Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (Mimulus)

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Abstract: Pollinator-mediated reproductive isolation is a major factor in driving the diversification of flowering plants. Studies of floral traits involved in reproductive isolation have focused nearly exclusively on visual signals, such as flower color. The role of less obvious signals, such as floral scent, has been studied only recently. In particular, the genetics of floral volatiles involved in mediating differential pollinator visitation remains unknown. The bumblebee-pollinated Mimulus lewisii and hummingbird-pollinated M. cardinalis are a model system for studying reproductive isolation via pollinator preference. We have shown that these two species differ in three floral terpenoid volatiles - D-limonene, -myrcene, and E- -ocimene - that are attractive to bumblebee pollinators. By genetic mapping and in vitro enzyme activity analysis we demonstrate that these interspecific differences are consistent with allelic variation at two loci – LIMONENE-MYRCENE SYNTHASE (LMS) and OCIMENE SYNTHASE (OS). M. lewisii LMS (MlLMS) and OS (MlOS) are expressed most strongly in floral tissue in the last stages of floral development. M. cardinalis LMS (McLMS) is weakly expressed and has a nonsense mutation in exon 3. M. cardinalis OS (McOS) is expressed similarly to MlOS, but the encoded McOS enzyme produces no E- -ocimene. Recapitulating the M. cardinalis phenotype by reducing the expression of MlLMS by RNAi in transgenic M. lewisii produces no behavioral difference in pollinating bumblebees; however, reducing MlOS expression produces a 6% decrease in visitation. Allelic variation at the OCIMENE SYNTHASE locus likely contributes to differential pollinator visitation, and thus promotes reproductive isolation between M. lewisii and M. cardinalis. OCIMENE SYNTHASE joins a growing list of “speciation genes” (“barrier genes”) in flowering plants.

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Floral volatile alleles can contribute to pollinator-mediated reproductive isolation in monkeyflowers (*Mimulus*)

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KM659024, *M. cardinalis LIMONENE-MYRCENE SYNTHASE* mRNA, exon3
KF857262, *M. lewisii OCIMENE SYNTHASE* mRNA, complete cds
AHI50306, *M. lewisii* OCIMENE SYNTHASE protein product
KF857263, *M. cardinalis OCIMENE SYNTHASE* mRNA, complete cds
AHI50307, *M. cardinalis* OCIMENE SYNTHASE protein product

Summary

Pollinator-mediated reproductive isolation is a major factor in driving the diversification of flowering plants. Studies of floral traits involved in reproductive isolation have focused nearly exclusively on visual signals, such as flower color. The role of less obvious signals, such as floral scent, has been studied only recently. In particular, the genetics of floral volatiles involved in mediating differential pollinator visitation remains unknown. The bumblebee-pollinated *Mimulus lewisii* and hummingbird-pollinated *M. cardinalis* are a model system for studying reproductive isolation *via* pollinator preference. We have shown that these two species differ in three floral terpenoid volatiles - D-limonene, β-myrcene, and *E*-β-ocimene - that are attractive to bumblebee pollinators. By genetic mapping and *in vitro* enzyme activity analysis we demonstrate that these interspecific differences are consistent with allelic variation at two loci – *LIMONENE-MYRCENE SYNTHASE* (*LMS*) and *OCIMENE SYNTHASE* (*OS*). *M. lewisii LMS* (*MlLMS*) and *OS* (*MlOS*) are expressed most strongly in floral tissue in the last stages of floral development. *M. cardinalis LMS* (*McLMS*) is weakly expressed and has a nonsense mutation in exon 3. *M. cardinalis OS* (*McOS*) is expressed similarly to *MlOS*, but the encoded McOS enzyme produces no *E*-β-ocimene. Recapitulating the *M. cardinalis* phenotype by reducing the expression of *MlLMS* by RNAi in transgenic *M. lewisii* produces no behavioral difference in pollinating bumblebees; however, reducing *MlOS* expression produces a 6% decrease in visitation. Allelic variation at the *OCIMENE SYNTHASE* locus likely contributes to differential pollinator visitation, and thus promotes reproductive isolation between *M. lewisii* and *M. cardinalis*. *OCIMENE SYNTHASE* joins a growing list of "speciation genes" ("barrier genes") in flowering plants.

Introduction

The rapid diversification of the world's estimated 275,000 species of flowering plants has often been attributed to their specialized association with different animal pollinators (Grant, 1949; Stebbins, 1970). Flowering plants use a variety of signals to advertise the presence (or illusion) of a reward to their associated pollinators; the association between pollinator type and suites of signals gives rise

to the concept of pollination syndromes (Fenster *et al.,* 2004). Perhaps the most well-known and easily studied signal is floral color, which has been investigated in a variety of pollination syndromes (Rausher, 2008). Other visual signals, such as texture, pattern, orientation, anthesis time, size, and shape have been investigated to some extent in a variety of systems (Harder and Johnson, 2009; Kay and Sargent, 2009; Yuan *et al.*, 2013a).

Floral scent – the amount, relative ratios, and identities of volatile compounds emitted by the flower – is a generally understudied signal, despite the long understanding that it may play a strong role in attracting pollinators (Raguso, 2008a). The recent development of techniques for studying floral scent, including chemical analysis of floral scent, analysis of pollinator neural activity at both the receptor and higher-order processing levels, and genetic and genomic tools, has allowed some progress in this area. However, although floral scent is frequently characterized, and genes responsible for the production of floral volatiles are occasionally identified, a synthesis of floral scent biochemistry, neurobiology, genetics, ecology, and evolution has been lacking. Those systems with well-characterized volatiles that affect pollination are separate from those with well-characterized genetics (Raguso, 2008a; Raguso, 2008b; Whitehead and Peakall, 2009; Parachnowitsch *et al.*, 2012).

Much of the work discussing speciation involving floral volatiles has been done in extremely specialized systems where scent is crucial to plant-pollinator interactions (Raguso, 2008b), most notably the sexually deceptive orchids in the genera *Chiloglottis* (Schiestl and Peakall, 2005; Peakall *et al.*, 2010) and *Ophrys* (Schiestl and Ayasse, 2002; Mant *et al.*, 2005; Vereecken *et al.*, 2010; Xu *et al.*, 2012), as well as the non-deceptive genus *Gymnadenia* (Huber *et al.*, 2005). Recent work has begun expanding this to non-orchid systems such as *Silene* (Waelti *et al.*, 2008), *Linanthus* (Chess *et al.*, 2008), and *Petunia* (Klahre *et al.*, 2011). While there are a growing number of studies demonstrating the importance of floral volatiles in mediating these largely specialized plant-

pollinator interactions, the genetic pathways controlling volatile production in these systems remain unknown.

