPOD, 9-HOT, or 9-HPOT ($n = 5$) presented as nmol/g fresh weight (FW) of roots. Asterisk (*) indicates significant difference ($P < 0.05$; *t* test) from the wild type for that time.

facilitated GPA infestation of the shoot (Figure 6A) and restored water content in insects collected from these plants (Figure 6B), thus confirming that a 9-LOX–derived oxylipin synthesized in roots facilitates infestation of the shoots by GPA.

DISCUSSION

Oxylipins are known for their participation in plant defense against biotic stress as antibiotic factors and as defense-stimulating signal molecules (Blée, 2002; Prost et al., 2005). Our results indicate that a 9-LOX–derived oxylipin, or a product thereof, is a susceptibility factor that facilitates the colonization of *Arabidopsis* by GPAs. We have shown that GPA infestation of the foliage results in the upregulation of *LOX5* expression in roots (Figure 4A). This was paralleled by an increase in levels of 9-HPs in roots and petiole exudates (Figures 4C and 5A). *LOX5* was required for this increase in content of 9-HPs in GPA-infested plants (Figures 4C and 5A). In agreement with a role of *LOX5* in plant susceptibility to GPA, in a no-choice assay, GPA population size was significantly smaller on the *lox5* mutants than on the wild-type plant (Figures 1A to 1C). Expression of LOX5 or a LOX5-GFP fusion from the *35S* promoter and exogenous application of the LOX5-derived oxylipin, 9-HOD, complemented the

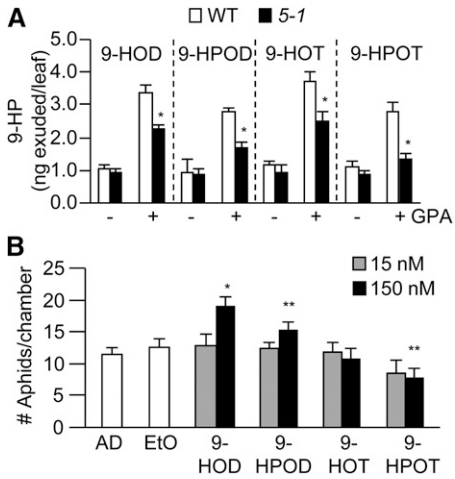

Figure 5. 9-LOX–Derived Oxylipins Accumulate in Petiole Exudates of GPA-Infested Plants and Enhance GPA Fecundity.

(A) Profile of 9-LOX–derived 9-HPs in petiole exudates collected from leaves of uninfested (−GPA) and GPA-infested (+GPA) wild-type (WT) and *lox5-1* plants. The insects were allowed to feed on plants for 48 h prior to harvesting leaves for petiole exudate collection. Data points show means (±SE) ($n = 3$). An asterisk (*) indicates significant difference from the wild type at the corresponding time point ($P < 0.05$; *t* test).

(B) Artificial diet assay with 9-HPs. Comparison of GPA numbers (mean ± SE) ($n = 4$) on an artificial diet supplemented with 9-HOD, 9-HPOD, 9-HOT, and 9-HPOT at final concentrations of 15 and 150 nM. Controls were an artificial diet (AD) alone and a diet supplemented with 0.1% ethanol (EtO), which was used as the solvent for the oxylipins. Significant differences from artificial diet and diet supplemented with 0.1% ethanol determined by GLM/ANOVA are indicated by asterisks (*, $P < 0.05$; **, $P < 0.1$).

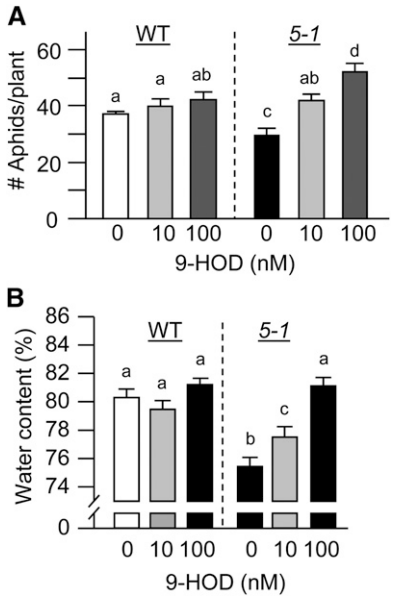

Figure 6. LOX5-Derived 9-HPs Complement *lox5-1* Phenotype.

