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RESEARCH ARTICLE

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Testing the effect of individual scent compounds on pollinator attraction in nature using quasi-isogenic *Capsella* lines

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Abstract

Premise: Floral scent, usually consisting of multiple compounds, is a complex trait, and its role in pollinator attraction has received increasing attention. However, disentangling the effect of individual floral scent compounds is difficult due to the complexity of isolating the effect of single compounds by traditional methods.

Methods: Using available quasi-isogenic lines (qILs) that were generated as part of the original mapping of the floral scent volatile-related loci *CNL1* (benzaldehyde) and *TPS2* (β-ocimene) in *Capsella*, we generated four genotypes that should only differ in these two compounds. Plants of the four genotypes were introduced into a common garden outside the natural range of *C. rubella* or *C. grandiflora*, with individuals of a self-compatible *C. grandiflora* line as pollen donors, whose different genetic background facilitates the detection of outcrossing events. Visitors to flowers of all five genotypes were compared, and the seeds set during the common-garden period were collected for high-throughput amplicon-based sequencing to estimate their outcrossing rates.

Results: Benzaldehyde and β-ocimene emissions were detected in the floral scent of corresponding genotypes. While some pollinator groups showed specific visitation preferences depending on scent compounds, the outcrossing rates in seeds did not vary among the four scent-manipulated genotypes.

Conclusions: The scent-manipulated *Capsella* materials constructed using qILs provide a powerful system to study the ecological effects of individual floral scent compounds under largely natural environments. In *Capsella*, individual benzaldehyde and β-ocimene emission may act as attractants for different types of pollinators.

KEYWORDS

benzaldehyde, β-ocimene, Brassicaceae, *Capsella*, floral scent, pollinator attraction, quasi-isogenic line

Floral trait diversity is one of the most striking features of angiosperms and considered to be linked to selection mediated by their pollinating agents (van der Niet et al., 2014; Farré-Armengol et al., 2015; Gervasi and Schiestl, 2017; Parachnowitsch et al., 2019; van der Kooi and Ollerton, 2020; Trunschke et al., 2021). Of the different agents involved, animal pollinators with their varying physical structures, sensory systems, and foraging behaviors have been shown to exert different selection pressures on visual and olfactory traits of plants, such as the size, color, and scent of flowers or inflorescences (Caruso et al., 2019; Chapurlat et al., 2019).

Floral scent is often a complex blend of various volatile organic compounds (Vereecken and Schiestl, 2008; Bouwmeester et al., 2019; Opedal et al., 2022). Many previous studies have documented an effect of the total blend of floral scent on pollinator visitation and behavior (Raguso and Willis, 2005; Riffell and Alarcón, 2013;

Russell et al., 2018). Several studies also documented phenotypic selection on total scent emission (Parachnowitsch et al., 2012; Chapurlat et al., 2019), and Gervasi and Schiestl (2017) found divergent evolution of scent emission in plants pollinated by bees versus hoverflies. However, it is typically unknown whether pollinators respond to the total blend or to specific compounds, because examining the effect of individual scent compounds or interactions between specific compounds is challenging. Also, emissions of different floral scent compounds are sometimes correlated with each other (multicollinearity) or the environment, which may complicate statistical inference and biological interpretation (Mitchell-Olds and Shaw, 1987). Augmentation of floral scent by adding single compounds or blends to living flowers could test the effect of emission of particular compounds on pollinator behaviors (Dobson et al., 2005), but is cumbersome when manipulating inflorescences that consist of large numbers of flowers and/or last for a long time. (Repeated small-dose augmentations are needed, or emissions would be inconstant.) Electroantennographic experiments can also be used to identify potential target floral scent compounds, as done in the orchid *Gymnadenia conopsea*, where pollinator-mediated selection later was identified on several single compounds (Chapurlat et al., 2019). However, this method is also time-consuming. Thus, metabolic inhibitors and gene-editing methods have been introduced to delete particular scent compounds from flowers (Raguso, 2006). For example, Junker et al. (2011) blocked monoterpene and sesquiterpene volatile biosynthesis using metabolic inhibitors in cut flowers of *Phlox paniculata* to reduce germacrene D, linalool and its derivatives from its floral scent, which resulted in an increased attraction of ants rather than its hoverfly pollinators. In a study of a North American tobacco, *Nicotiana attenuata* (Solanaceae), inverted repeat RNAi constructs were used to silence the production of floral nicotine in nectar, benzyl acetone emission in floral scent, or both (Kessler et al., 2008). The floral scent manipulation resulted in fewer pollinator visits because the deletion of benzyl acetone removed the attraction to the main moth visitors. However, because of legal issues with experiments involving genetically modified organisms (GMOs) in field settings (Turnbull et al., 2021), alternative ways of manipulating individual floral scent compounds would be helpful for understanding floral scent evolution in natural populations.

The genus *Capsella* (Brassicaceae) contains three diploid species, two of which (*C. rubella* and *C. orientalis*) are independently derived selfers diverged from an outbreeding *C. grandiflora*-like ancestor (Hurka et al., 2018; Bachmann et al., 2019; Koenig et al., 2019). *Capsella rubella* and *C. orientalis* show the classical selfing syndrome, with a reduction in traits associated with pollinator attraction (Sicard and Lenhard, 2011). In *C. rubella*, two loci have been identified that underlie the reduction in floral scent compared to *C. grandiflora* (Sas et al., 2016; Jantzen et al., 2019a; Wozniak et al., 2022). These, provide important genetic information for developing gene editing methods to investigate the ecological consequence of changes in pollinator-attraction due to alteration of individual floral scent volatiles. In particular, the loss of benzaldehyde (BAld) emission in *C. rubella* is due to inactivating mutations in the *CNL1* gene coding for cinnamate-CoA ligase, with all tested *C. rubella* accessions harboring one of two independently arisen inactive haplotypes (Sas et al., 2016; Jantzen et al., 2019a; Wozniak et al., 2022). CNL1 catalyzes the first committed step toward biosynthesis of benzenoids via the peroxisomal β-oxidative pathway. Similarly, the loss of β-ocimene emission in *C. rubella* results from inactivation of a key biosynthetic enzyme, TERPENOID SYNTHASE2 (TPS2) (Wozniak et al., 2022). In other plant species, the emission of BAld has been reported to be attractive to two types of pollinators (e.g., bees and syrphid flies) and herbivores (Theis, 2006). Likewise, β-ocimene is an effective attractant for bumblebees and honey bees (Pecetti et al., 2002; Granero et al., 2005) and has been proposed to act as a generalist pollinator attractant, given its high emission by plants with a generalist pollination syndrome (Filella et al., 2013). However, to our knowledge, the requirement for either of these compounds for efficient pollinator attraction has not been studied by selective manipulation their level of emission. Here, we have begun to address this with genetic recombinants generated as part of the original mapping of the two causal loci. If recombinants are available where the crossing-over events occurred on either side of the causal locus, these can be combined to generate quasi-isogenic lines (qILs), which differ only in a small chromosomal segment between the two recombination breakpoints; each side of the segregating segment is fixed. For *CNL1*, such a line has been developed, segregating for only 10 kb around the gene; a similar line is available for *TPS2*, albeit segregating for a larger segment of around 200 kb. Both lines have most of their remaining genome derived from *C. rubella*.

