Supporting information

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SI Materials and Methods

Generation of Plants with Inverted-Repeat Silencing of *AOS*, *AOC*, *OPR3*, and *ACX1*. *Nicotiana attenuata* plants silenced in the expression of the *AOS*, *AOC*, *OPR3*, and *ACX1* genes by inverted-repeat (ir) gene silencing were generated using *Agrobacterium*-mediated transformation as previously described (1). Partial sequences of Na*AOS* (240 bp), Na*AOC* (258 bp), Na*OPR3* (277 bp), and Na*ACX1* (369 bp) cDNAs were PCR amplified using the primers listed in Table S2 and were cloned in pSOL and pRESC vectors as ir constructs, respectively (2, 3). At least 10 independent transgenic lines per genotype were selected for homozygosity on agar plates supplemented with hygromycin (3). Homozygous plants were selected for the identification of lines efficiently silenced in *AOS*, *AOC*, *OPR3*, or *ACX1* mRNA accumulation and carrying one transfer DNA (tDNA) insertion as described below.

To determine the silencing efficiency of the transformed lines, total RNA was extracted from ∼0.1 g of leaf tissue with TRIzol (Invitrogen) and was treated with DNase-I (Fermentas) according to the manufacturer's instructions. Then 5 μg of total RNA were reverse-transcribed using oligo(dT)18 and the SuperScript-II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was conducted using the core reagent kit (qPCR Core Kit for SYBR Green I; Eurogentec) and gene-specific primer pairs (Table S2) in a Stratagene 500 Mx3005P (Stratagene) instrument. All quantitative PCR reactions were performed with three biological replicates. The eukaryotic elongation factor 1A-α (NaEF1A-α) was used as an internal standard for data normalization. The relative amounts of all mRNAs were calculated using the comparative threshold cycle method in accordance with the manufacturer's instructions (Applied Biosystems).

To determine the tDNA insertion number (Fig. S2), genomic DNA from the stably silenced lines and WT *N. attenuata* plants was isolated by the cetyltrimethylammonium bromide method. DNA samples (10 μg) were digested with XbaI (New England Biolabs) overnight at 37 °C according to the manufacturer's instructions and were separated on a 0.8% (wt/vol) agarose gel using standard conditions. DNA was blotted onto Gene Screen Plus Hybridization-Transfer membranes (Perkin-Elmer Life and Analytical Sciences) using the capillary transfer method. A gene-specific probe for the hygromycin resistance gene *hptII* was generated by PCR using the primer pairs HYG1-18 (5′-CCGGATCGGACGATTGCG-3′) and HYG3-20 (5′-CGTCTGTCGAGAAGTTTCTG-3′). The probe was labeled with [α-^{32}P]dCTP (Perkin-Elmer) using the RediprimeII kit (GE Healthcare) according to the manufacturer's instructions. For each construct, we chose two independently transformed homozygous lines harboring a single insertion for the transgene and exhibiting the strongest silencing of the expression of the endogenous targeted gene for further experimentation. These lines were lines A-04–414-3 and A-04–417-3 for ir-*aos*; lines A-07–457-1 and A-04–523-2 for ir-*aoc*; lines A-07–498-3 and A-04–499-3 for ir-*opr3*; and lines A-07–466-1 and A-07–468-3 for ir-*acx1*.