(A) Complementation of the *lox5-1* phenotype by 9-HOD applied to roots. No-choice test comparison of GPA numbers (mean ± SE) ($n = 6$) on wild-type (WT) and *lox5-1* plants that were irrigated with 9-HOD (10 and 100 nM). Controls were plants that did not receive 9-HOD (0 nM).

(B) Percentage water content in GPAs reared on *lox5-1* plants treated with 9-HOD. Each bar represents the percentage content of water (mean ± SE; n = six groups of 30 to 40 GPAs) in GPAs reared on wild-type and *lox5-1* mutant plants that were irrigated with 9-HOD (10 or 100 nM). Controls were wild-type and *lox5-1* plants that did not receive 9-HOD (0 nM).

In **(A)** and **(B)**, different letters above the bars indicate values that are significantly different ($P < 0.05$; GLM/ANOVA).

lox5 deficiency, resulting in the promotion of insect infestation (Figures 1B and 6A), thus confirming that a LOX5-synthesized oxylipin(s), or product thereof, has an important role in facilitating GPA infestation in *Arabidopsis*.

Aphid infestation is known to manipulate host physiology, leading to alterations in the composition of phloem sap (Sandström et al., 2000; Dinant et al., 2010; Wilson et al., 2011). Results presented here demonstrate that aphids also manipulate host physiology to promote the accumulation of an infestation-promoting factor in petiole exudates (Figure 2). This was in striking contrast with petiole exudates collected from uninfested *Arabidopsis* leaves, which contain an activity that adversely affected GPA population (Figure 2) (Louis et al., 2010a; Louis et al., 2010b). The infestation-promoting effect was significantly lower for petiole exudates collected from GPA-infested leaves of the *lox5-1* mutant compared with the wild type (Figure 2), suggesting that *LOX5* is required for the accumulation of this infestation-promoting activity in petiole exudates. In support of the involvement of *LOX5* in promoting insect infestation, 9-HOD and 9-HPOD were found to enhance the size of the GPA population on an artificial diet (Figure 5B). Furthermore, 9-HOD and 9-HPOD accumulated at elevated levels in petiole exudates from GPA-infested leaves of wild-type *Arabidopsis* (Figure 5A). By comparison, the levels of these oxylipins were lower in petiole exudates collected from GPA-infested *lox5-1* plants. Taken together, these results confirm that LOX5-synthesized oxylipins, or products thereof, promote GPA infestation.

As summarized in the model presented in Figure 7, our results indicate that *Arabidopsis LOX5* provides a factor that facilitates aphid feeding from the sieve elements and thus sap consumption. In the absence of *LOX5*, GPAs spent less time in SEP (Table 1). The sugar-rich phloem sap consumed by aphids has an osmotic potential that is considerably more negative than the aphid hemolymph, and the resulting gradient could result in loss of water from the hemolymph, leading to dehydration of the insect (Douglas, 2006). One mechanism that aphids use to avoid dehydration is the ingestion of xylem sap (Powell and Hardie, 2002; Douglas, 2006). The diluted composition of xylem sap as compared with phloem sap (Mattson, 1980; Taiz and Zeiger, 2002) has been suggested to enable aphids to osmoregulate their hemolymph and prevent dehydration (Pompon et al., 2010; Pompon et al., 2011). The importance of xylem sap consumption to the insect is evident from studies with bird cherry-oat aphid (*Rhopalosiphum padi*). On wheat (*Triticum aestivum*) plants treated with the neonicotinoid insecticide thiamethoxam, which disrupts XP, the water content in bird cherry-oat aphids was reduced, and the size of the insect population was adversely affected (Daniels et al., 2009). Our results show that *LOX5* also affects the ability of the insect to drink from the xylem. By comparison with the wild-type plant, when on the *lox5* mutant, the insects did not exhibit XP, and water content in the insects was lower than in insects reared on wild-type plants (Table 1, Figure 3). Water content was restored in GPAs reared on 9-HOD–treated *lox5* mutant plants (Figure 6B), confirming the importance of *LOX5*-derived oxylipin(s) in promoting hydration of GPA on *Arabidopsis*. At this stage, it is not clear whether the LOX5-dependent factor independently affects SEP and XP in GPA, or