In this study, we have combined these lines to generate a segregating population that includes four subpopulations that only differ in the two small causal *CNL1* and *TPS2* intervals and thus in their BAld and β-ocimene emission, but should be otherwise as phenotypically similar as possible, largely resembling *C. rubella*. In other words, we have specifically restored (albeit only partially) the emission of BAld and/or β-ocimene to a plant with a selfing-syndrome phenotype to study the effects of the scent compounds in isolation and their interaction. Since this system does not use transgene technology, we could examine the attractiveness to pollinators and the maternal outcrossing rates of these four genotypes in a common-garden setting similar to the natural environment. We specifically addressed two questions: (1) Does the restoration of BAld and/or β-ocimene emission to plants with a selfing-syndrome phenotype attract more pollinators? (2) How does the emission of BAld and/or β-ocimene affect the attraction of different pollinator groups in *Capsella* and the resulting outcrossing rates?

MATERIALS AND METHODS

Construction of scent-manipulated genotypes

The crossing scheme is illustrated in Appendix S1. We used two *Capsella* qILs (lines 11.3.2_R and 11.3.6_G), which only differ in about 10 kb around the *CNL1* locus that controls the emission of benzaldehyde (BAld, see Fig. S2 of Sas et al., 2016), and two near-isogenic lines, which differ in approximately 200 kb around the *TPS2* locus causing the loss of β-ocimene emission (Wozniak et al., 2022) (lines TPS02-I-234 with a recombination breakpoint between scaffold_7:9,330,436 and scaffold_7:9,354,677 and TPS02-III-145 with a recombination breakpoint between scaffold_7:9,452,872 and scaffold_7:9,569,768). The genetic background of the two lines was largely derived from near-isogenic lines after backcrossing to *C. rubella* for several generations. Plants with the *C. grandiflora* alleles at the *CNL1* or *TPS2* loci emit BAld or β-ocimene, respectively, while plants with the *C. rubella* alleles do not (Sas et al., 2016; Wozniak et al., 2022).

Seeds of the four lines were sown and germinated on 1/2 Murashige-Skoog (MS; Duchefa Biochemie, Haarlem, Netherlands) agar supplemented with 0.5 mM gibberellic acid. After about 7 days, seedlings were transplanted to soil in 10-cm pots and grown in a growth room under 120 μE/m^2 of light with 16-h light at 21°C/8-h dark at 16°C. The same conditions were also used for seedling and plant growth for headspace floral scent collection, floral trait measurement, and hand-pollination experiments. Plants from line 11.3.6_G were crossed with plants from line TPS02-I-234 (Cross 1a). Similarly, plants from line 11.3.2_R were crossed with TPS02-III-145 (Cross 1b). The F1 plants from Cross 1a and Cross 1b were then intercrossed (Cross 2). The F1 plants from Cross 2 were genotyped with two groups of markers: B_Cla129, B_Cla141 and B_Cla145 for the *CNL1* locus and oNW273/274, oAS1653/1654 and oAS1629/1630 for the *TPS2* locus (Appendix S2) (Sas et al., 2016; Wozniak et al., 2022). The desired genotype is fixed for the flanking markers B_Cla145 and B_Cla141, but remains heterozygous for B_Cla129 at the *CNL1* locus. Similarly, the desired genotype is fixed for the flanking markers oAS1653/1654 and oAS1629/1630, but still heterozygous for oNW273/274 in the *TPS2* interval. Two plants with this genotype were obtained in the F1 from Cross 2. These were allowed to self, and seeds were harvested for the next round of genotype selection.

To examine the effect of BAld, β-ocimene and their interaction on pollinator preference in the common-garden experiment (described later), we used markers B_Cla129 and oNW273/274 again to select for homozygous genotypes at both *CNL1* and *TPS2* loci in the F2 populations from the two focal F1 plants from Cross 2. These are the four scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2* and *cnl1*/*tps2*, where uppercase letters indicate loci from *C. grandiflora*, and lowercase indicate ones from *C. rubella*. Importantly, any additional heterozygous chromosome segments present in the two focal F1 plants from Cross 2 that are not linked to the *CNL1* and *TPS2* loci will segregate independently from them in the progeny, and the alleles at such additional segments should be evenly distributed between the four subpopulations of interest with alternative homozygous *CNL1* and *TPS2* genotype combinations. As such, the four selected subpopulations should only differ for the intervals around the two loci.

For the common-garden experiment, a self-compatible *C. grandiflora*-like line with different background genotypes was used as the pollen donor to enable progeny-based estimates of outcrossing (Sicard et al., 2016; described later).

Headspace floral scent collection and analysis

Volatile compounds emitted by plants were collected using a dynamic headspace sampling method as described by Edens-Meier et al. (2014). To adapt this method to the small organ size in *Capsella*, we designed a sampling chamber made of a polypropylene petri dish (10 mL). The sampling chamber has three openings on the side; two are attached to a vacuum pump to provide a constant charcoal-filtered air flow of 150 mL/min, which is adjusted by a flowmeter (Rotameter, Germany), and the inflorescence was passed through the other opening to collect the volatiles from living flowers. The emitted volatiles were collected by absorbent tubes filled with 50 mg Tenax (matrix Tenax TA, 60–80 mesh, Merck KGaA, Darmstadt, Germany) and sealed at both ends. To control any background interference from the air, a corresponding air sample was collected as a blank before each volatile collection. We numbered the collection devices and sampled the air for 3 h on the same day before the 3-h floral volatile sampling. The amount of BAld and β-ocimene emitted by the flowers was then calculated by subtracting the respective volatile amount in the air samples collected on the same collection device on the same date from the volatile amount in the floral headspace sample. Between 10 and 18 individuals from each of the four focal genotypes were sampled, and the number of flowers used per sample was recorded for the following quantitative analysis. We also collected floral volatiles from five individuals of the SC *C. grandiflora* (SC-*Cg*) line and from five outbred *C. grandiflora* individuals (population Cg-9; Josephs et al., 2015) as a BAld and β-ocimene emission control. Headspace air and floral volatile samples were collected between approximately 08:30 and 14:30 hours.

The collected scent samples were analyzed by coupled gas chromatography-mass spectrometry. We used a Gerstel thermal desorption unit (TDU) coupled to a PTV injector (both GERSTEL GmbH & Co., Mülheim, Germany), which served to desorb volatile flower-derived compounds from the Tenax TA adsorbent. The desorption started at 30°C, held for 1 min, then raised to 200°C at 100°C min^{-1} and kept at 200°C for 3 min. Helium (70 mL min^{-1}) carried the analytes in splitless mode during the heating period to the PTV unit kept at −75°C. At the end of the desorption

period, analytes were injected by heating the PTV from 12°C s^{-1} up to 220°C into a gas chromatograph (GC) Agilent-7890A (Agilent Technologies, Clara, CA, USA). The PTV conditions (with helium as carrier) were solvent vent mode, septum purge flow: 3 mL min^{-1}, purge flow to split vent: 70 mL min^{-1} at 1.01 min, vent flow: 30 mL min^{-1} with 0.48746 bar until 0.01 min, splitless time: 1 min. The GC was equipped with an HP-5ms column (Agilent Technologies) with a 30 m × 0.25 mm internal diameter and a 0.25 µm film thickness. The GC oven was programmed as 40°C for 4 min, 10°C min^{-1} to 150°C held for 1 min, then 50°C min^{-1} to 240°C and held for 10 min. Helium served as carrier gas at a constant flow rate of 1 mL min^{-1}. The GC was coupled to an Agilent-5975C mass spectrometer (MS). The MS was operated in electron-impact ionization mode (70 eV). Mass spectra were recorded scanning a range from 35 to 350 *m/z*. Compounds were identified using the Varian Workstation software with the NIST05 mass spectral library and verified using retention times of authentic standards from Sigma-Aldrich (St. Louis, MO, USA). BAld and β-ocimene emission rates were calculated as their peak area in the GC-MS total ion current chromatogram divided by the number of flowers enclosed in the headspace collection chamber and the sampling time (h).

