Ectopic Terpene Synthase Expression Enhances Sesquiterpene Emission in *Nicotiana attenuata* without Altering Defense or Development of Transgenic Plants or Neighbors[1][W]

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Sesquiterpenoids, with approximately 5,000 structures, are the most diverse class of plant volatiles with manifold hypothesized functions in defense, stress tolerance, and signaling between and within plants. These hypotheses have often been tested by transforming plants with sesquiterpene synthases expressed behind the constitutively active 35S promoter, which may have physiological costs measured as inhibited growth and reduced reproduction or may require augmentation of substrate pools to achieve enhanced emission, complicating the interpretation of data from affected transgenic lines. Here, we expressed maize (*Zea mays*) *terpene synthase10* (*ZmTPS10*), which produces (*E*)-α-bergamotene and (*E*)-β-farnesene, or a point mutant *ZmTPS10M*, which produces primarily (*E*)-β-farnesene, under control of the 35S promoter in the ecological model plant *Nicotiana attenuata*. Transgenic *N. attenuata* plants had specifically enhanced emission of target sesquiterpene(s) with no changes detected in their emission of any other volatiles. Treatment with herbivore or jasmonate elicitors induces emission of (*E*)-α-bergamotene in wild-type plants and also tended to increase emission of (*E*)-α-bergamotene and (*E*)-β-farnesene in transgenics. However, transgenics did not differ from the wild type in defense signaling or chemistry and did not alter defense chemistry in neighboring wild-type plants. These data are inconsistent with within-plant and between-plant signaling functions of (*E*)-β-farnesene and (*E*)-α-bergamotene in *N. attenuata*. Ectopic sesquiterpene emission was apparently not costly for transgenics, which were similar to wild-type plants in their growth and reproduction, even when forced to compete for common resources. These transgenics would be well suited for field experiments to investigate indirect ecological effects of sesquiterpenes for a wild plant in its native habitat.

Isoprenoids, derived from the five-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate, are the largest and most diverse group of plant metabolites (Thulasiram et al., 2007; Vranová et al., 2012). They are produced from two different biosynthetic pathways in the plant cell: the mevalonate (MVA) pathway in the cytosol and peroxisomes and the 2-*C*-methyl-D-erythritol 4-P/1-deoxy-D-xylulose 5-P pathway in the plastid (for review, see Rodríguez-Concepción, 2006; Tholl and Lee, 2011; Rodríguez-Concepción et al., 2013). These pathways are at an intersection of general and specialized metabolism. The plastidial 2-*C*-methyl-D-erythritol 4-P/1-deoxy-D-xylulose 5-P pathway produces the precursors of carotenoids and the chlorophyll side chain and in addition, precursors of, for example, abscisic acid (ABA) gibberellins, cytokinins, volatile monoterpenes, and in some plants, highly volatile isoprene. The largest metabolic sink for products of the MVA pathway is sterol biosynthesis (Rodríguez-Concepción, 2006), but substrate from the MVA pathway also provides precursors of sesquiterpene volatiles as well as signaling molecules, such as cytokinins and brassinosteroids, and prenyl moieties for protein modification among other things. The two pathways are, furthermore, able to exchange intermediates. Thus, the synthesis of any one terpene product may affect metabolic flux toward a large array of general and specialized plant metabolites and signaling molecules (for review, see Nagegowda, 2010; Vranová et al., 2012).

Of the volatile terpenes—isoprene (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), and a few homoterpenes (C_{11} and C_{16}) derived from the degradation of sesquiterpenes and diterpenes—sesquiterpenes and their derivatives are the most diverse, with approximately 5,000 structures (Seigler, 2014). Sesquiterpenes are often components of stress-induced plant volatile blends (Duhl et al., 2008; Holopainen and Gershenzon, 2010), and they have a

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multitude of possible functions in plant physiology and ecological interactions (Vickers et al., 2009; Dicke and Baldwin, 2010; Holopainen and Gershenzon, 2010). For example, several herbivore-induced sesquiterpenes have been shown to contribute to defense in multiple plant species by attracting predators or parasitoids of herbivores feeding on plant roots or shoots or deterring ovipositing herbivores in laboratory assays as well as field experiments (Kessler and Baldwin, 2001; Kappers et al., 2005; Rasmann et al., 2005; Schnee et al., 2006; Halitschke et al., 2008; Degenhardt et al., 2009a). Furthermore, there is evidence that herbivory-induced sesquiterpenes function as stress signals between leaves of single plants as well as between plants (Paschold et al., 2006; Heil and Silva Bueno, 2007; Ton et al., 2007; Heil and Karban, 2010; Zebelo et al., 2012). As has been shown for isoprene and some monoterpenes (for review, see Vickers et al., 2009), sesquiterpenes could also help to protect plants from oxidative stress resulting from abiotic factors.

The stress-induced emission of sesquiterpenes as well as their diversity among genotypes and species are thought to be largely controlled by the polymorphism, subcellular localization, and transcript levels of sesquiterpene synthase enzymes (for review, see Chen et al., 2011). These enzymes belong to the family of terpene synthases (TPSs), which includes members that produce both general and specialized metabolites, and are all thought to be derived from an ancestral kaurene synthase of general metabolism (Trapp and Croteau, 2001; Chen et al., 2011). Sesquiterpene synthases use the 15-carbon substrate farnesyl diphosphate (FDP) to produce a multitude of monocyclic and polycyclic sesquiterpene products and derivatives as well as linear products (Degenhardt et al., 2009b).

Hypotheses about the physiological and ecological functions of specific sesquiterpenes in plants have frequently been tested by ectopically expressing sesquiterpene synthases under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, resulting in constitutive emission of the engineered sesquiterpenes (Degenhardt et al., 2003, 2009a; Aharoni et al., 2005; Rasmann et al., 2005; Dudareva and Pichersky, 2008; Kos et al., 2013). These transgenic plants have helped to show a role for sesquiterpenes in resisting herbivore damage and attracting enemies of herbivores in several plant species. However, constitutive transgenic production of sesquiterpenes can result in negative physiological effects likely related to altering flux into essential terpenoid products, including stunted growth and reduced reproductive yield (Aharoni et al., 2003; Robert et al., 2013), complicating the use of such transgenic plants in ecological studies. In some cases, ectopic expression of TPSs has been thought to reveal a so-called silent metabolism because of the appearance of additional products derived from the ectopic terpenes (Nagegowda, 2010). Measures have sometimes been taken to increase available substrate pools to achieve enhanced emission, such as altered subcellular localization, or coexpression of *FARNESYL DIPHOSPHATE SYNTHASE* (Kappers et al., 2005; Nagegowda, 2010; Houshyani et al., 2013; Kos et al., 2013). These engineering efforts show that substrate flux can also regulate sesquiterpene emission (Vranová et al., 2012) and that ectopic engineering of sesquiterpenes cannot only relieve but also, produce stress in plants (Holopainen and Gershenzon, 2010; Nagegowda, 2010; Robert et al., 2013).

To learn more about the physiological and ecological roles of volatile sesquiterpenes in *Nicotiana attenuata*, we enhanced the emission of (E)-α-bergamotene and (E)-β-farnesene in this wild tobacco by ectopic expression of the sesquiterpene synthase maize (*Zea mays*) *TPS10* (*ZmTPS10*) or a point mutant *ZmTPS10M* producing primarily (E)-β-farnesene (Köllner et al., 2009) under the control of the CaMV 35S promoter. The sesquiterpene (E)-α-bergamotene is ubiquitously emitted by natural genotypes of *N. attenuata* in response to herbivore elicitation (Halitschke et al., 2000; Kessler and Baldwin, 2001), and natural variation in (E)-α-bergamotene emission is related to the variation in magnitude of endogenous jasmonate signaling response to herbivore attack (Schuman et al., 2009). (E)-α-bergamotene has been shown to reduce herbivore populations on *N. attenuata* plants in their native habitat by attracting predatory arthropods and deterring oviposition of the specialist herbivore *Manduca sexta* (Kessler and Baldwin, 2001; Halitschke et al., 2008). Furthermore, (E)-α-bergamotene regulates a subset of genes in *N. attenuata* plants exposed to herbivore-induced volatiles from conspecifics (Paschold et al., 2006). (E)-β-farnesene has occasionally been detected in small amounts in the headspace of *N. attenuata* plants and larger amounts from plants in which the JASMONATE ZIM DOMAIN H negative regulator of jasmonate responses was silenced (Oh et al., 2012) or wild-type plants that have been treated with strong jasmonate elicitors (Kallenbach et al., 2014). The engineered emission of these two sesquiterpenes, which are released on caterpillar damage to maize leaves, has been shown to attract a hymenopteran parasitoid of the caterpillar to Arabidopsis (*Arabidopsis thaliana*) plants constitutively emitting the sesquiterpenes but only after the parasitoids first had an oviposition experience in the presence of the sesquiterpenes (Schnee et al., 2006).

In this study, we found that enhanced emission of (E)-α-bergamotene and/or (E)-β-farnesene in *N. attenuata TPS10* and *TPS10M* plants had no measurable effects on plant growth, reproductive yield, or defense physiology, even when *TPS10* plants were forced to compete with wild-type plants for common resources. Moreover, the growth and defense physiology of wild-type plants were not affected by their neighbors' (E)-β-farnesene and (E)-α-bergamotene emissions. These data are not consistent with within-plant or between-plant signaling functions for (E)-α-bergamotene and (E)-β-farnesene in *N. attenuata*. However, given that enhanced levels of emission occurred without any alterations in growth and physiology, the engineered plants are well suited for use in ecological experiments.

RESULTS

Ectopic Expression of *TPS10* or *TPS10M* Results in Elevated Levels of (*E*)-β-Farnesene and (*E*)-α-Bergamotene in the Foliar Headspace

We ectopically expressed *ZmTPS10* or the point mutation *ZmTPS10M* under the control of the 35S promoter in multiple transgenic lines of *N. attenuata* and selected diploid, homozygous lines with single insertion events for additional screening (Supplemental Fig. S1). We then measured the abundance of the target volatiles (*E*)-β-farnesene and (*E*)-α-bergamotene in the headspace of unwounded leaves and leaves treated with wounding and addition of *M. sexta* oral secretions to wounds (W+OS), which elicits emission of (*E*)-α-bergamotene in wild-type plants (Halitschke et al., 2000). Headspace samples from transgenic lines contained (*E*)-β-farnesene, whereas samples from the wild type did not, and samples from transgenics also contained 2-fold to 25-fold as much (*E*)-α-bergamotene as those from the wild type (Supplemental Fig. S1). As expected, a *TPS10M* line (M-1) emitted more than 2 times as much (*E*)-β-farnesene and less than 0.5 times as much (*E*)-α-bergamotene compared with *TPS10* lines (10-1–10-5). Interestingly, emissions of both (*E*)-β-farnesene and (*E*)-α-bergamotene were enhanced by W+OS treatment (Supplemental Fig. S1): differences among transgenic lines in (*E*)-β-farnesene abundance were not significant in measurements of constitutive emission (Kruskal-Wallis test, χ^2 = 7.419, degrees of freedom [df] = 5, Bonferroni-corrected P = 0.3821) but became significant after W+OS treatment (χ^2 = 14.96, df = 5, corrected P = 0.0211), and the overall effect of W+OS treatment on (*E*)-β-farnesene abundance was also significant (Wilcoxon rank sum test, W = 143, corrected $P < 0.001$). Although (*E*)-α-bergamotene was detected in all headspace samples, its abundance increased after W+OS treatment (W = 317, corrected P = 0.0167); the effect of line on (*E*)-α-bergamotene abundance was significant in both measurements of constitutive emission (χ^2 = 21.16, df = 6, corrected P = 0.0034) and after W+OS treatment (χ^2 = 23.81, df = 6, corrected P = 0.0011).

From the lines having a single insertion, we selected two each of *TPS10* and *TPS10M* for additional experiments (10-3 = A-09-389, homozygote 4 or 6; 10-4 = A-09-391, homozygote 1; M-1 = A-09-596, homozygote 4; M-2 = A-09-334, homozygote 4). Because line M-2 was generated later than the other three lines, many experiments were conducted only with line M-1 of *TPS10M*, but line M-2 was included in experiments to show transgene transcript abundance and test for off-target effects.

