Empoasca leafhoppers attack wild tobacco plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations

Mario Kallenbach, Gustavo Bonaventure, Paola A. Gilardoni, Antje Wissgott, and Ian T. Baldwin[1]

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, D-07745 Jena, Germany

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Choice of host plants by phytophagous insects is essential for their survival and reproduction. This choice involves complex behavioral responses to a variety of physical and chemical characteristics of potential plants for feeding. For insects of the order Hemiptera, these behavioral responses involve a series of steps including labial dabbing and probing using their piercing mouthparts. These initial probing and feeding attempts also elicit a rapid accumulation of phytohormones, such as jasmonic acid (JA), and the induced defense metabolites they mediate. When *Nicotiana attenuata* plants are rendered JA deficient by silencing the initial committed step of the JA biosynthesis pathway, they are severely attacked in nature by hemipteran leafhoppers of the genus *Empoasca*. By producing *N. attenuata* plants silenced in multiple steps of JA biosynthesis and perception and in the biosynthesis of the plant's three major classes of JA-inducible insecticidal defenses, we demonstrate that the choice of plants for feeding by *Empoasca* leafhoppers in both nature and the glasshouse is independent of the accumulation of major insecticidal molecules. Moreover, this choice is independent of the presence of *Candidatus Phytoplasma* spp. and is not associated with detectable changes in plant volatiles but instead depends on the plant´s capacity to mediate JA signaling. We exploited this trait and used *Empoasca* leafhoppers to reveal genetic variation in JA accumulation and signaling hidden in *N. attenuata* natural populations.

Plants provide a variety of resources, such as food, mating and oviposition sites, and shelter for a majority of phytophagous insect species. Host-plant selection by insects involves complex behavioral responses to a variety of physical and chemical characteristics of the host plant that operate at different spatial scales and include long-range olfactory (e.g., plant-derived volatiles perceived by odor receptors) and visual (e.g., plant shape, size, and color) cues and short-range chemotactic and gustatory (e.g., surface metabolites perceived by chemoreceptors) cues (1–3). The physical and chemical characteristics of plants that insects use for host selection depend on the feeding guild and the dietary behavior (e.g., polyphagy or oligophagy) of the insect species (4). For example, *Drosophila melanogaster* flies (order Diptera) use a wide range of olfactory cues such as methyl-, ethyl-, and propyl esters of short-chain fatty acids generated by microorganisms growing on decaying fruit (5), whereas *Drosophila sechellia* flies use a specific molecule (methyl hexanoate) emitted by its exclusive food plant, *Morinda citrifolia* (6). Insects with mouthparts capable of piercing plant tissues and sucking out liquids (e.g., order Hemiptera) use labial dabbing and probing to perceive chemical cues (e.g., waxes, terpenoids, acyl sugars, and alkaloids) on tissue surfaces or internal cellular layers (1–3, 7). Interestingly, it has been shown that insects also can perceive phytohormones. *Helicoverpa zea* (order Lepidoptera) larvae can perceive jasmonic acid (JA) (8), which accumulates in the food plants during attack and induces de novo synthesis of plant defense metabolites (9, 10). Thus, one possible scenario is that insects can select plants for feeding based on the plant's capacity to produce JA (or to signal JA-mediated responses) by eavesdropping on the defensive capacity of a potential host plant.

In some cases insects can suppress the accumulation of plant defense metabolites as a mechanism of food plant selection (11, 12). These suppression mechanisms often are associated with the alteration of phytohormone biosynthesis or signaling pathways and may involve specific enzymes (e.g., glucose oxidase) produced by the insect (13) or vectored microorganisms (14, 15). Leafhoppers of the genus *Empoasca* are hemipterans (family Cicadellidae) that feed on phloem and cell contents of a broad range of host plants (16, 17). During feeding, the leafhoppers may induce "hopper burn" in the plant tissue, damage that is characterized by the yellowing of the tissue around the feeding site (18). *Empoasca* leafhoppers can also vector viruses, bacteria, and fungi and transmit them efficiently to plants as a consequence of their ingestion–egestion feeding behavior (18). For example, cell wall-lacking bacteria of the species *Candidatus Phytoplasma* (hereafter, *Ca*. *Phytoplasma*) can be transmitted by *Empoasca* leafhoppers (19, 20). Interestingly, the transmission of *Ca*. *Phytoplasma* spp. into the plant can affect the interaction of the plant with the transmitting insect via the modification of direct or indirect plant defenses. It has been shown that *Malus domestica* trees infected by *Ca*. *Phytoplasma mali* emit larger amounts of β-caryophyllene, a volatile that lures insect vectors to infected plants, than do noninfected trees, (14). A recent laboratory study performed with *Arabidopsis thaliana* and *Macrosteles quadrilineatus* leafhoppers showed that effector proteins produced by *Ca. Phytoplasma asteris* interfere with the activation of JA biosynthesis in the plant and thereby reduce the induction of JA-mediated defense responses (15).

Nicotiana attenuata (Solanaceae), an annual tobacco plant native to the southwestern United States, germinates after fires from long-lived seed banks to form monocultures and must cope with an unpredictable insect community (21). Populations of *N. attenuata* plants are known to harbor significant genetic diversity among individuals, and the genetic diversity frequently is larger within populations than among populations (22), probably because of the plant's fire-dependent germination and long-lived seed banks (21). In their natural environment, as well as in the glasshouse, *N. attenuata* plants respond strongly and specifically to attacks by insects of different feeding guilds (23, 24). A large number of these responses are governed by a strong burst of JA,

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[1]To whom correspondence should be addressed. E-mail: Baldwin@ice.mpg.de.

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which is amplified by elicitors in the insect's oral secretions (OS) (10, 25–27). The initial steps of JA biosynthesis involve the release of trienoic fatty acids [e.g., α-linolenic acid (18:3)] from membrane lipids in the chloroplast by the action of glycerolipases [GLA1 in *N. attenuata* (28, 29)]. The released trienoic fatty acids are oxidized by 13-lipoxygenases (LOX3 in *N. attenuata*) to form (13*S*)-hydroperoxy-18:3 (30). This molecule is the substrate for allene oxide synthase (AOS) that forms (9*Z*, 13*S*, 15*Z*)-12, 13-epoxy-18:3, which subsequently is cyclized to an isomeric mixture of 12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (AOC) (31). OPDA is transported into the peroxisome where the C_{10}–C_{11} double bond in (9*S*, 13*S*)-OPDA is reduced by an OPDA reductase (OPR) (32). The reduced OPDA (OPC-8:0) undergoes three cycles of β-oxidation involving acyl-CoA transferase (ACX) (33) and finally forming (3*R*, 7*S*)-JA. JA can be modified, e.g., by jasmonyl-O-methyl transferase (JMT) to form methyl-jasmonic acid (MeJA) (34) or by JASMONATE RESISTANT (JAR) that conjugates isoleucine to form JA-Ile (35, 36). JA-Ile activates the SCF^{COI1}–JAZ complex (37), which transcriptionally activates genes involved in the biosynthesis of defense molecules, among other responses (38, 39).

Previously, we have reported that *N. attenuata* plants rendered deficient in JA biosynthesis by silencing Na*LOX3* (as-*lox3*) are heavily damaged by *Empoasca* leafhoppers in nature (40). Here, we used a set of 11 *N. attenuata* transgenic lines deficient in specific steps of JA biosynthesis and perception and deficient in the accumulation of major insecticidal molecules to disentangle the mechanisms underlying the selection of plants for feeding by *Empoasca* leafhoppers. In addition, we used these insects to discover genetic variations in JA accumulation and signaling in natural *N. attenuata* populations.