Volatile Collection and Analysis. In 2009, volatiles from plants attacked by *Empoasca* spp. were collected from the genotypes empty vector (EV), A466, ir-*lox3*, ir-*aoc*, ir-*opr3*, ir-*coi1*, 35S-*jmt1*, ir-*ggpps*, and ir-*pmt*. Three adult leafhoppers were caged on the leaves between two 50-mL plastic containers (Huhtamaki). Leaves used were labeled with cotton string, and the *Empoasca* sp. damage caused in this experiment was determined and was subtracted from later determinations of damage. Volatiles were collected on charcoal traps (OrboM32; Sigma-Aldrich) by drawing air through the cage with a vacuum pump. To avoid UV-mediated oxidation of the emitted volatiles, charcoal traps were covered with aluminum foil. The constitutive volatile emission of each leaf used for the experiment was collected for 2 h before the start of the experiment. Herbivory-induced plant volatiles were trapped between 0 and 4 h and between 24 and 28 h after the start of the experiment. Charcoal traps were spiked with 400 ng of tetralin (Sigma-Aldrich) and were eluted into a GC vial with 500 μL of dichloromethane. Samples were analyzed on an GCxGC-ToF-MS (6890N GC; Agilent Technologies) coupled with an LECO Pegasus III ToF-MS (LECO), and data processing was performed as described in ref. 4. Sample mixes from each trapping period, containing an aliquot of all samples collected, were analyzed by GCxGC-ToF-MS. Raw data obtained after analysis first were deconvoluted using the LECO ChromaToF software (version 2.21). Known artifact peaks and contaminants were removed, and peak lists were combined to create a reference peak file containing 197 analytes. All samples were processed against the reference peak file using LECO ChromaToF software. Peak areas were corrected against the peak area of tetralin, and the mean of three independent replicates was normalized by autoscaling for statistical analysis. All analytes with a fold change (FC) of $1.5 \leq FC \leq 0.66$ and a P value <0.05 compared with untreated plants of the same genotype were considered as induced after *Empoasca* spp. feeding, resulting in a matrix of 83 different analytes (Table S3). Metaboanalyst software (5, 6) was used to perform principal component analysis (PCA). The grouping of the transgenic *N. attenuata* lines, necessary for PCA, was done by separating lines in which damage by *Empoasca* spp. in the field was similar to that in controls from lines in which *Empoasca* spp. damage was significantly greater than in controls.

Detection of *Candidatus Phytoplasma* Species. In 2009, when the first *Empoasca* spp. individuals were observed on *Cucurbita foetidissima* plants growing adjacent to the field plot, the first signs of *Empoasca* spp. feeding damage were observed on ir-*coi1* plants in the field plot. Several *Empoasca* spp. adults from *C. foetidissima* plants were collected to test for the presence of *Candidatus Phytoplasma* (hereafter, *Ca*. *Phytoplasma*) spp. in the first generation of leafhoppers of the 2009 field season. In parallel, leaves from field-grown ir-*coi1* and EV control plants without previous *Empoasca* spp. damage were collected as putative phytoplasma-free plants (control for negative-infected plants). After 1 d, three *Empoasca* spp. per plant (six replicate plants in total) were caged between two 50 mL plastic containers (Huhtamaki) on leaves of EV and ir-*coi1* plants without previous herbivore damage to force *Empoasca* spp. to feed on these plants. After 3 d, the insects were removed and frozen on dry ice. Cages remained on the leaves for another 5 d. Five days after the initial *Empoasca* spp. caging, we caged another three *Empoasca* spp. adults on three replicates of previously attacked EV and ir-*coi1* leaves. All leaf tissues and *Empoasca* spp. from the second caging were collected 8 d after the initial feeding and frozen on dry ice. Additionally, to control for any cage effects, ir-*coi1* plants were placed either inside the cage of the *Empoasca* sp. glasshouse colony or in an empty cage. After 7 d, more than 80% of ir-*coi1* leaves on plants inside the colony showed the characteristic signs of *Empoasca* sp. feeding. Adult *Empoasca* sp. were

collected, and leaf material was harvested from *Empoasca* sp-damaged and control leaves.

To determine the presence of *Ca*. *Phytoplasma* spp. in the choice assay (Fig. 4) 10 *Empoasca* sp. adults and leaf material from the most damaged leaves of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants were collected.

During the 2011 field season, leaf material from the five native *N. attenuata* showing *Empoasca* spp. damage was collected for phytoplasma detection. Additionally, *Empoasca* spp. adults were collected from the native *N. attenuata* populations that were screened.

To detect phytoplasma, DNA was isolated from leaf material and leafhoppers using the Agentcourt Cholopure Kit (Agencourt Bioscience Corporation) following the manufacturer's instructions. As controls, we included DNA material from uninfected (negative control for phytoplasma infection) and infected China aster (*Callistephus chinensis*) (positive control for *Ca*. *Phytoplasma* spp. infection) and from the phytoplasma strain Aster Yellow-Witches' broom (AY-WB; *Candidatus Phytoplasma asteris*), kindly provided by Saskia Hogenhout (John Innes Center, Norwich Research Park, Norwich, United Kingdom).