We analyzed the abundance of (*E*)-α-bergamotene and (*E*)-β-farnesene in the headspace of leaves on wild-type plants compared with lines 10-3 and 10-4 or compared with M-1 after no treatment or treatment with herbivory or jasmonate elicitors known to induce (*E*)-α-bergamotene emission from wild-type plants (Halitschke et al., 2000; Schuman et al., 2009; Fig. 1). Again, (*E*)-β-farnesene was detected in all headspace samples from transgenic plants but only a few samples from W+OS-treated wild-type plants; (*E*)-α-bergamotene was more abundant in headspace samples from 10-3 and 10-4 than the wild type, regardless of treatment, and enhanced in the headspace of M-1 compared with the wild type after wounding control (W+W), W+OS, or lanolin (Lan) treatment (Fig. 1; Supplemental Tables S1 and S2).

We also assayed the expression of the *TPS10* and *TPS10M* transgenes in lines 10-3, 10-4, M-1, and M-2 (Fig. 2) both constitutively and after the various treatments used to elicit (*E*)-β-farnesene and (*E*)-α-bergamotene emissions (shown for line M-1). There was a very low signal detected in wild-type plants from nonspecific amplification (judged from melting curves), but abundance of the specifically amplified *TPS10* and *TPS10M* transcripts was more than 100-fold the nonspecific abundance in the wild type (Welch's t tests followed by Holm-Bonferroni corrections for testing the wild type two times: 10-3 versus the wild type: t = 20.75, df = 3.129, P = 0.0004; 10-4 versus the wild type: t = 10.79, df = 4.644, P = 0.0002; M-1 versus the wild type: t = 5.596, df = 3.563, P = 0.0070; M-2 versus the wild type: t = 13.99, df = 5.866, $P < 0.0001$). Transgene transcript abundance was generally not affected by damage, herbivore, or jasmonate treatments (Welch's t tests, M-1 versus the wild type: constitutive [Con], t = 8.787, df = 2.018, P = 0.0124; W+W, t = 7.790, df = 3.458, P = 0.0026; W+OS, t = 1.126, df = 4.885, P = 0.3126; Lan, t = 1.765, df = 3.038, P = 0.1745; Lan + methyl jasmonate (MJ), t = 4.439, df = 3, P = 0.0213; treatment effect in M-1, Kruskal-Wallis test followed by a Holm-Bonferroni correction for testing both line and treatment: χ^2 = 3.414, df = 4, P = 0.4910).

Neither (*E*)-β-farnesene nor (*E*)-α-bergamotene were detected in the floral headspace of transgenics, which was dominated by benzyl acetone like in wild-type plants.

Emission of Nontarget Plant Volatiles Is Similar to the Wild Type in *TPS10* and *TPS10M* Lines

We determined the abundance of nontarget volatiles in the same experiment for which (*E*)-β-farnesene and (*E*)-α-bergamotene measurements are shown in Figure 1. To this end, we sampled the headspace of leaves for both 3 h immediately after treatment, when the rapidly emitted green leaf volatiles are most abundant (Allmann et al., 2010), and 24 to 32 h after treatment, when terpenoids are most abundant (Halitschke et al., 2000). All volatiles other than (*E*)-β-farnesene and (*E*)-α-bergamotene detected in the headspace were analyzed by principle component analyses (PCAs) for each time point, showing near-complete overlap of the profile of nontarget plant volatiles in the headspace of lines 10-3, 10-4, and M-1 with the wild type, regardless of treatment (Supplemental Figs. S2 and S3).

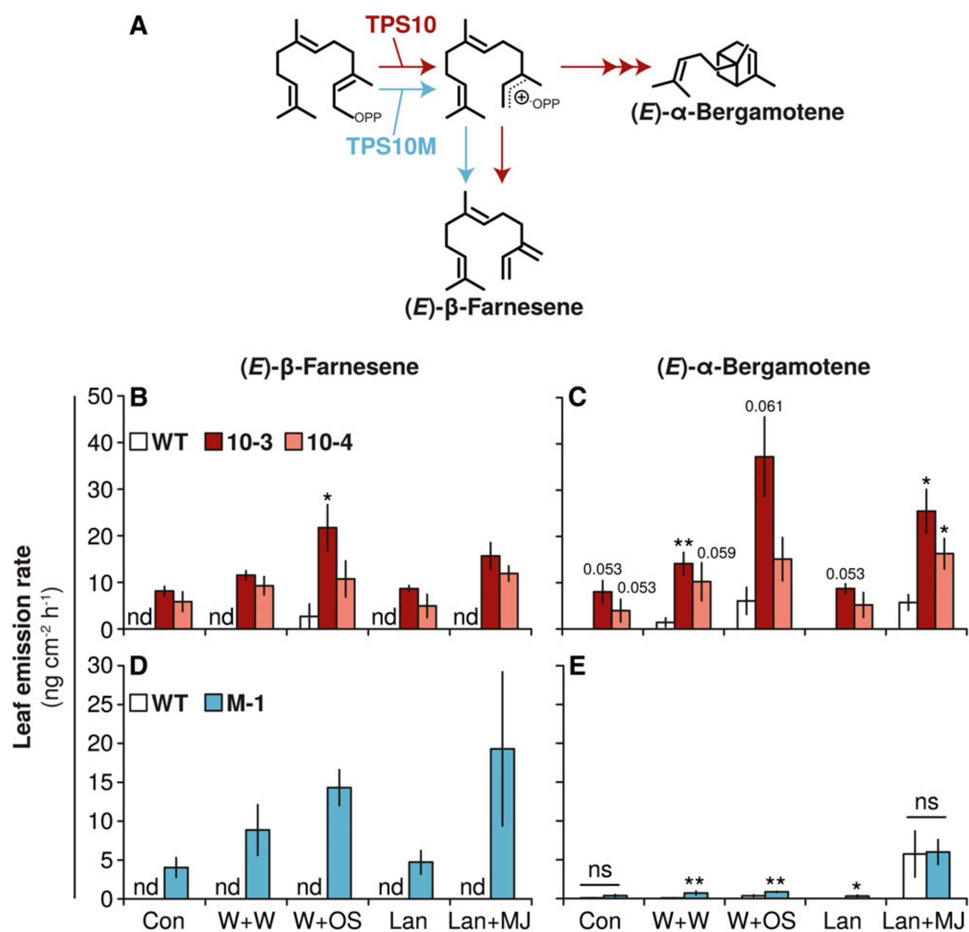

Figure 1. Ectopic expression of *TPS10* and the point mutant *TPS10M* results in greater headspace abundance of (*E*)-β-farnesene and/or (*E*)-α-bergamotene versus the wild type. A, Simplified mechanism of *TPS10* and *TPS10M* (modified from Köllner et al., 2009). *TPS10* (B and C) and *TPS10M* (D and E) lines consistently emit more (*E*)-β-farnesene (B and D) and/or (*E*)-α-bergamotene (C and E) than wild-type plants from leaves treated with several herbivory-related elicitors (mean $\pm$ SEM, n = 4). Note the different scales: data in B and C and data in D and E are from two separate experiments conducted within the same week; all plants were in the same growth stage (elongated). The headspace of the second fully expanded leaf (+2) on plants was collected without treatment (Con) or for 24 to 32 h after treatment with W+W (W+OS control), W+OS, Lan (Lan+MJ control), or Lan containing 150 μg of MJ (Lan+MJ). In the Con and Lan treatments, there was very low but detectable (*E*)-α-bergamotene emission from the wild type (<0.1 ng). Nontarget volatiles from these plants did not differ among transgenic lines and the wild type (PCAs in Supplemental Figs. S2 and S3). nd, Not detected; ns, not significant; WT, wild type. *, $P < 0.05$; **, $P < 0.01$ versus the wild type within each treatment after Holm-Bonferroni corrections for multiple testing if required (the wild type was tested against lines 10-3 and 10-4) after Wilcoxon rank sum tests or Welch's *t* tests (marginally significant corrected *P* values [<0.1] are written above bars).

Ectopic Expression of *TPS10* and *TPS10M* Does Not Alter the Jasmonate-Mediated Defense Response of Plants

There is evidence from *N. attenuata* and other plant species that sesquiterpenes elicited by herbivory, such as (*E*)-α-bergamotene, may be involved in the priming or elicitation of defense against herbivores (Baldwin et al., 2006; Paschold et al., 2006; Heil and Silva Bueno, 2007; Ton et al., 2007; Asai et al., 2009; Zebelo et al., 2012). We, therefore, hypothesized that lines producing elevated (*E*)-α-bergamotene and/or (*E*)-β-farnesene might respond to elicitation with elevated levels of defense. To determine whether *TPS10* and *TPS10M* plants showed an elevated response to treatment at the signaling level, we measured jasmonic acid (JA) and its active conjugate jasmonoyl Ile (JA-Ile), which largely regulate accumulation of defense metabolites after wounding or herbivory (Wang et al., 2008), as well as the antagonistically acting salicylic acid (SA) and the terpenoid ABA, which elicits responses to herbivory in synergy with JA (Gilardoni et al., 2011; Robert-Seilaniantz et al., 2011; Dinh et al., 2013; Vos et al., 2013). These phytohormones were measured both in untreated leaves and 1 h after treatment at the peak time of JA and JA-Ile (Gilardoni et al., 2011). Lines 10-3, 10-4, M-1, and M-2 all were similar to the wild type in their concentrations of JA, its active conjugate JA-Ile, SA, and ABA, regardless of treatment (Fig. 3; $P > 0.18$ in Wilcoxon

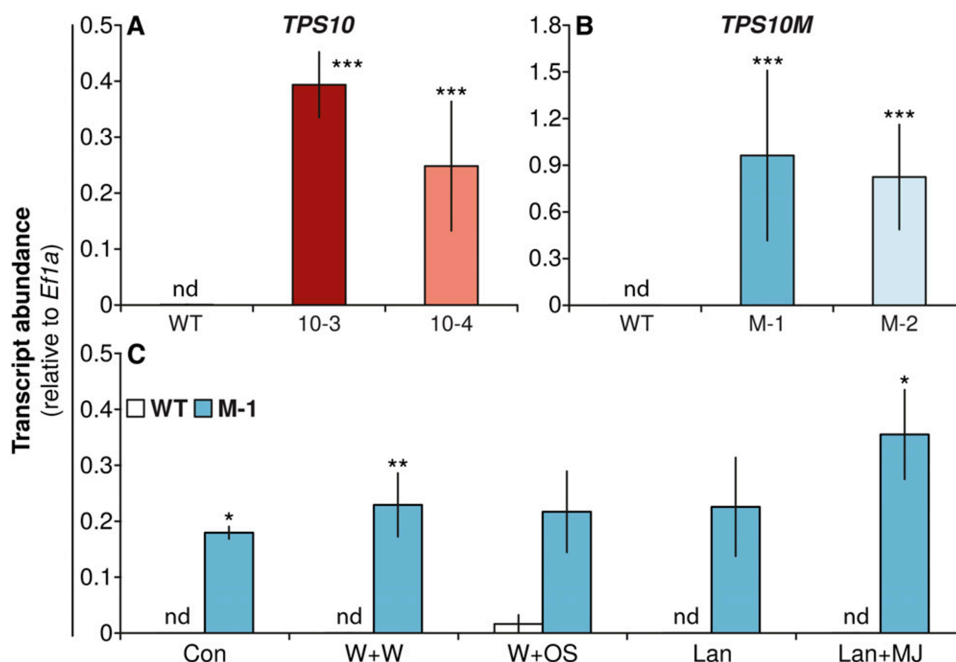

Figure 2. Transcript levels of *TPS10* and *TPS10M* are high in transgenic lines and stable across several treatments (mean ± SEM, $n = 4$). Transcripts were quantified in the first fully expanded leaf (+1) in two lines of *TPS10* (A), two lines of *TPS10M* (B), or line M-1 1 h after treatment with herbivory-related elicitors (C), which did not change *TPS10M* transcript abundance ($P = 0.491$ in a Kruskal-Wallis test across treatments). Similarly, no effect of treatment was observed on transcripts in the other lines in separate experiments (not shown). There was a very low signal caused by nonspecific amplification in wild-type (WT) negative controls (not detected [nd] < 0.001 units), which increased to a maximum of approximately 0.03 units after W+OS treatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ after Holm-Bonferroni correction of Welch's *t* tests versus the wild type (the wild type was tested two times in A and B, and M-1 was tested two times in C).

rank sum tests or Welch's *t* tests between wild-type and individual lines after Holm-Bonferroni corrections for testing the wild type two times).

