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

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KF657265, M. lewisii LIMONENE-MYRCENE SYNTHASE gene, complete cds.
KF657264, M. lewisii LIMONENE-MYRCENE SYNTHASE mRNA, complete cds.
AH50308, M. lewisii LIMONENE-MYRCENE SYNTHASE protein product.
KM659024, M. cardinalis LIMONENE-MYRCENE SYNTHASE mRNA, exon3.
KF657262, M. lewisii OCIMENE SYNTHASE mRNA, complete cds.
AH50306, M. lewisii OCIMENE SYNTHASE protein product.
KF657263, M. cardinalis OCIMENE SYNTHASE mRNA, complete cds.
AH50307, M. cardinalis OCIMENE SYNTHASE protein product.
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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 Mimulus 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 – α-limonene, β-myrcene, and E-β-ocimene – that are attractive to bumblebee pollinators. By genetic mapping and in vitro analysis of enzyme activity we demonstrate that these interspecific differences are consistent with allelic variation at two loci, LIMONENE-MYRCENE SYNTHASE (LMS) and OCIMENE SYNTHASE (OS). Mimulus lewisii LMS (MlLMS) and OS (MlOS) are expressed most strongly in floral tissue in the last stages of floral development. Mimulus cardinalis LMS (McLMS) is weakly expressed and has a nonsense mutation in exon 3. Mimulus 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 RNA interference 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 is likely to contribute to differential pollinator visitation, and thus promote reproductive isolation between M. lewisii and M. cardinalis. OCIMENE SYNTHASE joins a growing list of ‘speciation genes’ (‘barrier genes’) in flowering plants.

Keywords: terpene synthase, floral volatiles, speciation, pollination, Mimulus lewisii, Mimulus cardinalis.

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 the neural activity of pollinators 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,b; 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 to expand 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, 1998; Wang and Pichersky, 1998), Antirrhinum (Dudareva et al., 2000, 2003), Petunia (Koeduka et al., 2006; Orlova et al., 2006; Dexter et al., 2007), Silene (Gupta et al., 2012), Arabidopsis (Bohmann 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 these 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 Petunia axillaris and Petunia exserta differ in their production of methyl benzoate, a volatile attractive to the hawkmoth pollinators of P. axillaris. Through quantitative trait locus (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 production of methyl benzoate and the locus on chromosome VII substantially contributing to the quantity of methyl benzoate produced. 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 Mimulus lewisii and Mimulus 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 affecting differential pollinator attraction between the two species (Bradshaw and Schemske, 2003; Yuan et al., 2013b,c). 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% of the rate of release in M. lewisii (Byers et al., 2014).

Therefore, we ask some global questions, which we begin to address in this paper. 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, structural 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 F1 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 α-limonene for the F1 plants (mean = 47.2 ng flower⁻¹ h⁻¹; n = 3) was similar to that of the M. lewisii parental inbred line (mean = 55.1 ng flower⁻¹ h⁻¹, 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⁻¹ h⁻¹, n = 9; one-tailed P = 0.005, U = 27), suggesting that high levels of emission of α-limonene are inherited from the M. lewisii parent in a dominant manner. The pattern was similar for β-myrcene (F1, mean = 2.6 ng flower⁻¹ h⁻¹; M. lewisii, mean = 3.3 ng flower⁻¹ h⁻¹; M. cardinalis, mean = 0.0 ng flower⁻¹ h⁻¹; F1 versus M. lewisii two-tailed P = 0.600, U = 17; F1 versus M. cardinalis one-tailed P = 0.005, U = 27). For E-β-ocimene, the M. lewisii allele appears to be semi-dominant (F1, mean = 2.8 ng flower⁻¹ h⁻¹; M. lewisii, mean = 7.6 ng flower⁻¹ h⁻¹; M. cardinalis, mean = 0.0 ng flower⁻¹ h⁻¹; F1 versus M. lewisii two-tailed P = 0.036, U = 25; F1 versus 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 (F1 × 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 monoterpens between M. lewisii and M. cardinalis. Rates of emission of α-limonene and β-myrcene were very highly correlated (r = 0.975), but neither was particularly highly correlated with the rate of emission of E-β-ocimene (r = 0.474 versus α-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 α-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 Mimulus lewisii flowers

The locus associated with α-limonene and β-myrcene emission was mapped to a 15 cM interval between markers M02_510K and M02_1500K (Table S4 in Supporting Information), about 5.3 cM from M02_1500K. Using the assembled and annotated Mimulus 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 and 226 kbp there is a cluster of three terpene synthases/cyclases – excellent candidates for controlling emission of α-limonene and β-myrcene.

