Report

Current Biology

Tissue-Specific Emission of (*E*)-α-Bergamotene Helps Resolve the Dilemma When Pollinators Are Also Herbivores

Highlights

- In wild tobacco, both flowers and herbivory-induced leaves emit (*E*)-α-bergamotene
- Floral (*E*)-α-bergamotene increases *Manduca sexta* moth-mediated pollination success
- Herbivory-induced (*E*)-α-bergamotene mediates indirect defense against *M. sexta* larvae
- Expression of *NaTPS38* regulates both floral and herbivory-induced (*E*)-α-bergamotene

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In Brief

Plants face a dilemma when their pollinators are also herbivores. Zhou et al. find that wild tobacco resolves this dilemma through tissue-specific emission of a sesquiterpene, regulated by *NaTPS38*, which increases pollination success in flowers while defending against herbivores in leaves.

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Tissue-Specific Emission of (*E*)-α-Bergamotene Helps Resolve the Dilemma When Pollinators Are Also Herbivores

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SUMMARY

More than 87% of flowering plant species are animal-pollinated [1] and produce floral scents and other signals to attract pollinators. These floral cues may however also attract antagonistic visitors, including herbivores [2]. The dilemma is exacerbated when adult insects pollinate the same plant that their larvae consume. It remains largely unclear how plants maximize their fitness under these circumstances. Here we show that in the night-flowering wild tobacco *Nicotiana attenuata*, the emission of a sesquiterpene, (*E*)-α-bergamotene, in flowers increases adult *Manduca sexta* moth-mediated pollination success, while the same compound in leaves is known to mediate indirect defense against *M. sexta* larvae [3, 4]. Forward and reverse genetic analyses demonstrated that both herbivory-induced and floral (*E*)-α-bergamotene are regulated by the expression of a monoterpene-synthase-derived sesquiterpene synthase (*NaTPS38*). The expression pattern of *NaTPS38* also accounts for variation in (*E*)-α-bergamotene emission among natural accessions. These results highlight that differential expression of a single gene that results in tissue-specific emission of one compound contributes to resolving the dilemma for plants when their pollinators are also herbivores. Furthermore, this study provides genetic evidence that pollinators and herbivores interactively shape the evolution of floral signals and plant defense.

RESULTS AND DISCUSSION

Nicotiana attenuata Flowers and Herbivory-Induced Leaves Emit (*E*)-α-Bergamotene in the Night and Daytime, Respectively

A previous study showed that (*E*)-α-bergamotene, which is emitted from local and systemic leaves of herbivory-induced plants and mediates indirect defense [3, 4] by attracting predatory *Geocoris* spp., is also emitted from floral tissues [5]. We analyzed the kinetics of both herbivory-induced and floral (*E*)-α-bergamotene emission in *N. attenuata*. Flowers of *N. attenuata* mainly emit (*E*)-α-bergamotene at night. Their (*E*)-α-bergamotene emission levels increased rapidly immediately after corolla opening at 6 p.m. and reached the highest emission levels between 8 p.m. and 1 a.m., after which levels decreased during the subsequent morning (Figure 1). This kinetic of floral (*E*)-α-bergamotene emission was similar to another major floral attractant, benzyl acetone (BA), whose emission is coordinated with the activity of the *M. sexta* moth [6].

In leaves, constitutive (*E*)-α-bergamotene emission peaked in the morning and subsequently decreased until 2 a.m., but overall emission levels were very low. After simulated herbivory by wounding leaves with pattern wheels and adding *M. sexta* oral secretions (OSs) to the wounds, we found that the emission of (*E*)-α-bergamotene had two peaks during the daytime. The first peak was in the afternoon (12 p.m.–8 p.m.) on the day of elicitation, and the second one was during the following morning (6 a.m.–10 a.m.). Additional experiments also showed that the day emission pattern of herbivory-induced (*E*)-α-bergamotene was largely independent of the elicitation time (Y. Joo, M.C.S, J.K. Goldberg, S.-G. Kim, F.Y., C. Brütting, and I.T.B., unpublished data). Furthermore, the timing of herbivory-induced (*E*)-α-bergamotene emission matched the activity and