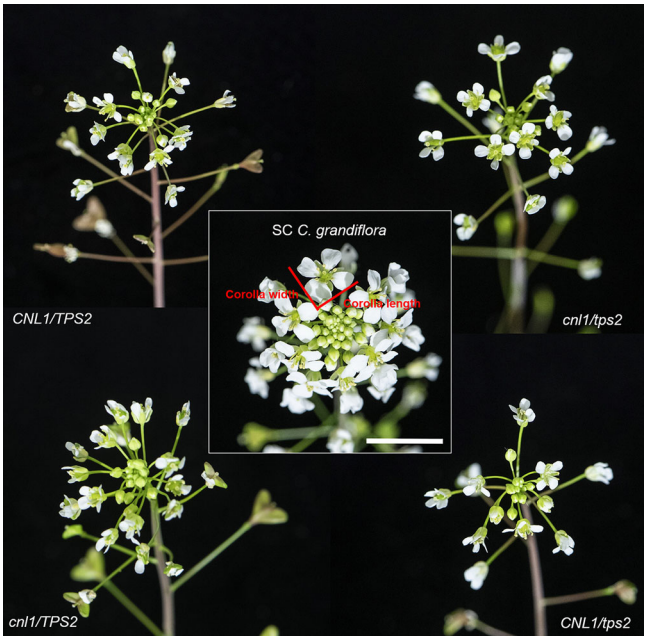

FIGURE 1 Flowers of pollen donor, self-compatible (SC) *Capsella grandiflora*, and scent-manipulated genotypes *CNL1/TPS2*, *CNL1/tps2*, *cnl1/TPS2*, and *cnl1/tps2*. Scale bar = 10 mm.

Floral trait measurement

To compare floral traits among the four scent-manipulated genotypes and self-compatible *C. grandiflora* (SC-*Cg*), we measured one flower each from randomly labeled 20 plants per genotype (*CNL1/TPS2*, *CNL1/tps2*, *cnl1/TPS2* and *cnl1/tps2*) and SC-*Cg*. We measured the length and width of the opened corolla when viewed facing the flower (Figure 1) and of petals, the height of stigma, and the length of the floral tube with digital calipers to an accuracy of 0.01 mm. We also harvested 15 flower buds per genotype to estimate pollen production. The whole flower bud was put in a 1.5-mL centrifuge tube, finely ground using a plastic grinding rod and suspended in 500 µL 5% v/v Tween 80. Pollen grains of three 5-µL drops were counted using a light microscope. The final pollen production per flower was calculated as the average number of pollen grains per drop multiplied by 100.

Field experiment and pollinator observation

Selfed seeds of the two focal F1 plants from Cross 2 and the SC-*Cg* line were germinated on 1/2 MS medium supplemented with 0.5 mM GA. After about 7 days, one cotyledon was removed from each seedling and used for DNA extraction and PCR genotyping for markers B_Cla129 and oNW273/274. Plants with homozygous genotypes at both loci were transplanted to soil in 10-cm pots and grown in a growth room under 120 µE m^{-2} of light with a 16-h light at 21°C/8-h dark at 16°C.

In mid May 2021, when the seedlings were around 4 weeks old, around 40 seedlings per genotype (*CNL1/TPS2*, *CNL1/tps2*, *cnl1/TPS2*, and *cnl1/tps2*) and 100 seedlings of the SC-*Cg* line were transferred to the Botanical Garden of the University of Potsdam just before they started to flower.

We set up four 2 m × 2 m experimental arrays in four sampling plots each 10 m away from the others. Twenty individuals of SC-*Cg* were assigned to the center of each array as pollen donors, and one of the four scent-manipulated genotypes was randomly assigned to one of the four corners (clockwise, plot1: *CNL1/tps2*, *cnl1/tps2*, *CNL1/TPS2*, and *cnl1/TPS2*; plot2: *cnl1/tps2*, *cnl1/TPS2*, *CNL1/tps2*, and *CNL1/TPS2*; plot3: *cnl1/TPS2*, *CNL1/tps2*, *CNL1/TPS2*, and *cnl1/tps2*; plot4: *cnl1/TPS2*, *CNL1/TPS2*, *cnl1/tps2*, and *CNL1/tps2*), with seven individuals per genotype (because only around 30 individuals survived per genotype until the start of flowering); as a result, there were 28 potential pollen recipients of the focal genotypes and 20 pollen donors per plot. On 14 June 2021, when all plants were flowering, we removed any developing siliques on the four genotypes of interest and placed them into the arrays for 2 weeks. During this time, we determined pollinator visitation rates (see below). At the end of the 2 weeks, we removed any unopened buds and inflorescence meristems and placed the plants in a sheltered location in the Botanical Garden. When the upper fruits started to ripen, we removed any remaining flowers that had opened after the end of the 2-week period, and the whole plants were bagged and left in the pots to ripen for another 2 weeks. Seeds from all flowers on the same individual were pooled and cleaned. To be able to define parental

haplotypes, we also collected leaf material from the SC-*Cg* line and the four scent-manipulated genotypes and subjected them to the same analysis as the seed samples.

Pollinators were observed for a total of 10 h between 09:00 to 17:00 hours on sunny days during the peak flowering time from 18 to 23 July 2021 (4 h on 18 July, 3.5 h on 21 July, and 2.5 h on 23 July). To obtain one sample of the visitation rate, the number of visits by all visitors of different categories was recorded during one 15-min observation period (40 total periods). All plants of one plot (7 individuals per genotype for four scent-manipulated genotypes and 20 individuals of SC-*Cg*) were observed in parallel during one 15-min observation period. The visitation rate per flower per hour was estimated as the number of visits per observation period multiplied by four and divided by the number of observed flowers.

The seeds of the remaining genotyped homozygous plants that had not been transferred to the Botanical Garden (see above) and from the SC-*Cg* line were harvested after selfing for later volatile collection experiments and measurements of floral morphology. These plants were again grown as above, but kept in the growth room under 120 $\mu E/m^2$ of light with a 16-h light at 21°C/8-h dark at 16°C until flowering, when they were used for headspace collection, floral trait measurements and pollination treatments as described above and below.

Pollination treatment

As mentioned above, the genetic background of the four scent-manipulated genotypes is largely derived from *C. rubella*, while the genotype of the pollen donor is largely derived from *C. grandiflora*. Crosses between *C. rubella* and *C. grandiflora* have been reported to result in seed abortion due to endosperm cellularization defects (Rebernig et al., 2015). If such seed abortion occurred in crosses between our genotypes of interest, then the number of developing outcrossed seeds would not reflect the rate of cross-pollination. Therefore, we tested for the presence and strength of a hybridization barrier between the four scent-manipulated genotypes and the SC-*Cg* line.

We randomly selected 20 flower buds on 10 different individuals for each of the four scent-manipulated genotypes, divided them in two treatment groups (10 buds per group): (1) untreated flowers as control; (2) flowers were emasculated and hand-pollinated with pollen from the SC-*Cg* line. For the SC-*Cg* line, we randomly selected 100 flower buds on 20 different individuals for treatment as follows: (1) 20 untreated flowers as control; (2) 80 flowers were emasculated and 20 were hand-pollinated with pollen from each of the scent-manipulated genotypes. When fruits matured, labeled fruits were harvested to count the number of developed and aborted ovules to calculate seed set as number of developed ovules/(number of developed ovules + number of aborted ovules).