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 Experimental Procedures). The very tight linkage among the candidate terpene synthases within the cluster made it impractical to resolve the identity of the α-limonene and β-myrcene synthases by recombination. Reverse transcriptase (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 emissions of α-limonene in M. lewisii and M. cardinalis and 98% of the difference in β-myrcene emissions, consistent with a single-locus model for α-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 binding Mg²⁺ during the process of terpene synthesis (Nieuwenhuizen et al., 2013), as well as the RRxW motif required for cyclic terpene formation (Dudareva et al., 2003).

The M. lewisii cDNA (KF857264) orthologous to mgv1a003660m, designated TS321K, was overexpressed in Escherichia 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 α-limonene and β-myrcene in the same proportions as observed in the authentic headspace collection from M. lewisii flowers (Table S1, Figure 2a). This suggests that the high correlation between α-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 (M/LMS). 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 M/LMS 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 emission of α-limonene and β-myrcene between M. lewisii and M. cardinalis; it is formally possible that a different, tightly linked gene instead might be responsible. Transgenic complementation of the non-functional 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 *Mimulus lewisii* flowers

The locus associated with emission of \( E-\beta \)-ocimene was mapped to a 7.5-cM interval midway between markers sc4_2325 and M13_2620 (Table S4). The marker genotype at sc4_2325 accounted for 98% of the difference in emissions of \( E-\beta \)-ocimene between *M. lewisii* and *M. cardinalis*, consistent with a single-locus model for production of \( E-\beta \)-ocimene. No transgressive segregation was observed in the backcross population. The orthologous region of the *M. guttatus* genome lies in a 484-kbp interval (2325–2809 kbp) on scaffold 4. There is a cluster of five terpene biosynthesis genes on scaffold 4 at position 2538–2577 kbp. The gene at position 2538 kbp (mgv1a003660m) is annotated as a terpene synthase, while the other four genes are annotated as sesquiterpene cyclases (Figure 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 Experimental Procedures).

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) (Figure 2a, 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 (Figure S1). Both sequences contain the same DDxx(D/E) and NSE/DTE Mg\(^{2+}\)-binding motifs, as well as the RRx8W cyclase motif, which are unaltered by the 19 non-synonymous amino acid substitutions (Figure 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 \( E-\beta \)-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 (8 days prior to anthesis, 5 mm) to open flower (see Yuan et al., 2013c), we found that both MILMS and MIOS are expressed in the last 3 days prior to anthesis (15 and 20 mm) of floral development, as well as in the open flower (Figure 2b). McLMS is weakly expressed in late-stage floral buds but not in open flowers. McOS is expressed similarly to MIOS.

**Construction of RNA interference knockdowns of MILMS and MIOS in stably transformed *Mimulus lewisii***

Using RNA interference (RNAi) via *Agrobacterium tumefaciens*-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 MILMS and MIOS to produce much lower levels of floral volatiles, 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) T1 plants carrying the *MILMS*-RNAi transgene and assayed each transgenic plant in triplicate for production of floral volatiles. All T1 plants had lower emission rates of α-limonene and β-myrcene relative to the wild-type *M. lewisii* LF10 (α-limonene, range 1.2–56.1%, mean 10.2%; β-myrcene, range 4.1–50.0%, mean 12.8%). Interestingly, most T1 plants showed a decrease in emission of terpinolene (range 0.0–132.6%, mean 18.0%), indicating that MILMS may be responsible for synthesizing an additional minor compound in *M. lewisii*. One of these T1 transgenics (LMS321K-8) was selfed as the parent of T2 plants used for pollinator studies (for data on three other T2 lines from independent T1 transgenics see Table S3). The original T1 plant LMS321K-8 had very low rates of emission of α-limonene and β-myrcene, with a mean production of 2.8% α-limonene and 9.1% β-myrcene relative to the *M. lewisii* LF10 T0 parent. Notably, LMS321K-8 had an increase in E-β-ocimene of 452.8% compared with the *M. lewisii* T0 parent (Table 1, Figure 3). All other T1 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 T2 plants from the self-pollinated progeny of T1 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 α-limonene and β-myrcene than the wild-type *M. lewisii* ancestor (α-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 levels of α-limonene and β-myrcene were similar to those found in *M. cardinalis* (α-limonene, range 0.03–2.8%, mean 0.9% of wild-type *M. lewisii*; β-myrcene is absent from *M. cardinalis*).