We used different group-specific and universal primers for the detection of phytoplasma based on the 16S rRNA sequence (Table S1*A*), using either direct or nested PCR as described in previous studies (7–9). The PCRs were performed in a final volume of 50 μL containing 100–200 ng of template, 1× PCR buffer (Sigma-Aldrich), 0.5 μM of each primer, 100 μM of the four dNTPs (Invitrogen), and 1 U JumpStart Taq DNA Polymerase (Sigma-Aldrich). All primer pair combinations and PCR conditions are listed in Table S1*B*. For nested PCR, a 1:50 (vol/vol) dilution in water of the products of the preceding direct PCR was used as a template. All PCR products were resolved in 1% (wt/vol) agarose gels, and the extracted bands were cloned into the pGEM-T Easy Vector (Promega) and sequenced on an ABI Prism 377 XL DNA sequencer using the BigDye terminator kit (PE-Applied Biosystems). Sequence data were analyzed using the Lasergene software package (DNASTAR). Sequence-similarity searches were performed in both GenBank using the BLAST algorithms and in the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/).

1. Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* 12(4):177–183.
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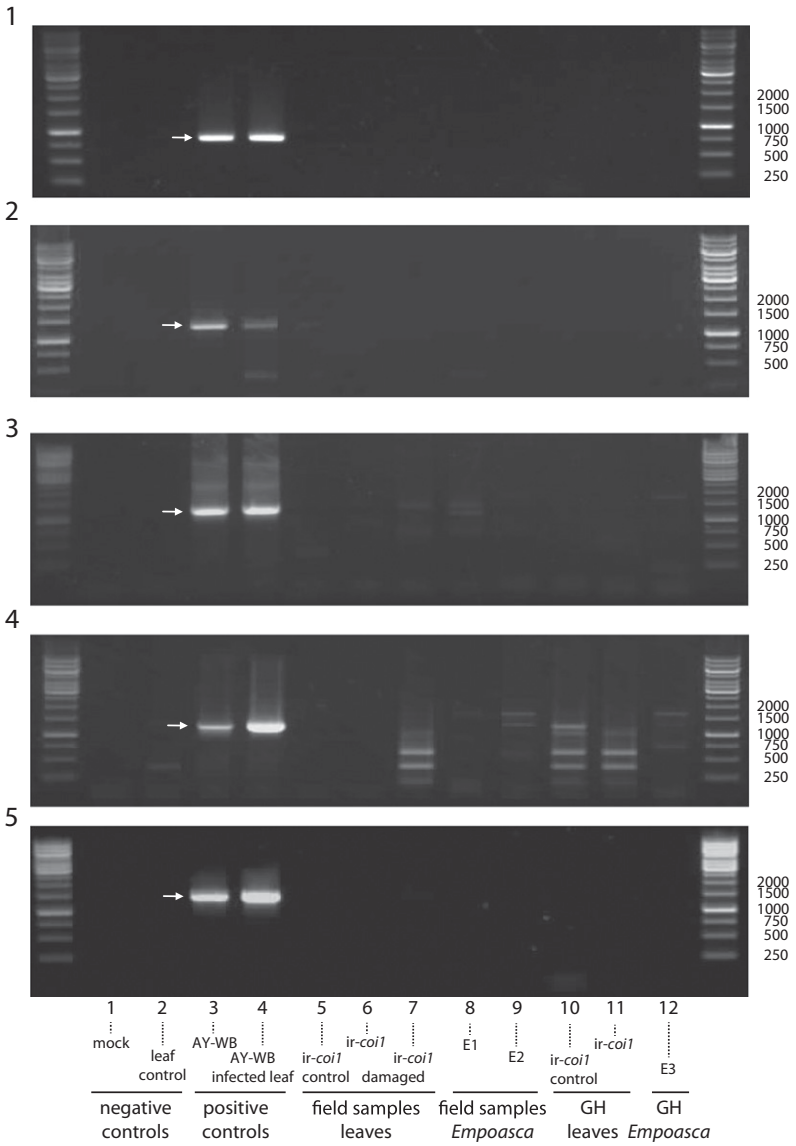