Results

Ca. Phytoplasma Species Are Not Found in _Empoasca_ Leafhoppers or the Plants They Attack in the Field. A previous study showed that *Ca. Phytoplasma asteris* transmitted by *Macrosteles quadrilineatus* leafhoppers can affect JA biosynthesis in infected *A. thaliana* plants in the laboratory (15). Thus, we first examined whether the interaction between *Empoasca* leafhoppers and *N. attenuata* plants involved *Ca. Phytoplasma* species. For this purpose we carried out two different sets of experiments. In the 2009 field season, we observed that *N. attenuata* plants silenced in the expression of *COI1* (ir-*coi1*) were severely attacked by adult *Empoasca* leafhoppers. These adult leafhoppers originated from *Cucurbita foetidissima* plants growing adjacent to our field plot and are referred to hereafter as "*Empoasca* spp." *Empoasca* spp. adults and leaves from *Empoasca* spp.–damaged and undamaged ir-*coi1* plants were collected for analysis (first set). In parallel, adult *Empoasca* leafhoppers were collected and used to establish a colony in our glasshouse (see *Materials and Methods* for details). This colony was composed of a single *Empoasca* species and is referred to hereafter as "*Empoasca* sp." Adult leafhoppers from this in-house colony were used to challenge inverted repeat (ir)-*coi1* plants for 7 d under glasshouse conditions. Adult leafhoppers and attacked and unattacked (control) leaves were collected and used for analysis (set 2). The presence of *Ca. Phytoplasma* spp. was analyzed in adult *Empoasca* leafhoppers and leaf samples from both sets. A PCR approach using universal primers for the amplification of 16S rRNA (Table S1) (14, 41, 42) was used for the detection of *Ca. Phytoplasma* spp. (Fig. S1). *Ca. Phytoplasma* spp. were detected in the positive control samples (isolated genomic DNA from *Ca. Phytoplasma asteris* and *Callistephus chinensis* leaves infected with *Ca. Phytoplasma asteris*) but not in the negative control samples or in samples collected from field and glasshouse experiments (Fig. S1; see *SI Materials and Methods* for details). These results demonstrate that the interaction between *Empoasca* leafhoppers and *N. attenuata* plants was independent of the presence of *Ca. Phytoplasma* spp.

Generation of a Toolbox of Transformed Plants to Examine _Empoasca_ Leafhopper Plant-Choice Mechanisms. To examine the mechanisms underlying plant choice by *Empoasca* leafhoppers, we transformed *N. attenuata* plants with RNAi ir constructs to silence (*i*) specific steps of JA biosynthesis [ir-*lox3* (43), ir-*aos*, ir-*aoc*, ir-*opr3*, and ir-*acx1*; see *SI Materials and Methods* for details]; (*ii*) JA perception (ir-*coi1*) (44); and (*iii*) accumulation of JA-dependent defense molecules [i.e., nicotine (ir-*pmt*); trypsin proteinase inhibitor (PI) (ir-*pi*); nicotine and PIs (ir-*pmt/pi*) (45); and diterpene glycosides (ir-*ggpps*) (46)] (Fig. 1*A*). Additionally, we ectopically expressed JA methyl transferase 1 (JMT1; 35S-*jmt1*) to deplete jasmonate accumulation metabolically by redirecting the flux of JA into methyl-JA (MeJA) (47). Compared with control plants, 35S-*jmt1* plants have reduced levels of JA-Ile after simulated herbivory, and therefore their COI1-mediated JA-signaling capacity is reduced (47).

Plants silenced in the expression of the *AOS* (ir-*aos*), *AOC* (ir-*aoc*), *OPR3* (ir-*opr3*), and *ACX1* (ir-*acx1*) genes were newly generated for this study, and two homozygous, independently transformed lines harboring a single transfer DNA insertion (Table S2 and Fig. S2) were selected for each genotype. In unelicited leaves, the transcript levels of Na*AOS*, Na*AOC*, Na*OPR3*, and Na*ACX1* were silenced by 10- to 100-fold in the respective lines compared with WT (Fig. 1*B*). In leaves treated with synthetic fatty acid–amino acid conjugate (FAC) to amplify the induction of the JA biosynthesis pathway (28), the target transcripts were reduced by five- to 100-fold in the respective lines compared with WT at 60 min after treatment (Fig. 1*C*). Consistent with the reduced expression of the JA biosynthesis genes, all lines showed significantly reduced levels of JA and JA-Ile after FAC treatment (Fig. S3). ir-*acx1* plants lost their capacity to suppress JA accumulation in the third generation, and therefore one line (line A466) was used as a control in addition to the plants transformed with the empty vector (EV) construct.

This set of transgenic lines, deficient in JA accumulation, JA perception, and JA-dependent defense molecules, allowed us to study in detail the steps of the JA biosynthesis and signaling pathways that are responsible for feeding-choice decisions of *Empoasca* leafhoppers.

Defense Molecules or Volatiles Do Not Direct Initial _Empoasca_ Leafhopper Feeding in Nature. To examine *Empoasca* leafhopper plant choice in nature, all the transgenic *N. attenuata* lines mentioned above were grown in a fully randomized design in a field plot in the Great Basin Desert during the 2009 field season (Fig. 2 *A* and *B*). ir-*aos* plants did not survive transplantation to the field and were not included in the analysis. An adjacent alfalfa (*Medicago sativa*) field served as a source of *Empoasca* spp. adults that were encouraged to move into the *N. attenuata* plantation by mowing the alfalfa field (Fig. 2*C*). Eight days after mowing, we quantified *Empoasca* spp. attack as the percentage of total canopy area damaged (Fig. 2*D*). Importantly, although the degree of damage was expressed per total canopy area for normalization, we observed that 80–90% of the damage inflicted by *Empoasca* leafhoppers occurred on stem leaves.

The ir-*lox3*, ir-*aoc*, 35S-*jmt1*, and ir-*coi* plants were heavily damaged by *Empoasca* spp. (Fig. 2*D*). Interestingly, *opr3* plants had WT levels of *Empoasca* spp. damage (Fig. 2*D*) even though the levels of JA and JA-Ile in these plants were strongly reduced (Fig. S3). The reduced levels of *Empoasca* spp. damage on ir-*opr3* plants can be explained by their reduced number of stem leaves resulting from decelerated growth in the field (Fig. S4). Thus, when the experiment was conducted, the canopy of ir-*opr3* plants was dominated by rosette leaves. An alternative explanation for these observations is that OPDA may play a role in the mecha-

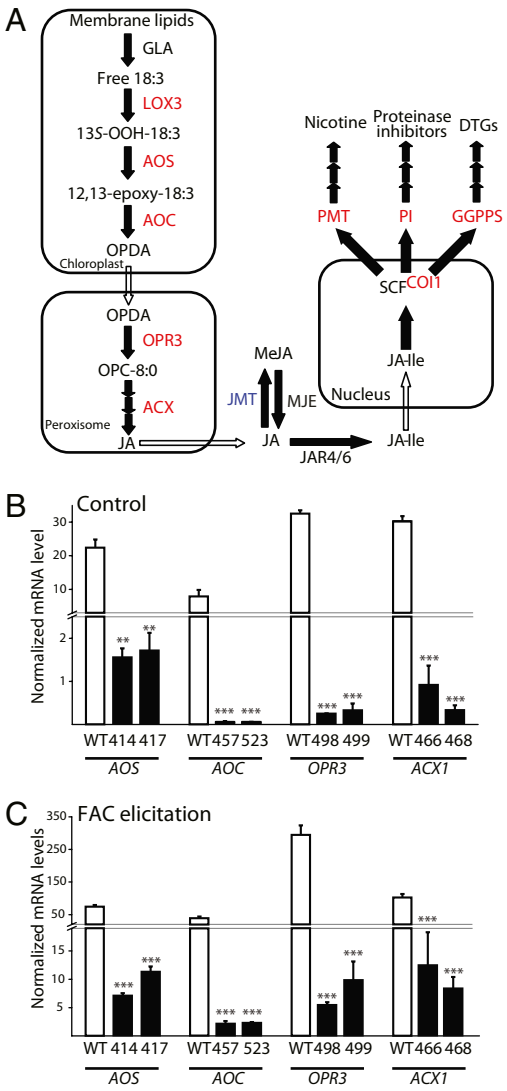

Fig. 1. Lines of transformed plants generated to dissect the mechanisms of *Empoasca* spp. feeding choice. (*A*) Enzymes of the jasmonate biosynthetic and signaling pathway and of jasmonate-regulated direct defenses in *N. attenuata*. All enzymes in red font were silenced by RNAi. *JMT* (blue font) was expressed ectopically to generate a toolbox of transformed plants to examine the feeding choice of *Empoasca* spp. in nature. RNAi lines were generated by transforming *N. attenuata* plants with constructs harboring an ir fragment of each gene (Table S2). (*B* and *C*) Silencing efficiency of two independently transformed homozygous ir lines with a single transfer-DNA insertion was determined by qPCR analysis in control leaves (*B*) and leaves harvested 60 min after FAC elicitation (*C*). Transcript levels were quantified by comparing the levels of corresponding genes with the eukaryotic elongation factor 1A-α (NaEF1A-α) (average ± SE, $n = 3$). Asterisks indicate statistically significant differences in WT plants versus the ir line. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; Student's *t* test with Welch correction.

nisms underlying the interaction between *Empoasca* leafhoppers and *N. attenuata* plants. However, this possibility was ruled out by additional experiments (see below). The damage to plants with reduced levels of JA-dependent defense molecules (ir-*pmt*, ir-*pi,* ir-*pmt*/*pi*, and ir-*ggpps*) was not distinguishable from that of control plants (Fig. 2*D*). These results demonstrated that *Empoasca* leafhoppers feed preferentially on plants with reduced jasmonate accumulation and reduced ability to perceive feeding rather than on plants with reduced accumulation of nicotine, PIs, and diterpene glycosides (DTGs) (ir-*pmt*, ir-*pi,* ir-*pmt*/*pi*, and ir-*ggpps*).