Outcrossing rate estimation

The outcrossing rate was estimated using the protocol of Jantzen et al. (2019b). From each of seven individuals for the four scent-manipulated genotypes and the SC-*Cg* line per sampling plot, approximately 300 progeny seeds per individual were counted and pooled, then DNA was extracted from the pooled seeds using the Qiagen DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany); thus, a total of 28 samples per genotype was processed. Eleven pairs of polymorphic markers designed on conserved sites within the *Capsella* genus (see primer sequences of Jantzen et al., 2019b) were used to amplify the gene-specific sequences. An indexing PCR was then conducted to add indices and i5/i7 sequences to the corresponding ends of the amplicons from the first PCR amplification (Jantzen et al., 2019b). The indexed outputs were normalized and pooled using the protocol of Gohl et al. (2016) and then sequenced on a HiSeq2000 sequencing platform at Novogene (Novogene Co., Cambridge, UK).

Correct read pairs were confirmed by checking for the presence of i5/i7 sequences at the end of reads using cutadapt version 2.1 (Martin, 2011). From these reads, the forward and reverse primer sequences were removed, and only fragments corresponding to the expected length (around 300 bp) were kept and treated as haplotypes later. The sums of fragments per sample and amplicon were used as a baseline to calculate the proportions of nonmaternal haplotypes per sample and amplicon. Given the diploid nature of the plants, the frequency of nonmaternal haplotypes has to be multiplied by two to obtain an estimate for the fraction of outcrossed seeds. The outcrossing rate of a single seed DNA sample was calculated as the average of outcrossing rate estimated from nine informative amplicons (see below). For each genotype, seeds samples from 23 or 24 individuals could be successfully analyzed (see below). Data analyses were done using R version 4.2 (R Core Team, 2022). Illustrations were done using the R package Lattice (http://lmdvr.r-forge.r-project.org; Sarkar, 2008).

Data analyses

To compare floral traits among all genotypes, we used a generalized linear model (GLM) with normal distribution and identity link function (data were ln-transformed before analysis to achieve normal distribution, except for flower number and pollen production, which were compared using a GLM with Poisson distribution and log linear link function). To compare number of visits among all genotypes, we used a GLM with Poisson distribution and log linear link function, with number of visits per observation period (15 min) as the response variable and the natural log of flower number as an offset in the model (Zuur et al., 2009). To further examine whether pollinator groups show different visitation preference to the four scent-manipulated genotypes, we used a GLM with Poisson distribution and log linear link function, with the ability to

emit BAld and β-ocimene and their interaction as fixed factors, number of visits by each pollinator group per bout (15 min) as the response variable and the natural log of flower number as an offset in the model. To compare seed set between different pollination treatments, we used a GLM with binomial distribution and logit link function for each genotype separately. To compare the outcrossing rate among four scent-manipulated genotypes, we ran a generalized linear mix model (GLMM) with normal distribution and identity link function and sampling plot as a random factor (there were only few samples per genotype in each sampling plot, so we did not compare the outcrossing rate in each sampling plot separately). The least-significant difference method was used for multiple pairwise comparisons to determine significant differences.

Because β-ocimene is not detected in floral samples of *CNL1*/*tps2* and *cnl1*/*tps2*, only the emission rate BAld of four scent-manipulated genotypes was compared with 0 using a one-sample *t*-test (single tailed) separately to examine whether plants emitted BAld as their genotypes predicted. To compare the emission rate of BAld among scent-manipulated genotypes, a GLM with normal distribution and identity link function was used. To compare the emission rate of BAld and β-ocimene among scented genotypes (for BAld, *cnl1*/*tps2* and *cnl1*/*TPS2* were not included; for β-ocimene, *CNL1*/*tps2* and *cnl1*/*tps2* were not included), SC and WT *C. grandiflora*, we used a GLM with normal distribution and identity link function. The least-significant difference method was used for multiple pairwise comparisons to determine significant differences.

GLM analyses and one-sample *t*-tests were conducted in SPSS version 20.0 (IBM, Armonk, NY, USA).

RESULTS

Construction of scent-manipulated genotypes

From 87 F1 plants of Cross 2, we identified two individuals with the desired genotype, i.e., heterozygous for both *CNL1* and *TPS2* loci and homozygous at the flanking regions of both loci. We then screened 728 selfed progeny seedlings of these two plants for homozygous genotypes at both target loci, and 42 *CNL1*/*TPS2*, 47 *CNL1*/*tps2*, 44 *cnl1*/*TPS2*, and 44 *cnl1*/*tps2* individuals were obtained for respective frequencies of 5.8%, 6.5%, 6.0%, and 6.0%, very close to their predicted frequency of 6.25%. The frequencies of the seedlings with homozygous *CNL1*, *cnl1*, *TPS2* and *tps2* loci were 24.7%, 26.4%, 22.0%, and 25.4%, respectively, consistent with their predicted frequencies of 25.0%, indicating that there is no segregation distortion in the population.

Floral scent

BAld with a retention time of around 9.46 min was detected in all samples including the air blank, suggesting its widespread presence in the air. Therefore, we subtracted the BAld amount in the blank air sample collected on a given collection device and day from the BAld amount in the floral headspace sample collected immediately after to determine the BAld emission by the flowers and divided this value by the number of flowers sampled and the collection time (Appendix S3). The resulting emission rate of BALD in flowers from the *cnl1*/*TPS2* and *cnl1*/*tps2* genotypes was not significantly higher than 0 (one-tailed *t*-test, $t = -0.701$, df = 16, $P = 0.247$ and $t = -0.483$, df = 17, $P = 0.318$, respectively, and both measures also did not deviate from 0). By contrast, both *CNL1*/*TPS2* and *CNL1*/*tps2* genotypes emitted BAld, with emission rates significantly larger than 0 (one-tailed $t = 3.394$, df = 9, $P = 0.004$, and $t = 3.994$, df = 14, $P = 0.001$, respectively) and also larger than that for *cnl1*/*TPS2* and *cnl1*/*tps2* (Wald $\chi^2 = 32.929$, $P < 0.001$), as predicted by their genotypes. The BAld emission in the SC-*Cg* line was significantly higher than that from the *CNL1* genotypes (*CNL1*/*TPS2* and *CNL1*/*tps2*) and did not differ significantly from that in outcrossed *C. grandiflora* (Figure 2; Wald $\chi^2 = 26.133$, $P < 0.001$).

Two peaks of β-ocimene were observed in floral scent samples but not in the air control at a retention time of around 10.96 min and 11.17 min, representing *cis*- and *trans*-β-ocimene, respectively. In scent-manipulated genotypes, β-ocimene was not detected in samples from flowers of *CNL1*/*tps2* and *cnl1*/*tps2* but was present from flowers of both *CNL1*/*TPS2* and *cnl1*/*TPS2* (Figure 2), consistent with the prediction from their genotypes. The emission rate of β-ocimene emission was not significantly different between the SC-*Cg* line and the *CNL1*/*TPS2* and *cnl1*/*TPS2* genotypes, but all were lower than that from the outcrossed *C. grandiflora* (Figure 2, Wald $\chi^2 = 14.834$, $P = 0.002$).

In conclusion, we used our nontransgenic *Capsella* materials to build an effective scent-manipulated system that consists of four near-isogenic subpopulations that only differ in their genotypes for BAld and β-ocimene emission. Their emission phenotypes closely follow their genotypes, making these lines suitable for testing pollinator preference for floral scent in a common-garden setting.