Fig. S1. *Empoasca* spp. collected in Utah during the 2009 field season do not harbor or transmit *Ca. Phytoplasma* spp. to *N. attenuata* plants during feeding. *Ca. Phytoplasma* spp. detection assays were performed with different combinations of PCR conditions and primers (Table S1*B*): (1) direct PCR using fU5/rU3 primer pairs; (2) direct PCR using P1/P7 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; (3) direct PCR using R16mF2/R16R1 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; (4) direct PCR using R16F2n/R16R2 primer pairs; and (5) direct PCR using R16mF2/R16R1 primer pairs. Sample reference: lane 1, no template sample, mock; lane 2, DNA extracted from uninfected China aster (*Callistephus chinensis*) leaves, leaf control; lane 3, DNA extracted from Aster Yellow-Witches' broom (AY-WB, *Candidatus* phytoplasma *asteris*), AY-WB; lane 4, DNA extracted from AY-WB–infected China aster leaves, AY-WB–infected leaves; lane 5, DNA extracted from ir-*coi1* plants grown in Utah showing no signs of *Empoasca* spp. feeding, ir-coi1 control; lane 6, DNA extracted from ir-coi1 plants grown in Utah and enclosed with three *Empoasca* spp. adult leafhoppers, ir-coi); lane 7, DNA extracted from ir-*coi1* plants grown in Utah and twice subjected to *Empoasca* spp. leafhopper feeding over 8 d, ir-*coi1* damaged; lane 8, DNA extracted from one *Empoasca* spp. adult fed on sample 6, E1; lane 9, DNA extracted from one *Empoasca* spp. adult fed on ir-*coi1* leaves which previously had been attacked by leafhoppers (sample 7), E2; lane 10, DNA extracted from undamaged ir-coi1 plants grown in the glasshouse, ir-*coi1* control; lane 11, DNA extracted from ir-*coi1* plants grown in glasshouse on which three adult *Empoasca* sp. leafhoppers were clip-caged for 7 d, ir-*coi1*; lane 12, DNA extracted from three *Empoasca* sp. adults collected from the glasshouse colony, E3. All PCR products were purified from the gel and sequenced. Only the bands labeled in samples 3 and 4 (positive controls) matched *Ca. phytoplasma* spp. 16S-ribosomal DNA sequences.

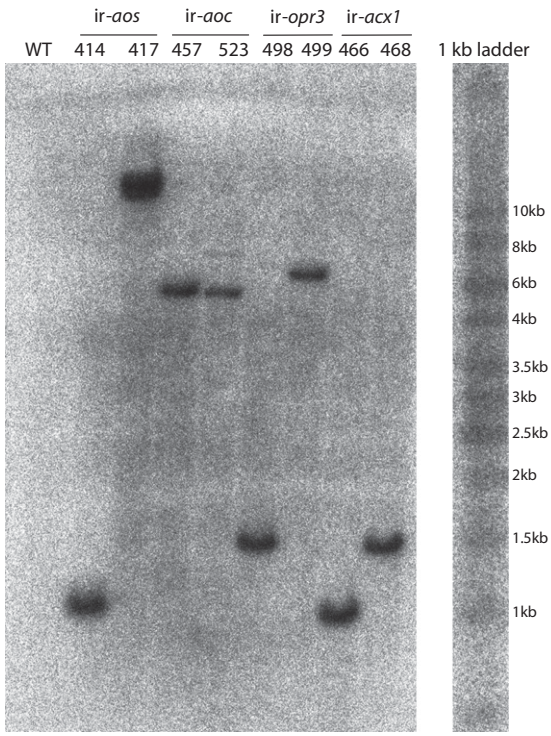


Fig. S2. Southern blot analysis of stably transformed ir lines. The number of tDNA insertions in the genomic DNA of stably silenced *N. attenuata* plants was assessed by digesting 10 μg of genomic DNA extracted from plants of the T2 generation with XbaI and separation of the digested DNA fragments on a 0.8% (wt/vol) agarose gel. The DNA was blotted onto Gene Screen Plus Hybridization Transfer membranes using the capillary transfer method. A gene-specific probe for the hygromycin resistance gene *hptII* was generated by PCR using the primer pairs HYG1-18 (5′-CCGGATCGGACGATTGCG-3′) and HYG3-20 (5′-CGTCTGTCGA-GAAGTTTCTG-3′). The probe was labeled with [α- 32P] dCTP using the Rediprime II kit (GE Healthcare) according to the manufacturer's instructions.