To test whether *Empoasca* leafhopper feeding choice in nature was driven by constitutively emitted volatiles from *N. attenuata* plants, we first trapped leaf volatiles from unattacked ir-*lox3*, ir-*aoc*, ir-*opr3*, 35S-*jmt1*, ir-*coi1*, ir-*pmt*, ir-*ggpps*, EV, and A466 plants grown in the field. Ultra-high-resolution GC analysis detected 197 volatiles constitutively released from these plants. These 197 volatiles were subjected to principal component analysis (PCA) (48) for which the genotypes were grouped in two classes based on their significant differences in *Empoasca* spp. damage (Fig. 2*D*). Principal components (PCs) 1 and 2 explained 44% of the variation but did not separate the two plant classes (Fig. S4*C*). Second, we tested whether herbivory-induced plant volatiles (HIPVs), which consist of green leaf volatiles released immediately upon insect attack and terpenoids released during the following photoperiod (48), affected *Empoasca* spp. feeding choices. For this purpose, we caged three adult *Empoasca* leafhoppers on leaves of the genotypes mentioned above and trapped HIPVs for 4 h during two different periods, immediately after the leafhoppers were caged (0–4 h) and 24 h after the leafhoppers were caged (24–28 h). HIPVs differentially emitted by *Empoasca* spp. feeding from the different transgenic lines were defined as compounds with a fold change (FC) of $1.5 \leq FC \leq 0.66$ and a P value <0.05 compared with unattacked plants of the same genotype. Of the 197 volatiles detected, 83 HIPVs were identified as accumulating differentially among all the genotypes (Table S3). These 83 HIPVs were used for PCA analysis (48) in which the two volatile-trapping periods were analyzed individually. As mentioned above, the genotypes were grouped in two classes based on their significant differences in *Empoasca* spp. damage (Fig. 2*D*). PCs 1 and 2 explained 50% of the variation within the first trapping period and 52% of the variation within the second trapping period, but in neither trapping period did PC1 and PC2 separate the two plant classes (Fig. 2 *E* and *F*). In summary, these results demonstrate that with an ultra-high-resolution analysis of plant volatiles neither the detectable constitutively released volatiles nor HIPVs were associated with *Empoasca* spp. feeding preferences in the field.

***Empoasca* Leafhopper Damage Correlates with Reduced Levels of OPDA and JA Accumulation.** The field observations showed clearly that *Empoasca* spp. preferentially attacked plants with reduced jasmonate accumulation and signaling capacity. To evaluate more directly the association between the *Empoasca* leafhopper feeding preferences observed in the field (Fig. 2*D*) and the jasmonate (OPDA, JA, and JA derivatives)-producing capacities of the transgenic *N. attenuata* lines used, we first assessed the capacity of EV, A466, ir-*lox3*, ir-*aoc*, ir-*opr3*, ir-*coi1*, 35S-*jmt1*, ir-*ggpps*, ir-*pi*, ir-*pmt*, and ir-*pmt*/*pi* plants to accumulate jasmonates after standardized mechanical wounding under glasshouse conditions (Fig. 3*A*). PCA separated the transgenic *N. attenuata* lines deficient in JA accumulation and perception from controls and from lines deficient in JA-dependent defense molecules (Fig. 3*B*). PC 1 explained almost all (99.7%) variance present in the data and was influenced positively by jasmonate levels (loading factors, 0.2–0.4) but negatively by *Empoasca* spp. damage (loading factor, −0.34) (Fig. 3*B*). A correlation analysis between the amount of *Empoasca* spp. damage quantified in the field and the capacity of the plants to accumulate jasmonates after standardized mechanical wounding revealed that damage correlated negatively with the capacity of the plants to accumulate OPDA and JA (JA vs. damage: Pearson's $R^2 = 0.36$, $P = 0.03$; OPDA vs. damage: Pearson's $R^2 = 0.50$, $P = 0.01$) (Fig. 3 *C* and *D*). We therefore hypothesized that the extent of initial *Empoasca* leafhopper feeding on *N. attenuata* plants depended either on OPDA or JA accumulation or on their respective signaling capacities.

***Empoasca* Leafhopper Feeding Preferences Depend on the Plants' Capacity to Mediate JA Signaling.** To examine which jasmonate triggers initial feeding of *Empoasca* leafhoppers on *N. attenuata* plants, we first analyzed the induction of jasmonate biosynthesis