Floral traits

The floral morphology and other floral traits of the four scent-manipulated genotypes were compared between each other and with the SC-*Cg* line. For these measurements, we used F3 plants derived from genotype-selected F2 plants. Flowers of the four scent-manipulated genotypes were fewer and smaller than those of the SC-*Cg* line (Figure 3), with lower values of all measured traits including number of opening flowers per inflorescence, flower size, petal size, and pollen production. The visual signals of the genotyped individuals clearly differed from flowers of the SC-*Cg* line (Figure 1). Floral traits either did not differ significantly between the four scent-manipulated genotypes, or they differed by less than 20% (Figure 3); an exception was pollen

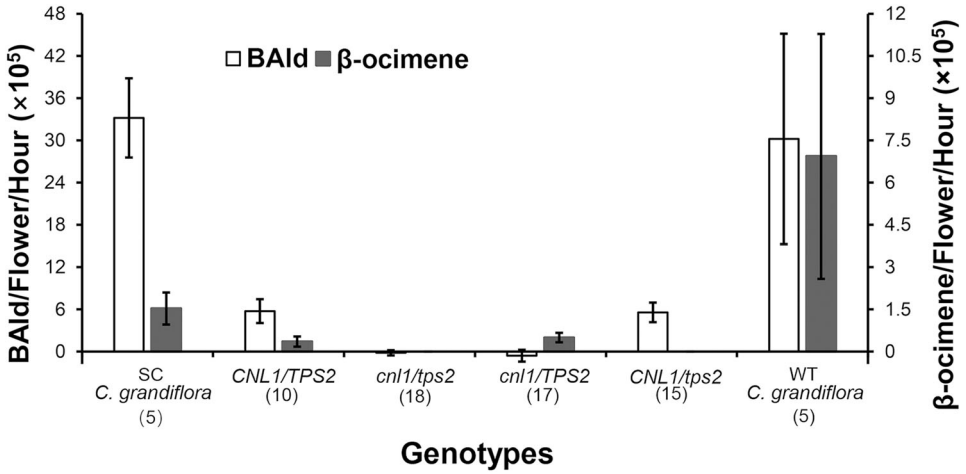

FIGURE 2 Benzaldehyde (BAld) and β-ocimene emission in self-compatible (SC) *Capsella grandiflora*, four scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2*, and *cnl1*/*tps2* and outcrossed *C. grandiflora* (WT *C. grandiflora*). High variance among outcrossed *C. grandiflora* likely reflects the high level of genetic variation in this outbred species. BAld and β-ocimene emission rate was calculated as peak area in the GC-MS spectrum divided by sampling flower number and time. Values in parentheses indicate number of samples for GC-MS analysis.

number that differed by 23% between the lines with the highest and lowest values. Thus, consistent with their genetic background being mostly derived from *C. rubella*, the four focal genotypes form very similar flowers that resemble those of their selfing parental lines.

Pollinators and visitation rates

A total of 615 floral visits were recorded by six groups of insect visitors (Table 1; Appendix S4), including 55 visits from three fly species (*Cylindromyia brassicaria*, *Phania funesta*, *Dinera grisescens*), 117 from a hover fly species (*Sphaerophoria scripta*), 192 from solitary bees (*Ceratina* sp. and *Sphecodes* sp.), 123 from wasps (*Ectemnius* sp. and *Chrysis* sp.), 106 from honey bees (*Apis mellifera*), and 22 from butterflies (*Coenonympha pamphilus*). All these visitors were seen exploring nectar and/or pollen on the flowers after landing (Figure 4). The mean visitation frequency (±SE) to flowers of SC-*Cg* and the four scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2*, *cnl1*/*tps2* was 0.048 ± 0.013, 0.050 ± 0.018, 0.017 ± 0.007, 0.055 ± 0.020 and 0.043 ± 0.015, respectively, and no significant difference among them was found (Table 2), implying no preference of pollinators at an overall level for the flowers of different genotypes with differing floral scent emission. However, when the visitation frequency data were compared separately by pollinator groups, butterflies visited plants emitting BAld more frequently than they did the *cnl1* mutant genotypes, while flies had the opposite behavior and avoided BAld-emitting plants (Tables 1 and 2). Hoverflies, wasps, and butterflies visited plants emitting β-ocimene more frequently than those that did not (Tables 1 and 2). Interestingly, solitary bees, the most frequent pollinators, showed no visitation preference to plants with BAld and/or β-ocimene emission (Table 2). Since solitary bees, hoverflies, and flies contributed the most visits to the four scent-manipulated genotypes, their opposite visitation preference to BAld and/or β-ocimene emitting plants may explain the lack of significant difference in visitation frequencies to the four genotypes when analyzing the combined data for all pollinators.

Pollination treatments

All flowers from the four scent-manipulated genotypes and the SC-*Cg* line could set a high percentage (>70%) of seeds if left untreated (Table 3). The outcrossing pollination treatment did not significantly decrease or affect the seed set in the four focal genotypes, when they received pollen from the SC-*Cg* line (Table 3). For SC-*Cg* line, we outcrossed them with pollen from the four focal genotypes, and again no significant effect on seed set was found (Table 3). These results indicate that there are no hybridization barriers between our five genotypes of interest, ensuring that the rate of cross-pollination in our common-garden experiment can be reliably estimated from the genotypes of the developed seeds.

Outcrossing rates

In total, we harvested seeds from 28 individuals per genotype, from seven plants each for all four sampling plots. Due to failure of DNA extraction and very low representation in the sequencing libraries for some seed samples, we obtained useable results for 23 *CNL1*/*TPS2* and SC-*Cg* plants and for 24 plants each for the other three genotypes (see Appendix S5). All 11 primer pairs for gene-specific sequences amplified well (Appendix S2). However, for two amplicons (amplicons Carubv10023818m and Carubv10005658m, see Appendix S5),