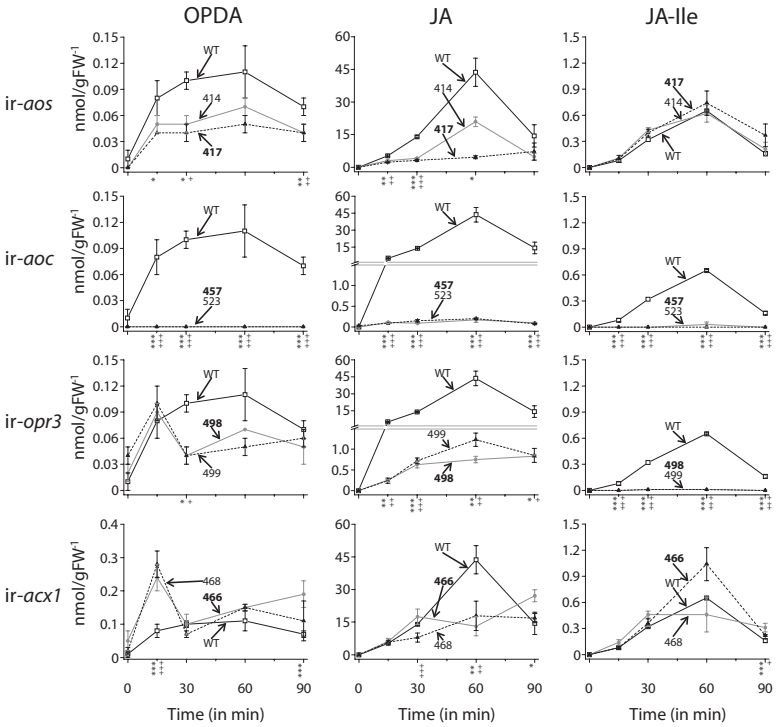


Fig. S3. Kinetics of 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and JA-isoleucine (JA-Ile) accumulations in stably transformed ir lines and WT plants after elicitation of fatty acid-amino acid conjugate (FAC). We examined the consequences of silencing the different genes of the JA biosynthetic pathway in plants of the T2 generation of RNAi-silenced lines by comparing the accumulation of OPDA, JA and JA-Ile with those in WT in FAC-elicited leaves at different times after elicitation (average ± SE, $n = 4$). Asterisks indicate statistically significant differences for WT versus the silenced line (Student's t test). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for lines with bold font; +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ for lines presented in regular font. One independently transformed line of each construct was used for further experiments (bold font: ir-*aos* line 417, ir-*aoc* line 457, ir-*opr3* line 498, and ir-*acx1* line 466).