In contrast, the genes underlying volatile production are known in a diverse range of angiosperm systems (Gang, 2005), including *Clarkia* (Pichersky *et al.*, 1995; Dudareva *et al.*, 1996; Dudareva *et al.*, 1998; Wang and Pichersky, 1998), *Antirrhinum* (Dudareva *et al.*, 2000; Dudareva *et al.*, 2003), *Petunia* (Koeduka *et al.*, 2006; Orlova *et al.*, 2006; Dexter *et al.*, 2007), *Silene* (Gupta *et al.*, 2012), *Arabidopsis* (Bohlmann *et al.*, 2000; Chen *et al.*, 2003), and many species of agricultural importance. Although our knowledge of the genetic underpinnings of volatile production and emission has grown as a result of these systems, there is a paucity of research linking floral volatiles and plant speciation with the genetic and molecular basis for those effects.

Petunia is the only well-developed model demonstrating the role that a specific volatile plays in differential attraction of pollinators between sister species (Klahre *et al.*, 2011). The sister species *P. axillaris* and *P. exserta* differ in their production of methyl benzoate, a volatile attractive to the hawkmoth pollinators of *P. axillaris*. Through QTL mapping, two regions underlying this difference were identified in the *Petunia* genome on chromosomes II and VII, with the *P. axillaris* allele at the locus on chromosome II being absolutely required for methyl benzoate production and the locus on chromosome VII substantially contributing to methyl benzoate quantity. *ODO1*, one of the genes hypothesized to underlie the locus on chromosome VII, encodes a MYB transcription factor that is differentially expressed in the two species. The hawkmoth *Manduca sexta* is attracted more strongly to near isogenic lines with high levels of methyl benzoate production, suggesting that a change in volatile production mediated by a single gene can lead to differential pollinator attraction; however, the underlying genetic mechanisms mediating species-specific volatile emission in this system have not been completely described.

The sister species *M. lewisii* and *M. cardinalis* have served as a model system for studying pollinator-mediated reproductive isolation for several decades (Hiesey *et al.*, 1971), and the combination of ecological and genetic resources has led to the discovery of multiple loci impacting differential pollinator attraction between the two species (Bradshaw and Schemske, 2003; Yuan *et al.*, 2013b; Yuan *et al.*, 2013c). *Mimulus lewisii* is a bumblebee-pollinated alpine species, while its sister, *M. cardinalis*, is a lower-elevation hummingbird-pollinated species (Hiesey *et al.*, 1971; Schemske and Bradshaw, 1999). Within areas of sympatry, pollinator fidelity is responsible for 98% of reproductive isolation between *M. lewisii* and *M. cardinalis* (Ramsey *et al.*, 2003). Previous work has shown that three floral volatiles produced by *M. lewisii* – D-limonene, β-myrcene, and *E*-β-ocimene – are important for the attraction of bumblebee pollinators, including *Bombus vosnesenskii*, the native pollinator of *M. lewisii* in the central Sierra Nevada mountains of California. Of the three volatiles, *M. cardinalis* produces only D-limonene, released at just 0.9% the rate of *M. lewisii* (Byers *et al.*, 2014).

Therefore, we ask some global questions, which we begin to address in this manuscript: What are the genetic underpinnings of the differential emission of floral volatiles between *M. lewisii* and *M. cardinalis*? How many genes are responsible, and how do the species differ in gene expression and protein function? What role, if any, do these scent differences play in differential pollinator visitation, and through this, reproductive isolation? In keeping with other discussions of "speciation genes" in plants (Rieseberg and Blackman, 2010), are these genes of large effect or small effect, coding or regulatory genes? *Mimulus*, with its known attractive volatiles, genetic and genomic tools, and well-studied ecology, is an obvious choice for filling in this missing piece of the floral scent-speciation link.

Results

Genetic mapping of species-specific differences in floral volatiles

Construction of an F_1 cross between *M. lewisii* inbred line LF10 and *M. cardinalis* inbred line CE10 revealed patterns of inheritance of loci controlling the emission of D-limonene, β-myrcene, and *E*-β-ocimene. The emission rate of D-limonene for the F_1 (mean = 47.2 ng/flower/hr; *N* = 3) was similar to that of the *M. lewisii* parental inbred line (mean = 55.1 ng/flower/hr, *N* = 9; two-tailed Mann-Whitney p = 0.864, U = 15) and much higher than that of the *M. cardinalis* parental inbred line (mean = 0.5 ng/flower/hr, *N* = 9; one-tailed p = 0.005, U = 27), suggesting that high levels of D-limonene emission are inherited from the *M. lewisii* parent in a dominant manner. The pattern was similar for β-myrcene (F_1: mean = 2.6 ng/flower/hr, *M. lewisii*: mean = 3.3 ng/flower/hr, *M. cardinalis*: mean = 0.0 ng/flower/hr; F_1 vs. *M. lewisii* two-tailed p = 0.600, U = 17; F_1 vs. *M. cardinalis* one-tailed p = 0.005, U = 27). For *E*-β-ocimene, the *M. lewisii* allele appears to be semidominant (F_1: mean = 2.8 ng/flower/hr; *M. lewisii*: mean = 7.6 ng/flower/hr; *M. cardinalis*: mean = 0.0 ng/flower/hr; F_1 vs. *M. lewisii* two-tailed p = 0.036, U =25; F_1 vs. *M. cardinalis* one-tailed p = 0.005, U =27). Complete or partial dominance of the *M. lewisii* alleles for these floral volatiles is consistent with other traits that differ between the species (Bradshaw *et al.*, 1998).

When a backcross (F_1 x *M. cardinalis*) population of 100 plants was scored for the presence or absence of emission of β-myrcene and *E*-β-ocimene, it segregated approximately 1:1 for both volatiles (0.52:0.48 β-myrcene present:absent; 0.38:0.62 *E*-β-ocimene present:absent), suggesting that alleles at Mendelian loci might control the difference in emission of these monoterpenes between *M. lewisii* and *M. cardinalis*. D-limonene and β-myrcene emission rates were very highly correlated (r = 0.975), but neither was particularly highly correlated with *E*-β-ocimene emission rate (r = 0.474 versus D-limonene, r = 0.574 versus β-myrcene). Therefore, we considered a two-locus model for the difference in these three compounds between the two species – one locus controlling

the production of D-limonene and β-myrcene, and another, unlinked, locus controlling *E*-β-ocimene. A larger backcross population (N = 768) was constructed to map the two loci with greater precision.

Identification and characterization of a bifunctional LIMONENE-MYRCENE SYNTHASE (LMS) in *M. lewisii* flowers

The locus associated with D-limonene and β-myrcene emission was mapped to a 15 cM interval between markers M02_510K and M02_1500K (Table S4), *ca*. 5.3 cM from M02_1500K. Using the assembled and annotated *M. guttatus* genome v1.1 as a reference (http://www.phytozome.net/cgi-bin/gbrowse/mimulus/), the ortholog of M02_1500K maps to *M. guttatus* scaffold 89 at position 201 kbp. On scaffold 89 between positions 206 kbp and 226 kbp there is a cluster of three terpene synthases/cyclases – excellent candidates for controlling D-limonene and β-myrcene emission.