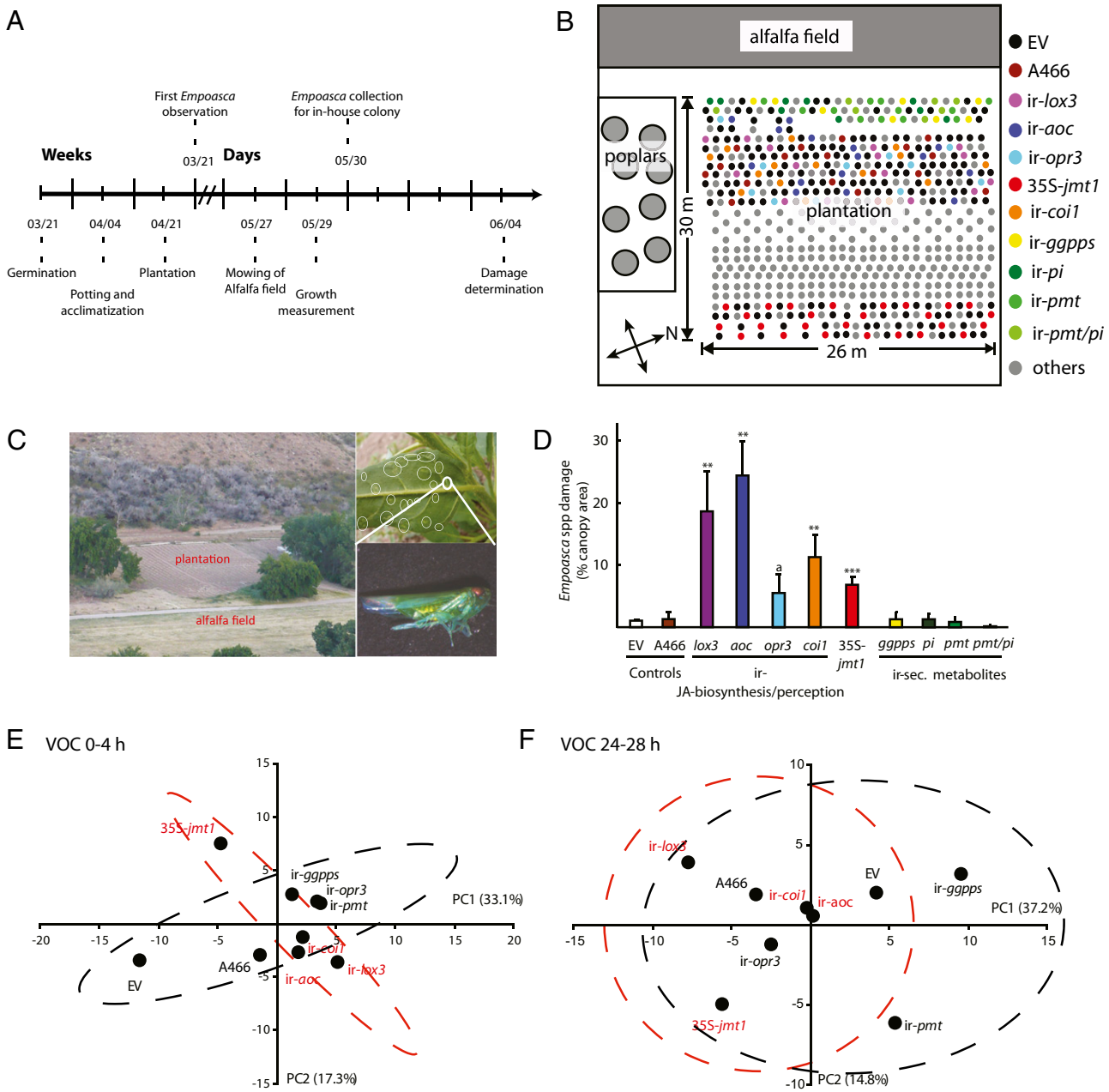

Fig. 2. *Empoasca* spp. select plants deficient or modified in JA accumulation or perception for feeding in the field, independently of released HIPVs. (*A*) Timeline of experimentation during the 2009 Utah field season. (*B*) Design of the field plot and the location of the different *N. attenuata* genotypes within the field plot. (*C*) (*Left*) In the field, *N. attenuata* plants were grown adjacent to an alfalfa (*M. sativa*) field, a source of *Empoasca* spp. (*Right*) *Empoasca* spp. damage on *N. attenuata* was determined as the percentage of canopy area exhibiting the characteristic damage (white circles) resulting from *Empoasca* spp. attack. (*D*) Eight days after the mowing of the alfalfa field, damage was quantified on EV and A466 controls, lines silenced in JA biosynthesis (ir-*lox3*, ir-*aoc*, ir-*opr3*) and perception (ir-*coi1*), lines silenced in JA-dependent defense molecules [i.e., diterpene glycosides (ir-*ggpps*), trypsin PIs (ir-*pi*), nicotine (ir-*pmt*), and both nicotine and PIs,(ir-*pmt/pi*)], and lines ectopically expressing a jasmonic acid methyl transferase (35S-*jmt1*). Asterisks indicate significant differences compared with control plants ($n = 7–10$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; a, $P = 0.07$; Student's *t* test. (*E* and *F*) After *Empoasca* leafhoppers were caged on single leaves, HIPVs were collected for 4 h during two different periods: immediately after the leafhoppers were caged (0–4 h) (*E*) and 24 h after the leafhoppers were caged (24–28 h) (*F*). Of 197 detected volatiles released with *Empoasca* spp. feeding, 83 HIPVs had a fold change (FC) of $1.5 \leq FC \leq 0.66$ (P value <0.05) compared with volatiles released from unattacked plants of the same genotype. These 83 HIPVs were used for PCA analysis in which the two volatile-trapping periods were analyzed individually. *N. attenuata* plants were grouped in two classes based on *Empoasca* spp. damage (black indicates no significant differences in *Empoasca* spp. damage compared with controls; red indicates significant differences), and PCs 1 and 2 of the transgenic lines were plotted against each other.

in WT leaves after *Empoasca* sp. feeding. For this experiment, 25 adult *Empoasca* sp. from the glasshouse colony were caged on single leaves of *N. attenuata* WT plants. After 24 h, the levels of OPDA, JA, JA derivatives, and MeJA were quantified. The levels of OPDA, JA, JA-Ile, 11/12-hydroxy-JA-Ile, and 11/12-carboxy-JA-Ile were increased significantly (three- to eightfold) in leaves attacked by *Empoasca* sp. compared with control leaves (Fig. 4 *A* and *B* and Table S4*A*). The 11/12-hydroxy-JA, MeJA, and JA-amino acid conjugates other than JA-Ile were not detected in leaves. Thus, the induction of OPDA and JA accumulation by *Empoasca* sp. feeding is consistent with a potential role of these two jasmonates in the plant-selection process.

A

Field		Glasshouse							
% canopy area		pmol g^{-1} FM							
Damage *		OPDA	JA	JA-Ile	JA-Val	OH-JA	OH-JA-Ile	COOH-JA-Ile	JA-glucose
1.0 ± 0.2	EV	70 ± 6	4803 ± 443	106 ± 8	18 ± 1	128 ± 23	282 ± 45	124 ± 15	131 ± 19
1.3 ± 1.2	A466	130 ± 21	4922 ± 859	80 ± 9	6 ± 1	nd	424 ± 101	290 ± 73	257 ± 44
18.6 ± 6.5	ir-*lox3*	77 ± 8	3293 ± 324	69 ± 5	11 ± 1	116 ± 20	263 ± 30	142 ± 15	87 ± 13
24.4 ± 5.5	ir-*aoc*	7 ± 1	41 ± 7	nd	nd	nd	nd	10 ± 1	nd
5.5 ± 3.0	ir-*opr3*	54 ± 7	255 ± 40	nd	nd	nd	nd	16 ± 1	nd
11.3 ± 3.6	ir-*coi1*	63 ± 6	2026 ± 231	354 ± 32	15 ± 1	nd	44 ± 6	94 ± 6	17 ± 10
6.8 ± 1.3	35S-*jmt1*	74 ± 6	2252 ± 188	16 ± 2	6 ± 1	nd	18 ± 2	58 ± 6	128 ± 14
1.2 ± 0.8	ir-*ggpps*	85 ± 3	2884 ± 146	68 ± 4	4 ± 0	52 ± 22	221 ± 12	197 ± 13	31 ± 13
1.2 ± 0.6	ir-*pi*	98 ± 3	3663 ± 346	67 ± 3	18 ± 1	67 ± 15	310 ± 16	231 ± 15	85 ± 12
0.8 ± 0.4	ir-*pmt*	147 ± 12	4804 ± 609	89 ± 5	18 ± 1	39 ± 8	411 ± 38	301 ± 17	119 ± 23
0.1 ± 0.2	ir-*pmt/pi*	124 ± 11	4032 ± 413	74 ± 6	17 ± 1	41 ± 20	388 ± 56	239 ± 28	156 ± 11

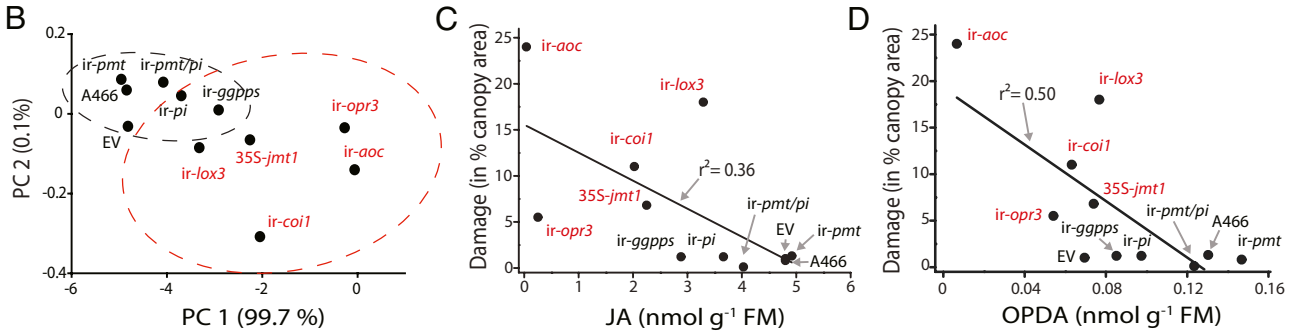

Fig. 3. Wound-elicited JA and OPDA levels correlate with *Empoasca* spp. damage. (*A*) All lines analyzed in the field were grown in the glasshouse, and jasmonate accumulations in treated leaves were analyzed 1 h after wounding. Numbers represent average ± SE, $n = 5$. (*Damage data are the same as shown in Fig. 2*D* and have been included here for comparison and to assist with the understanding of the results in this figure.) (*B*) PCA separated the transgenic *N. attenuata* lines deficient in JA accumulation and perception from controls and from lines deficient in JA-dependent defense molecules. (*C* and *D*) *Empoasca* spp. damage observed in the field correlated negatively with the wound-induced JA and OPDA levels observed in the glasshouse (Pearson correlation; JA vs. damage: Pearson's $R^2 = 0.36$, $P = 0.03$; OPDA vs. damage: $R^2 = 0.50$, $P = 0.01$).

To determine the predominant factor (i.e., OPDA or JA accumulation or mediated signaling) influencing plant selection by *Empoasca* sp., we performed feeding-choice experiments using *N. attenuata* WT (control), ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants in the glasshouse. Leaves were treated either with lanolin (control treatment) or with lanolin containing MeJA. Two days after the treatment, the plants were challenged with 150 adult *Empoasca* sp., and the percentage of leaf damage was determined after 7 d (Fig. S5*A*). Lanolin-treated ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants were damaged significantly more than lanolin-treated WT plants by *Empoasca* sp. (Fig. 4*C*). Treatment with MeJA decreased *Empoasca* sp. damage ir-*lox3* plants significantly, by eightfold compared with lanolin treatment, but did not decrease damage in ir-*coi1* and 35S-*jmt1* plants (Fig. 4*C*).