We, furthermore, measured the levels of several defense metabolites: nicotine, several phenolic compounds, and hydroxygeranyllinalool diterpene glycosides (HGL-DTGs; Steppuhn et al., 2004; Heiling et al., 2010; Kaur et al., 2010), in leaves from the wild type and lines 10-3, 10-4, and M-1 at peak accumulation 72 h after elicitation or simultaneously collected leaves from untreated plants (Con; Fig. 4; Supplemental Tables S3 and S4). There were few significant differences among lines ($P > 0.08$ in Wilcoxon rank sum tests or Welch's *t* tests between wild-type and individual lines after Holm-Bonferroni corrections for testing the wild type two times against 10-3 and 10-4), except for in Con samples of M-1, which had slightly reduced rutin levels compared with the wild type (<20% reduction, $t = -4.100$, df = 2.268, $P = 0.0440$). In addition, there were only a few significant differences in individual HGL-DTGs (Welch's *t* tests followed by Bonferroni corrections for testing the wild type versus 10-3 and 10-4 and testing individual as well as total HGL-DTGs; 10-4 versus the wild type, W+W treatment: nicotianoside V, $t = -4.330$, df = 5.408, $P = 0.0190$; nicotianoside VII, $t = -5.514$, df = 4.557, $P = 0.0110$; Lan treatment: nicotianoside I, $t = 5.131$, df = 5.528, $P = 0.0080$; all other comparisons, $P > 0.1$).

TPS10-Expressing Plants Maintain Elevated (*E*)-*β*-Farnesene and (*E*)-*α*-Bergamotene Emissions throughout Growth and Development

To investigate the effects of (*E*)-*β*-farnesene and (*E*)-*α*-bergamotene on plants' ecological interactions and assay potential functions—which are defined by their contribution to plant fitness—it is essential to use plants that maintain emission through several developmental stages. We enclosed the entire aboveground portions of wild-type and 10-3 plants in a push-pull trapping setup and measured the headspace 24 to 32 h after no treatment (Con), W+OS treatment, or Lan+MJ treatment at four stages starting with establishment of the rosette stage and ending with maturation of seed capsules (Fig. 5). At every stage, the headspace around 10-3 plants contained (*E*)-*β*-farnesene, which was not detected in the wild-type headspace, as well as 2-fold to more than 10-fold the amount of (*E*)-*α*-bergamotene measured in the wild-type headspace (Welch's *t* tests or Wilcoxon rank sum tests between the wild type and 10-3 followed by Bonferroni corrections for also testing emission by treatment: rosette, Lan+MJ, $t = 4.558$, df = 5.974, $P = 0.0078$; elongated, Con, $t = 3.240$, df = 6.344, $P = 0.0327$; W+OS, $t = 4.220$, df = 6.999, $P = 0.0079$; Lan+MJ, $t = 3.329$, df = 6.967, $P = 0.0254$; flowering, Con, $W = 25$, $n = 5$, $P = 0.0159$; W+OS, $t = 4.380$, df = 7.617, $P = 0.0053$; Lan+MJ,

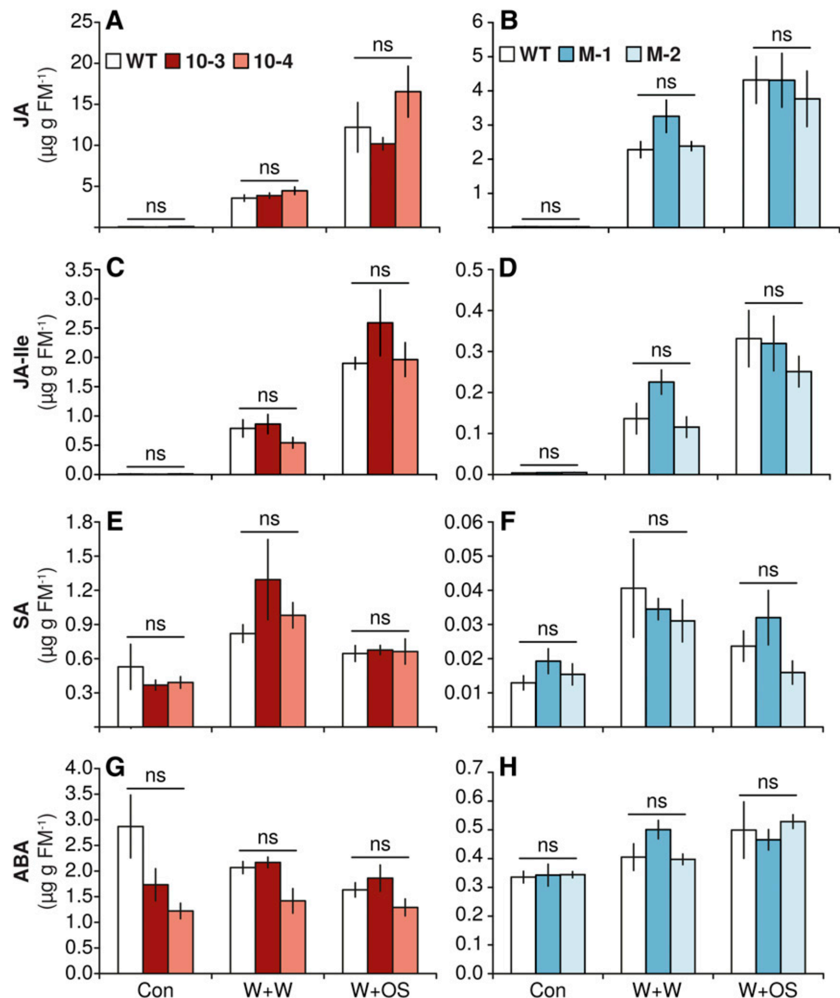

Figure 3. Expression of *TPS10* and *TPS10M* does not alter levels of defense-related phytohormones constitutively or after elicitation (mean ± SEM, $n = 4$). Data in A, C, E, and G and B, D, F, and H are from two separate experiments with elongated plants (legends are in A and B). JA (A and B), JA-Ile (C and D), SA (E and F), and ABA (G and H) were quantified in the first fully expanded leaf (+1) after no treatment (Con) or 1 h after treatment with W+W or W+OS, which is within the peak of elicited jasmonates in *N. attenuata*. A very small amount of JA and JA-Ile was detected in constitutive measurements (<0.1 μg for JA and <0.01 μg for JA-Ile). There were no significant differences (corrected $P > 0.05$) in Welch's *t* tests or Wilcoxon rank sum tests between lines and the wild type within each treatment; the Holm-Bonferroni method was used to correct for multiple testing (the wild type was tested two times within each treatment). FM, Fresh mass; ns, not significant; WT, wild type.

$t = 6.300$, df = 7.729, $P = 0.0005$; seed set, Con, $W = 25$, $n = 5$, $P = 0.0239$; W+OS, $t = 3.198$, df = 7.787, $P = 0.0262$; Lan+MJ, $t = 2.752$, df = 7.842, $P = 0.0510$). Treatment did not significantly alter the emission of (*E*)-β-farnesene or (*E*)-α-bergamotene in line 10-3 at any growth stage [Kruskal-Wallis tests followed by Bonferroni corrections for testing (*E*)-α-bergamotene emission two times; $P > 0.2$]. After normalization to grams of fresh mass, the headspace of flowering plants contained the most (*E*)-α-bergamotene: about 2 times as much as rosette stage and elongated plants and about 10 times as much as plants setting seed. (*E*)-β-farnesene abundance did not vary strongly with plant growth stage (Fig. 5).

TPS10 Plants Do Not Alter the Defense Responses of Their Neighbors

Although *TPS10* and *TPS10M* plants did not alter their own defense responses to herbivory-related elicitation, we hypothesized that they might modify the defense responses of neighboring plants, because priming and defense elicitation effects have frequently been shown in plants exposed to externally supplied volatiles either as pure compounds or from the headspace of an elicited plant or plant part. Thus, we tested whether wild-type plants growing in pots together with either of two independently transformed *TPS10* lines differed in their levels of defense metabolites and herbivore-induced volatiles. To assay both primed and directly elevated defenses, we sampled *TPS10* plants and their neighbors both before elicitation and after multiple applications of W+OS to the second fully expanded leaf (position + 2) for both plants in the pot. The same procedure was conducted with plants of the wild type and both *TPS10* lines grown individually in pots of the same size as a control for competition effects.

As expected, before elicitation, (*E*)-β-farnesene and (*E*)-α-bergamotene could be quantified in the headspace around *TPS10* plants but inconsistently and in very low amounts in wild-type plants (<10% of the amounts detected in the *TPS10* headspace), even for wild-type plants with *TPS10* neighbors (thus indicating little or no headspace contamination by neighbors). However, multiple rounds of elicitation resulted in greatly elevated emission of (*E*)-α-bergamotene in all plants, with the wild type becoming similar to both *TPS10* lines; in contrast, (*E*)-β-farnesene emission was less affected by