Indel markers developed for two of the *M. lewisii/cardinalis* candidate genes in the terpene synthase cluster (Table S5) revealed no recombinations (in 768 backcross plants) between themselves or the putative *LIMONENE-MYRCENE SYNTHASE* (see Methods). The very tight linkage among the candidate terpene synthases within the cluster made it impractical to resolve the identity of the D-limonene and β-myrcene synthases by recombination. RT-PCR showed that, of the three candidates, only the *M. lewisii* ortholog (KF857265) of the *M. guttatus* terpene synthase gene on scaffold 89 at position 321 kbp (mgv1a003660m) is transcribed in *M. lewisii* flowers. The marker genotype at M02_1500 accounted for 92% of the difference between *M. lewisii* and *M. cardinalis* emissions of D-limonene and 98% of the difference in β-myrcene emissions, consistent with a single-locus model for D-limonene and β-myrcene production. No transgressive segregation was observed in the backcross population. The predicted AHI50308 gene product contains the conserved DDxx(D/E) and (N,D)Dxx(S,T,G)xxxE (NSE/DTE) motifs required for Mg^{2+} binding during the terpene synthesis process (Nieuwenhuizen *et al.*, 2013), as well as the RRx_8W motif required for cyclic terpene formation (Dudareva *et al.*, 2003).

The *M. lewisii* cDNA (KF857264) orthologous to mgv1a003660m, designated *TS321K*, was overexpressed in *E. coli* (as in Bohlmann *et al.*, 2000). A crude lysate from the *E. coli* culture was supplied with geranyl pyrophosphate (GPP) as a substrate, yielding D-limonene and β-myrcene in the same proportions as observed in the authentic headspace collection from *M. lewisii* flowers (Table S1, Fig. 2*A*). This suggests that the high correlation between D-limonene and β-myrcene emission in the backcross mapping population is due to the pleiotropic effect of a bifunctional LIMONENE-MYRCENE SYNTHASE (LMS) encoded by a single *LMS* gene in *M. lewisii* (*MlLMS*). This is consistent with the frequent occurrence of multi-product terpene synthases (Dudareva *et al.*, 2004).

The *M. cardinalis LMS* (*McLMS*) coding sequence was not expressed *in vitro* because there is a G66T transversion mutation in exon 3 of *McLMS* (KM659024) that results in a nonsense mutation in the McLMS protein (G201X, using MlLMS AHI50308 as the reference allele).

Of note, this is not a definitive demonstration that *LMS* is the gene underlying the locus responsible for the difference in D-limonene and β-myrcene emission between *M. lewisii* and *M.cardinalis*; it is formally possible that a different, tightly-linked gene instead might be responsible. Transgenic complementation of the nonfunctional *M. cardinalis LMS* allele by the *M. lewisii* allele would be necessary to show conclusively that *LMS* is the causal gene for the phenotypic difference.

Identification and characterization of OCIMENE SYNTHASE (OS) in *M. lewisii* flowers

The locus associated with *E*-β-ocimene emission was mapped to a 7.5 cM interval midway between markers sc4_2325K and M13_2620 (Table S4). The marker genotype at sc4_2325 accounted for 98% of the difference between *M. lewisii* and *M. cardinalis* emissions of *E*-β-ocimene, consistent with a single-locus model for *E*-β-ocimene production. No transgressive segregation was observed in the backcross population. The orthologous region of the *M. guttatus* genome lies in a 484 kbp interval

(2325 kbp – 2809 kbp) on scaffold 4. There is a cluster of five terpene biosynthesis genes on scaffold 4 at position 2538 kbp – 2577 kbp. The gene at position 2538 kbp (mgv1a020487m) is annotated as a terpene synthase, while the other four genes are annotated as sesquiterpene cyclases (Fig. 1). An indel marker developed for the *M. lewisii/cardinalis* ortholog of mgv1a003660m, designated *TS2538* (Table S5), revealed no recombinations (in 768 backcross plants) with the putative *OCIMENE SYNTHASE* (see Methods).

When overexpressed in *E. coli* and supplied with GPP as a substrate, the *M. lewisii TS2538* cDNA (KF857262) encodes a functional OCIMENE SYNTHASE (MlOS, AHI50306) (Fig. 2*A*, Table S1). However, under the same conditions the *M. cardinalis TS2538* cDNA (KF857263) does not encode an enzyme (McOS, AHI50307) capable of synthesizing any monoterpene that we could detect. McOS differs from MlOS at 19 amino acid residues, including insertion of a leucine residue at position 238 in McOS and deletion of an arginine residue at position 308 in McOS (Fig. S1). Both sequences contain the same DDxx(D/E) and NSE/DTE Mg^{2+} binding motifs, as well as the RRx_8W cyclase motif, which are unaltered by the 19 nonsynonymous amino acid substitutions (Fig. S1).

As with *LMS*, in the absence of a transgenic complementation test this is not a strict demonstration that *MlOS* is the gene underlying the locus responsible for the difference in *E*-β-ocimene emission between *M. lewisii* and *M. cardinalis*.

LMS* and *OS* expression *in vivo

Using RT-PCR with six different stages of flowering tissue from early bud (8d prior to anthesis, 5mm) to open flower (see Yuan *et al.*, 2013c), we found that both *MlLMS* and *MlOS* are expressed in the last three days prior to anthesis (15mm and 20mm) of floral development, as well as in the open flower (Fig. 2*B*). *McLMS* is weakly expressed in late-stage floral buds but not expressed in open flowers. *McOS* is expressed similarly to *MlOS*.

Construction of RNAi knockdowns of *MlLMS* and *MlOS* in stably transformed *M. lewisii*

Using RNA interference *via Agrobacterium*-mediated *in planta* transformation of hairpin RNAi constructs into *M. lewisii* (Yuan *et al.*, 2013c), we were able to knock down the expression of both *MlLMS* and *MlOS* to produce much lower floral volatile levels, comparable to those produced in plants homozygous for the *M. cardinalis* alleles at *LMS* and *OS* (Table S2). This allowed us both to verify *LMS* and *OS* gene function *in vivo* and to determine the effect of decreased emission of specific floral volatiles on pollinating bumblebees.