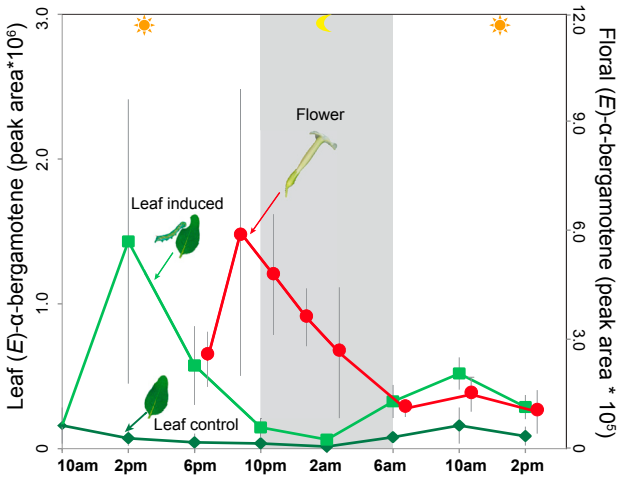

Figure 1. *Nicotiana attenuata* Emits (*E*)-α-Bergamotene in Both Flowers and Herbivory-Induced Leaves

Emission kinetics of (*E*)-α-bergamotene in the leaf (green) and the flower (red) are shown. The x axis indicates the sampling time, and the y axis denotes the abundance of (*E*)-α-bergamotene level in leaves (left) and flowers (right). Dark-green- and light-green-colored lines refer to control (unwounded and uninduced) and OS-induced (wounded with *M. sexta* oral secretion added) leaf samples, respectively. The elicitations were carried out at 8 a.m. The leaf volatiles were collected for 4 hr, and the middle time points are shown. The floral volatiles were collected for 1 hr, and the middle time points are shown. Data are presented as mean ± SD.

abundance of *Geocoris* spp. predators in the field (Y. Joo, M.C.S, J.K. Goldberg, S.-G. Kim, F.Y., C. Brütting, and I.T.B., unpublished data).

The Terpene Synthase *NaTPS38* Is Involved in the Emission of Both Herbivory-Induced and Floral (*E*)-α-Bergamotene

To examine whether herbivory-induced and floral (*E*)-α-bergamotene share the same biosynthetic machinery, we correlated herbivory-induced and floral (*E*)-α-bergamotene emissions among 23 natural accessions of *N. attenuata*. We reasoned that, if the same molecular machinery controls both herbivory-induced and floral (*E*)-α-bergamotene, the emission levels from these two tissues should co-vary among different genotypes. Indeed, the results showed that the levels of (*E*)-α-bergamotene in flowers and herbivory-induced leaves are highly variable and significantly correlated among 23 genotypes ($p = 3.2E{-}6$; $R^2 = 0.65$; Figure 2A), indicating that natural variations of (*E*)-α-bergamotene in flowers and herbivory-induced leaves share the same genetic basis.

To identify the genetic basis underlying the variation in (*E*)-α-bergamotene emission, we performed quantitative trait loci (QTL) mapping using an *N. attenuata* advance intercross recombinant inbred line (AI-RIL) population developed by crossing two inbred lines originating in Arizona (AZ) and Utah (UT), USA (Supplemental Information), which differ in both herbivory-induced and floral (*E*)-α-bergamotene emissions (Figure 2B). We measured the herbivory-induced (*E*)-α-bergamotene emission among 256 individuals of the AI-RIL population (Supplemental Information). The analysis showed that, within a 95% confidence interval, the genetic control of herbivory-induced (*E*)-α-bergamotene was mapped to two QTL loci on linkage groups 1 and 2 (Figure 2C, QTL1 and QTL2). By fitting a multiple QTL model, QTL1 and QTL2 showed significant dominant and additive effects, respectively (Table S1).