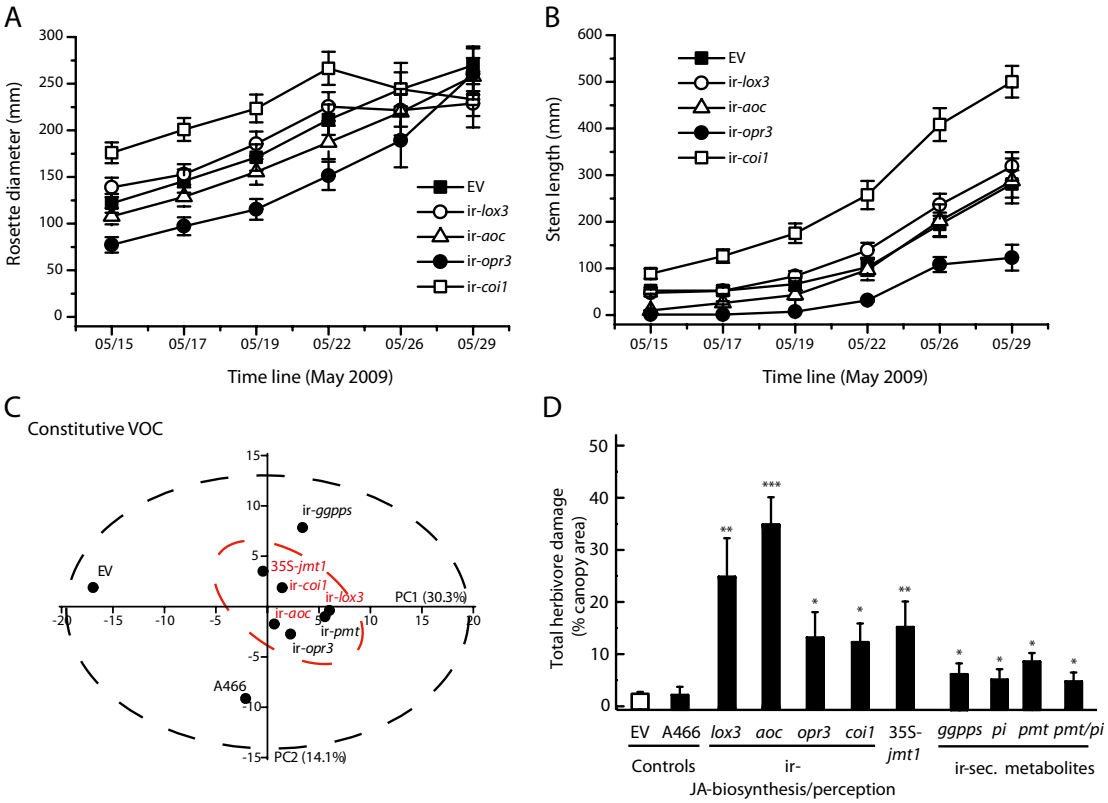
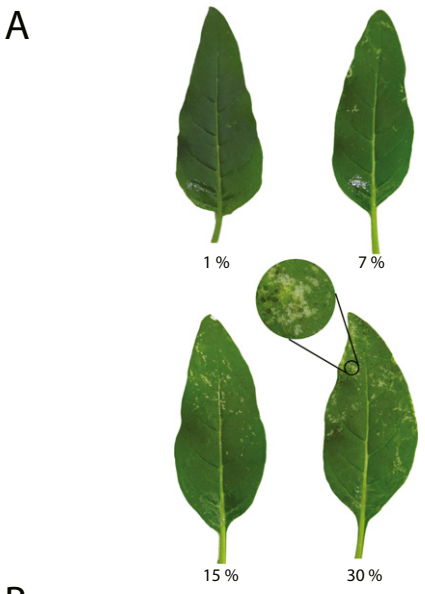
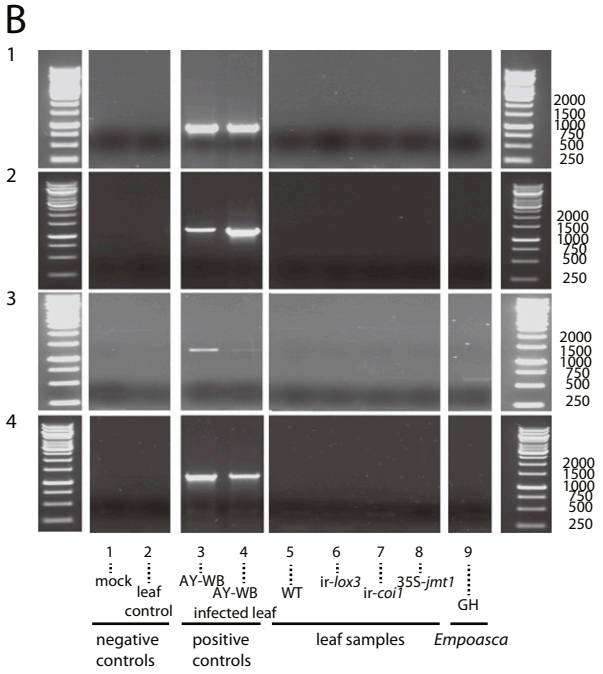


Fig. S4. Growth parameters, analysis of volatiles released from unattacked leaves, and total herbivore damage in plants deficient in JA biosynthesis and perception in the 2009 field season. (*A* and *B*) The rosette diameter (*A*) and stem length (*B*) of EV, ir-*lox3*, ir-*aoc*, ir-*opr3*, and ir-*coi1* plants were measured May 15–19, 2009 (24–38 d after plants were transplanted into the field plantation). (*C*) Leaf volatiles were collected for 2 h from unattacked leaves (Table S3). *N. attenuata* plants were grouped in two classes based on *Empoasca* spp. damage (black: no significant differences in *Empoasca* spp. damage compared with controls; red: significant differences compared with controls). One hundred ninety-seven volatiles were detected and analyzed by PCA, and principal components (PCs) 1 and 2 of the transgenic lines were plotted against each other. (*D*) Total herbivore damage was quantified on EV and A466 controls, lines silenced in JA biosynthesis (ir-*lox3*, ir-*aoc*, and ir-*opr3*) and perception (ir-*coi1*), lines silenced in JA-dependent defense molecules [i.e., diterpene glycosides (ir-*ggpps*), trypsin proteinase inhibitors (PIs; ir-*pi*), nicotine (ir-*pmt*), and both nicotine and PIs (ir-*pmt/pi*)], and lines ectopically expressing a JA methyl transferase (35S-*jmt1*). Asterisks represent significant differences compared with control plants (n = 7–10). *P < 0.05, **P < 0.01, ***P < 0.001; Student's t test.