We recovered 24 *M. lewisii* (inbred line LF10) T_1 plants carrying the *MlLMS*-RNAi transgene, and assayed each transgenic plant in triplicate for floral volatile production. All T_1 plants had lower emission rates of D-limonene and β-myrcene relative to the wild-type *M. lewisii* LF10 (D-limonene: range = 1.2%-56.1%, mean = 10.2%; β-myrcene: range = 4.1-50.0%, mean = 12.8%). Interestingly, most T_1 plants showed a decrease in emission of terpinolene (range = 0.0%-132.6%, mean = 18.0%), indicating that MlLMS may be responsible for synthesizing an additional minor compound in *M. lewisii*. One of these T_1 transgenics (LMS321K-8) was selfed as the parent of T_2 plants used for pollinator studies (for data on three other T_2 lines from independent T_1 transgenics, see Table S3). The original T_1 LMS321K-8 had very low emission rates of D-limonene and β-myrcene, with a mean of 2.8% D-limonene production and 9.1% β-myrcene production relative to the *M. lewisii* LF10 T_0 parent. Notably, LMS321K-8 had an increase in *E*-β-ocimene of 452.8% compared with the *M. lewisii* T_0 parent (Table 1, Fig. 3). All other T_1 plants had a similar increase in *E*-β-ocimene production relative to the wild-type parent (range = 190.4-493.9%, mean = 383.4%).

A total of 71 T_2 plants from the self-pollinated progeny of T_1 LMS321K-8 were assayed using headspace collection of floral volatiles to select the greenhouse population for the bumblebee pollinator behavioral experiment. The 24 individuals selected for the experiment produced much

less D-limonene and β-myrcene compared to the wild-type *M. lewisii* ancestor (D-limonene: range = 0.1-2.4%, mean = 1.9%; β-myrcene: range = 0.0-4.8%, mean = 0.6%), and more *E*-β-ocimene than the *M. lewisii* wild-type ancestor (range = 93.0-510.5%, mean = 247.2%). The D-limonene and β-myrcene levels were similar to those found in *M. cardinalis* (D-limonene: range = 0.03-2.8%, mean = 0.9% of wild-type *M. lewisii*; β-myrcene is absent from *M. cardinalis*).

Only two T_1 plants carrying the *MlOS*-RNAi transgene were recovered, but both had the desired *E*-β-ocimene knockdown phenotype relative to *M. lewisii* LF10 (*E*-β-ocimene: range = 0.8%-2.9%, mean = 1.8%; D-limonene: range = 39.6%-58.9%, mean = 49.2%; β-myrcene: range = 28.3%-41.2%, mean = 34.8%). T_1 plant TS2538-1, which was self-pollinated to create a T_2 population for pollinator studies, had a much lower emission rate of *E*-β-ocimene (0.8%) relative to *M. lewisii* LF10, as well as lower emission rates of D-limonene (39.6%) and β-myrcene (28.3%)(Fig. 3). T_1 plant TS2538-2 flowered substantially later than TS2538-1, so T_2 lines were not created from this plant. A total of 80 T_2 plants were produced from TS2538-1, and these produced similar amounts of D-limonene and β-myrcene as the *M. lewisii* LF10 ancestor (D-limonene: range = 61.3-127.4%, mean = 83.6%; β-myrcene: range = 57.3-144.5%, mean = 88.7%), but much less *E*-β-ocimene (range = 0.9-3.9%, mean = 1.9%).

Effects of *MlLMS* and *MlOS* knockdowns on bumblebee pollinator behavior

Two experiments, one for the *MlLMS*-RNAi transgenics and one for the *MlOS*-RNAi transgenics, were performed to assay the impact of reduced monoterpene production on bumblebee (*Bombus impatiens*) visitation in a captive greenhouse setting. During each experiment, both preference (expressed as the proportion of total visits to each flower type) and constancy (expressed as the tendency of an individual bumblebee to deviate from random choices, exclusive of preference, see Waser, 1986) were measured.

A total of 1682 visits were observed to flowers in the *MlLMS*-RNAi knockdown experiment. Visits were defined as observable contact with the sexual organs of the flower – *i.e.,* the bumblebee entered the flower fully, as required to effect pollination. Of 1682 visits, 833 (49.52%) were to the wild-type *M. lewisii* and 849 (50.48%) were to the *M. lewisii MlLMS*-RNAi transgenic plants, showing no significant difference ($X^2 = 0.15$, $p = 0.70$, Fig. 4). Bumblebees appeared to show no overall qualitative behavioral difference towards either flower type.

A total of 39 bumblebee foraging bouts were assayed for constancy, with an average Bateman's index of -0.0114 (-1 indicates complete inconstancy – regular switching between types; 0 indicates random visitation patterns; +1 indicates complete constancy, always within types). To determine whether this constancy was significantly different from random visitation, the same bumblebee foraging bouts were used with 100,000 simulated runs of randomly permuted plant locations, resulting in an average Bateman's index of -0.1141. A total of 96,648 simulations had more divergent Bateman's index values than the actual data, showing that bumblebees demonstrated no constancy when presented with these flowers ($p = 0.97$).

For the *MlOS*-RNAi knockdown experiment, a total of 2202 visits were observed. Of these visits, 1166 (52.95%) were to wild-type *M. lewisii* and 1036 (47.05%) were to the *M. lewisii MlOS*-RNAi plants, showing a significant preference for the wild-type *M. lewisii* flowers ($X^2 = 7.67$, $p = 0.0056$, Fig. 4). Bumblebees approaching the *MlOS*-RNAi flowers were noted to frequently wave their antennae and contact the flower with their antennae prior to aborting a potential visit, suggesting that *E*-β-ocimene may operate as a near-field olfactory cue, but this behavior was not noted for the wild-type flowers in this experiment nor for either flower type in the *MlLMS*-RNAi experiment. Constancy was also absent in the *MlOS*-RNAi experiment, with a total of 46 bumblebee foraging bouts showing an average Bateman's index of 0.0149; the simulation described above was repeated using these foraging bouts, with an average Bateman's index of -0.1142 ($p = 0.95$).

Discussion

Mimulus lewisii produces three floral volatiles with significant neurophysiological and behavioral effects on bumblebees – D-limonene, β-myrcene, and *E*-β-ocimene, while *M. cardinalis* produces only D-limonene at much lower levels (0.9% of *M. lewisii*) (Byers *et al.*, 2014). These differences are likely due to mutations in two genes: *LIMONENE-MYRCENE SYNTHASE* (*MlLMS*, *McLMS*) and *OCIMENE SYNTHASE* (*MlOS*, *McOS*). In quantitative genetic terms, allelic variation at loci containing *LMS* and *OS* accounts for 92-98% of the phenotypic difference between *M. lewisii* and *M. cardinalis* in floral emission of D-limonene, β-myrcene, and *E*-β-ocimene. The very low level of volatile emission from *M. cardinalis* flowers can be explained at the molecular genetic level; *McLMS* is a null allele due to a nonsense mutation in exon 3, while *McOS* has multiple coding sequence differences that eliminate its ability to produce *E*-β-ocimene.