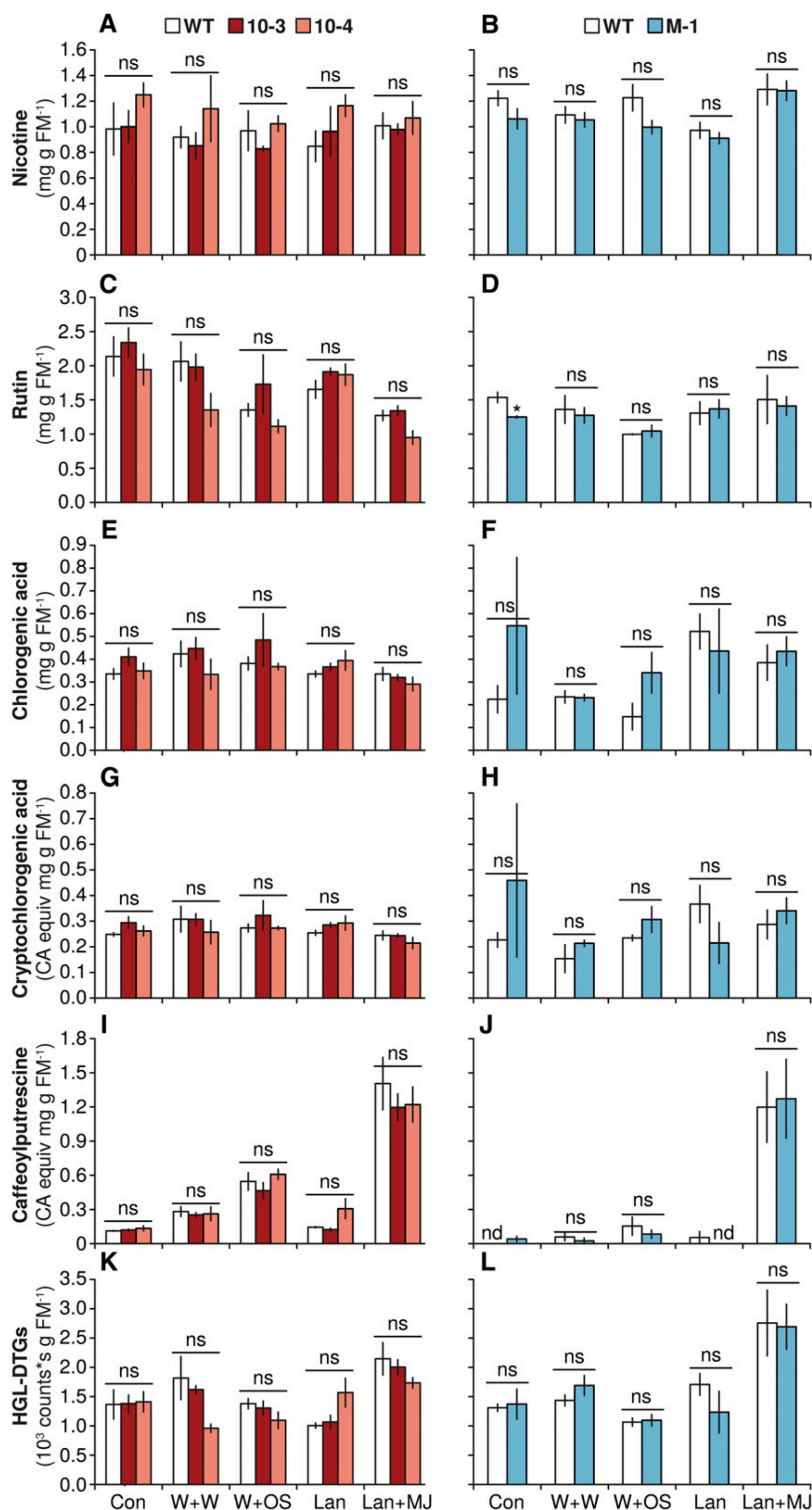

Figure 4. Expression of *TPS10* and *TPS10M* does not alter levels of defense metabolites constitutively or after elicitation (mean ± SEM, n = 4). Data in A, C, E, G, I, and K and B, D, F, H, J, and L are from two separate experiments with elongated plants (legends are in A and B). Samples were taken from the 0 leaf (source to sink transition) 3 d after treatment to permit elicited metabolite synthesis and accumulation. Nicotine (A and B), rutin (C and D), CA (E and F), chryptochlorogenic acid (G and H), caffeoylputrescine (I and J), and total HGL-DTGs (K and L) were quantified after no treatment (Con) or treatment with W+W, W+OS, Lan, or Lan+MJ. Cryptochlorogenic acid and caffeoylputrescine were quantified as CA equivalents (CA equiv). Peak areas of individual HGL-DTGs are shown in Supplemental Tables S1 and S2. There were no other significant differences ($P > 0.05$) after Holm-Bonferroni correction of Welch's t tests or Wilcoxon rank sum tests between lines and the wild type within each treatment (the wild type was tested two times versus lines 10-3 and 10-4; individual and total HGL-DTGs tested). FM, Fresh mass; nd, not detected; ns, not significant; WT, wild type. *, $P < 0.05$ in a Welch's t test between line M-1 and the wild type in the Con treatment (rutin; D).

treatment and remained undetected in the wild-type headspace (Fig. 6). Emissions of both engineered volatiles were more robust in line 10-3 than 10-4. There was no significant effect of competition or neighbor identity on the abundance of either (E)-β-farnesene or (E)-α-bergamotene ($P > 0.3$ in Wilcoxon rank sum tests or Kruskal-Wallis tests after Bonferroni corrections for between two and five tests of each data set). There was, furthermore, no difference among lines in the abundance of other volatiles either before or after elicitation,

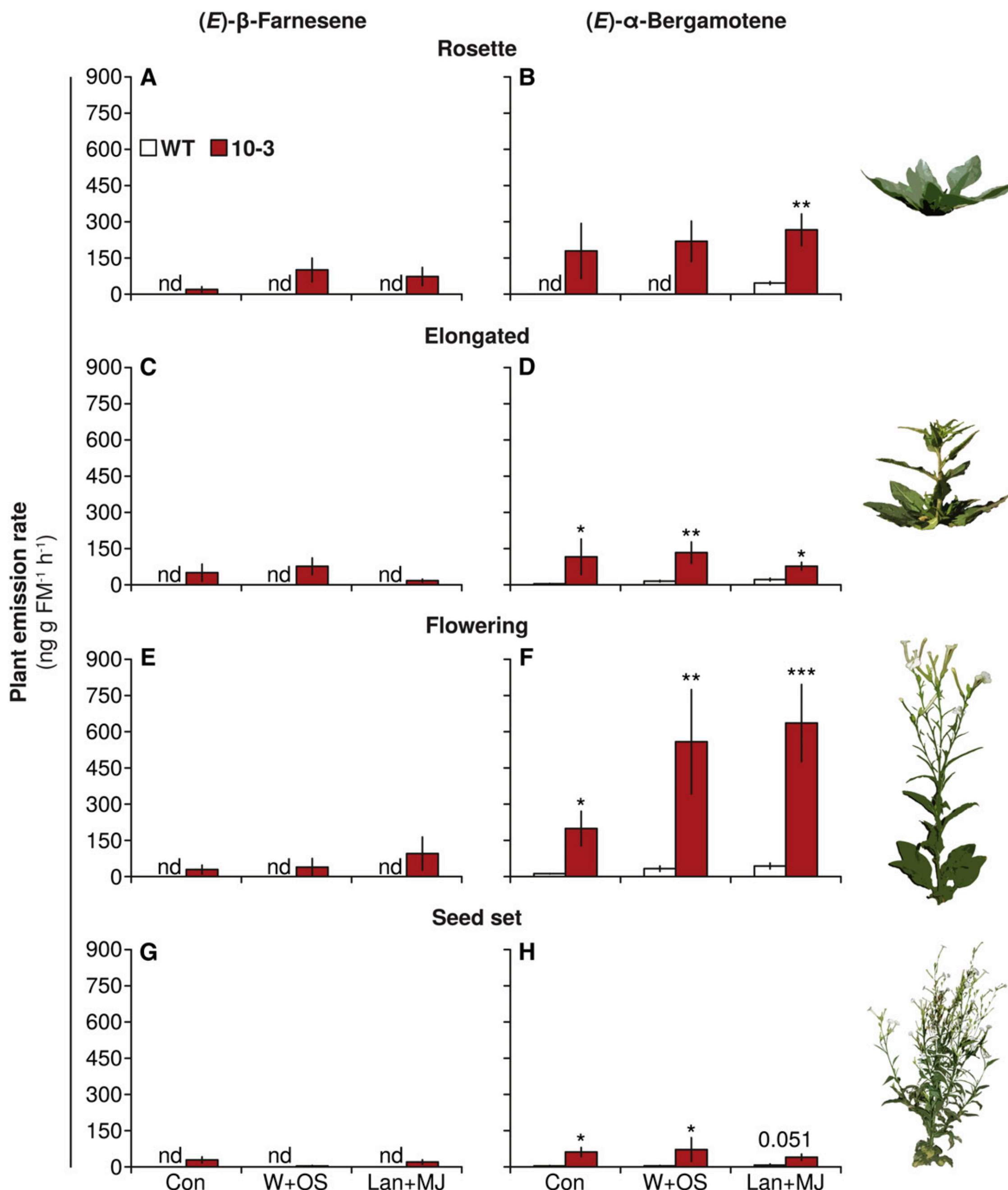

Figure 5. *TPS10* expression results in elevated (*E*)-β-farnesene and (*E*)-α-bergamotene emission throughout plant growth (mean $\pm$ SEM, $n = 5$). The legend in A applies to all panels. Volatiles were collected from whole shoots of rosette-stage (A and B), elongated (C and D), early flowering (E and F), or late flowering plants (G and H) either left untreated (Con) or treated with W+OS or Lan+MJ. In these measurements, W+OS and Lan+MJ elicited larger amounts of (*E*)-α-bergamotene emission from the wild type but not from 10-3. $P = 0.051$ for the wild type vs. 10-3 after Lan+MJ treatment in plants setting seed. Plant growth stage icons are modified from photos by Danny Kessler. FM, Fresh mass; nd, not detected; WT, wild type. *, $P < 0.05$; **, $P < 0.01$ after Bonferroni corrections after Welch's *t* tests or Wilcoxon rank sum tests between lines within a treatment (tests were additionally conducted among treatments).

regardless of whether plants were grown alone or in competition; likewise, there was no effect of neighbor identity on the emission of nontarget volatiles (PCAs; Supplemental Fig. S4).

We also measured trypsin protease inhibitor (TPI) activity, caffeoylputrescine, two abundant HGL-DTGs, and nicotine in the same plants before and 48 h after the first of the multiple W+OS elicitations. Most of

these metabolites reach peak levels 72 to 96 h after elicitation (Steppuhn et al., 2004; Wu et al., 2006; Heiling et al., 2010), although priming effects were detected in *N. attenuata* plants within 3 d of exposure to sagebrush volatiles and 48 h after the onset of *M. sexta* feeding (Kessler et al., 2006). Thus, we chose the time point 48 h after the first of multiple W+OS elicitations to most sensitively detect the results of priming effects on increasing metabolite levels (Fig. 7). Treatment strongly elevated the levels of all defensive metabolites measured except for nicotine, which is suppressed by the addition of *M. sexta* OS to wounds (von Dahl et al., 2007). However, lines did not differ, except that competing *TPS10* plants had slightly lower levels of caffeoylputrescine after elicitation (ANOVA minimal model, significant effect of line: $F_{2,47}$ = 4.947, corrected P = 0.0224, Tukey's honestly significant difference [HSD] mean-separation test: the wild type versus 10-4: corrected P = 0.0163; P values were adjusted using the Bonferroni correction for additionally testing plants grown individually versus in competition for each line, two to three tests total).

Although constitutive levels of the HGL-DTG nicotianoside VI were low overall, there was significant variance in competing 10-3 plants before treatment (ANOVA minimal model: neighbor, $F_{2,40}$ = 4.697, corrected P = 0.0294; line, $F_{2,40}$ = 0.570, corrected P = 1.000; neighbor to line interaction, $F_{4,40}$ = 3.260, corrected P = 0.0418; Tukey's HSD mean-separation test: the wild type versus 10-3: corrected P = 0.0374; Tukey's HSD mean-separation test: 10-3 with the wild type versus 10-3 with 10-4: corrected P = 0.0351). However, there were no other significant effects of neighbor identity: during model reduction, neighbor and neighbor to line interactions were the most insignificant factors.

Individuals differed significantly from competing pairs in some measures of caffeoylputrescine (the wild type before treatment, W = 13, n = 5–6, corrected P = 0.0386; 10-4 after treatment, t = 4.643, df = 10.07, corrected P = 0.0018), nicotianoside VI (the wild type before treatment, W = 12.5, n = 5–6, corrected P = 0.0392; 10-4 before treatment, W = 11, n = 5, corrected P = 0.0297; 10-3 after treatment, t = −4.213, df = 10.86, corrected P = 0.0030), and nicotine accumulation (10-4 before treatment, t = −2.689, df = 14.99, corrected P = 0.0337). There were no other significant effects of line, neighbor, or growth in competition on metabolite levels either before or after multiple W+OS treatments.

Ectopic Expression of *TPS10* Does Not Reduce Growth or Reproduction, and *TPS10* Plants Do Not Affect the Growth and Reproduction of Neighbors

Costs of producing defense compounds are best quantified in plants forced to compete for common resources (van Dam and Baldwin, 2001; Schwachtje et al., 2008). We, therefore, measured vegetative growth and counted the production of flowers and seed capsules for the wild type and both lines of *TPS10* grown separately and in competition. These measurements were conducted with the same plants used for the chemical measurements described above (4 replicates were used for headspace analysis and 6 replicates were used for analysis of defense compounds from a total of 10 replicates for which growth and reproduction were monitored). Rosette diameter was measured from the time that rosettes were established until they stopped expanding at 40 d postgermination (dpg), and stem length was measured from the time that plants began to elongate at 25 dpg until the end of the experiment (Fig. 8). Multiple W+OS treatments for defense metabolite measurements were conducted from 36 to 37 dpg; thus, growth measurements represent relative plant sizes both before and after elicitation.

Throughout their growth, *TPS10* plants were similar in size to the wild type, with the exception that competing plants of line 10-4 had slightly smaller rosettes on day 34; this difference disappeared within 1 week, at which point rosette growth had nearly ceased (Fig. 8). There was a strong effect of competition on both rosette diameter and stalk length, with competing plants being smaller than individuals, but there was no effect of line (except for the transient difference observed in line 10-4) or neighbor identity on either growth measure (Supplemental Table S5).

Reproduction was monitored by counting buds, flowers, and ripe and unripe seed capsules 72 to 73 d after germination when all plants were ripening seed. *TPS10* plants were similar to the wild type in their production of buds, flowers, and ripe and unripe seed capsules, and neighbors of *TPS10* plants were similar to neighbors of wild-type plants (Fig. 9). Overall, plants in competition produced fewer buds, flowers, and unripe and ripe seed capsules than individuals (statistical analysis in Supplemental Table S6), and there were a few small differences among lines in seed capsule numbers when plants competed, but the only significant difference from the wild type was that line 10-4 produced approximately 25% fewer ripe seed capsules by the end of the experiment. There were no other significant effects of line or neighbor (Supplemental Table S6).