We searched candidate genes in the corresponding genomic information of the two candidate loci in *N. attenuata* and their syntenic genomic region in tomato. The major QTL locus (QTL2) overlapped with a terpene synthase (TPS) cluster that contains members of the TPS-b subclade. In tomato, this cluster includes *SlTPS25*, *SlTPS26* (pseudogene), *SlTPS27*, and *SlTPS38* [7]. Among these, whereas *SlTPS25* and *SlTPS27* are highly similar to the grape *VvTPS47* that produces either (*E*)-β-ocimene using geranyl pyrophosphate (GPP) or (*E*)-α-farnesene using farnesyl pyrophosphate (FPP), *SlTPS38* produces (*E*)-α-bergamotene using FPP in vitro [7]. Searching the available genomic and transcriptomic information of *N. attenuata* (both UT and AZ genotypes), we identified *NaTPS38* as the ortholog of *SlTPS38* and *NaTPS25*—which was not found in UT but was found in the AZ genome—as the ortholog of *SlTPS25/27*.

Examining the expression profile of herbivory-induced transcriptomic responses in *N. attenuata* (UT genotype) showed that *NaTPS38* is highly induced after herbivory in leaves (Figure 3A). Among different floral tissues, *NaTPS38* was highly expressed in the corolla tube, but not in the floral limb (Figure 3B), consistent with the previously reported tissue-specific emission of (*E*)-α-bergamotene in flowers [5].

To measure the TPS activity of *NaTPS38* and *NaTPS25* in vitro, we heterologously expressed these two genes in *Escherichia coli*. The recombinant NaTPS25 converted the substrate GPP into (*E*)-β-ocimene as the only product (data not shown); NaTPS38 converted the substrate (*E*,*E*)-FPP into (*E*)-α-bergamotene as the sole product (Figures 3C and 3D). Additional tests with (*E*,*E*)-FPP revealed that NaTPS25 does not convert (*E*,*E*)-FPP into sesquiterpenes. These results indicate that *NaTPS38* is likely the gene involved in (*E*)-α-bergamotene biosynthesis.

To determine the function of *NaTPS38* in vivo, we silenced the expression of this gene in *N. attenuata* UT using virus-induced gene silencing (VIGS) (Supplemental Information). Because the emission of herbivory-induced (*E*)-α-bergamotene is mediated by jasmonate signaling [10], we measured both constitutive and methyl jasmonate (MeJA)-induced (*E*)-α-bergamotene emission in leaves. Silencing *NaTPS38* reduced the emission of (*E*)-α-bergamotene in flowers and in uninduced and MeJA-induced leaves (Figures 3E–3H).

We further measured the transcript abundance of *NaTPS38* in both flowers and herbivory-induced leaves among the 23 natural accessions using qPCR. *NaTPS38* transcripts were quantifiable in all 23 accessions, but relative transcript abundance varied. *NaTPS38* transcript abundance in flowers and herbivory-induced leaves were significantly correlated with the emission of (*E*)-α-bergamotene in these two tissues among the 23 accessions (Figure S1; flowers: $p = 0.0098$, $R^2 = 0.28$; herbivory-induced leaves: $p = 1.0E{-}8$, $R^2 = 0.77$). These results showed that the emissions of both herbivory-induced and floral (*E*)-α-bergamotene are mediated by *NaTPS38* in *N. attenuata*.