RNAi knockouts show that the loss-of-function *LMS* and *OS* alleles can recapitulate the *M. cardinalis* volatile emission phenotypes, and that functional copies of both genes are necessary to produce D-limonene, β-myrcene, and *E*-β-ocimene *in vivo*. However, there remains the formal possibility that the allelic variants producing differences in floral volatile emissions between *M. lewisii* and *M. cardinalis* are not in *LMS* or *OS*, but in genes very tightly linked to them. To show that the *M. lewisii* alleles of *LMS* and *OS* are sufficient (since we have shown that they are necessary) to produce D-limonene, β-myrcene, and *E*-β-ocimene, we would have to transform *M. cardinalis* with constructs containing the *M. lewisii* alleles. However, *M. cardinalis* is very difficult to transform, so we have not performed these definitive experiments. Although we lack conclusive evidence that these are the genes underlying these loci, we present strong circumstantial evidence (the nonsense mutation in *McLMS* and the lack of product from McOS activity *in vitro*) that is consistent with this, and RNAi

knockouts show that the loss-of-function *LMS* and *OS* alleles can recapitulate the *M. cardinalis* volatile emission phenotypes necessary to test for differential pollinator visitation.

Surprisingly, despite the high level of production of D-limonene and β-myrcene in *M. lewisii* flowers, substantially knocking down emission of these two compounds produces no significant effect on bumblebee visitation in the greenhouse. In contrast, knocking down emission of *E*-β-ocimene results in a modest (6%) but significant decrease in bumblebee visitation, suggesting that alternative alleles of *OCIMENE SYNTHASE* can contribute to reproductive isolation between the bumblebee-pollinated *M. lewisii* and the hummingbird-pollinated *M. cardinalis*. Although 6% is a modest effect size in molecular genetic terms, in evolutionary genetic terms a selection coefficient (*s*) of 0.06 (130 greater visits to the wild-type plant / 2202 total visits) would sweep the beneficial allele to fixation very quickly in natural populations (Hartl and Clark, 1997), so we designed our pollinator visitation experiments to detect a difference in visitation as small as 5%. Assuming an infinite population size, the probability of fixation of the allele is 2*s*, or 12%; an effective population size greater than five individuals would allow selection to exceed drift as an evolutionary force at this locus ($N_e = 1/(4s) = 4.17$).

Why does the loss of D-limonene and β-myrcene have no effect on bumblebee visitation? First, the T_2 plants used in the greenhouse experiment had surprisingly high levels of *E*-β-ocimene, perhaps due to rerouting of a common pool of the shared precursor geranyl pyrophosphate (GPP). Terpene synthesis is a flexible but complex process, and buildups of precursors can be utilized by alternate metabolic pathways (Gang, 2005). Given the much higher emission of *E*-β-ocimene in the *MlLMS*-RNAi transgenic plants, *M. lewisii* may be prone to this effect. As the RNAi technique used here is an analogous (but weaker) representation of the phenotypes resulting from a loss-of-function mutation

in a wild population, fluctuations in volatile production as found here are reflective of the system's physiology and the effects that might occur in a natural setting.

It is also possible that the high production of D-limonene and β- myrcene in *M. lewisii* serves another function within the plant, such as defense against herbivores, nectar robbers, or disease (Kessler *et al.*, 2013), as these volatiles are known anti-herbivory compounds (Levin, 1976). Although the three volatiles have similar physical properties, D-limonene and β-myrcene may serve to mediate long-distance attraction at the patch level rather than at the level of the individual flower; long-distance attraction has been shown to be important for honeybee (*Apis mellifera*) navigation (Bogdany and Taber, 1979). The high production of D-limonene and β-myrcene may be a remnant of some previous pollination syndrome, environmental context, or merely the byproduct of some other metabolic process within the plant. Similarly, although a significant effect on bumblebee visitation was seen with the loss of *E*-β-ocimene, it is possible that the main role of this volatile may lie elsewhere (Kessler *et al.*, 2013), for example in herbivory defense (Arimura *et al.*, 2004) with a secondary role in the attraction of bumblebee pollinators. Data on herbivory, florivory, or pathogen infestation in wild populations of *M. lewisii* and *M. cardinalis* are currently lacking, limiting our ability to speculate on these possibilities. Future field experiments will increase our understanding of the multiple roles these volatiles may be playing in *M. lewisii* and *M. cardinalis*.

Finally, it is possible that these effects differ from those that would be found with wild *Bombus vosnesenskii*. However, both species are generalist floral visitors, and the *M. lewisii* scent elicits similar olfactory responses in both bee species. Moreover, *B. impatiens* has been used as a model for bumblebee-flower interactions in other systems, including those involving *B. vosnesenskii* (Bodbyl Roels and Kelly, 2011), thus we feel that *B. impatiens* is an excellent model for these experiments

(see SI Materials and Methods for a full explanation). Although these results differ in detail from those we found in previous behavioral experiments with artificial and extracted floral scents, in which all three monoterpenes were required for maximum bumblebee response (Byers *et al.*, 2014), the greenhouse experiments offer a more realistic assay for the effect of scent on pollinators by allowing them to integrate multiple floral cues.

What role does scent play in pollinator interaction within this system? Many studies have shown that scent plays a strong role in landing decisions by diurnal pollinators such as bumblebees and honeybees (Butler, 1951; Galen and Kevan, 1980; Galen and Kevan, 1983; Lunau, 1992; Majetic *et al.*, 2009; Dötterl and Vereecken, 2010) – the initial approach may be guided by patch-level visual signals, followed by a visually-guided approach to an individual flower. At that point, the final landing decision may be influenced by floral scent, especially in relatively weakly scented flowers such as *M. lewisii* (Dötterl and Vereecken, 2010; Parachnowitsch *et al.*, 2012). Therefore, even in the densely-flowered greenhouse experiments, signals such as the presence or absence of *E*-β-ocimene may play a significant role in final landing decisions. Additionally, densities in the greenhouse experiments were similar to those found in wild populations of *M. lewisii*, which grows along montane streambeds in large clusters, so the dense greenhouse conditions are a better indicator of the potential effect of a single change in scent in a wild population.

How might a loss-of-function allele of *OS* promote a pollinator switch from bumblebees to hummingbirds? Hummingbirds have a very limited sense of smell (Ioalé and Papi, 1989), and retain scent information very poorly (Goldsmith and Goldsmith, 1982), so the loss of scent in a hummingbird-pollinated flower such as *M. cardinalis* (an “anti-bee” but not “pro-bird” shift, to use the language in Castellanos *et al.*, 2004) would likely have no fitness cost, and might even increase

fitness by discouraging bumblebee visitors from transferring heterospecific pollen to the stigma and carrying away nectar or pollen. In combination with the difference in visual signals and mechanical access found between *M. lewisii* and *M. cardinalis*, such a loss of *E*-β-ocimene might serve to reinforce visitation behavior. Whether these changes in floral volatiles evolved in allopatry or as reinforcement during secondary contact is unclear; investigating the volatile profiles and orthologous terpene synthase genes of other species in *Mimulus* section *Erythranthe* may provide some insight into this question.