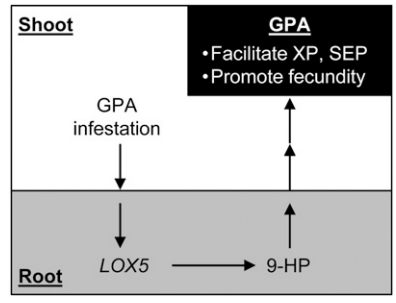

Figure 7. Model Depicting the Role of Root-Derived LOX5-Synthesized Oxylipin in *Arabidopsis* Interaction with GPAs.

GPA infestation on *Arabidopsis* shoots induces expression of *LOX5* in roots within 3 h of infestation. The model suggests that a plant-derived and/or insect-derived signal originating in the shoot is delivered to the roots, leading to the stimulation of *LOX5* expression and increased synthesis of 9-HPs. These 9-HPs are subsequently transported to the shoots, most likely through the vasculature, where they are utilized by GPAs as cues that facilitate both insect feeding from the sieve elements and water consumption from xylem and insect fecundity. Alternatively, these 9-HPs may counter and/or attenuate defenses targeting GPAs.

whether the reduced phloem sap consumption by insects reared on the *lox5* mutant results in a lowered need for the insect to consume xylem sap.

Oxylipins synthesized by LOX5 may have a direct effect on GPA behavior and physiology, thus affecting time spent by the insect in SEP and XP and fecundity. 9-HOD is present in GPAs reared on plants, as compared with GPAs reared on an artificial diet (Harmel et al., 2007). Because GPAs lacks a 9-HOD synthesizing activity, it has been suggested that GPAs derive 9-HOD from the plant host (Harmel et al., 2007). Indeed, 9-HOD and other 9-LOX oxylipins are present in phloem sap (Harmel et al., 2007), and our results show that the content of 9-HOD and other 9-LOX oxylipins increases in petiole exudates collected from GPA-infested leaves compared with petiole exudates from uninfested leaves of *Arabidopsis* (Figure 5A). The fact that 9-HOD added to an artificial diet resulted in an increase in size of the insect population (Figure 5B) suggests that LOX5-synthesized oxylipins have the potential to directly affect GPA. The stimulation of insect feeding by LOX5-synthesized oxylipins and the resultant increase in nutrient consumption could be the cause of the larger GPA population on the wild type compared with *lox5* mutant.

However, a more direct effect of 9-LOX products on insect fecundity cannot be ruled out. Because *LOX5*-synthesized oxylipins affect physiological processes in plants (Vellosillo et al., 2007; Vicente et al., 2011), it is also possible that the effect of LOX5-synthesized oxylipins on facilitating GPA infestation is mediated through their influence on molecular and physiological mechanisms in *Arabidopsis*. For example, 9-LOXs are known to influence activation of plant defense (Hwang and Hwang, 2010; López et al., 2011; Vicente et al., 2011). An antagonistic relationship between LOX5 oxylipins and *Arabidopsis* defense against GPAs could facilitate colonization of *Arabidopsis* by GPAs. It was recently shown that the 9-LOX oxylipin 9-keto-10 (*E*),12(*Z*),15(*Z*)-octadecatrienoic acid modulates expression of JA- and salicylic acid–regulated genes (Vicente et al., 2011). Because JA signaling promotes defense against insects, and salicylic acid is known to antagonize the activation of JA signaling in plant–insect interaction (Walling, 2008), we monitored expression of the *PATHOGENESIS-RELATED1* (*PR1*) and *PDF1.2* genes, which are molecular markers for the activation of salicylic acid and JA signaling, respectively. As shown in Supplemental Figure 5 online, although expression of *PR1* and *PDF1.2* were elevated in GPA-infested plants, no discernible differences were observed in the temporal pattern and extent of expression of these genes between GPA-infested wild-type and *lox5* mutant plants, suggesting that the *lox5* phenotype is not associated with any obvious changes in the activation of salicylic acid and JA signaling. However, additional genetic studies are required to determine whether these and other hormones have a role in the *lox5*-mediated enhanced resistance to GPAs.