To evaluate the accumulation of jasmonates after external MeJA application, the levels of OPDA, JA, JA derivatives, and MeJA were quantified in unchallenged WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants treated with lanolin or MeJA (Table S4*B*). None of the jasmonates analyzed were detected in lanolin-treated leaves. JA levels accumulated to 2.5–4.6 nmol/g fresh mass (FM) in MeJA-treated leaves of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants (Fig. 4*D* and Table S4*B*), indicating that the JA levels did not affect feeding damage by *Empoasca* sp.. JA-Ile was detected in low amounts (0.01–0.02 nmol/g FM) in MeJA-treated leaves of WT, ir-*lox3*, and 35S-*jmt1* plants but was detected in significantly higher amounts in MeJA-treated ir-*coi1* leaves (0.14 nmol/g FM), as is consistent with the lower metabolism of JA-Ile in ir-coi1 plants (44, 49, 50). Thus, because *Empoasca* sp. feeding damage was similar on lanolin- and MeJA-treated ir-*coi1* plants, JA-Ile levels did not affect *Empoasca* sp. feeding choice directly. In MeJA-treated leaves, MeJA was detected only in the leaves of 35S-*jmt1* plants (1 nmol/g FM) (Fig. 4*D* and Table S4*B*) but did not directly affect *Empoasca* sp. feeding. OPDA was detected in similar amounts in MeJA-treated leaves of control and 35S-*jmt1* plants (0.01–0.02 nmol/g FM) (Fig. 4*D*) but was not detected in ir-*lox3* and ir-*coi1* plants, as is consistent with the activation of the JA biosynthetic pathway by exogenous MeJA (23). Although OPDA levels in MeJA-treated 35S-*jmt1* plants were similar to those of MeJA-treated WT leaves, *Empoasca* sp. feeding on 35S-*jmt1* was not affected by MeJA treatment. This result demonstrated that OPDA or its associated signaling cascade was not involved in the plant-selection process by *Empoasca* sp. The results revealed that *Empoasca* sp. feeding on *N. attenuata* can be reduced by external MeJA application to lines in which JA biosynthesis is silenced but not in lines in which JA perception is silenced (ir-*coi1*) or in plants in which the flux of JA is redirected to an inactive jasmonate (35S-*jmt1*).

To provide independent evidence of the deficiency in JA-mediated defense signaling in ir-*coi1* and 35S-*jmt1* plants, the induction of trypsin PI activity [as an indicator of defenses induced by JA-mediated COI1 signaling (51)], was quantified in WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants treated with MeJA. Compared with lanolin-treated leaves, PI activity was induced to an activity of 2–2.5 nmol/mg protein in MeJA-treated leaves of control and ir-*lox3* plants but was not induced in leaves of ir-*coi1* plants and was reduced by 60% (0.9 nmol/mg protein) in 35S-*jmt1* plants (Fig. 4*E*). Although MeJA treatment of 35S-*jmt1* plants partially induced JA-mediated defenses (i.e., PI activity), damage by *Empoasca* sp. on MeJA-treated 35S-*jmt1* plants was similar to that on lanolin-treated 35S-*jmt1* plants. These experiments demonstrated that MeJA treatment induced defense responses (i.e., PI activity) in *N. attenuata* and were consistent with the results obtained from the field with *N. attenuata*

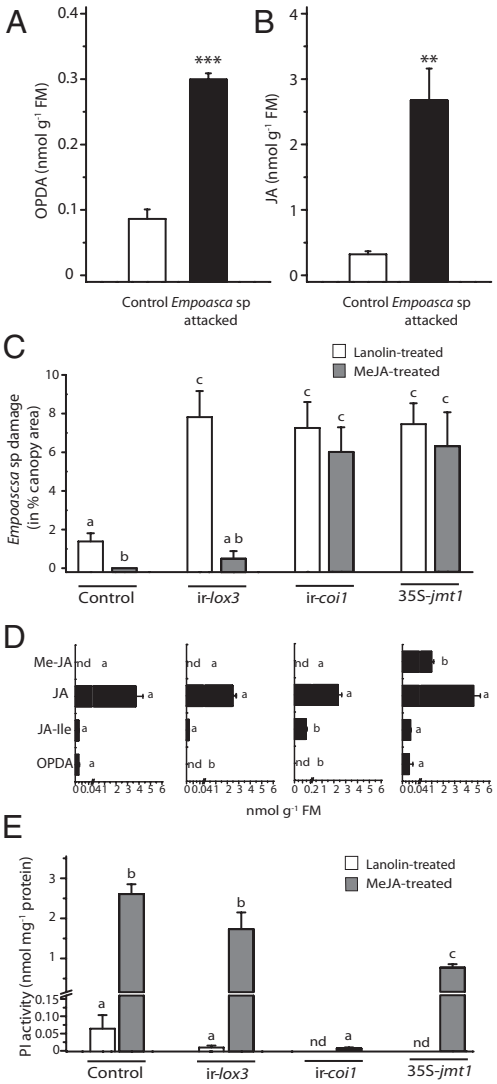

Fig. 4. Initial *Empoasca* sp. feeding induces jasmonate accumulation and is triggered by JA signaling capacities. (*A* and *B*) Twenty-five *Empoasca* sp. were caged on *N. attenuata* WT leaves, and jasmonate accumulation was measured. OPDA and JA levels were measured in control and attacked leaves 24 h after *Empoasca* sp. feeding. Bars represent average ± SE; $n = 4$. Asterisks represent significant differences compared with control plants. $^{**}P < 0.01$; $^{***}P < 0.001$; Student's *t* test. (*C* and *D*) In glasshouse bioassays, MeJA treatment of leaves enhances the resistance to *Empoasca* sp. attack of WT (control) and ir-*lox3* plants but not of ir-*coi1* and 35S-*jmt1* plants. Leaves of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants were treated with lanolin or with lanolin containing MeJA. (*C*) Two days after the treatment, plants were challenged with 150 *Empoasca* sp. adults, and damage was recorded after 7 d. Damage is shown as the percentage of canopy area; data shown are average ± SE; $n = 15$–18. Different letters indicate statistically significant differences. $P < 0.05$; Student's *t* test. (*D*) Levels of MeJA, JA, JA-Ile, and OPDA were quantified in MeJA-treated leaves of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants 3 d after the treatment (Table S4). Data shown are average ± SE; $n = 4$–9. Different letters indicate statistical significance among lines and within analytes. $P < 0.05$; Student's *t* test. nd, not detected. (*E*) To analyze JA signaling capacities, we determined the levels of trypsin PI activity in lanolin- and MeJA-treated leaves 3 d after the treatment (average ± SE; $n = 6$). Different letters indicate statistically significant differences. $P < 0.05$; Student's *t* test. nd, not detected.

ir-*pi* lines (Fig. 2*D*) (i.e., the choice of plants for feeding by *Empoasca* leafhoppers was independent of PI levels).

Finally, to evaluate if the *Empoasca* sp. in-house colony used for these experiments was free of *Ca*. *Phytoplasma* spp, we analyzed the presence of *Ca*. *Phytoplasma* spp. in severely *Empoasca* sp-damaged leaves from WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants and in 10 adult *Empoasca* leafhoppers from the colony. Phytoplasma were not detected in either leaf or leafhopper samples (Fig. S5*B*; see *SI Materials and Methods* for details).

***Empoasca* Leafhopper Attack Identified Variations in JA Accumulation in Nature.** The work presented above demonstrated that *Empoasca* spp. damage could be used to identify genetically modified *N. attenuata* plants deficient in JA accumulation and signaling in the field and glasshouse. We next asked if *Empoasca* spp. attack also could identify natural variation in JA accumulation and signaling. During the 2009 field season, a natural population of 100 *N. attenuata* plants was screened using *Empoasca* spp. (collected from *C. foetidissima* plants) to discover genetic variation in JA accumulation hidden in natural populations. Two of the plants in the population showed *Empoasca* spp. damage; when JA was elicited by treating leaves with *Manduca sexta* OS in the field, these plants accumulated less JA than neighboring unattacked plants of the same population (Fig. 5 *A* and *B*). Elicitation by OS provides a standard method to assess the maximal capacity of the plants to produce JA after an insect-associated response. Moreover, because the OS was collected from *M. sexta* larvae, the analysis allowed us to exclude the possibility that factors (other than phytoplasma) introduced by *Empoasca* spp. attack were responsible for the suppression of the JA burst. Self-pollinated seeds from the two plants showing *Empoasca* spp. damage and their closest undamaged neighbors were collected and grown in the glasshouse. Consistent with the field results, elicitation of JA through treatment of the leaves with *M. sexta* OS elicited smaller JA bursts in the progeny of the two plants previously showing *Empoasca* spp. damage than in the progeny of previously unattacked plants (Fig. 5 *C* and *D*).