The fact that the *OS* polymorphism between *M. lewisii* and *M. cardinalis* is in a structural gene contradicts the current thinking that genes involved in prezygotic reproductive isolation – often referred to as "speciation genes" (Coyne, 1992) or "barrier genes" (Noor and Feder, 2006) – are nearly always regulatory genes (Rieseberg and Blackman, 2010). However, the limited number of genes with known effects in prezygotic reproductive isolation should preclude any general conclusions from being drawn about this process. We would suggest, however, that the potential for structural genes to contribute to this process should not be ignored.

Although many systems used in the study of floral volatiles have relatively strong scents that are detectable by the human nose, scent can also be a factor in reproductive isolation in systems where it is easily missed, as in *Mimulus*. The role of strong emissions of floral volatiles in attracting nighttime pollinators from a distance is well documented (Raguso and Willis, 2003). The potential role of changes in floral scent in pollinator-mediated reproductive isolation involving generalist, daytime pollinators such as bumblebees is largely unknown, and no examples integrating floral scent genetics and pollinator reproductive isolation in sister species with generalist pollinators have been reported. Some authors have commented that the role of floral scent in reproductive isolation is

questionable in generalist cases, as floral scents thus serve less as "private channels" and pollinators are attracted to multiple floral scent profiles (Schiestl and Ayasse, 2002).

Floral scent should be considered as an attractive factor even in generalist systems, along with more easily-measured visual signals such as floral color and pattern. Here, the sister species *M. lewisii* and *M. cardinalis* can be used as a model for the study of reproductive isolation involving floral volatiles – one can begin by looking at species-specific differences, identifying critical volatiles within a complex mixture *via* electrophysiological and behavioral assays (Riffell *et al.*, 2013; Byers *et al.*, 2014). Then, studies can proceed by determining the genetic basis of these phenotypic differences, creating high-resolution genetic materials (near-isogenic lines, transgenics), and, finally, assaying of the results of these genetic changes in ecologically relevant greenhouse or field settings. Nearly all previous studies of the role of floral volatiles in speciation have only answered a subset of these questions, but our work with *Mimulus*, an emerging model system, shows that a comprehensive, integrative study is possible.

Experimental Procedures

QTL and fine mapping

Volatiles were first assayed in triplicate in an F_1 cross of *M. lewisii* inbred line LF10 and *M. cardinalis* inbred line CE10 (LF10 x CE10) and compared with previous results for the parent lines (9 samples each; see Byers *et al.*, 2014) using a Mann-Whitney *U* test. A coarse mapping population consisting of 100 individuals of a cross between LF10 and CE10, backcrossed to CE10 [(LF10 x CE10) x CE10], was then constructed. Headspace volatiles were collected in the manner described in Byers *et al.*, 2014 (see also SI Materials and Methods and methods below) and assayed for emission rates of D-limonene, β-myrcene, and *E*-β-ocimene. Pearson correlation coefficients were calculated pairwise

for the three scents to investigate potential linkage or pleiotropy. A subset of 24 backcross plants with the two most divergent phenotypes (high D-limonene/β-myrcene and low *E*-β-ocimene; low D-limonene/β-myrcene and high *E*-β-ocimene) were screened at 34 indel markers evenly spaced across the genome (Table S4) with the intent of creating a low-resolution quantitative trait locus (QTL) map. However, it was clear from inspection of the genotypic and phenotypic data that the emission of D-limonene/β-myrcene and *E*-β-ocimene were, to a first approximation, segregating as Mendelian traits.

A larger backcross population (N = 768) was constructed and screened with markers flanking the putative *LIMONENE-MYRCENE SYNTHASE* (M02_510 and M02_1500), and flanking the putative *OCIMENE SYNTHASE* (sc4_2325K and M13_2620) (Table S4). Markers used in the mapping process were developed from *M. lewisii* and *M. cardinalis* genome sequences, and amplify codominant markers in the backcross. To reduce the effort required to score flowers for volatile production, only those backcross plants with informative recombinations between markers flanking *LMS* (N = 107) or *OS* (N = 52) were phenotyped for scent, using a direct extraction assay from flowers rather than the more labor- and time-intensive headspace collection method. For further details, see Supplemental Information.

The *Mimulus guttatus* genomic region corresponding to the *M. lewisii* region containing *LMS* or *OS* was examined, and candidate genes were identified based upon their map position relative to the flanking molecular markers and the *M. guttatus* annotation. For the *LMS* locus controlling D-limonene and β-myrcene emission, primers were designed to amplify indel polymorphisms in two of the terpene synthases/cyclases on *M. guttatus* scaffold 89. The candidate genes were designated LC250K and TS306K (see Table S5 for all primers). No recombination events were observed among

the two candidate genes and the putative *LMS*, defining a candidate region of less than 0.1 cM. For the *OS* locus controlling *E*-β-ocimene emission, primers were designed to amplify an indel polymorphism in a terpene synthase designated TS2538 on *M. guttatus* scaffold 4. No recombination events were observed between TS2538 and *OS*, defining a candidate region of less than 0.1 cM.

In vitro assay for terpene synthase activity

For details, see Supplemental Information and Fäldt *et al.* (2003).

Terpene synthase expression *in vivo*

Total RNA was extracted from flower buds collected at 5, 8, 10, 15, and 20mm stages in *M. lewisii* and equivalent stages in *M. cardinalis*. Total RNA was extracted from open flowers of both species. cDNA was prepared from total RNA extracts using the SuperScript III First-Strand Synthesis System (Invitrogen). RT-PCR for both loci for both species was performed with these cDNA, using *MlUBC* as a control for background expression levels as described in Yuan *et al.* (2013c). Primers used were the following: *MlLMS* RNAi forward/reverse for *MlLMS, McLMS* forward/reverse for *McLMS*; *MlOS* RNAi sense forward/reverse for *MlOS* and *McOS*; and *MlUBC* foward/reverse for *MlUBC*.

Construction of *MlLMS* and *MlOS* RNAi transgenic *M. lewisii*

Transgenesis was done in the *M. lewisii* background, as insect pollination is inferred to be the ancestral state in this clade (Beardsley *et al.*, 2003). Hairpin RNA interference (RNAi) transgenes targeted to knock down the expression of *MlLMS* or *MlOS* were constructed in pFGC5941 (Kerschen *et al.*, 2004; Arabidopsis Biological Resource Center, CD3-447) as described in (Yuan *et al.*, 2013c). In

each case, target specificity of the RNAi fragment was assured by BLAST search against the *M. lewisii* LF10 genome sequence. For *MlLMS*, a 106 bp fragment of *M. lewisii* cDNA was amplified and directionally cloned into the pFGC5941 *Nco*I/*Asc*I (sense) and *Bam*HI/*Xba*I (antisense) sites. For *MlOS*, a 289 bp sense fragment was amplified and directionally cloned into the *Nco*I/*Asc*I site of pFGC5941. A 180 bp antisense fragment (entirely within the 289 bp *Nco*I/*Asc*I amplicon) was amplified and directionally cloned into the *Bam*HI/*Xba*I site. Constructs were verified by sequencing, then electroporated separately into *Agrobacterium tumefaciens* strain GV3101 and used for *in planta* transformation of *M. lewisii* LF10 following (Yuan *et al.*, 2013c).