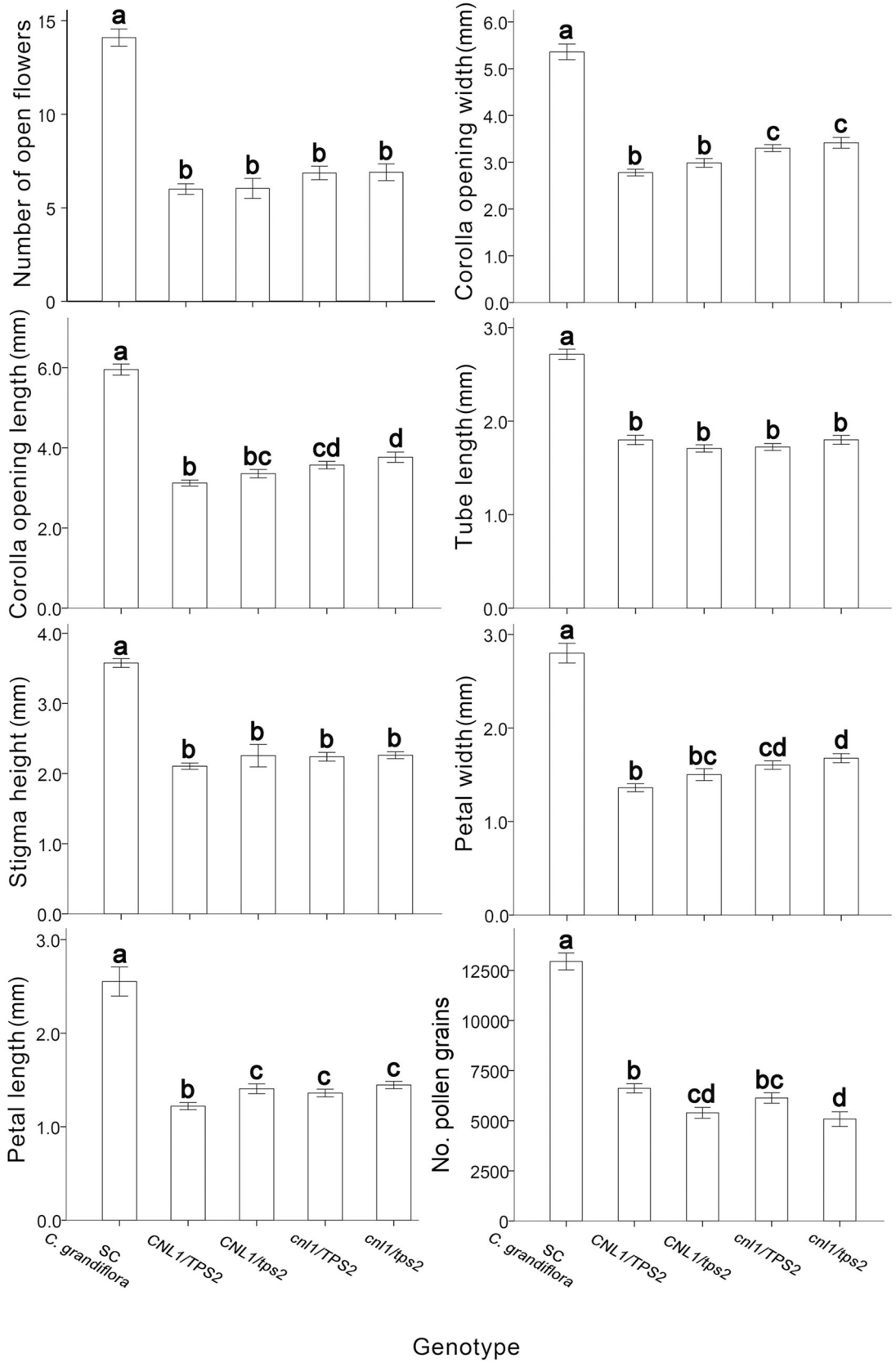

FIGURE 3 Means (±SE) for floral traits self-compatible *Capsella grandiflora* (SC-Cg) and four scent-manipulated genotypes (*CNL1/TPS2*, *CNL1/tps2*, *cnl1/TPS2* and *cnl1/tps2*). Different lowercase letters following mean values indicate significant differences among five groups using the least-significant difference method ($P < 0.05$). $N = 20$ flowers for each genotype.

TABLE 1 Mean (±SE) number of visits of different pollinator groups and visitation frequency (visits per flower per hour) to self-compatible (SC) *Capsella grandiflora* (SC-*Cg*) plants and scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2*, and *cnl1*/*tps2*. No significant difference in visitation frequencies among the five genotypes was found using a generalized linear model (Wald $\chi^2 = 2.319$, $P = 0.667$).

Pollinator group	SC-*Cg*	*CNL1*/*TPS2*	*CNL1*/*tps2*	*cnl1*/*TPS2*	*cnl1*/*tps2*
Flies	20	6	0	11	18
Hover flies	59	15	13	28	2
Solitary bees	86	34	14	21	37
Wasps	93	7	1	21	1
Honey bees	101	4	1	0	0
Butterflies	10	9	2	1	0
Total	369	75	31	82	58
Visitation frequency	0.048 ± 0.013	0.050 ± 0.018	0.017 ± 0.007	0.055 ± 0.020	0.043 ± 0.015

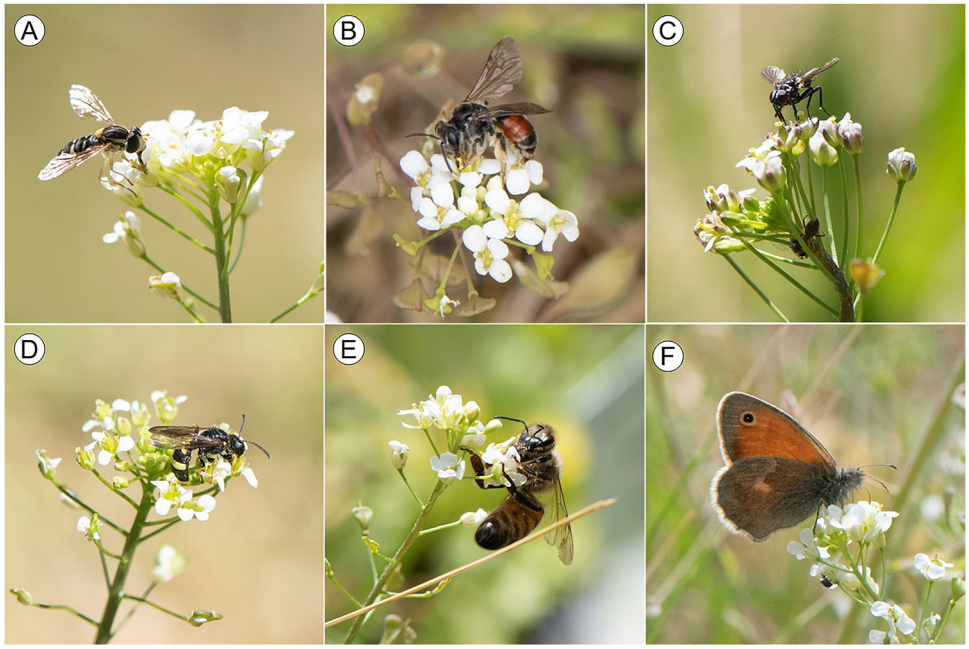

FIGURE 4 Pollinators visiting flowers of (A, B) self-compatible *Capsella grandiflora* plants and (C–F) scent-manipulated genotypes. (A) *Sphaerophoria scripta* (Syrphidae); (B) *Sphecodes* sp. (Halictidae); (C) *Phania funesta* (Tachinidae); (D) *Ectemnius* sp. (Crabronidae); (E) *Apis mellifera* (Apidae); (F) *Coenonympha pamphilus* (Nymphalidae).

the four scent-manipulated genotypes and the SC-*Cg* line shared a haplotype; therefore, these two amplicons were excluded from further analysis.

Across the remaining nine amplicons, the frequencies of nonmaternal haplotypes derived from the SC-*Cg* line as pollen donor were largely consistent for the individual samples (see Appendix S5), supporting the use of the mean value across all amplicons as the estimate for the sample-specific outcrossing rate. This value varied greatly across the individual samples and was unexpectedly high in many of them (see Appendix S5), in contrast to the low visitation frequencies in our previous pollinator observations (Table 1). In the generalized linear mixed model used, no significant effect of sampling plot (random factor) was found ($Z = 1.015$, $P = 0.310$), and more importantly, no significant difference in outcrossing rate was found between the four scent-manipulated genotypes (Figure 5; $F_{3,91} = 0.728$, $P = 0.538$), consistent with the lack of a significant difference in pollinator-visitation frequency in the previous observations. In summary, flowers of the four scent-manipulated genotypes differ in the emission of BAld and β-ocimene, and some groups of pollinators in our common-garden setting discriminated among them. However, at an overall level across all pollinators the visitation frequencies to the four genotypes did not differ significantly; thus, the estimates of outcrossing rates did not differ among them.

TABLE 2 Results of generalized linear models to test whether total pollinator visits differed between plant genotypes SC-*C. grandiflora* and scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2*, and *cnl1*/*tps2*, and whether number of visits of different pollinator groups to the four scent-manipulated genotypes are affected by BAld and β-ocimene emission and their interaction. The natural log of flower number is included as an offset in the model. Number of visits by honey bees were too few to be analyzed and the effect of BAld × β-ocimene interaction could not be examined in fly, wasp and butterfly visitation frequency comparisons because some convergence criteria are not satisfied in these analyses.