DISCUSSION

We specifically enhanced both constitutive and inducible emissions of two sesquiterpene volatiles, (E)-β-farnesene and (E)-α-bergamotene, by ectopically expressing *ZmTPS10* and the point mutant *TPS10M* (Köllner et al., 2009) under the control of the CaMV 35S promoter in *N. attenuata* (Figs. 1 and 2; Supplemental Fig. S1; Supplemental Tables S1 and S2). We profiled the herbivory-inducible and jasmonate-inducible defense responses of the transformed plants at the level of phytohormone signaling and metabolite accumulation and showed that transformants were similar to wild-type plants constitutively and in response to several herbivory-related elicitors (Figs. 3 and 4; Supplemental Figs. S2 and S3; Supplemental Tables S2 and S3). Our results are inconsistent with either volatile serving as a within-plant

stress signal. To determine whether effects of ectopic emission could be assayed throughout plant growth, we measured the emission of (*E*)-β-farnesene and (*E*)-α-bergamotene from rosette stage through seed set and saw that *TPS10* plants consistently had higher levels of emission (Fig. 5).

We then forced *TPS10* plants to compete with other plants for resources and measured growth, reproduction, and defense responses in transgenic plants and their neighbors. There was no effect of transformants' (*E*)-β-farnesene and (*E*)-α-bergamotene emissions on the defense chemistry of their neighbors, even after multiple elicitation events; the sole exception was a small change in constitutive levels of a single HGL-DTG for neighbors of only one of two independently transformed lines (Figs. 6 and 7; Supplemental Fig. S4). This is inconsistent with a role for either sesquiterpene in eliciting or priming defense in neighboring *N. attenuata* plants. Furthermore, by eliciting plants multiple times with W+OS from the specialist herbivore *M. sexta*, we were able to produce (*E*)-α-bergamotene but not (*E*)-β-farnesene emission from wild-type plants that was similar in magnitude to the emission from *TPS10* plants (Fig. 6). Finally, we observed little to no cost of ectopic *TPS10* expression in terms of growth and reproduction of transformants versus their wild-type competitors (Figs. 8 and 9; Supplemental Tables S5 and S6). We discuss the significance of these results below.

Constitutive Expression of *TPS10* or *TPS10M* Enhances Emission of (*E*)-β-Farnesene and/or (*E*)-α-Bergamotene, Even after Elicitation

Transcription of the transformed TPSs was constitutive (Fig. 2), which was expected given that the transgenes were under control of a 35S promoter. However, emissions of (*E*)-α-bergamotene and (*E*)-β-farnesene were altered by induction treatment (Fig. 1; Supplemental Fig. S1) and growth stage (Fig. 5). TPS10 plants roughly doubled emissions of both target volatiles when treated with MJ (Fig. 1, B and C); in *TPS10M*, MJ treatment tripled (*E*)-β-farnesene emission (Fig. 1D). Likewise, (*E*)-α-bergamotene varied over a 4-fold range between elongated and flowering growth stages (Fig. 5). The independence of emission rate from transcript level suggests that production of volatiles is also regulated by substrate supply or posttranscriptional processes.

The lack of close transcriptional control over emission contrasts with studies of terpene emission from other plant species, including poplar (*Populus canescens*), hop (*Humulus lupulus*), citrus (*Citrus unshiu*), strawberry (*Fragaria* spp.), and *Clarkia breweri*, in which emission rate and *TPS* transcript level were tightly correlated (Nagegowda, 2010). However, studies of *Picea abies* and Arabidopsis (*Arabidopsis thaliana*) showed that levels of substrate-supplying enzymes, including 1-DEOXY-D-XYLULOSE 5-PHOSPHATE SYNTHASE, 1-DEOXY-D-XYLULOSE 5-PHOSPHATE REDUCTOISOMERASE, GERANYL DIPHOSPHATE SYNTHASE, and GERANYL GERANYL DIPHOSPHATE SYNTHASE, can also be up-regulated during times of increased isoprenoid emission (Nagegowda, 2010). Hence, in wild-type plants, production of TPSs and their substrates may be coordinated across ontogeny and environmental conditions by well-regulated genetic networks (Vranová et al., 2012). Constitutive expression of an ectopic TPS as in this study may disturb this network because of the occurrence of high TPS levels accompanied by low substrate supply. Meeting this increased substrate demand could negatively affect primary metabolic processes, such as sterol biosynthesis, that depend on supply of the sesquiterpene precursor FDP (Vranová et al., 2012). However, the normal growth, development, and emission of nontarget volatiles from our transgenic lines indicate that they were able to compensate for this increased demand, perhaps by up-regulating FDP production. The increased sesquiterpene production from both wild-type and transgenic plants after herbivore-related elicitation may be at least partially attributable to increased FDP supply (Fig. 1; Supplemental Fig. S1).

Production of (*E*)-β-Farnesene and (*E*)-α-Bergamotene Does Not Induce or Prime Defense Responses in Transgenic Plants or Their Neighbors

Previous studies have implicated plant volatiles in within-plant and between-plant signaling. In a variety of species, exposure to the volatiles of herbivore-damaged tissues results in induction of defense against herbivores, increased transcription of defense-related genes, and priming: increased responsiveness to subsequent herbivore challenge (Heil and Karban, 2010). Terpenes specifically have been shown to alter transcriptional profiles in Arabidopsis (Godard et al., 2008). Likewise, volatile-mediated induction of extrafloral nectar production by *Phaseolus lunatus* is compromised by destruction of several terpenoid components under high-ozone conditions (Blande et al., 2010). About 60% of genes regulated in neighboring *N. attenuata* plants after 48 h of exposure to the headspace of W+OS-elicited neighbors may be regulated by induced terpenes, including (*E*)-α-bergamotene (Paschold et al., 2006), although no direct or priming effects were observed in neighbors as a result of these changes in gene expression.

To test whether (*E*)-β-farnesene and (*E*)-α-bergamotene induced defenses in emitters themselves or their neighbors, we measured constitutive levels of secondary metabolites in transgenic plants and neighboring wild-type plants. If exposure to these sesquiterpenes induced defenses directly, we would have expected increased levels of secondary metabolites and phytohormones in both the transgenics, which were exposed to their own volatile emissions, and neighboring wild-type plants growing close to the emitters. In contrast, our experiments showed that, in uninduced plants, emitters did not differ from the wild type in levels of phytohormones (Fig. 3), defense metabolites (Fig. 4), or nontarget volatiles (Supplemental Figs. S2 and S3). When transgenic plants were grown in competition with the wild type, neither

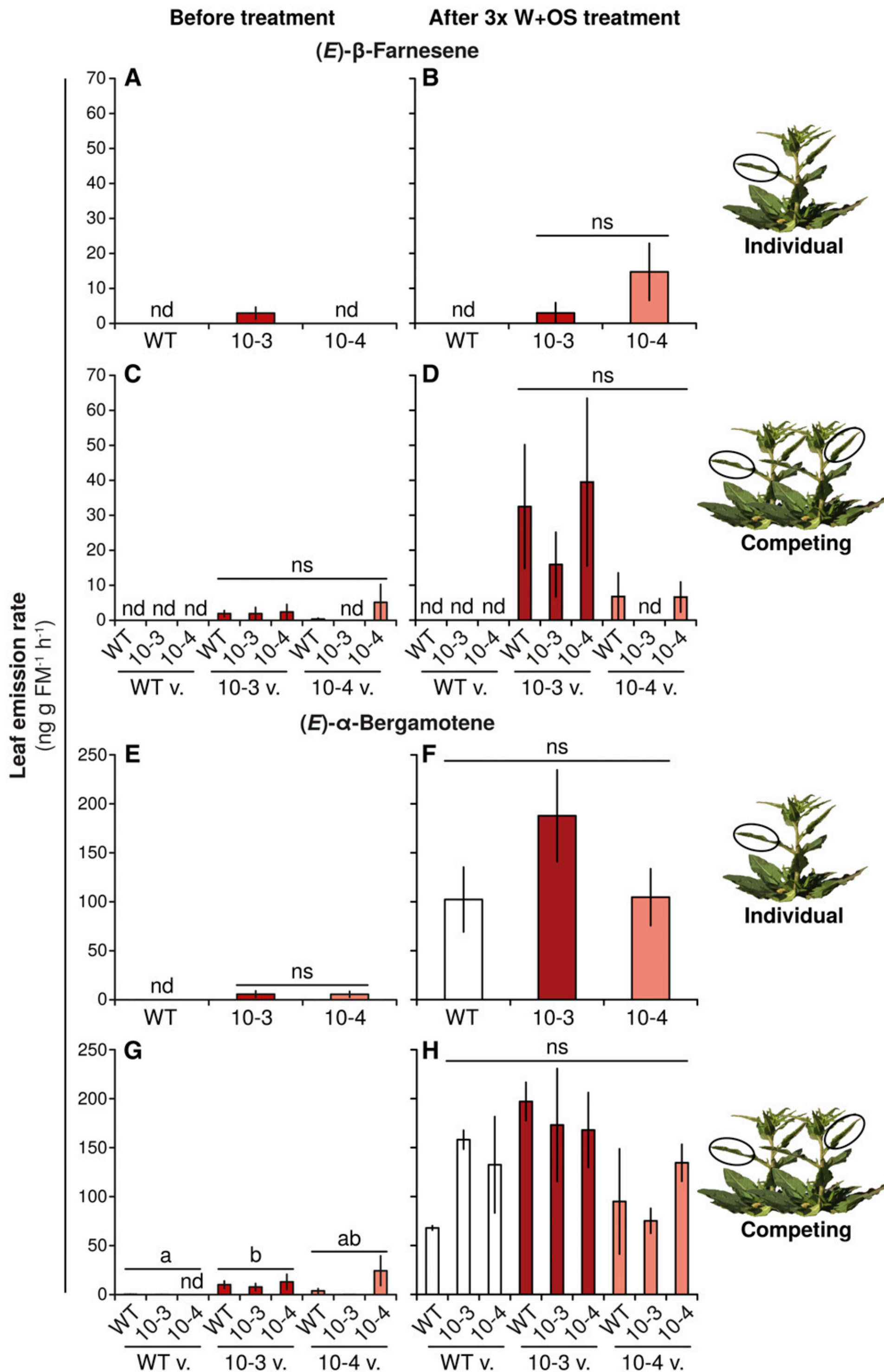

Figure 6. Emission of (E)-α-bergamotene but not (E)-β-farnesene is as great from wild-type plants as from *TPS10* lines after multiple elicitations, but neither volatile is affected by growth of plants in close competition with the wild type or *TPS10* (mean ± SEM, n = 4). The headspace around +2 leaves on plants, grown either alone or in competing pairs, was sampled for 6 h before treatment (A, C, E, and G) or 0 to 6 h after the last of three elicitations with W+OS over 18 h across 2 d (B, D, F, and H). Multiple elicitations were conducted so that differences in emission caused by either direct induction or priming of the induced response could be amplified and detected. (E)-β-farnesene (A–D) and (E)-α-bergamotene (E–H) emission rates are shown here, whereas PCA analyses of nontarget volatiles measured in the same samples are shown in Supplemental Figure S4. Different letters indicate significant differences ($P < 0.05$) in Wilcoxon rank sum tests or Welch's t tests between lines after significant Kruskal-Wallis tests and after Bonferroni corrections for multiple testing ($P > 0.05$). FM, Fresh mass; nd, not detected; ns, not significant; v., versus; WT, wild type.

emitters nor their neighbors differed in levels of defense metabolites (Fig. 7) or nontarget volatiles (Supplemental Fig. S4) when compared with the wild type grown with other wild types.