C

Plant	Location	Neighbors	Damage (%)	Distance to E (cm)
1	N 37.14363 W 114.01902		n/a	
		N1	0	32
		N2	0	35
2	N 37.14346 W 114.01902		1	
			0	28
3	N 37.14391 W 114.01879		10	
		N1	1	770
		N2	3	280
4	N 37.13770 W 114.02616		5	
		N1	0.5	143
5	N 37.13770 W 114.02612		5	
		N1	n/a	80



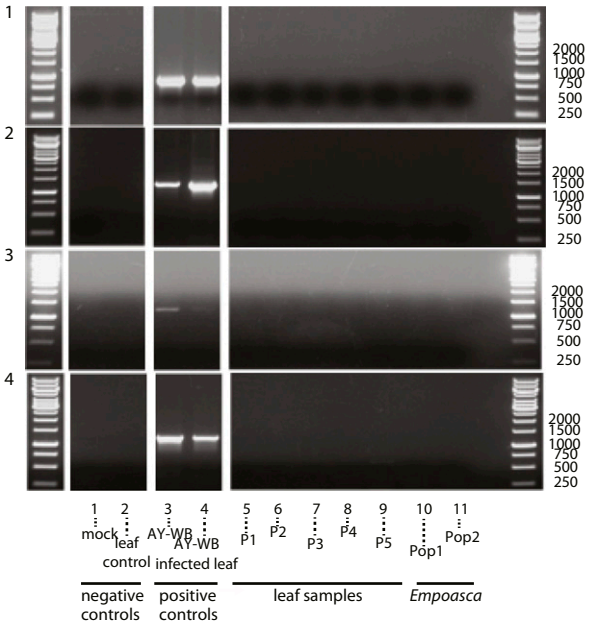


Fig. S5. *Empoasca* sp. feeding preferences are independent of *Ca. Phytoplasma* spp. in the glasshouse and on five jasmonate-deficient accessions identified by *Empoasca* spp. damage during the 2011 field season. (*A*) During feeding-choice assays, severely *Empoasca* sp.-damaged *N. attenuata* leaves were used to analyze the presence of *Ca. phytoplasma* spp. Examples of leaves showing damage of 1, 7, 15, and 30% of the canopy area are shown. (*B*) *Empoasca* sp. did not harbor or transmit *Ca. Phytoplasma* spp. to *N. attenuata* plants. Assays to detect *Ca. Phytoplasma* spp. were performed with different combinations of PCR conditions and primers: (1) direct PCR using fU5/rU3 primer pairs; (2) direct PCR using P1/P7 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; (3) direct PCR using R16mF2/R16R1 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; and (4) direct PCR using R16F2n/R16R2 primer pairs. Sample reference: lane 1, no template sample, mock; lane 2, DNA extracted from ir-*coi1* plants grown in Utah showing no signs of *Empoasca* sp. feeding, leaf control; lane 3, DNA extracted from Aster Yellow- Witches' broom (AY-WB) (*Ca. Phytoplasma asteris*) AY-WB; lane 4, DNA extracted from AY-WB–infected China aster leaves, AY-WB infected leaf; lane 5, DNA extracted from leafhopper-damaged WT leaves, WT; lane 6, DNA extracted from leafhopper-damaged ir-*lox3* leaves, ir-lox3; lane 7, DNA extracted from leafhopper-damaged ir-*coi1* leaves, ir-coi1; lane 8, DNA extracted from leafhopper-damaged 35S-jmt1 leaves, 35S-jmt1; lane 9, DNA extracted from 10 adult *Empoasca* sp. collected from the in-house colony, GH. *Empoasca* spp. damage to plants in native populations provides a rapid visual screening tool for plants deficient in jasmonate signaling and identified five plants with reduced JA accumulations among ~600 plants in native populations during the 2011 field season (Fig. 5 *E* and *F*). (*C*) For all plants attacked by *Empoasca* spp.(E), their location, their characteristic *Empoasca* spp. damage, and the distance to the nondamaged neighboring plants, which served as controls, were recorded. n/a, quantification of *Empoasca* spp. damage was not possible because plants had been damaged extensively by other herbivore species, particularly *Tupiocoris notatus*). (*D*) Detection of *Ca. Phytoplasma* spp. in these plants and in adult *Empoasca* spp. from the corresponding areas was performed using different combinations of PCR conditions and primers: (1) direct PCR using fU5/rU3 primer pairs; (2) direct PCR using P1/P7 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; (3) direct PCR using R16mF2/R16R1 primer pairs followed by nested PCR using R16F2n/R16R2 primer pairs; and (4) direct PCR using R16F2n/R16R2 primer pairs. Sample reference: lane 1, no template sample, mock; lane 2, DNA extracted from ir-*coi1* plants grown in Utah showing no signs of *Empoasca* spp. feeding, leaf control; lane 3, DNA extracted from Aster Yellow- Witches' broom (AY-WB) (*Ca. Phytoplasma asteris*), AY-WB); lane 4, DNA extracted from AY-WB–infected China aster leaves, AY-WB–infected leaf; lane 5, DNA extracted from the leaves of native plant 1 (P1); lane 6, DNA extracted from the leaves of native plant 2 (P2); lane 7 (DNA extracted from the leaves of native plant 3 (P3); lane 8, DNA extracted from leaves of native plant 4 (P4); lane 9, DNA extracted from leaves of native plant 5 (P5); lane 10, DNA extracted from 10 adult *Empoasca* spp. collected in the area around native plants 1–3 (Pop1); lane 11, DNA extracted from 10 adult *Empoasca* spp. collected in the area around native plants 4 and 5 (Pop2).