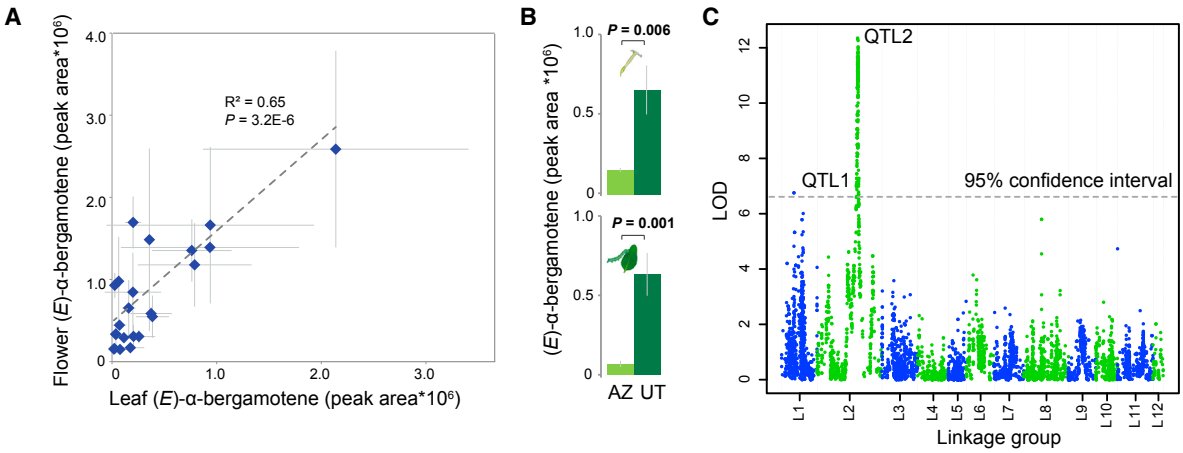

Figure 2. Genetic Basis of (*E*)-α-Bergamotene Emission in *N. attenuata*

(A) Floral (y axis) and herbivory-induced leaf (x axis) (*E*)-α-bergamotene emissions are correlated among natural accessions. Data are shown as mean ± SD.

(B) Both floral (top panel) and herbivory-induced (bottom panel) (*E*)-α-bergamotene emissions are significantly different between AZ and UT genotypes. Data are shown as mean ± SEM. For both (A) and (B), the measurement of herbivory-induced and floral emission (*E*)-α-bergamotene was performed for the periods of 9 p.m.–10 p.m. and 12 p.m.–4 p.m., respectively.

(C) Herbivory-induced (*E*)-α-bergamotene is mapped to two QTL loci. Two QTL loci on linkage group 1 (QTL1) and 2 (QTL2) are marked. The 95% confidence interval is indicated with a dashed line.

See also Table S1.

NaTPS38 Evolved from a Monoterpene Synthase and Was Likely under Positive Selection

Whereas the product of *NaTPS38* is a sesquiterpene, *NaTPS38* is located in the monoterpene synthase gene cluster (TPS-b clade). To understand the molecular evolution of *NaTPS38*, we constructed a phylogenetic tree using *NaTPS38* and members of TPS-b clade from *N. attenuata*, tomato, grape, and *Arabidopsis thaliana*. The results showed that *TPS38* can be found in both *Nicotiana* and tomato but is absent in grape and *A. thaliana*, suggesting that *TPS38* is Solanaceae-specific (Figure S2A). Indeed, we found a single copy of *TPS38* in available genomic datasets from all diploid solanaceous species, including *Petunia axillaris* and *N. obtusifolia* (Table S2).

Whereas monoterpene synthases are often localized in plastids and contain chloroplast transit peptides [11], sesquiterpenes are predominantly synthesized in the cytosol. To investigate the localization of TPS38 in *N. attenuata*, we measured the subcellular localization of NaTPS38 fused to yellow fluorescent protein (YFP) by transient expression assays in leaves of *N. attenuata*. The fusion protein of YFP-NaTPS38 was localized to the cytosol (Figure S3), suggesting that TPS38 lost its chloroplast transit peptide (cTP) from its ancestor.

In addition to changes in subcellular localization, in vitro assays also showed that both tomato and *Nicotiana* TPS38 specifically use (*E*,*E*)-FPP as a substrate, which differed from their ancestors, which use GPP as their primary substrate [7]. This indicated that amino acid changes resulting in an altered substrate specificity might have occurred before the speciation event between *Nicotiana* and *Solanum*. To examine whether this change in substrate utilization was under positive selection, we performed a maximum-likelihood-based analysis of synonymous mutations (d_S; preserving the amino acid sequence) versus nonsynonymous mutations (d_N; altering the amino acid sequence). We found that the ratio of d_N/d_S on the *TPS38* clade before the split of *Solanum* and *Nicotiana* was significantly higher than that of other branches (p = 9.8E−14), indicating that the evolution of the *TPS38* branch was under either positive or relaxed purifying selection.

To further understand the evolution of *TPS38* at a population level, we analyzed the whole-genome re-sequencing data from 23 natural accessions of *N. attenuata*. Based on sliding-window analyses, we found a sharp decrease of nucleotide diversity at the genomic location of *NaTPS38*, as measured by Tajima's D, Fu, and Li's F and site frequency spectrum statistics (Figures S2B–S2D). This suggests that *NaTPS38* was likely under positive selection in *N. attenuata* populations.