If exposure to (E)-β-farnesene and (E)-α-bergamotene had primed defenses rather than inducing them directly, we would have expected greater responsiveness to elicitation in volatile-producing or volatile-exposed plants (i.e. in transgenics themselves and wild-type plants competing with transgenic neighbors). On the contrary, responses to induction were similar between the wild type and transgenics in terms of phytohormones (Fig. 3) and

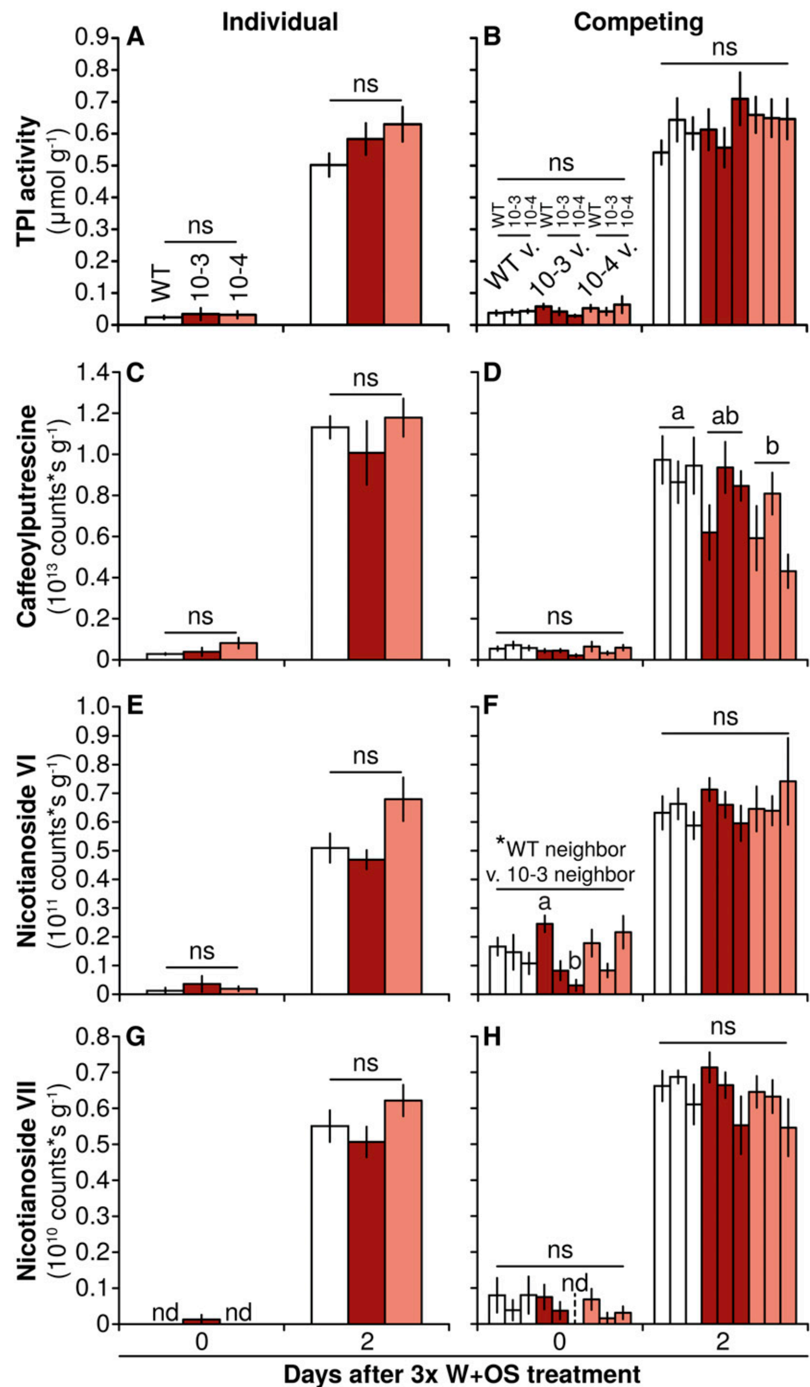

Figure 7. *TPS10* plants and the wild type accumulate similar levels of defense metabolites after multiple elicitations when grown alone or in competition with wild-type or *TPS10* neighbors (mean ± SEM, n = 6). Labels in A apply to A, C, E, and G, whereas labels in B apply to B, D, F, and H. Leaves were harvested from the same plants before (+1) and 48 h after the first of three elicitations with W+OS over 18 h across 2 d (+2); differences in metabolite accumulation caused by either direct induction or priming of the induced response could thus be amplified and detected. Levels of several defense metabolites elicited by herbivory, including TPIs (A and B), caffeoylputrescine (C and D), and two malonylated HGL-DTGs nicotianoside VI (E and F) and VII (G and H), showed few or no differences among lines or in response to different neighbors. The Holm-Bonferroni method was used to correct for two to three tests of each data set (effect of line within individuals or competing pairs, effect of neighbor within competing pairs, effect of competition, and post hoc pairwise tests); each ANOVA and its accompanying post hoc tests were counted as single tests for P value correction. Different letters indicate significant differences ($P < 0.05$) after Holm-Bonferroni correction of Tukey's HSD mean-separation tests of genotype for caffeoylputrescine at 2 d or neighbor identity (wild-type neighbor or 10-3 neighbor) and the interaction between line and neighbor identity (10-3 versus the wild type and 10-3 versus 10-4) for nicotianoside VI at 0 d. nd, Not detected; ns, not significant, v., versus; WT, wild type.

defense metabolites (Fig. 4) as well as nontarget volatiles (Supplemental Figs. S2 and S3). Likewise, when the wild type was grown with transgenic neighbors, responses to induction were similar in magnitude to the wild type grown with other wild types, which was measured by volatile emission (Fig. 6) and defense metabolite levels (Fig. 7). Although *N. attenuata* has been shown to respond to both conspecific (Paschold et al., 2006) and heterospecific plant volatiles (Kessler et al., 2006), (E)-β-farnesene and (E)-α-bergamotene do not seem to alter defense responses in this species.

There Is Little or No Physiological Cost of the Enhanced Emission of (*E*)-*β*-Farnesene and (*E*)-*α*-Bergamotene

Our experiments did not show any consistent physiological costs of either transformation or emission of (E)-β-farnesene and (E)-α-bergamotene. Production of secondary metabolites is believed to be costly, which may explain why many compounds are synthesized under specific environmental conditions rather than constitutively (Karban and Baldwin, 1997). Physiological costs result from diversion of resources away from growth and

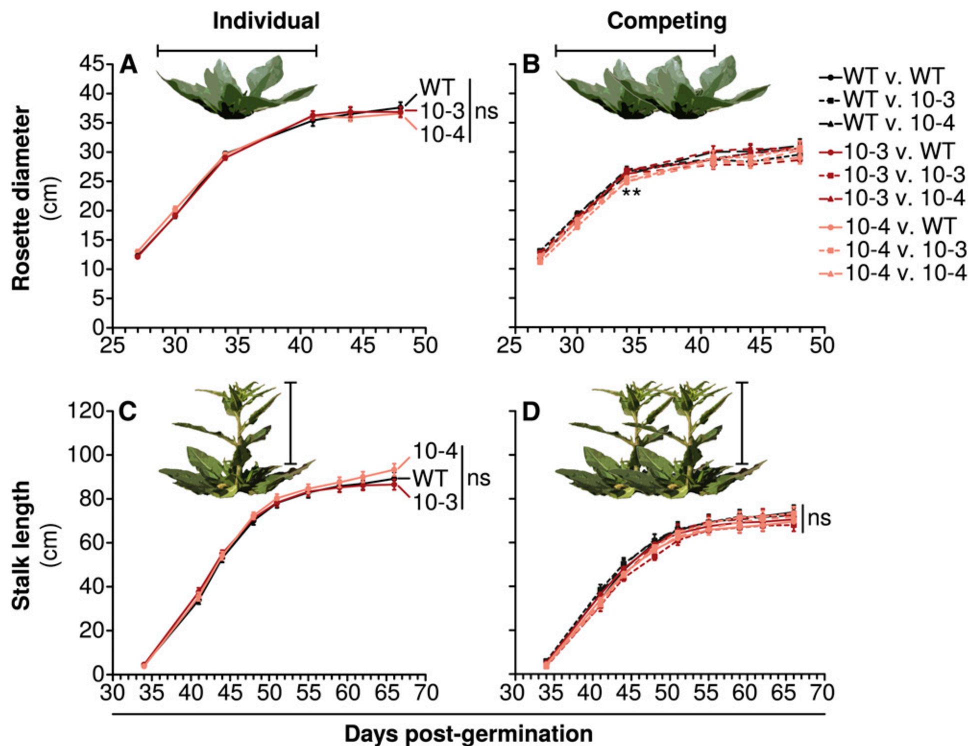

Figure 8. *TPS10* plants grow similarly to wild-type plants both alone and in competition and do not affect their neighbors' growth differently than wild-type competitors (mean ± SEM, $n = 10$). Rosette diameter (A and B) and stalk length (C and D) of plants grown individually (A and C) were greater than for plants grown in competition (B and D) for all lines (statistical analyses in Supplemental Table S5) but differed little between either of two independent lines of *TPS10* and the wild type. Within competing pairs, neither rosette diameter nor stalk length were affected by neighbor identity (Supplemental Table S5). Line 10-4 had smaller rosettes than the wild type or *TPS10* line 10-3 on day 34 postgermination when grown in competition. The Holm-Bonferroni method was used to correct for two to four tests of each data set (effect of line within individuals or competing pairs, effect of neighbor within competing pairs, effect of competition, and post hoc pairwise tests). ns, Not significant, v., versus; WT, wild type. **, Corrected P value < 0.01 in a Wilcoxon rank sum test after a significant result (corrected $P = 0.0024$) in a Kruskal-Wallis test among lines.

reproduction. If production of sesquiterpenes imposed a high physiological cost, we would have expected impaired vegetative growth or reproductive output from transformed plants. For example, transformation of maize (*Zea mays*) with a constitutively expressed sesquiterpene synthase resulted in reduced seed germination, growth, and yield under field conditions (Robert et al., 2013).

However, our transgenic plants grew and developed normally: when plants were grown alone, neither rosette diameter nor stalk length differed (Fig. 8), and reproductive output was also similar to that of wild-type plants (Fig. 9, A and C). Because physiological costs may be more readily apparent under competition (van Dam and Baldwin, 1998), we also assessed growth and reproduction with wild-type and transgenic plants grown together in the same pot. Even under direct competition, transgenic plants had no consistent disadvantages in rosette growth, stalk elongation, or flower or seed production compared with the wild type (Fig. 9, B and D).

The seemingly negligible physiological costs of (E)-β-farnesene and (E)-α-bergamotene emission combined with their shown benefits raise the question of why these volatiles are not produced constitutively in nature. One possible explanation may be that physiological costs only become apparent under adverse environmental conditions. Our characterization of the transgenic plants was conducted on amply watered and fertilized plants grown in a climate-controlled glasshouse environment, and under these conditions, growth was likely limited by rooting volume and not by other factors. Results might differ under field conditions that present stresses, such as water scarcity, nutrient limitation, and variable temperature, as well as leaf area removal from herbivores or possible attack by pathogens.