A second screening of native *N. attenuata* populations was carried out during the 2011 field season. In this case, and in contrast to 2009, we used natural infestations of *Empoasca* spp. originating from *C. foetidissima* plants growing within natural *N. attenuata* populations. We screened two populations consisting of ~400 and 200 *N. attenuata* plants, respectively, (Fig. 5*E*) and found three plants in one population and two plants in the other that were highly damaged by *Empoasca* spp. compared with neighboring plants at similar growth stages (Fig. S5*C*). Undamaged leaves from the five plants showing *Empoasca* spp. damage and from undamaged neighbor plants were treated with *M. sexta* OS for JA elicitation and were harvested 60 min after the treatment. All five plants showing *Empoasca* spp. damage accumulated significantly lower JA levels than their undamaged neighbors within the same population (Fig. 5*F*). Again, the analysis of phytoplasma in leaves and *Empoasca* spp. collected in both plant populations was negative (Fig. S5*D*; see *SI Materials and Methods* for details). These results demonstrated that the initial *Empoasca* leafhopper feeding choice can be used to identify variations in JA accumulation or signaling in *N. attenuata* populations. In addition, these experiments allowed us to exclude any role of *Empoasca* spp. feeding in the suppression of the JA signaling in these plants.

Discussion

Volatile Release Is Not Associated with *Empoasca* Leafhopper Damage. Plant selection by *Empoasca* leafhoppers can be guided by perceiving nonvolatile molecules during the first feeding but also by perceiving specific volatile cues released from plants appropriate for feeding. Several studies have highlighted the fundamental role of plant volatiles in plant selection by herbivore insects (52, 53). For example, *Manduca* moths can distinguish *N. attenuata* plants already infested by *M. sexta* larvae and preferably lay eggs on uninfested plants (54). Furthermore, *Empoasca fabae* leafhoppers are more arrested by volatiles emitted from an alfalfa genotype susceptible to *E. fabae* than by volatiles from a resistant

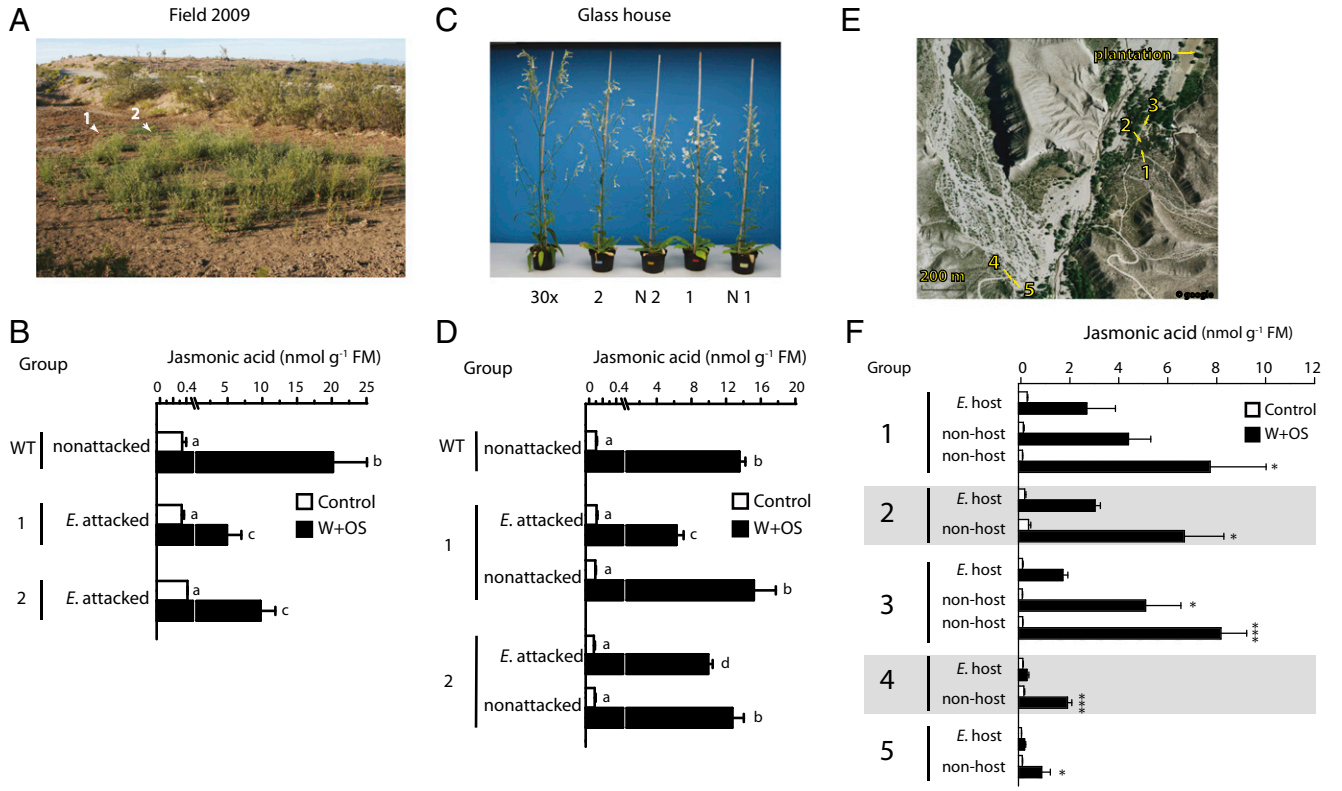

Fig. 5. *Empoasca* spp. identified natural variation in JA accumulation in genetically diverse native *N. attenuata* populations. (*A*) During the 2009 field season, ~50 *Empoasca* spp. were released into a native *N. attenuata* population (*ca*. 100 plants), and *Empoasca* spp. damage on all plants was determined after 2 d. Two plants (arrowheads marked I and 2) with detectable *Empoasca* spp. damage were found. (*B*) Leaves from both plants and from a nonattacked control plant were treated with *M. sexta* OS to elicit JA and were harvested for jasmonate analysis after 60 min. Both plants showing *Empoasca* spp. damage had lower OS-elicited amounts of JA than did control plants (average ± SE, $n = 3$–4). Bars sharing same the letters are not significantly different. $P < 0.05$; Student's *t* test. (*C*) Self-pollinated seeds from these plants (1 and 2) and from neighboring undamaged plants (N1, N2) were grown in the glasshouse, treated with OS to elicit JA, and harvested for jasmonate analysis after 60 min. (*D*) JA accumulations in plants from two accessions showing *Empoasca* spp. damage in the field were significantly lower than in undamaged neighbors (average ± SE, $n = 4$). Bars sharing same the letters are not significantly different. $P < 0.05$; Student's *t* test. (*E*) During the 2011 field season, two areas with ~400 and 200 native *N. attenuata* plants, respectively, were surveyed to identify *Empoasca* spp.-damaged plants. We found five plants with increased *Empoasca* spp. damage compared with neighboring plants in the same developmental stage. (*F*) Leaves of these five plants and undamaged neighbor plants were treated with OS to elicit JA and harvested for JA analysis after 60 min. All plants with *Empoasca* spp. damage had lower elicited JA values than did their undamaged neighbors (average ± SE, $n = 3$). Asterisks indicate statistical significance of the plants attacked by *Empoasca* spp. compared with their respective neighbors. $*P < 0.05$; $***P < 0.001$; Student's *t* test.

alfalfa genotype (55). Additionally, phytopathogen infections can cause dramatic changes in volatile emissions, and these emissions can lure insect vectors to infected plants (14). Our studies focused on the initial selection of plants for feeding by *Empoasca* leafhoppers. We therefore analyzed *N. attenuata* volatile emissions before and during the first stages of leafhopper attack rather than tracking long-term changes. Changes in plant volatiles released from both unattacked and *Empoasca* spp-attacked plants and analyzed by ultra-high-resolution GCxGC-ToF mass spectrometry were not associated with *Empoasca* spp. feeding preferences. Although we cannot exclude the possibility that either unmeasured volatiles or nonlinear behavioral responses to emitted volatiles might play a role, our results are consistent with the conclusion that the choice of plants for feeding by *Empoasca* leafhoppers is based on plant characteristics perceived during the initial probing process (see below).