Grafting experiments confirmed that GPA performance on *Arabidopsis* foliage is influenced by the *LOX5* genotype of roots (Figure 4B). Indeed, the level of *LOX5*-derived 9-HPs was elevated in roots of GPA-infested plants (Figure 4C), and application of 9-HOD to roots complemented the *lox5-1* defect to restore fecundity and water content of GPAs (Figures 6A and 6B). Although the xylem is the potential route of transport of 9-LOXs from root to shoot, in the foliage, there is significant flow of xylem content into the phloem. Hence, whether these 9-LOX products are delivered to GPAs during XP or SEP is not clear and requires further investigation.

Expression of *LOX5* was upregulated in roots of GPA-infested plants (Figure 4A), suggesting (as summarized in Figure 7) that an unknown factor travels from the GPA-infested foliage to stimulate *LOX5* expression in the roots. Further research is necessary to identify the responsible signal(s). This shoot-to-root signal could be of plant origin, similar to the involvement of shoot-derived JA in nicotine production by roots of insect-infested *Nicotiana sylvestris* plants (Baldwin et al., 1994). It is equally possible that the shoot-to-root signal(s) that stimulates *LOX5* expression in roots is derived from the aphid. Aphids inject saliva containing numerous proteins into the plant. Recent studies have indicated that salivary proteins are capable of eliciting responses in plants (De Vos and Jander, 2009; Bos et al., 2010). Furthermore, some salivary proteins have been shown to be required for efficient colonization of the host plant by the aphid (Mutti et al., 2008).

Although both LOX1 and LOX5 potentially synthesize the same oxylipins, our results (Figure 1A) indicate that only LOX5 had a discernible role in facilitating GPA infestation. This could be because of differences in the intracellular location of these two enzymes. Whereas LOX1 localizes to the chloroplast, LOX5 exhibits an extraplastidic pattern (see Supplemental Figure 1 online). This suggests that 9-HPs synthesized outside the chloroplast could have a different physiological and biological function than 9-HPs synthesized within the chloroplast. Although not much is known about the tissues in which *LOX1* and *LOX5* are expressed, any differences in these could also contribute to the differences in their involvement in facilitating GPA infestation.

In summary, evidence presented here indicates that roots are a critical source of oxylipins that promote colonization of the foliage by GPA. Although oxylipins are inducers of plant defense against pathogens and insects, and although volatile products of the LOX pathways are utilized by plants to attract predatory insects (Feussner and Wasternack, 2002), evidence presented here suggests that the GPA has adapted to cue in on specific 9-LOX–derived oxylipins for facilitating infestation. These results have broader ramifications to agriculture, because of the economic importance of the GPA as an insect pest of more than 50 families of plants (Blackman and Eastop, 2000) and a vector of several viral diseases in plants (Brault et al., 2010).