Greenhouse experiments

For details of experimental design, see Supplemental Information. The layout of the greenhouse experiments, including randomized plant positions, is depicted in Figure S2.

Observations of bumblebee behavior were recorded for the first six hours of the first three days by two observers using voice recorders, each following one or two bumblebees at a time. At the start of each day prior to the first bumblebee activity, old flowers were removed and newly opened flowers were counted and equalized between the two types of plants (wild-type and RNAi transgenic) to ensure that bumblebees had an equal chance of encountering a given flower of each type on each day.

Data were transcribed and analyzed for preference (proportion of total visits) and constancy. For constancy, visits were "collapsed" to the plant level – *i.e.*, multiple visits to one plant in sequence were reduced to a single visit, since flower numbers were unequal between plants and flowers were

often tightly clustered. Bumblebees were only used for constancy analysis if they visited ten or more plants in a foraging bout. Constancy was calculated using Bateman's method (described in Waser, 1986), which is independent of preference; equalizing flowers at the start of the day gave pollinators equal access to each type, as required by this metric. To determine if observed constancy was different from the null expectation, the same foraging data were used in a permutation test with shuffled plant identities, repeated 100,000 times; the fraction of the simulations with a greater than observed deviation from zero (complete randomness) was used to estimate the *p*-value.

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Legends for Supporting Information

Supplemental Materials and Methods: Description of volatile headspace collection and analysis, volatile extraction collection and scoring, *in vitro* assays for terpene synthase activity, and greenhouse experiments.

Table S1: Products of the *in vitro* terpene synthase assays.

Table S2: Volatile production in the 2-4 best T1 plants recovered from RNAi experiments.

Table S3: Volatile production in T2 plants from four separate T1 parents recovered from RNAi knockdown of *MlLMS.*

Table S4: Molecular markers used during QTL and fine mapping.

Table S5: Sequences of oligonucleotide primers used.

Figure S1: Protein sequences of MlOS and McOS with differences highlighted.

Figure S2: Schematic of the greenhouse experimental setup.

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Tables:

Table 1: Volatile production in transgenic (T_1 parent plants of greenhouse experiment lines) and wild-type *M. lewisii* and *M. cardinalis*. Values are an average of 2-3 independent headspace samples. Emission values in parentheses represent the 90% confidence interval. See Table S2 for complete data on all volatiles produced by *M. lewisii*.

Volatile	LF10 (ng/hr)	CE10 (ng/hr)	*MlLMS*-RNAi (ng/hr)	*MlOS*-RNAi (ng/hr)	*MlLMS*-RNAi (% LF10)	*MlLMS*-RNAi (% CE10)	*MlOS*-RNAi (% LF10)	*MlOS*-RNAi (% CE10)
β-myrcene	2.837 (2.056, 3.793)	Absent (0.000, 0.000)	0.257 (0.194, 0.339)	1.116 (1.047, 1.185)	9.06%	n/a	39.34%	n/a
D-limonene	43.228 (35.539, 50.708)	1.024 (0.649, 1.419)	1.216 (0.765, 1.757)	23.820 (23.285, 24.354)	2.81%	118.75 %	55.10%	2326.17 %
***E*-β-ocimene**	5.563 (4.320, 7.049)	Absent (0.000, 0.000)	25.125 (20.591, 30.120)	0.054 (0.042, 0.074)	451.64 %	n/a	0.97%	n/a

Figures:

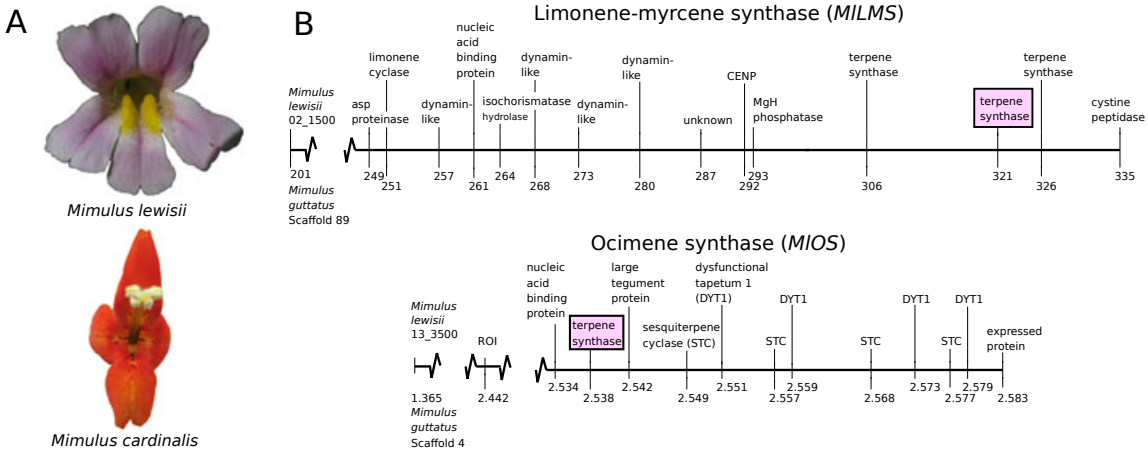
Figure 1: *Mimulus lewisii* and *M. cardinalis* and their terpene synthases. (A) *Mimulus lewisii* and *M. cardinalis*. (B) Genetic maps of *MlLMS* and *MlOS* and the homologous regions in *M. guttatus*. Positions on the lower half of each are from the *M. guttatus* genome scaffolds; annotations are from queries of the *M. guttatus* transcripts with BLASTx. Putative terpene synthases are highlighted. *M. lewisii* limonene-myrcene synthase is homologous to a terpene synthase at 319,982 bp on *M.*