***Empoasca* Leafhopper Plant Choice Is Not Associated with the Presence of *Ca. Phytoplasma* Species in Nature or in the Glasshouse.** Recent laboratory studies have shown that *Ca. Phytoplasma asteris* infection reduces JA biosynthesis in *A. thaliana* plants (15) and that *Ca*. *Phytoplasma mali* infections induce *Malus domestica* to emit higher amounts of β-caryophyllene to lure insect vectors (14). In nature, the proportion of leafhoppers infected by *Ca*. *Phytoplasma* spp. is low [~2% (56)], and in our study the choice of *N. attenuata* plants by *Empoasca* leafhoppers was not associated with the presence of *Ca*. *Phytoplasma* spp. in either natural and laboratory environments.

Major Defense Molecules of *N. attenuata* Do Not Affect Initial *Empoasca* Leafhopper Feeding. Since Fraenkel (57) first argued that plant secondary metabolites have a defensive function, many laboratory studies have described defensive metabolites induced by herbivore attack (7). We demonstrate that *Empoasca* leafhopper feeding on *N. attenuata* plants is independent of the accumulation of the three major classes of insecticidal compounds in *N. attenuata* (i.e., nicotine, trypsin PIs, and DTGs). The biosynthesis of these molecules is regulated by JA signaling, and they are known to have a strong influence on the herbivore community on *N. attenuata* in field experiments (45, 46, 58, 59). Although the total herbivore damage on ir-*pmt*, ir-*pi*, ir-*pmt*/*pi*, and ir-*ggpps* plants was significantly higher than on control plants (Fig. S4*D*), damage by *Empoasca* spp. was similar to that on control plants (Fig. 2*D*) in the field. Furthermore, in the glasshouse, MeJA treatment of 35S-*jmt1* plants significantly induced the activity of PIs, but *Empoasca* leafhoppers attacked these plants and lanolin-treated 35S-*jmt1* plants similarly. *Empoasca* leafhopper performance may be affected by nicotine, PIs, or

DTGs, but the initial feeding choice of *Empoasca* leafhoppers (as reflected by the initial feeding damage) clearly is not affected by these molecules.

JA Signaling Mediates Plant Choice by *Empoasca* Leafhoppers. In nature, *Empoasca* leafhoppers preferentially select *N. attenuata* plants for feeding when the plants are rendered deficient in JA accumulation or JA perception (Fig. 2*D*). Eleven ir lines deficient in JA accumulation, JA perception, and JA-regulated defense molecules were analyzed in the field to disentangle the mechanisms responsible for the feeding preferences of the *Empoasca* leafhopper. We also reassessed as-*aos* plants, which previously were shown to be attacked similarly to WT plants by *Empoasca* spp. (Fig. S6*A*) (40). We found that the wound-induced accumulation of JA was similar to that in EV control plants (Fig. S6*B*), providing a likely explanation for why *Empoasca* spp. did not attack as-*aos* plants in the 2003 field season (40). Our results demonstrate that initial *Empoasca* spp. feeding on *N. attenuata* plants in the field correlates negatively with the accumulation of jasmonates (Fig. 3). To examine these correlations, wound-induced jasmonate accumulation rather than *Empoasca* spp.-induced JA accumulation was used because (*i*) *Empoasca* spp. feed differentially on the different genotypes analyzed, and (*ii*) *Empoasca* spp. feed at irregular times. Therefore, we used a single wounding elicitation to provide rigorous quantitative measures for comparisons of JA bursts among genotypes.

The results demonstrated that in the glasshouse and in the field the plant's capacity to induce defense responses mediated by JA signaling dictates the initial feeding choice of *Empoasca* spp. In ir-*coi1* plants, treatment with MeJA elicits the accumulation of JA but not of JA-mediated defenses and therefore did not decrease *Empoasca* spp. feeding damage compared with lanolin-treated plants (Fig. 4). In 35S-*jmt1* plants, MeJA treatment induces JA-mediated defenses, but attack from *Empoasca* spp. was not decreased (Fig. 4). Thus, initial *Empoasca* spp. feeding can be mediated either directly by the plant's JA signaling capacity or, most likely, by JA-dependent responses via COI1. The identification of single molecules or a combination of molecules (including signaling complexes) that *Empoasca* spp. perceives in the plant will require experiments in which the response of *Empoasca* spp. to the exposure of these molecules can be determined. These responses can range from behavioral responses (e.g., attraction, repulsion) to the recording of electrical signals from the precibarial sensilla (60) that likely are used by Cicadellidae leafhoppers in assessing the JA bursts elicited by their ingestion–egestion mode of feeding. Imaging techniques, e.g., those commonly used to study calcium signaling in *Drosophila melanogaster* neurons upon excitation with specific molecules, can be developed for *Empoasca* spp. (5). These experiments must be coupled with the isolation of molecules from the plant and, in later steps, with the generation of transgenic plants deficient in the accumulation of specific molecules.

JA is a chemical and functional analog of eicosanoids in animals, and during the divergence of plants and animals the function of these oxygenated derivatives of fatty acids in mediating defense responses against sucking insects has been conserved. Hematophagous insects induce the accumulation of eicosanoids in the bite zone that elicits inflammation and defensive behavioral responses in the host (61). Thus, as for blood-feeding dipterans, the responses induced by phytophagous hemipterans are associated with the production of oxygenated forms of fatty acids. During the course of evolution, *Empoasca* spp. have acquired the capacity to select appropriate hosts with diminished defensive response by indirectly perceiving JA signaling (e.g., the SCF^{COI1}-JA-Ile complex) after feeding. As an evolutionary counterresponse, animal and plant hosts may have amplified their attack-elicited signaling to function as an aposematic signal, warning these eavesdropping potential grazers of impending defense responses.

In summary, we demonstrate that *Empoasca* spp. leafhoppers select plants for feeding in nature by eavesdropping on JA-mediated signaling. Given their high mobility, *Empoasca* spp. leafhoppers may probe plants at random in the field and settle on those with lower levels of JA-mediated signaling for sustained feeding. This behavior can be used to identify natural variation in JA accumulation in native populations. This native tobacco uses fire to synchronize its germination from long-lived seed banks to grow in dense populations characterized by intense intraspecific competition and variable herbivore pressures (21). Hence, we hypothesize that growth–defense tradeoffs for this plant likely are severe, and these tradeoffs likely provide the selective pressure to maintain these JA-signaling mutants in native populations, despite the clear disadvantages of being defense impaired. Once we have completed the sequencing of the *N. attenuata* genome, we will characterize in greater detail these JA-signaling mutants that *Empoasca* leafhoppers have identified for us from natural populations.

Materials and Methods

Plant Material and Growth. Seeds of the 30th and 31st generations of an inbred line of *N. attenuata* were used as the WT genotype in all experiments. The inbred line originated from seeds collected in 1988 from a natural population at the DI Ranch in southwestern Utah. Seeds were germinated and plants were grown as described (62).

We used plants harboring an EV construct [line A-03–9-1 (63)] as controls in all field experiments. We used the stably transformed lines [ir-*lox3* line A-03–562-2 (43), ir-*aos*, ir-*aoc*, ir-*opr3*, ir-*acx1*, ir-*coi1* line A-04–249-A-1 (44), and 35S-*jmt1* line A-07–291-2 (47)] as plants that are silenced to various degrees in their JA production and JA signaling and perception capabilities and lines silenced in the expression of JA-mediated direct defenses, namely, DTGs [ir-*ggpps* line A-07–231-3 (46)], nicotine [r-*pmt* line A-03–108-3 (45)], PIs [ir-*pi* line A-04–186-1 (45)], and nicotine and PIs together [ir-*pmt*/*pi* line A-04–103-3 (45)].

Plant Treatments. In the glasshouse, tissue was collected from the first fully elongated leaf at nodes +1 (64) of rosette-stage (~30-d-old) *N. attenuata* plants. Wounding was performed by rolling a fabric pattern wheel three times on each side of the midvein. To analyze differences in jasmonate accumulation between plants, leaves were treated with water, synthetic FAC, or *Manduca sexta* OS. For FAC elicitation, the wounds were supplied immediately with 0.6 pmol of synthetic *N*-linolenoyl-glutamic acid [18:3-Glu; 20 μL of a 0.03 nmol/mL solution in 0.02% (vol/vol) Tween 20/water]. For OS elicitation we used *M. sexta* OS, stored under argon at −20 °C immediately after collection until use. The wounds were supplied with 20 μL of 1:5 (vol/vol) diluted OS. Leaf tissue was collected at different times and was frozen immediately in liquid nitrogen for subsequent analysis.