METHODS

Plant and Insect Cultivation

GPAs (*Myzus persicae* Sülzer) (Kansas State University, Museum of Entomological and Prairie Arthropod Research, voucher specimen 194) were reared on a 1:1 mixture of commercially available radish (*Raphanus sativus*) (Early Scarlet Globe) and mustard (*Brassica juncea*) (Florida Broadleaf) under a 14-h light (100 μE m^{-2} s^{-1}) and 10-h dark regime at 22°C. For aphid hydration studies, adult apterous (wingless) aphids were starved by confining them in a Petri dish for 4 to 6 h at 22°C. *Arabidopsis thaliana* accession Col-0 plants were cultivated under a 14-h light (100 μE m^{-2} s^{-1}) and 10-h dark regime at 22°C in an autoclaved peat-based planting mixture (Premier Pro Mix-PGX; Premier Tech

Horticulture, http://www.pthorticulture.com/). Plants that were ~4 weeks old were used for all experiments. The *lox1-1* (SALK_059431), *lox1-3* (SALK_012188), *lox5-1* (SALK_044826), and *lox5-3* (SALK_050933) mutants are all in the *Arabidopsis* accession Col-0. To generate the *lox1 lox5* double mutant, pollen from the *lox1-1* plant was used to pollinate *lox5-1* flowers. F2 segregants that were doubly homozygous for the *lox1-1* and *lox5-1* mutant alleles were identified using PCR (see below).

Transgenic Plants

To generate *35S*:*LOX1* and *35S*:*LOX5* constructs in which the *LOX1* and *LOX5* coding regions are cloned downstream of the *Cauliflower mosaic virus 35S* promoter, RNA extracted from roots was used to amplify the coding region of *LOX1* and *LOX5* using the following primer combinations: LOX1-C-F and LOX1-C-R (full length) and LOX5-C-F and LOX5-C-R (full length) (see Supplemental Table 1 online). The PCR products were cloned into the pCR8/GW/TOPO vector (Invitrogen) and subsequently used in a LR recombination reaction with the destination vectors pMDC32 (Curtis and Grossniklaus, 2003) to yield pMDC32-*35S*:*LOX1* and pMDC32-*35S*:*LOX5* plasmids. To generate *35S*:*LOX1-GFP* and *35S*:*LOX5-GFP* constructs in which the GFP coding region is fused to the C terminus (last amino acid) of LOX1 and LOX5, RNA extracted from roots was used to amplify the coding region of *LOX1* and *LOX5* using the following primer combinations: LOX1-C-F and LOX1-NS-R (full length without stop codon) and LOX5-C-F and LOX5-NS-R (full length without stop codon) (see Supplemental Table 1 online). The PCR products were cloned into the pCR8/GW/TOPO vector (Invitrogen) and subsequently used in a LR recombination reaction with the destination vectors pMDC83 (Curtis and Grossniklaus, 2003) to yield pMDC83-*35S*:*LOX1-GFP* and pMDC83-*35S*:*LOX5-GFP* plasmids. The pMDC32-*35S*:*LOX1*, pMDC32-*35S*:*LOX5*, pMDC83-*35S*:*LOX1-GFP*, and pMDC83-*35S*:*LOX5-GFP* plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis* by the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected for their resistance to hygromycin. To generate *35S*:*LOX1* and *35S*:*LOX1-GFP* plants, the pMDC32-*35S*:*LOX1* and pMDC83-*35S*:*LOX1-GFP* constructs, respectively, were transformed into the *lox1-1* mutant, whereas the pMDC32-*35S*:*LOX5* and pMDC83-*35S*:*LOX5-GFP* constructs were transformed into the *lox5-1* and *lox5-3* mutants to generate transgenic *35S*:*LOX5* and *35S*:*LOX5-GFP* plants, respectively.

No-Choice Assay

For no-choice assays, 20 apterous adult aphids were released on each plant, and the total numbers of insects (adults + nymphs) were counted 2 d later (Pegadaraju et al., 2005).

EPG Analysis

The EPG (Walker, 2000) technique was used to monitor GPA feeding behavior as previously described (Pegadaraju et al., 2007). A total of 16 replicates were conducted each with a different pair of GPA and plant. Data obtained from EPG were analyzed by nonparametric Kruskal-Wallis test (Minitab v15).