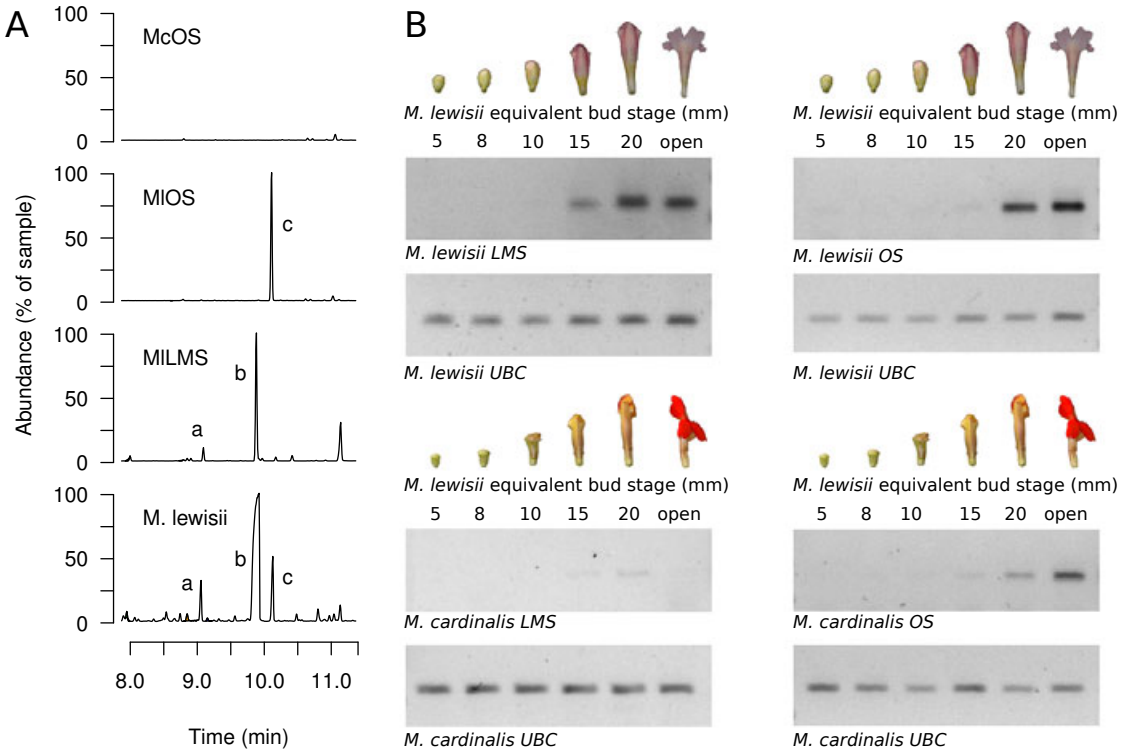
guttatus scaffold 89, and *M. lewisii* ocimene synthase is homologous to a terpene synthase at 2,538,727 bp on scaffold 4.

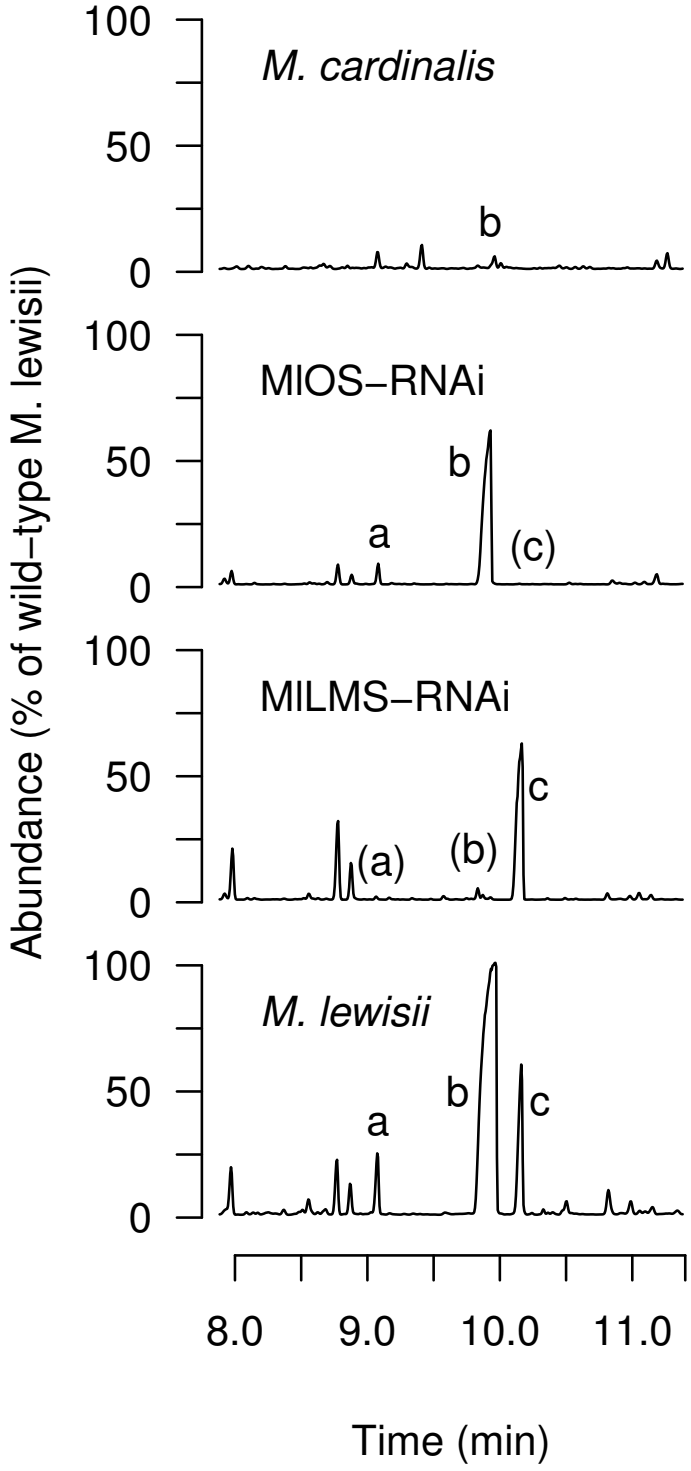
Figure 2: Terpene synthase activity *in vitro* and *in vivo*. (A) Products of terpene synthases using a bacterial overexpression system and *in vitro* enzyme assay. Using geranyl pyrophosphate (GPP, the common monoterpene precursor), the MlLMS enzyme produces D-limonene (b) and β-myrcene (a) in the same relative proportion as in the floral volatile emission. *M. lewisii* MlOS produces *E*-β-ocimene (c), but *M. cardinalis* McOS does not. (B) Temporal expression of terpene synthases *in vivo.* *MlLMS* is expressed just prior to flowering and in open flowers, but *McLMS* is not expressed in open flowers. *MlOS* shows a similar expression pattern to *MlLMS*, and *McOS* is expressed at the same stages, despite producing no terpenoid volatile that we could detect. Developmental staging is according to bud size of *M. lewisii*, and the corresponding stage of *M. cardinalis* is pictured for *McLMS*, *McOS*, and *McUBC*.

Figure 3: Stable RNAi knockdowns of *MlLMS* and *MlOS* in *M. lewisii* produce plants with low emission levels of D-limonene (b)/β-myrcene (a) and *E*-β-ocimene (c), respectively.

Figure 4: Greenhouse experiments with *Bombus impatiens* and *M. lewisii* wild-type and transgenic lines. (A) Image of a typical bumblebee visit. (B) Response of bumblebees to *MlLMS* RNAi knockdowns, *MlOS* RNAi knockdowns, and the wild-type parent. Bumblebees show the same visitation response to *MlLMS* knockdown transgenics as to wild-type *M. lewisii*. Bumblebees preferentially visit wild-type *M. lewisii* over *MlOS* knockdown transgenics.







A

B