Floral (*E*)-α-Bergamotene Increases Pollination Success in *N. attenuata*

Because the expression of *NaTPS38* is associated with both herbivory-induced and floral (*E*)-α-bergamotene, the evolution of this gene is likely shaped by the ecological functions of (*E*)-α-bergamotene in both flowers and herbivory-induced leaves. Whereas herbivory-induced (*E*)-α-bergamotene is known to improve the fitness of *N. attenuata* by attracting predators of *M. sexta* larvae, thereby acting as an indirect defense [3, 4], the ecological functions of floral (*E*)-α-bergamotene remained largely unknown.

The night-emission pattern of floral (*E*)-α-bergamotene—which is similar to that of benzyl acetone (BA), the major attractant of *M. sexta* moths as pollinators in *N. attenuata* [6, 12]—suggests that floral (*E*)-α-bergamotene might be involved in modifying *M. sexta* moth pollination behavior. Tests based on electroantennograms showed weak responses of *M. sexta* antennae to (*E*)-α-bergamotene at the concentration present in the floral headspace (Table S3), which was consistent with its

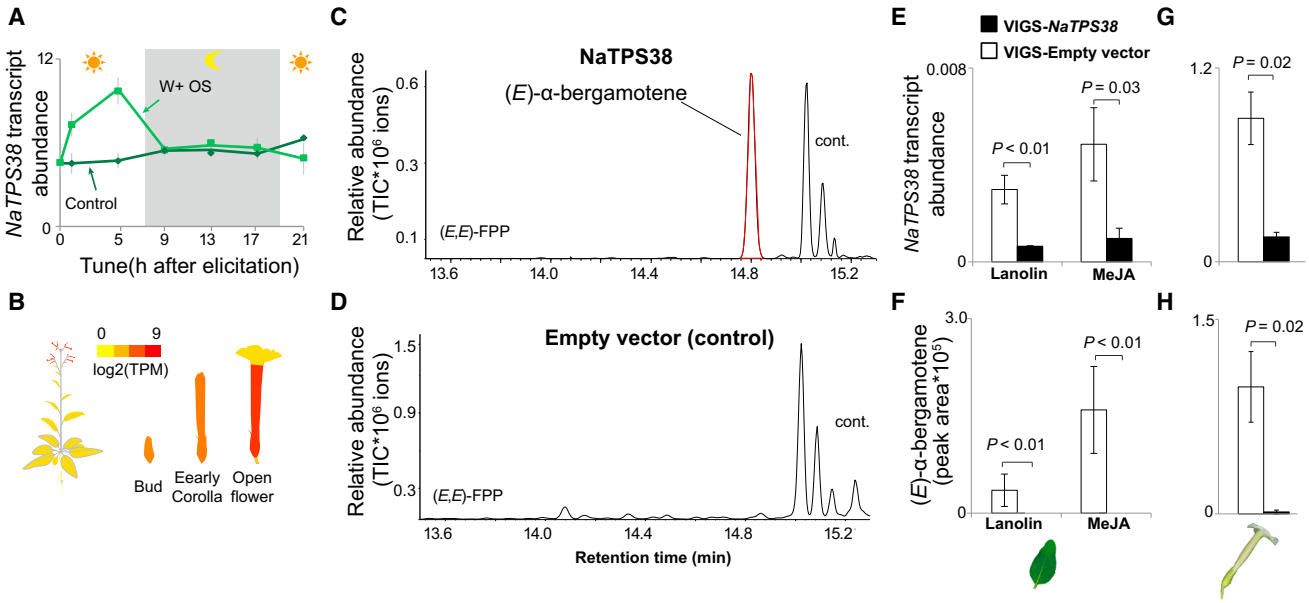

Figure 3. *NaTPS38* Is Required for Both Herbivory-Induced and Floral (*E*)-α-Bergamotene Emission in *N. attenuata*

(A) The expression kinetics of *NaTPS38* in leaves. Dark-green- and light-green-colored lines refer to control (undamaged) and OS-induced leaves, respectively. The y axis shows the relative expression level measured by microarray. Data are shown as mean ± SEM. The data were extracted from the microarray dataset in Kim et al. [8].