Petiole Exudate Collection

Petiole exudates were collected from *Arabidopsis* leaves using a previously described method (Chaturvedi et al., 2008) with a few modifications. Leaves were cut at the base of their petioles, and the petiole was immediately dipped in 50% ethanol, followed by 0.0005% bleach, and then was immersed in 1 mM EDTA (pH 8.0) solution. After 20 min, the base of the petiole was cut, and the petiole end was immediately placed in a fresh solution (3 mL) of 1 mM EDTA (pH 8.0) contained in a well of a 24-well tissue culture plate (ICN Biochemical; http://www.mpbio.com), six leaves to each well. High humidity was maintained by placing the entire set-up on wet paper towels under a transparent dome. The leaves were allowed to exude for a period of 12 h. After the exudation period, the contents of each well were filtered through a 0.45-μm syringe filter (Fisherbrand 25-mm Syringe Filter; http://www.fishersci.com). Petiole exudates from six wells were pooled, lyophilized, and resuspended in 2 mL of 1 mM EDTA (pH 8.0) for use in all experiments. Petiole exudates were collected from uninfested (−GPA) and GPA-infested (+GPA) plants, 48 h after infestation. For collection of petiole exudates from GPA-infested plants, only leaves that had a minimum of five aphids were used.

Artificial Diet Assays

An artificial diet (Mittler et al., 1970) was used for all feeding trial bioassays. A feeding chamber was constructed as previously described (Louis et al., 2010a). Each feeding chamber contained a total volume of 500 μL, which included the artificial diet with or without petiole exudates or chemicals. Six adult apterous GPAs were placed on each feeding chamber for 2 d, at the end of which the total number of aphids (adults + nymphs) in each chamber was determined.

Measurement of GPA Body Water Content

Aphids were starved for 14 to 16 h by placing them in a Petri dish without water. Twenty starved aphids were allowed to feed on each *Arabidopsis* plant for 12 h. To estimate water content, 30 to 40 adult aphids from two plants were collected and immediately weighed. Dry weights were obtained after drying the aphids at 60°C for 24 h. The water content of aphids was determined by subtracting the dry weight from the fresh weight of the aphids.

Micrografting

Single-hypocotyl grafts were constructed as previously described (Turnbull et al., 2002). *Arabidopsis* seedlings were germinated on one-half-strength Murashige and Skoog plates with 2% agar and 1% Suc under a 14-h light (100 $\mu E\ m^{-2}\ s^{-1}$)/10-h dark regime at 22°C. One d before grafting, the 10-d-old seedlings were moved to 27°C. The scion and rootstock were cut transversely and aligned precisely under sterile conditions without a grafting collar. The grafted seedlings were placed back at 27°C for 3 to 4 d to promote graft union and were monitored every day, and any adventitious roots that were observed were crushed with forceps. After 7 to 12 d, grafts that were functional were transplanted to soil. No-choice assays were performed on plants that were 4 to 5 weeks old. Genotype of the rootstock and scion for each graft was confirmed by PCR performed on DNA extracted from root and shoot for each set of plants, respectively.

Confocal Laser Scanning Microscopy

Protoplasts from leaves of the wild-type, *35S*:*GFP*, *35S*:*LOX1-GFP*, and *35S*:*LOX5-GFP* plants were isolated as previously reported (Jin et al., 2001) and observed with a Ziess 200M inverted optical microscope fitted with a CSU-10 Yokogawa Confocal scanner (McBain Instruments) and captured with a digital camera (Hamamatsu). Excitation wavelengths and emission filters were 488 nm/bandpass 505 to 535 nm for GFP and 488 nm/bandpass 672 to 712 nm for chloroplast autofluorescence. Three-dimensional images were obtained using Imaris 6.2 (BitPlane) software and are presented as stacks of neighboring sections.

Genomic PCR

All primers used in this study are listed in Supplemental Table 1 online. Plants homozygous for *lox1-1* (SALK_059431), *lox1-3* (SALK_012188),

lox5-1 (SALK_044826), and *lox5-3* (SALK_050933) were identified using a multiplex PCR involving the corresponding gene-specific primers and the T-DNA left border primer LBb1.3. PCR using the three primers was performed under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 2 min, with a final extension of 72°C for 5 min.