Source of variation	Wald χ^2	df	*P*
Total visits			
Intercept	944.839	1	0.000
Genotype	2.337	4	0.674
Fly visits to scent-manipulated genotypes			
Intercept	634.424	1	0.000
BAld	8.869	1	**0.003**
β-ocimene	0.337	1	0.562
Hover fly visits to scent-manipulated genotypes			
Intercept	473.901	1	0.000
BAld	1.006	1	0.316
β-ocimene	5.028	1	**0.025**
BAld × β-ocimene	3.348	1	0.067
Solitary bee visits to scent-manipulated genotypes			
Intercept	1005.853	1	0.000
BAld	1.831	1	0.176
β-ocimene	0.001	1	0.975
BAld × β-ocimene	0.187	1	0.666
Wasp visits to scent-manipulated genotypes			
Intercept	309.525	1	0.000
BAld	2.594	1	0.107
β-ocimene	8.997	1	**0.003**
Butterfly visits to scent-manipulated genotypes			
Intercept	439.888	1	0.000
BAld	11.715	1	**0.001**
β-ocimene	9.786	1	**0.002**

For the SC-*Cg* samples, the mean frequency of nonmaternal haplotypes was only 0.83 ± 0.18%, indicating that almost no pollen had been transferred from scent-manipulated flowers in the corners to the SC-*Cg* plants in the centers of the four arrays. Conversely, the mean frequency of haplotypes from the SC-*Cg* plants in the progeny of the scent-manipulated samples was relatively high (13.29 ± 0.96%), indicating a strongly asymmetric pollen flow among the genotypes, with most pollen grains transferred from the SC-*Cg* plants to the scent-manipulated genotypes, but not vice versa.

DISCUSSION

Using quasi-isogenic lines (qILs) generated as part of the original mapping of the two floral scent volatile-related loci *CNL1* and *TPS2*, we generated a segregating population that includes four subpopulations that differ in these two locus intervals. Most floral traits of these four subpopulations are similar, largely resembling the selfing species *Capsella rubella*, and thus are significantly smaller than the outbreeding *C. grandiflora*. The emissions of BAld and β-ocimene in floral scent detected in corresponding subpopulations were significantly higher than in the air blank, but still lower than the emissions in the reference species, *C. grandiflora*. In other words, we specifically (albeit only partially) restored the emissions of BAld and/or β-ocimene to a plant with a selfing-syndrome phenotype, helping us to study the effects of these scent compounds in isolation. However, when the four scent-manipulated genotypes were introduced to a common garden, neither the overall pollinator visitation frequencies to flowers nor the maternal outcrossing rates in their seeds differed significantly among the four subpopulations. When visitation frequencies were compared separately by pollinator groups, different pollinators had inconsistent or even opposite preference for BAld and β-ocimene, which may explain the lack of significant differences in the comparison of visitation frequencies and of outcrossing rates.

In our previous work in *C. rubella*, we identified several loci that are associated with the reduction in floral scent compared to *C. grandiflora*, including the *CNL1* and *TPS2* genes that govern the benzaldehyde (BAld) and β-ocimene emission by the flowers, respectively (Sas et al., 2016; Jantzen et al., 2019a; Wozniak et al., 2022). During the mapping of these two floral scent volatile-related loci, we had already generated two quasi-isogenic lines (qILs) (*CNL1* and *TPS2*), which only segregated for the small chromosomal segments around the corresponding gene; these qILs resemble *C. rubella* for most floral traits except for BAld and/or β-ocimene emission. By hybridizing these two qILs, we generated a segregating population that included four subpopulations, which only differed in the small intervals around these two loci and thus in their BAld and/or β-ocimene emissions (Figure 2). Most importantly, most floral traits of these four subpopulations are phenotypically similar (Figure 1); still, some significant differences were detected between some pairs (no more than 20%), which were much smaller than their differences with SC-*Cg* (about 100%, Figure 3). The detected differences may represent variation in the genomic background between the four F2 plants from which the F3 families used for phenotyping were derived, and so may not reflect differences between the four focal genotypes in the segregating F2 population. In summary, here we developed a reductionist system for only

TABLE 3 Mean (±SE) seed set between selfing and outcrossing treatments for self-compatible *Capsella grandiflora* (SC-*Cg*) and scent-manipulated genotypes *CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2*, and *cnl1*/*tps2*).

	Mean seed set ± SE (*N*)				
Genotypes	**Selfing**	**Outcrossing**	**Pollen donor**	**Wald χ^2**	***P***
SC-*Cg*	73.22 ± 5.44 (20)	70.82 ± 3.95 (11)	*CNL1*/*TPS2*	0.02	0.750
		57.71 ± 7.12 (10)	*CNL1*/*tps2*	0.03	0.059
		70.08 ± 3.92 (10)	*cnl1*/*TPS2*	0.15	0.672
		72.93 ± 3.74 (12)	*cnl1*/*tps2*	0.00	0.975
CNL1/*TPS2*	90.59 ± 3.66 (10)	91.08 ± 2.50 (12)	SC-*Cg*	0.001	0.981
CNL1/*tps2*	86.41 ± 3.23 (10)	87.12 ± 3.73 (12)	SC-*Cg*	0.040	0.842
cnl1/*TPS2*	85.64 ± 5.29 (10)	93.57 ± 1.65 (12)	SC-*Cg*	2.017	0.156
cnl1/*tps2*	87.95 ± 2.97 (10)	93.22 ± 2.28 (12)	SC-*Cg*	2.192	0.139

Notes: For SC-*Cg*, no significant difference in seed set among treatments was found (generalized linear model, Wald $\chi^2 = 4.419$, $P = 0.352$). Multiple comparisons between selfing and every outcrossing treatment were performed using the least-significant difference method, which are reported here as mean difference values instead of Wald χ^2 values.

assaying individual scent compounds and their interactions, while keeping other floral signals (display size and morphology) essentially constant. The genotypes in this system built by genetic mapping lines are heritable, allowing the system to be studied repeatedly; and most importantly, it does not use transgenes, making it compatible with common-garden experiments under largely natural environments even in countries with restrictive laws on release of GMOs.

When the plants were growing in the field site of the Botanical Garden of the University of Potsdam, insect visitors were seen exploring nectar and/or pollen on the flowers of all five genotypes (Figure 4). Although the pollen donor SC *C. grandiflora* received significantly more visitations overall than did the other four groups of scent-manipulated plants (*G*-test of goodness-of-fit, $G = 497.425$, $P < 0.001$), the huge number of flowers produced by SC-*Cg* greatly reduced the difference in per-flower visitation frequencies among them (Table 1), indicating the pollinators had no preference for the flowers with different floral scent emissions (Figure 2) in the field. The seed analysis of outcrossing rate also confirmed this lack of preference, showing no significant difference among the four focal genotypes (Figure 5, Wald $\chi^2 = 1.973$, $P = 0.578$). Since outcrossing rate analysis could only detect cross events in developed seeds, the original outcrossing rates would need calibration if four scent-manipulated genotypes had different seed abortion rates when receiving pollen from SC-*Cg*. However, all flowers from the four scent-manipulated genotypes set a high and similar percentage of hybrid seeds after crossing with SC-*Cg* (Table 3). This similar seed set indicates that there were no apparent hybridization barriers between these five genotypes, thus ensuring unbiased outcrossing rate estimates from using developed seeds from flowers in the common-garden experiment. In conclusion, although flowers of the four scent-manipulated genotypes differed in the emission of BAld and β-ocimene in their floral scent, overall pollinators do not appear to discriminate consistently among them, resulting in similar outcrossing rates for seed after open pollination.