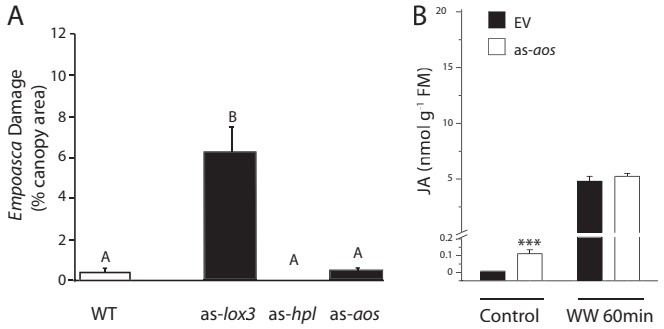


Fig. S6. Previously published levels of *Empoasca* spp. leaf damage quantified on as-*lox3* and as-*aos* plants during the 2003 field season and wound-elicited JA accumulation in as-*aos* leaves. (*A*) Previous research in our group demonstrated that *Empoasca* spp. leafhoppers fed only on plants silenced by antisense RNA in Na*LOX3* expression (as-*lox3*) but not on plants silenced by antisense RNA in Na*AOS* expression (as-*aos*) (1). (*B*) We reassessed as-*aos* plants and found that silencing of *AOS* expression in as-*aos* plants was not sufficient to reduce the wound-elicited JA burst (average ± SE, $n = 5$). Asterisks represent significant difference compared with EV leaves. ***$P < 0.001$; Student's t test.

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Table S1. Primers and PCR conditions used for analysis of *Ca. Phytoplasma* spp.

Table S1

Table S2. Primers used for cDNA synthesis to generate constructs for Na*AOS*, Na*AOC*, Na*OPR3*, and Na*ACX1* and to quantify silencing efficiency

Table S2

Table S3. Constitutive and *Empoasca* spp. feeding-induced volatile emissions on transgenic *N. attenuata* lines in the field

Table S3

Volatiles emitted from untreated plants were collected for 2 h before the start of the experiment. Herbivory-induced plant volatiles (HIPVs) were collected for 4 h (0–4 h) immediately after the start of leafhopper feeding and for 4 h after the following photoperiod (24 h after the start of the experiment). Volatiles analysis was performed on a GCxGC-ToF-MS instrument. Numbers represent the average of the normalized peak area ($n = 3$). The relative SD is below 40% and is not shown for clarity. nd: not detected. A total of 83 HIPVs emitted by *Empoasca* spp. feeding with a fold change (FC) of $1.5 \leq FC \leq 0.66$ ($P < 0.05$) compared with untreated plants of the same genotype.

Table S4. *Empoasca* spp. feeding and methyl-JA treatment induce jasmonate accumulation

Table S4

(*A*) 25 *Empoasca* spp. were caged on *N. attenuata* WT leaves to elicit jasmonate accumulation. Jasmonate levels were measured 24 h after *Empoasca* spp. feeding in control and attacked (*Emp*. fed) leaves. Values represent average ± SE, $n = 4$. nd, not detected. (*B*) Leaves of WT, ir-*lox3*, ir-*coi1*, and 35S-*jmt1* plants were treated by exogenous methyl-JA (MeJA) application. Jasmonates levels were measured 2 d after MeJA treatment (average ± SE, $n = 6$). nd, not detected.