A second possibility is that sesquiterpene production carries ecological costs that can only be observed in the plant's natural habitat (Karban and Baldwin, 1997). One ecological cost of emission could be the attraction of specialists. For example, maize plants that constitutively emitted (E)-β-caryophyllene and (E)-α-humulene attracted more larvae of the specialist herbivore *Diabrotica virgifera* (Robert et al., 2013). A second possible ecological cost results from crying wolf—plants that emit large amounts of herbivore-induced volatiles independently of prey

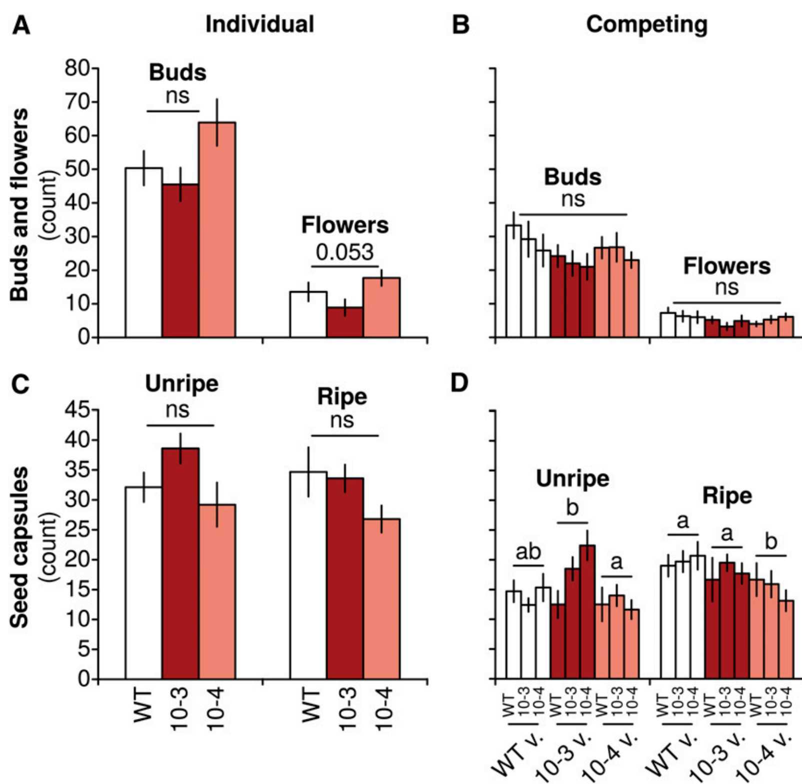

Figure 9. *TPS10* plants produce similar numbers of buds, flowers, and seed capsules as the wild type when grown alone or in competition and do not affect their neighbors' reproduction differently than wild-type competitors (mean ± SEM, $n = 10$). Plants of all lines produced more buds and flowers (A and B) and ripe and unripe seed capsules (C and D) when grown individually (A and C) versus in competition (B and D), but reproductive measures differed little between either of two independent lines of *TPS10* and the wild type; within competing pairs, none of these parameters were affected by neighbor identity (statistical analyses in Supplemental Table S6). Different letters indicate significant differences (corrected $P < 0.05$) in Tukey's HSD mean-separation tests between lines conducted after a significant effect of line (corrected $P < 0.05$) in an ANOVA minimal model. The Holm-Bonferroni method was used to correct for two to four tests of each data set (effect of line within individuals or competing pairs, effect of neighbor within competing pairs, effect of competition, and post hoc pairwise tests). ns, Not significant, v., versus; WT, wild type.

abundance may attract naïve natural enemies of herbivores, but experienced natural enemies may learn that those volatiles are unreliable indicators of prey presence (Shiojiri et al., 2010). Thus, it would be interesting to learn the extent to which the inducibility of sesquiterpene emission is frequency dependent in wild populations, and the transgenic plants described here could be used to set up otherwise isogenic experimental populations to test hypotheses about frequency-dependent costs and benefits of constitutively enhanced sesquiterpene emission.

A third ecological cost could include repellence of beneficial insects, such as pollinators. Herbivore-induced changes in volatile emission have been shown to decrease pollinator services to *Solanum peruvianum* (Kessler et al., 2011), and pollinating moths of the herbivore *M. sexta* have been shown to avoid oviposition on—and perhaps, also pollination of—*N. attenuata* plants supplemented with (*E*)-α-bergamotene (Kessler and Baldwin, 2001). Suggestive evidence that pollinators are specifically repelled by sesquiterpenes comes from a comparison of Arabidopsis with its sister species, *Arabidopsis lyrata*. In the self-pollinating Arabidopsis, an (*E*)-β-caryophyllene synthase is expressed constitutively in floral tissue, whereas in the outcrossing *A. lyrata*, the homologous sesquiterpene synthase is expressed only after herbivory (Abel et al., 2009). This phylogenetic comparison suggests that (*E*)-β-caryophyllene and possibly, other herbivore-induced sesquiterpenes could decrease floral attractiveness to pollinators as well as herbivores. Finally, a fourth possible ecological cost is the deterrence of beneficial microbes. Sesquiterpenes have shown antimicrobial properties both in vitro and in planta that can protect plants from pathogens (Huang et al., 2010). However, these antimicrobial properties might be undesirable if they reduce colonization by mutualist or commensalist flora. In *Tropaeolum majus* and *Carica papaya*, for example, jasmonate treatment impaired colonization of roots with mycorrhizae (Ludwig-Müller et al., 2002), and monoterpenes and a sesquiterpene from *Pinus sylvestris* inhibited mycorrhizal growth in vitro (Melin and Krupa, 1971). Because the transgenic lines described here seem to bear few or no physiological costs, they may be useful tools with which to test hypotheses about ecological costs of sesquiterpene production under field conditions.

CONCLUSION

In conclusion, we propose that, in *N. attenuata*, (*E*)-α-bergamotene and (*E*)-β-farnesene have ecological rather than physiological functions in plants' responses to herbivory and furthermore, that these ecological effects are mediated by the response of arthropods, such as herbivores and their predators, to these volatiles. The transgenic lines described here are excellent candidates for experiments to test the ecological consequences of constitutively enhanced emission of both volatiles, especially because the ectopic expression seems to have little or no direct effect on plant defense, growth, or reproduction, permitting the straightforward interpretation of ecological data.

MATERIALS AND METHODS

Plants and Growth Conditions

Nicotiana attenuata (Solanaceae) wild-type plants were from a 31st generation inbred line described by Krügel et al. (2002). The full-length maize (*Zea mays*) *TPS10* B73 allele (Schnee et al., 2006; GenBank accession no. AY928078) or *TPS10M* (TPS10-B73 L356F in Köllner et al., 2009) was transferred out of plasmids provided by Eran Pichersky and Tobius Köllner into the pSOL9 plasmid after first confirming correct gene sequences by Sanger sequencing of BigDye reactions (www.lifetechnologies.com) using vector primers and primers designed midsequence to obtain full sequence coverage. Vector construction and the pSOL9 plasmid have been described previously (Gase et al., 2011). The *Agrobacterium tumefaciens* (strain LBA 404)-mediated transformation procedure described by Krügel et al. (2002) was used to generate multiple stably transformed *N. attenuata* lines expressing *TPS10* and *TPS10M* under control of the CaMV 35S promoter in the pSOL9 plasmid.

Homozygosity of T2 plants was determined by screening for resistance to hygromycin (Gase et al., 2011) provided by the *HYGROMYCIN PHOSPHOTRANSFERASE II* (*HPTII*) gene from pCAMBIA-1301 in the pSOL9 vector. High-quality genomic DNA was extracted using a modified cetyl-trimethyl-ammonium bromide method (Bubner et al., 2004) from nonsenescent leaf tissue collected from transformed plants, flash frozen, and ground as described below. Southern blotting was performed to identify lines with single transgene insertions using a probe for *HPTII* as previously described (Gase et al., 2011) after digestion of genomic DNA with *Xba*I or *Eco*RI (Supplemental Fig. S1). A PCR was conducted with the genomic DNA template to confirm full transfer DNA insertions in these lines as previously described (Gase et al., 2011). Flow cytometric analysis (described in Bubner et al., 2006) confirmed that all lines were diploid. Two independently transformed diploid, homozygous lines of each construct with single transgene insertions and the predicted pattern of (*E*)-β-farnesene and (*E*)-α-bergamotene emission (Supplemental Fig. S1) were chosen for additional characterization.

Germination and seedling growth conditions were as described by Krügel et al. (2002). Petri dishes with 20 to 30 germinated seeds each were kept in a growth chamber (Percival Scientific CU-36L; www.percival-scientific.com) at 26°C for 16 h of 105.4 μmol s^{-1} m^{-2} of light (measured using an LI-COR LI-250A Light Meter und Sensor LI-COR Quantum Q 35195; www.licor.com) 24°C for 8 h of dark for 10 d and then transferred to TEKU JP 3050 104 pots (www.poeppelmann.com) with plug soil (www.klasmann-deilmann.com). From that time on, plants were cultivated in the glasshouse with a 16-h/8-h day-night cycle under daylight supplemented with 1,000 to 1,300 μmol m^{-2} s^{-1} of photosynthetically active radiation supplied by sodium vapor high-intensity discharge bulbs (Master Sun-T PIA Agro 400 W or Master Sun-T PIA Plus 600 W sodium lights; www.philips.com). Air temperatures in the glasshouse were 23°C to 35°C during the day and 19°C to 23°C at night; relative humidity was 40% to 55%. Twenty days after germination, individual small rosette plants were transplanted to 1-L plastic pots, or for the competition experiment, two size-matched plants were paired in a 2-L pot as described by Schwachtje et al. (2008). Pots contained Fruhsdorfer Nullerde (www.hawita-gruppe.de) with 0.5 g L^{-1} of PG mix (www.yara.co.uk), 0.9 g L^{-1} of Superphosphat (www.triferto.org), 0.35 g L^{-1} of $MgSO_4 \times 7H_2O$ (www.sigmaaldrich.com), and 0.055 g L^{-1} of Micro Max (www.everris.com). Water and fertilizer were supplied daily using an automatic flood irrigation system: at 21 dpg, 0.0075 g L^{-1} of Bor-Folicin (www.jost-group.com) was added to the 400-L watering tank; and at 27 dpg, 0.05 g L^{-1} of Peters Allrounder 20:20:20 (www.scottsprofessional.com) and 0.005 g L^{-1} of Folicin-Bor were added to the tank. At 34 dpg, 0.1 g L^{-1} of Peters Allrounder 20:20:20 and 0.0025 g L^{-1} of Folicin-Bor were added; from 41 dpg, conductivity was maintained by weekly addition of 0.0625 to 0.075 g L^{-1} of Peters Allrounder 20:20:20.

Treatment with Herbivory and Jasmonate Elicitors

The leaf undergoing the source to sink transition (position 0) and the first two fully expanded leaves (positions +1 and +2) were used to assay defense metabolites, transcript and phytohormone abundance, and volatile emission, which are known to respond strongly to local elicitation in leaves at these positions (Halitschke et al., 2000; van Dam et al., 2001; Wang et al., 2008). Single elicitations with W+OS were performed to reproducibly mimic herbivore elicitation by making three rows of holes on either side of the leaf midvein along the adaxial surface using a pattern wheel, applying 20 μL of *Manduca sexta* oral secretions (from 4th–5th instar larvae fed on wild-type *N. attenuata* plants) diluted 1:5 with distilled water, and rubbing diluted oral secretions into holes with a gloved finger as described (Halitschke et al., 2001; Schittko et al., 2001; Schuman et al., 2012). The same treatment using distilled water instead of W+OS served as a wounding control (W+W). Multiple W+OS treatment of plants grown in competition was used as a standardized method to provide multiple herbivore elicitation events: leaves were wounded by creating two rows of holes from the adaxial surface of the +2 leaf (one on each side of the leaf midvein) and adding 5 μL of 1:5 diluted *M. sexta* oral secretions to each row; this was done three times over 18 h across 2 d from the edge of the leaf toward the midvein. MJ, which is demethylated by plants to generate JA (Wu et al., 2008), was applied to treated leaves by dissolving 150 μg of racemic MJ in 20 μL of Lan (www.sigmaaldrich.com) and gently applying the Lan paste to the base of the adaxial leaf surface by the petiole (Lan+MJ); application of Lan paste in the same manner served as the Lan control (Preston and Baldwin, 1999; Kessler and Baldwin, 2001; Heiling et al., 2010). Plant treatments were always begun in the morning, except for multiple W+OS treatments which began in the early afternoon.

Headspace Analyses

For Con measurements during the prescreening of transformed lines (Supplemental Fig. S1), headspaces were collected for 8 h during the day from the +2 leaf on rosette-stage plants. The next morning, the neighboring +1 leaf was treated with W+OS, and the headspace was collected from 24 to 32 h to measure induced sesquiterpenes (Halitschke et al., 2000). For the volatile collections in Figure 1 and Supplemental Figures S2 and S3, +2 leaves of plants were treated as described above, and the headspaces were immediately collected for 3 h and then again for 24 to 32 h after treatment. For whole-plant volatile collections (Fig. 5), treatments were applied to the +2 leaf in the rosette and elongating stages or on the first true stem leaf (S1) in the early and late flowering stages. For plants in the competition experiment (Fig. 6), the headspace was collected from +2 leaves for 6 h during the day, after which leaves were treated three times with W+OS as described above, and then, the headspace was immediately collected again for 6 h during the next day.