(B) A heatmap depicts tissue-specific expression of *NaTPS38* in *N. attenuata*. The relative expression levels of *NaTPS38* among different tissues were extracted from the *N. attenuata* data hub [9]. The color gradient represents the relative expression value (log_2(TPM)). TPM, transcripts per million.

(C and D) Heterologously expressed NaTPS38 produces (*E*)-α-bergamotene as the sole product in vitro (C). The empty vector control is shown in (D). Gas chromatography (GC) chromatograms of enzyme products from recombinant NaTPS38 expressed in *E. coli* and incubated with the substrate (*E*,*E*)-FPP are depicted. Cont. refers to contaminations. The peak of the target product is marked in red.

(E–H) Silencing the expression of *NaTPS38* using VIGS in flowers and leaves. For leaves, both constitutive (control, with lanolin) and MeJA (lanolin + MeJA)-induced levels of *NaTPS38* expression and (*E*)-α-bergamotene emissions were measured. p values were calculated using Student's t tests. Data are shown as mean ± SEM.

(E and G) In comparison to the empty vector (EV) control, the relative transcript abundance of *NaTPS38* was reduced in both leaves (E) and flowers (G) of *VIGS-NaTPS38* plants.

(F and H) In comparison to EV plants, the levels of (*E*)-α-bergamotene emission in both leaves and flowers of VIGS-*NaTPS38* plants were significantly lower.

See also Figures S1–S3 and Table S2.

inability to attract moths in wind tunnel assays (data not shown). We hypothesized that (*E*)-α-bergamotene might act as a stimulus to the chemosensory sensilla at the tip of *M. sexta*'s proboscis, which also house chemosensory receptors detecting BA [13]. Repeating these recordings with purified (*E*)-α-bergamotene indeed revealed the neuronal responses of *M. sexta*'s proboscis at concentrations present in the floral headspace (Figure 3A, insert; Table S3). Therefore, we specifically examined the effects of (*E*)-α-bergamotene on proboscis-based attraction in a Y-maze setup [13].

We first examined the effect of the pure compound on the probing behavior of *M. sexta* moths. In comparison to the solvent, (*E*)-α-bergamotene significantly increased the probing time (Figure 4A; p = 0.002) of *M. sexta* moths, which is associated with increased pollination success [13]. To further examine the effects of (*E*)-α-bergamotene on the probing behavior of *M. sexta* moths in the natural floral volatile background, we tested the probing preference of *M. sexta* moths between the headspaces of (*E*)-α-bergamotene-emitting (VIGS-EV) and non-emitting (VIGS-*NaTPS38*) flowers. Probing time of *M. sexta* moths was significantly longer in (*E*)-α-bergamotene-emitting flowers compared to the (*E*)-α-bergamotene-deficient flowers (Figures 4B and S4; p = 0.016; Supplemental Information). To test whether the observed probing time behavior changes have direct consequences on plant fitness, we further performed an experiment under semi-natural conditions in a large outdoor tent [6], offering individual *M. sexta* moths antherectomized flowers with and without biologically relevant amounts of (*E*)-α-bergamotene supplemented in the corolla tube (Supplemental Information). Supplementation of (*E*)-α-bergamotene significantly increased both the likelihood of capsule formation (p = 0.05; binomial test) and the total seed numbers per flower (Figure 4C; p = 0.03; Wilcoxon signed-rank test). Together, these results show that floral (*E*)-α-bergamotene increases *M. sexta* moth-mediated pollen transfer between plants and thus outcrossing rate in *N. attenuata*.