Only two T1 plants carrying the *MIOs*-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%; α-limonene, range 39.6–58.3%, mean 49.2%; β-myrcene, range 28.3–41.2%, mean 34.8%). The T1 plant TS2538-1, which was self-pollinated to create a T2 population for pollinator studies, had a much lower rate of emission of E-β-ocimene (0.8%) relative to *M. lewisii* LF10, as well as lower rates of emission of α-limonene (39.6%) and β-myrcene (28.3%) (Figure 3). The T1 plant TS2538-2 flowered substantially later than TS2538-1, so T2 lines were not created from this plant. A total of 80 T2 plants were produced from TS2538-1, and these produced similar amounts of α-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%).

### Table 1  Volatile production in transgenic (T₁ parent plants of greenhouse experiment lines) and wild-type *Mimulus lewisii* and *Mimulus cardinalis*. Values are an average of two to three independent headspace samples. Emission values in parentheses represent the 90% confidence interval. See 2 for complete data on all volatiles produced by *M. lewisii*

<table>
<thead>
<tr>
<th>Volatile</th>
<th>LF10 (ng h⁻¹)</th>
<th>CE10 (ng h⁻¹)</th>
<th>MILMS-RNAi (ng h⁻¹)</th>
<th>MIOS-RNAi (ng h⁻¹)</th>
<th>MILMS-RNAi (%LF10)</th>
<th>MIOS-RNAi (%CE10)</th>
<th>MILMS-RNAi (%LF10)</th>
<th>MIOS-RNAi (%CE10)</th>
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<tr>
<td>β-myrcene</td>
<td>2.837</td>
<td>Absent</td>
<td>0.257</td>
<td>1.116</td>
<td>9.06</td>
<td>n.a.</td>
<td>39.34</td>
<td>n.a.</td>
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<td>(2.056, 3.793)</td>
<td>(0.000, 0.000)</td>
<td>(0.194, 0.339)</td>
<td>(1.047, 1.185)</td>
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<tr>
<td>d-limonene</td>
<td>43.228</td>
<td>1.024</td>
<td>1.216</td>
<td>23.820</td>
<td>2.81</td>
<td>118.75</td>
<td>55.10</td>
<td>2326.17</td>
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<td></td>
<td>(35.539, 50.708)</td>
<td>(0.649, 1.419)</td>
<td>(0.765, 1.757)</td>
<td>(23.285, 24.354)</td>
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<td><em>E</em>-β-ocimene</td>
<td>5.563</td>
<td>Absent</td>
<td>25.125</td>
<td>0.054</td>
<td>451.64</td>
<td>n.a.</td>
<td>0.97</td>
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<td>(0.000, 0.000)</td>
<td>(20.591, 30.120)</td>
<td>(0.042, 0.074)</td>
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n.a., not applicable.

### Effects of MILMS and MIOS knockdowns on bumblebee pollinator behavior

Two experiments, one for the MILMS-RNAi transgenics and one for the MIOS-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 MILMS-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* MILMS-RNAi transgenic plants, showing no significant difference ($\chi^2 = 0.15$, $P = 0.70$, Figure 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 $/C0_0.0114$ ($/C0_1$ 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 MIOS-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
**DISCUSSION**

*Mimulus lewisii* produces three floral volatiles with significant neurophysiological and behavioral effects on bumblebees - $\alpha$-limonene, $\beta$-myrcene, and $E$$\beta$-ocimene – while *M. cardinalis* produces only $\alpha$-limonene at much lower levels (0.9% of *M. lewisii*) (Byers et al., 2014). These differences are probably due to mutations in two genes, LIMONENE-MYRCENE SYNTHASE (*MlLMS*, *McLMS*) and OCIMENE SYNTHASE (*MlOS*, *McOS*). In quantitative genetic terms, allelic variation at loci containing *LMS* and *OS* accounts for 92-98% of the phenotypic difference between *M. lewisii* and *M. cardinalis* in floral emission of $\alpha$-limonene, $\beta$-myrcene, and $E$$\beta$-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$$\beta$-ocimene.