Headspace collection of single leaves was conducted by enclosing leaves on plants in two 50-mL polyethylene tetraphthalate cups (www2.huhtamaki.com) lined on the edges with foam to protect leaves and an activated charcoal filter attached to one side for incoming air and secured with miniature claw-style hair clips. Headspace volatiles were collected on 20 mg of Porapak Q (Tholl et al., 2006; www.sigmaaldrich.com) in self-packed filters (bodies and materials from www.ars-fla.com) by drawing ambient air through these clip cages at 300 mL min^{-1} using a manifold with screw-close valves set to provide equal outflow by pushing air at 2 to 3 bar through a Venturi aspirator as described previously by Oh et al. (2012) and based on procedures by Halitschke et al. (2000) and Schuman et al. (2009). Background volatiles present in ambient air were collected using empty trapping containers, and background signals were later subtracted if necessary from raw intensities of plant samples before additional processing. To sample from whole shoots (Fig. 5), additional pressurized air was filtered through activated charcoal and pushed into custom-cut clean PET bags of equal volumes for each growth stage (rosette, push 500 mL min^{-1}; all other stages, push 1 L min^{-1}), and 400 mL min^{-1} were pulled through the Porapak Q filter. The ratio of air in to air out was factored into the final quantification of these headspace samples: signals from rosette-stage plants were multiplied by 5/4 and signals from other stages were multiplied by 5/2 to account for loss caused by overpressure.

Porapak Q filters were either immediately spiked with 320 ng of tetralin as an internal standard and eluted with 250 μL of dichloromethane into a 1.5-mL gas chromatography (GC) vial containing a 250-μL glass insert or stored at −20°C until tetralin addition and elution. Samples were analyzed on one of two different GC-mass spectrometry (MS) instruments from Varian with columns from Phenomenex (30 m × 0.25 mm i.d., 0.25 μm film thickness; www.phenomenex.com). One microliter of each sample was injected in splitless mode, and then the injectors were returned to a 1:70 split ratio from 2 min after injection through the end of each run. Helium carrier gas was used with a column flow of 1 mL min^{-1}. Samples collected from 0 to 3 h after treatment were analyzed by a CP-3800 GC Saturn 2000 Ion Trap MS with a polar ZB wax column and a CP-8200 autoinjector; the GC and MS were programmed as previously described for this instrument (Schuman et al., 2012), and compounds were separated by a temperature ramp of 5°C min^{-1} between 40°C and 185°C. All other headspace samples were analyzed on a CP-3800 GC coupled to a Saturn 4000 ion-trap mass spectrometer with a nonpolar ZB5 column and a CP-8400 autoinjector. The GC and MS were programmed as previously described for this instrument (Oh et al., 2012), and compounds were separated by a temperature ramp of 5°C min^{-1} between 40°C and 180°C.

Individual volatile compound peaks were quantified using the combined peak area of two specific and abundant ion traces per compound using MS Work Station Data Analysis software (Varian) and normalized by the 104 + 132 ion trace peak area from tetralin in each sample. The identification of compounds was conducted by comparing GC retention times and mass spectra with those of standards and mass spectra databases: Wiley version 6 (www.wiley.com) and National Institute of Standards and Technology (www.nist.gov) spectral libraries. Values in nanograms for (*E*)-α-bergamotene and (*E*)-β-farnesene in Figure 1 were determined using standard curves with a coinjected tetralin standard curve to determine response factors versus tetralin. The standard curve for (*E*)-α-bergamotene used another bicyclic sesquiterpene olefin, (*E*)-β-caryophyllene, because no pure standard of (*E*)-α-bergamotene was available. These response factors were used to calculate nanogram values for (*E*)-β-farnesene and (*E*)-α-bergamotene in all other data sets analyzed on the same instrument (Figs. 5 and 6, Supplemental Fig. S1).

The area of trapped leaves was quantified for comparison by scanning and calculating areas in pixels using SigmaScan (www.systat.com) and then converting pixels to centimeters^2 using a size standard that was scanned with leaves. Volatiles were expressed in either nanograms of tetralin per centimeter^2 of leaf area or absolute nanograms per centimeter^2 of leaf area.

Collection and Processing of Plant Tissue Samples

Treated whole leaves were cut at the petiole, wrapped securely in aluminum foil, and flash frozen in liquid nitrogen within less than 1 min after removal from the plant. Frozen tissue samples were stored at −80°C until additional processing. Leaf tissue was ground over liquid N_2 by crushing and transfer to 2-mL microcentrifuge tubes followed by grinding with small plastic pestles or a Genogrinder 2000 (www.spexsampleprep.com) to a fine homogeneous powder, which was weighed out over liquid N_2 for extraction and analysis of hormones, transcripts, or metabolites or stored at −80°C.

Determination of Transcript Abundance

Transcript abundance was determined in +1 leaves harvested 1 h after treatment as described above. RNA was extracted from approximately 100 mg of ground leaf tissue using TRI reagent (www.sigmaaldrich.com) according to the manufacturer's instructions. RNA quality was checked on a 1% (w/v) agarose gel (agarose from www.sigmaaldrich.com), and concentration was measured at 260 nm using a NanoDrop ND-1000 spectrophotometer (www.nanodrop.com). Synthesis of complementary DNA (cDNA) from 0.5 μg of total RNA per sample and SYBR Green quantitative PCR (qPCR) analyses were conducted as in Wu et al., 2007 using reagents from www.thermoscientificbio.com/fermentas, a Mastercycler (www.eppendorf.com), an Mx3005P qPCR system (www.stratagene.com), and a qPCR Core Kit for SYBR Green I (www.eurogentec.com). We designed a primer pair to amplify a 158-bp product from *TPS10* and *TPS10M*: NaTPS10_FWD1 (TTGTTGGGATGGGTGACA) and NaTPS10_RVS1 (TTGGACCGTGGACACA), which was previously used by Fragoso et al. (2011). Transcript abundance was normalized to the abundance of *N. attenuata ELONGATION FACTOR 1a* (*NaEF1a*) transcripts in each sample (Kaur et al., 2012) using primers Nt_EF1a_FWD2 (CCACACTTCCCACATTGCTGTCA) and Nt_EF1a_RVS2 (CGCATGTCCCTCACAGCAAAAC). Samples of RNA used to make cDNA were pooled to the same dilution as in cDNA samples and run alongside cDNA in all qPCRs to control for genomic DNA contamination; no contamination was detected.

Quantification of Phytohormones

Phytohormone accumulation was determined in +1 leaves harvested 1 h after treatment as described above. Phytohormones were extracted from approximately 100 mg of ground leaf tissue using ethyl acetate spiked with internal standards and quantified by liquid chromatography (LC)-electrospray ionization (ESI)-triple quadrupole tandem MS (Varian) as described by Oh et al. (2012), with the modification that 100 ng rather than 200 ng of [$^2H_2$]JA and 20 ng rather than 40 ng of [$^{13}C_6$]JA-Ile, [$^2H_4$]SA, and [$^2H_6$]ABA were used as internal standards. Individual hormones were quantified in nanograms by comparison to the corresponding internal standard peak area and normalized per gram of leaf tissue fresh mass.

Quantification of Defense Metabolites

Frozen ground tissue (100 mg) from 0 leaves collected 3 d after treatment (Fig. 4; Supplemental Tables S3 and S4) as described above was extracted and analyzed as described by Oh et al. (2012) for a combined analysis of nicotine and phenolic metabolites by HPLC-UV diode array detector (Keinänen et al., 2001) and individual diterpene glycosides by LC-ESI-MS/MS (Heiling et al., 2010). Nicotine, chlorogenic acid (CA), and rutin were quantified in micrograms using external standard curves, and the CA standard curve was used to quantify cryptochlorogenic acid and caffeoylputrescine as CA equivalents; for HGL-DTGs, peak areas were normalized to the peak area of the internal standard glycyrrhizinic acid. Values (micrograms; CA equivalents or normalized peak areas) were normalized to grams of leaf tissue fresh mass.

Frozen ground tissue (100 mg) from +1 leaves harvested before or +2 leaves collected from the same plants 48 h after multiple W+OS treatments (Fig. 7) was used to extract and profile metabolites by LC-ESI-microtime of flight-MS in positive mode as described by Gaquerel et al. (2010) with specific modifications described by Kim et al. (2011) and a 2-μL injection volume. This permitted quantification of nicotine, phenolics, and individual HGL-DTGs in the same run. Metabolites having a robust signal-to-noise ratio (>10) were selected, and peaks were integrated using Data Analysis v 4.0 and Quant Analysis (www.bruker.com). Peak areas were normalized to grams of leaf tissue fresh mass. TPI activity levels were determined relative to milligrams of protein in 100 mg of frozen ground tissue from the same leaf samples (Fig. 7) using the extraction, Bradford assay, and radial diffusion assay described by van Dam et al. (2001) based on the protocol by Jongsma et al. (1993) and materials from Sigma-Aldrich (www.sigmaaldrich.com).

Measurements of Plant Growth and Reproduction

Plant size (maximum rosette diameter and stalk length) was monitored for plants grown in competition and singly grown controls: rosette diameter was measured as the maximum diameter found by gently laying a ruler over the rosette, and stalk length was measured from the base of the stem to the tip of the apical inflorescence by placing a ruler beside the stem. Reproductive output was quantified by counting the total number of buds (>2 mm long), flowers (corolla visible and turgid), unripe seed capsules (green and visible past calyx), and ripe seed capsules (browning and dry).

Statistical Analyses

Summary statistics were calculated in Excel (www.microsoft.com). Additional statistical tests were performed in R version 3.0.2 using RStudio version 0.98.501 (R Core Team, 2012). We checked treatment groups graphically (quantile-quantile plots and residual versus fitted plots) and statistically (Shapiro-Wilk test and Bartlett test) for normality and homoscedasticity. Multiple groups were compared by ANOVA when requirements of normality and homoscedasticity were met for raw or natural log-transformed data using the stepwise model simplification approach in R to determine the minimal adequate model in which either all factors contributed significantly or otherwise, only the least insignificant factor remained (Crawley, 2013) followed by Tukey's HSD mean-separation post hoc tests. Binary comparisons were made using Welch's *t* tests when at least the requirement of normality was met. Nonparametric data were analyzed using Kruskal-Wallis tests when comparing more than two groups or Wilcoxon rank sum tests for binary comparisons.

Bonferroni *P* value corrections, using the Holm modification when applicable, were calculated in Excel (www.microsoft.com) for families of tests on the same data. Both corrections control the familywise error rate and are simple to calculate, but the Holm-Bonferroni method is more powerful (Holm, 1979): briefly, all *P* values in a family of tests conducted on the same data sets and subsets are listed from smallest to largest. The smallest *P* value is multiplied by the total number of tests (n) conducted on that data. If the resulting corrected *P* value is <0.05, then the next smallest *P* value is multiplied by $n - 1$. If the resulting corrected *P* value is <0.05, then the third smallest *P* value is multiplied by $n - 2$, and so on. At the first correction resulting in a P value ≥ 0.05, that *P* value and all larger *P* values are considered nonsignificant. The corrected *P* values reported are the products of Bonferroni or Holm-Bonferroni corrections as noted: e.g. (*P* value) × (n tests) or (second smallest *P* value) × ($n - 1$).

Sequence data from this article can be found in the GenBank data library under accession number AY928078.

Supplemental Data

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

Supplemental Figure S1. Selection of *TPS10* and *TPS10M* lines with a single transgene copy and enhanced emission of the target volatiles (*E*)-β-farnesene and (*E*)-α-bergamotene.

Supplemental Figure S2. Green leaf volatiles are similar to the wild type in *TPS10* and *TPS10M* lines.

Supplemental Figure S3. Plant volatiles other than (*E*)-β-farnesene and (*E*)-α-bergamotene are similar to the wild type in *TPS10* and *TPS10M*.

Supplemental Figure S4. Plant volatiles other than (*E*)-β-farnesene and (*E*)-α-bergamotene are similar to the wild type in *TPS10* and are not affected by competition.

Supplemental Table S1. Statistical analysis of (*E*)-β-farnesene and (*E*)-α-bergamotene emission for two lines of *TPS10* and wild-type plants (to accompany Fig. 1).

Supplemental Table S2. Statistical analysis of (*E*)-β-farnesene and (*E*)-α-bergamotene emission for one line of *TPS10M* and wild-type plants (to accompany Fig. 1).

Supplemental Table S3. Hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) in two lines of *TPS10* and the wild type.

Supplemental Table S4. Hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) in one line of *TPS10M* and the wild type.

Supplemental Table S5. Statistical analysis of growth for two lines of *TPS10* and wild-type plants grown alone or in competition (to accompany Fig. 8).

Supplemental Table S6. Statistical analysis of reproduction for two lines of *TPS10* and wild-type plants grown alone or in competition (to accompany Fig. 9).

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