For the screening of native *N. attenuata* plants in the field, the least damaged nonsenescing leaves available on each plant were used. The leaves were chosen randomly as either control or elicited leaves. Wounding and OS elicitation were performed as described above for the glasshouse treatments. Tissue was collected 60 min after elicitation and was frozen immediately between dry ice blocks.

For MeJA treatment, 34.6 μL of pure MeJA (Sigma-Aldrich) was dissolved in 5 mL of pure lanolin (Roth) to attain a final concentration of 7.5 μg/μL. Twenty microliters of pure lanolin or MeJA-containing lanolin were applied to the abaxial side of the bases of the first three fully elongated leaves (positions +1, +2, +3) (64) of rosette-stage plants. Choice experiments were performed 2 d after treatments. For analysis of jasmonate and PI activity, tissue was collected from the untreated leaf portion 3 d after the treatment and was frozen immediately in liquid nitrogen.

To analyze the changes in jasmonate levels after *Empoasca* sp. attack, 25 adult leafhoppers were caged on WT leaves in two 50-mL plastic containers (Huhtamaki); two empty 50-mL plastic containers were placed on WT leaves as controls. After 24 h, the plastic containers together with leaves and insects were removed from the plant and flushed with CO_2 for 15 s to anesthetize the leafhoppers. All *Empoasca* sp. were removed, and the leaves were frozen immediately in liquid nitrogen. Control leaves were treated similarly in the absence of *Empoasca* sp.

Insect Collection and Treatment. For all experiments in the field, *Empoasca* leafhoppers were collected from infested *C. foetidissima* plants growing adjacent to the field plot at the Lytle Ranch Preserve in southwest Utah (N 37.146301, W 114.019795) during the 2009 and 2011 field seasons. For field experiments, we collected *Empoasca* spp. adults on the day of the experiments, between 4:00 AM and 6:00 AM, when the leafhoppers are relatively immobile and are easier to collect. Leafhopper adults were kept in plastic containers until the start of the experiment.

To establish a glasshouse colony of *Empoasca* sp, ~400 adults were collected from *C. foetidissima* growing at the Lytle Ranch Preserve on May 30, 2009 and on June 16, 2011, were placed in an adapted Plexiglas container (1 × 2 × 1.5 m) in the glasshouse, and were reared on *Cucurbita moschata*, *Cucurbita maxima*, and *Cucurbita pepo* plants, which were replaced weekly. (So far, we have not been able to identify the *Empoasca* species used in this study because the genus is poorly understood (18). We assume that the species is *Empoasca fabae*, but further corroboration is required to ascertain the actual species. We would greatly appreciate expert knowledge regarding the identity of the leafhopper species. We can provide males as voucher specimens, stored in 80% (vol/vol) ethanol/water, as well as living individuals as long as our in-house colony remains viable) For the glasshouse experiments, *Empoasca* sp. adults were collected from the colony immediately before use, anesthetized with CO_2 for 15 s, and placed in 50-mL plastic containers in different numbers, according to the experiment.

Field Experiments. Seeds of the *N. attenuata* genotypes EV, ir-*lox3*, ir-*aos*, ir-*aoc*, ir-*opr3*, A466 (ir-*acx1*), ir-*coi1*, 35S-*jmt1*, ir-*ggpps*, ir-*pi*, ir-*pmt*, and ir-*pmt/pi*, were imported under US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) notification number 07–341-101n, and the field experiments were conducted under notification number 06–242-3r-a2. All transformed *N. attenuata* genotypes mentioned above were used for experiments in the experimental field plot at the Lytle Ranch Preserve near Santa Clara, Utah in 2009.

For germination, seeds were treated with 1 mM gibberellic acid (GA_3) in 1:50 (vol/vol) diluted liquid smoke (House of Herbs), germinated on agar plates containing Gamborg's B5 medium (Duchefa), and grown in a shade house. After 2 wk, young seedlings were transplanted into Jiffy 703 pots (Jiffy Products), fertilized with ~300 μg Borax (7.5 mg/L $Na_2[B_4O_5(OH)_4]$*8 H_2O in water), and grown outdoors for 2 wk. At 28–30 d after germination, plants were planted in the field plot, and a plastic label carrying the APHIS identification number of each genotype was buried under the roots of each plant to ensure unambiguous genotype identifications. A 10-cm bamboo stick with the APHIS identification code was placed in the soil beside each plant. The field plot consisted of 26 rows separated by open irrigation troughs which allowed plants to be watered every second day until they were established rosette-stage plants.

Size-matched EV, ir-*pi*, ir-*pmt*, ir-*pmt/pi*, and ir-*ggpps* plants (10–15 plants per genotype) were planted in a fully randomized design. A466, ir-*lox3*, ir-*aos*, ir-*aoc*, ir-*opr3*, and ir-*coi1* plants were planted in a randomized design, each paired with a size-matched EV plant (15 pairs per genotype). 35S-*jmt1* plants were planted pairwise with a size-matched EV plant (26 pairs). All plants were monitored daily during reproductive growth, and all flowers of each genotype were removed before their corollas opened and could release pollen. Rosette diameter and stem length were determined continuously between May 15–29, 2009 (24–38 d after plantation).

An alfalfa field adjacent to the field plot provided a source of *Empoasca* spp. leafhoppers. We mowed this field on May 27, 2009 to encourage movement of leafhoppers to the *N. attenuata* plantation. We quantified the damage caused by *Empoasca* spp. feeding as the percentage of the leaf area damaged normalized to the total plant area 8 d after the mowing of the alfalfa field (44 d after transplantation).

Jasmonate Analysis. The analysis of OPDA, JA, JA-Ile, MeJA, JA-Val, 11/12-hydroxy-JA, 11/12-hydroxy-JA-Ile, and 11/12-carboxy-JA-Ile was performed as previously described (29, 47). The PCA (Fig. 3*B*) was performed using the Metaboanalyst software (65, 66). The grouping of the transgenic *N. attenuata* lines, necessary for PCA analysis, was done by separating lines deficient in JA accumulation and perception from controls and from lines deficient in JA-dependent defense molecules. For this analysis levels of jasmonates and *Empoasca* spp. damage were normalized using autoscaling.

Analysis of PI Activity. Leaf tissue from 40-d-old ir-*lox3*, ir-*coi1*, 35S-*jmt1*, and WT plants was harvested 2 d after treatment with either lanolin or MeJA containing lanolin. The analysis of PI activity was performed as previously described (67).

***Empoasca* Species Choice Experiments.** Six leaves from three plants each of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* were treated with pure lanolin or with MeJA containing lanolin (7.5 μg/μL) as described above. Two days after the treatment, the plants were placed in a fully randomized design within a containment cage and were challenged with 150 *Empoasca* sp. adults. After 7 d, the percentage of canopy leaf area damaged by *Empoasca* sp. of treated leaves (Fig. S5*A*) was determined.

Identification of Natural Variation in Capacity for JA Accumulation in Native Populations of *N. attenuata*. Native *N. attenuata* plants growing in the Great Basin Desert, southwestern Utah, were selected for analysis (22). In 2009 we selected one population consisting of ~100 plants (Fig. 5*A*, N 37.08844, W 113.93228), and in 2011 we selected two populations consisting of ~200 and 400 plants, respectively (Fig. 5*E* and Fig. S5*C*).

In 2009, all plants in the population were inspected carefully; plants showed no evidence of *Empoasca* spp. feeding damage or the presence of *Empoasca* spp. adults or nymphs on the plants. Fifty *Empoasca* spp. adults were collected from infested *C. foetidissima* plants growing adjacent to our field plot and were released into the native population. Two days after this release, the plants were rescreened for *Empoasca* spp.-damaged leaves, and two plants that had been attacked were found. Leaves from these two plants and from an unattacked control plant were treated with OS to elicit JA as described, harvested 60 min after elicitation, and frozen immediately on dry ice for subsequent jasmonate analysis. Flowers from these plants were bagged to exclude flower visitors, and seed capsules were collected from the self-pollinated flowers of both *Empoasca* spp.-attacked plants and from their closest undamaged neighbors. Seeds from each plant and from 30× inbred WT plants were grown in the glasshouse. Leaves were left untreated (control) or were treated with OS to elicit JA as described above and were harvested after 60 min for jasmonate analysis.

In 2011, we screened all 600 plants of the two populations and found five plants with visible *Empoasca* spp. damage. Leaves from these plants and from their nearest undamaged neighbors either were left untreated or were treated with OS as described, harvested 60 min after elicitation, and frozen immediately on dry ice for subsequent jasmonate analysis.

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