In conclusion, whereas *N. attenuata* flowers express *NaTPS38* and emit (*E*)-α-bergamotene in the night to facilitate pollination by *M. sexta* moths [3, 4], these same pollinators often lay eggs on the leaves of the same plant [6]; when the leaves are attacked by *M. sexta* larvae, they express the same gene and emit the same compound during the day to attract the predators

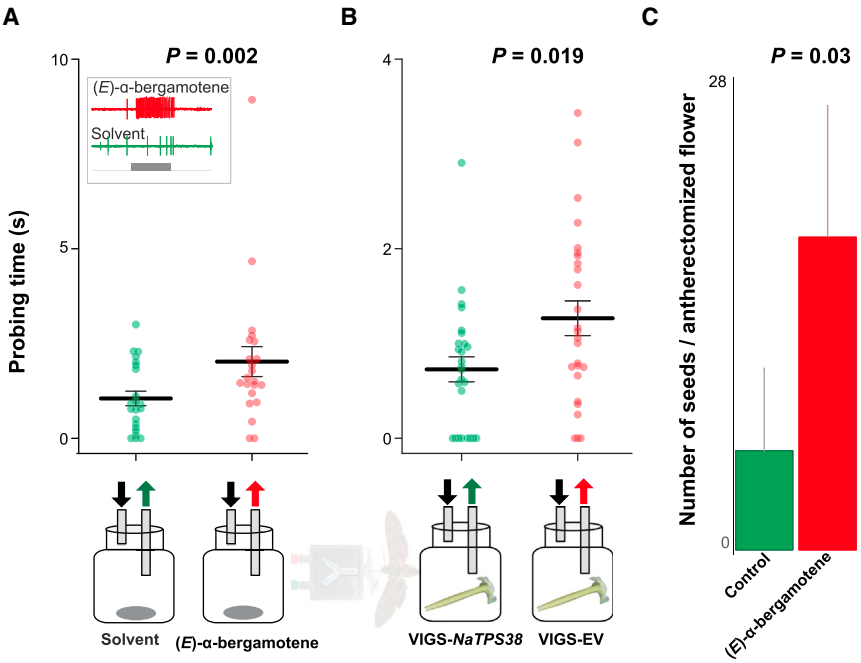

Figure 4. *N. attenuata* Floral (*E*)-α-Bergamotene Increases Probing Time and *M. sexta* Moth-Mediated Pollination Success

(A) *M. sexta* moths probed significantly longer in the Y-maze arm in which (*E*)-α-bergamotene was present than in the arm with solvent present (p = 0.002; Wilcoxon signed-rank test). Inset in (A) shows a representative response of a sensillum on the tip of *M. sexta*'s proboscis to (*E*)-α-bergamotene (up) and the solvent control (low). Black bar at the bottom indicates stimulus duration (0.5 s).

(B) *M. sexta* moth probed significantly longer in the Y-maze arm with the floral headspace from VIGS-empty vector (EV) flowers than in the one from VIGS-*NaTPS38* flowers (p = 0.019; Wilcoxon signed-rank test).

(C) (*E*)-α-bergamotene supplementation in corolla tubes increased *M. sexta* moth-mediated pollination success (p = 0.03; Wilcoxon signed-rank test). Data are shown as mean ± SEM.

See also Figure S4 and Tables S3 and S4.

of *M. sexta* larvae, which subsequently reduce herbivore damage [3, 4, 14]. Thus, tissue- and time-specific expression of *NaTPS38* and its product (*E*)-α-bergamotene mediate both defense and pollinator attraction and contribute to resolving the dilemma that *N. attenuata* plants face because its preferred pollinator is also a voracious herbivore. Furthermore, these results suggest that interactions between herbivores and pollinators can act synergistically or additively in shaping plant evolution [2].

ACCESSION NUMBERS

The accession number for the GBS data of the AI-RIL population reported in this paper is NCBI: PRJNA378521.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.03.017.

AUTHOR CONTRIBUTIONS

Conceptualization, S.X.; Methodology, W.Z., S.X., E.M., A.H., F.B., M.K., F.Y., R.L., M.C.S., B.S.H., and D.K.; Investigation, W.Z., S.X., A.K., E.M., A.H., H.G., F.B., N.L., T.G.K., J.B., and D.K.; Writing – Original Draft, S.X. and W.Z.; Writing – Review and Editing, S.X., W.Z., I.T.B., A.H., and M.C.S.; Funding Acquisition, S.X., I.T.B., and B.S.H.; Resources, S.X., B.S.H., I.T.B., and D.K.; Supervision, S.X.

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