RT-PCR and Quantitative Real-Time PCR

RNA extraction from leaves and roots and cDNA synthesis was performed as previously described (Pegadaraju et al., 2005). DNA contamination in samples was removed by treatment with RNase-free DNase (Sigma-Aldrich). For each time point and treatment, RNA was collected from three biological replicates, each consisting of leaves pooled from four plants. Gene-specific primers used for RT-PCR and quantitative real-time PCR are listed in Supplemental Table 1 online and were identified using the AtRTPrimer database (http://atrtprimer.kaist.ac.kr/) (Han and Kim, 2006). The PCR conditions for all RT-PCR reactions were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s, followed by a final extension step of 72°C for 5 min. Real-time PCR was performed with Sybr Green PCR Master Mix (Applied Biosystems) on an Eco qPCR system (Illumina) using the following amplification protocol: 10 min polymerase activation and denaturation at 95°C, 40 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s. This was followed by a product melt to confirm a single PCR product. The level of *LOX5* expression was normalized to that of *EF1*α (At5g60390) by subtracting the cycle threshold value of *EF1*α from the cycle threshold value of *LOX5*.

Oxylipin Profiling

Lipids were extracted from plant samples as previously described (Göbel et al., 2002). The extracted lipids were resuspended in 100 μL of methanol:water (80:20 v/v). Oxylipins in the lipid extract were first purified by reverse-phase HPLC using an Agilent 1100 HPLC coupled to a UV diode array detector. A Nucleosil 120-5 C18 column (4.6 $\times$ 150 mm, 5 μm; Macherey-Nagel) with a binary gradient solvent system [solvent A: methanol:water:acetic acid (80:20:0.1 v/v/v) and solvent B: methanol: acetic acid (100:0.1 v/v)], flow rate of 0.18 mL/min, was used as previously described (Kilaru et al., 2011). Subsequently, the oxylipins were resolved by normal-phase HPLC over a Zorbax Rx-SIL column (2.1 $\times$ 150 mm, 5 μm; Agilent) with linear solvent systems (hexane:isopropyl alcohol: trifluoroacetic acid 100:1:0.02 v/v/v) and a flow rate of 0.125 mL/min. To determine whether the oxylipin products were formed by enzymatic reaction and/or autooxidation, chiral-phase HPLC analysis was used to determine the *S* and *R* isomer composition of the oxylipins. A chiral OD-H column (150 $\times$ 2.1 mm, 5 μm particle size; Diacel) along with a socratic solvent system of *n*-hexane:2-propanol:acetic acid (100:12.5:0.05, v/v/v) and a flow rate of 0.1 mL/min were used. A strong abundance of *S*-isomer over *R*-isomer was observed, suggesting that the vast majority of the oxylipins detected were synthesized by enzymes.

Statistical Analysis

For all assays, *t* test or analysis of means using the general linear model (GLM)/Analysis of Variance (ANOVA) was used to separate the means. For the EPG analysis, the mean time spent by the aphids on various activities was analyzed using the nonparametric Kruskal-Wallis test (Minitab v15).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative and GenBank/EMBL data libraries under accession numbers *LOX1* (At1g55020), *LOX5* (At3g22400), and *EF1*α (At5g60390).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. LOX1 and LOX5 Are Localized to Different Subcellular Compartments.

Supplemental Figure 2. 9-LOX Mutants of *Arabidopsis*.

Supplemental Figure 3. *LOX5* Expression in *Arabidopsis* Leaves.

Supplemental Figure 4. Verification of Graft Integrity.

Supplemental Figure 5. *PR1* and *PDF1.2* Expression in Wild-Type and *lox5-1* Mutant Plants.

Supplemental Table 1. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

V.J.N. and J.S. designed the research; V.J.N., J.K., and S.S. performed the research; V.J.N., J.K., and J.S. analyzed data; and V.J.N. and J.S. wrote the article.

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