The 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 $\alpha$-limonene, $\beta$-myrcene, and $E$$\beta$-ocimene in vivo. However, there remains the formal possibility that the allelic variants producing differences in floral volatile emissions between *M. lewisii* and *M. cardinalis* are not in *LMS* or *OS*, but in genes very tightly linked to them. To show that the *M. lewisii* alleles of *LMS* and *OS* are sufficient (since we have shown that they are necessary) to produce $\alpha$-limonene, $\beta$-myrcene, and $E$$\beta$-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 $\alpha$-limonene and $\beta$-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$$\beta$-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 more visits to the wild-type plant out of
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 2s, or 12%; an effective population size greater than five individuals would allow selection to exceed drift as an evolutionary force at this locus (Ne = 1/4s = 4.17).

Why does the loss of α-limonene and β-myrcene have no effect on bumblebee visitation? First, the T2 plants used in the greenhouse experiment had surprisingly high levels of E-β-ocimene, perhaps due to re-routing of a common pool of the shared precursor 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 MILMS-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 α-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, α-limonene and β-myrcene can 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 α-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 B. 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 Appendix S1 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 monoterp enes 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, 1983; Lunau, 1992; Majetic et al., 2009; Dotterl 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 (Dotterl 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 (Ioal and Chen, 1984) 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 be likely to 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 pre-zygotic 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 maps, and, finally, assaying 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**

**Quantitative trait loci and fine mapping**

Volatiles were first assayed in triplicate in an F1 cross of *M. lewisii* inbred line LF10 and *M. cardinalis* inbred line CE10 (LF10 × CE10) and compared with previous results for the parent lines (nine 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 (ILF10 × CE10) × CE10, was then constructed. Headspace volatiles were collected in the manner described in Byers et al. (2014) (see also Appendix S1 and below) and assayed for emission rates of δ-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 δ-limonene/β-myrcene and low E-β-ocimene; low δ-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 QTL map. However, it was clear from inspection of the genotypic and phenotypic data that the emission of δ-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 LIMONE-MYRCENE SYNTHASE (M02_510 and M02_1500) and the putative OCIMENE SYNTHASE (ss4_2325K and M13_2620) (Table S4). Markers used in the mapping process were developed from *M. lewisii* and *M. cardinalis* genome sequences, and amplify co-dominant 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 Appendix S1.

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 emission of δ-limonene and β-myrcene, 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 TS208K (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 ~0.1 cM. For the OS locus controlling emission of E-β-ocimene, primers were designed to amplify an indel polymorphism in a putative *LIMONENE-MYRCENE SYNTHASE* candidate region of ~0.1 cM.

**In vitro assay for terpene synthase activity**

For details see Appendix S1 and Fäldt et al. (2003).

**Terpene synthase expression in vivo**

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

**Construction of MILMS and MIOS RNAi transgenic *Mimulus 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 RNAi transgenes targeted to knock down the expression of *MILMS* or *MIOS* were constructed in pFGC5941.
For details of experimental design see Appendix S1. The layout of the greenhouse experiments, including randomized plant positions, is depicted in Figure S2.

Observations of bumblebee behavior were recorded for the first 6 h of the first 3 days by two observers using video 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 10 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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Figure S1. Schematic of the experimental greenhouse setup.

Table S1. Products of the in vitro terpenoid synthase assays.

Table S2. Volatile production in the two to four best T1 plants recovered from RNA interference experiments.

Table S3. Volatile production in T2 plants from four separate T1 parents recovered from RNA interference knockdown of MILMS.

Table S4. Molecular markers used during quantitative trait locus analysis.

Table S5. Sequences of oligonucleotide primers used.

Appendix S1. Description of volatile headspace collection and analysis, volatile extraction collection and scoring, in vitro assays for terpene synthase activity, and greenhouse experiments.

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Floral scent and reproductive isolation in Mimulus


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