Four possible reasons may explain the unexpected results above. First, pollinators may fail to sense the difference in BAld and/or β-ocimene emissions from the four scent-manipulated genotypes because of the abundant BAld and β-ocimene produced by SC-*Cg* individuals in the center of the study patches. Although three of the scent-manipulated genotypes emitted BAld and/or β-ocimene, their emission levels were lower (significantly so for BAld) than those in SC-*Cg* (Figure 2). The higher floral scent emissions in SC-*Cg* may have caused a high patch-wide baseline level for BAld and β-ocimene, which may make it difficult for pollinators to detect the difference in amount of BAld and/or β-ocimene emitted by the scent-manipulated plants in each of the corners against the background. In an open field, the olfactory environment is complex and filled with various natural volatile compounds, setting up a big challenge for pollinators to successfully discriminate and locate important scents (Thiery and Visser, 1986; Schröder and Hilker, 2008; Riffell et al., 2014), which are usually emitted by flowers as honest reward signals (Papaj and Lewis, 1993; Daly et al., 2001; Skiri et al., 2005; de Boer and Dicke, 2006; Dukas, 2008; Wright and Schiestl, 2009). For example, in *Datura wrightii* (Solanaceae), extra benzaldehyde (a volatile in *Datura* plume) mixed in the volatile background significantly decreased the ability of its pollinator moth, *Manduca sexta*, to locate the *Datura* floral scent plume (Riffell et al., 2014). In our common-garden experiment, a similar scenario may apply: SC-*Cg* filled the volatile environment in the patches with high levels of BAld and β-ocimene, which may have decreased the ability of the most often observed solitary bee pollinators to detect difference in BAld and β-ocimene emission levels in four scent-manipulated genotypes (Table 2). Although a "scented background" is likely the norm in natural populations, which suggests that our negative results are representative,

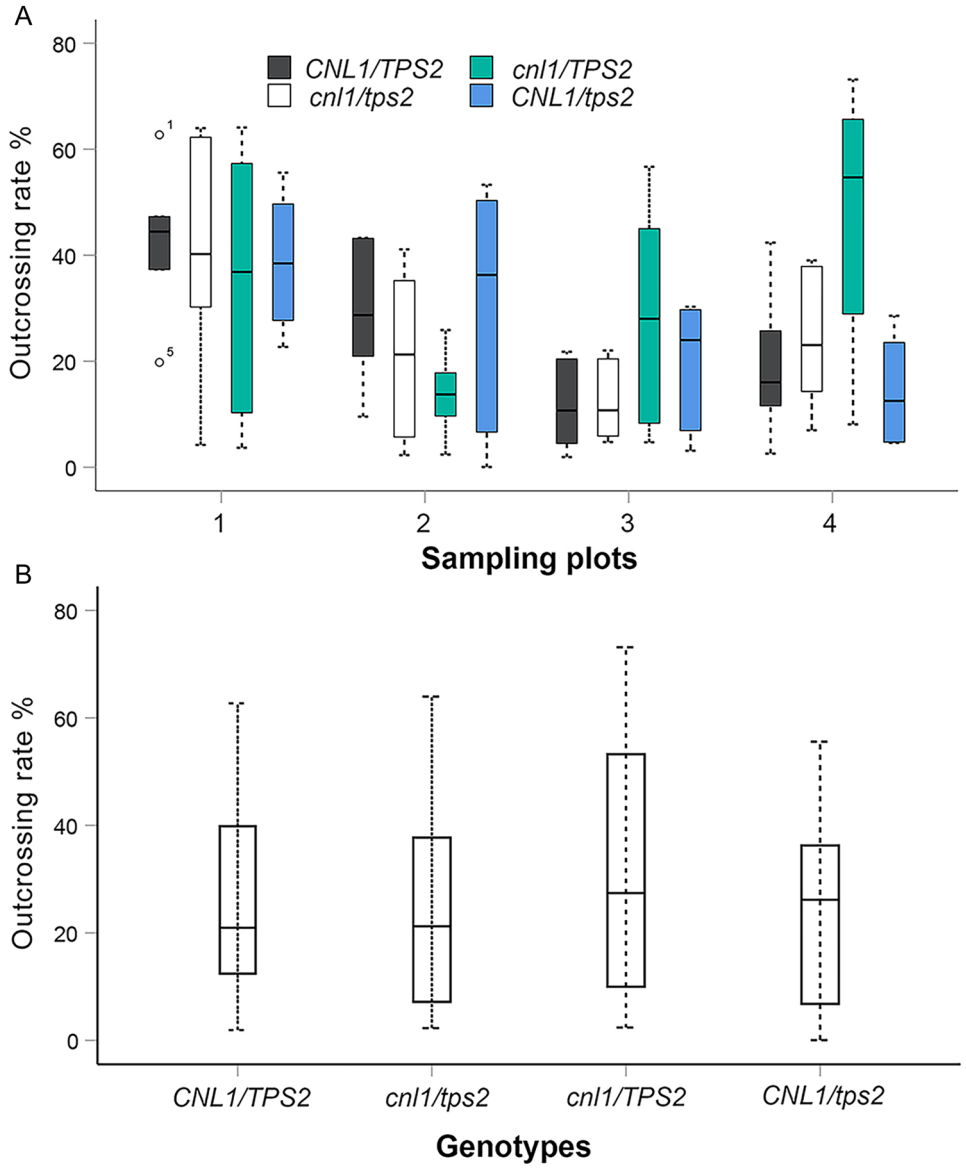

FIGURE 5 Outcrossing rate (%) between four scent-manipulated genotypes (A) in each sampling plot ($N = 6$, except for *CNL1/TPS2* in sampling plot 1: $N = 5$) separately and (B) using pooled data across the four plots ($N = 24$, except for *CNL1/TPS2*: $N = 23$). The box shows data from the first quartile to the third quartile. The horizontal line in the box indicates the median. The vertical bars go from each quartile to the minimum and maximum.

repeating the experiment with a pollen-donor line that does not emit BAld and β-ocimene may yet reveal overall pollinator discrimination between the four test genotypes.

Second, the inconsistent preference of different pollinator groups for BAld and β-ocimene scent may be responsible for the lack of significant differences in overall visitation frequencies to flowers and outcrossing rates among the four subpopulations. Of the six groups of insect pollinators observed, four had a significant preference for BAld or β-ocimene scent. While butterflies visited plants emitting BAld or β-ocimene more frequently than non-emitting ones, hoverflies and wasps visited plants emitting β-ocimene more frequently than the non-emitting, and flies avoided BAld-emitting plants (Table 2). In *Brassica rapa* (also from the Brassicaceae family), Gervasi and Schiestl (2017) also detected opposite directions of selection on floral volatile emission by different pollinator groups. In our study, the inconsistent or opposite preferences for BAld and β-ocimene between different insect groups appear to have cancelled out at the level of overall visitation rates. Combined with the absence of any detectable preference amongst solitary bees as the most frequent visitors, these inconsistent or opposite preferences may contribute to explaining the lack of a difference in outcrossing rates between the four genotypes.

Third, our failure to find a difference in pollinator attraction between the four focal genotypes could conceivably reflect a discrepancy between the distances over which visual versus odor signals act on pollinators, combined with the spatial arrangement in our plots. In other words, if olfactory signals acted at a closer distance than visual ones, pollinators

attracted to patches by visual signals over a long distance may distinguish and choose one of the four corners according to the volatile emission by the plants there. However, this scenario appears less likely based on what is known about the interplay of visual and olfactory cues in pollinator attraction in other systems. For example, for muscid and anthomyiid flies, floral attraction depends more on olfactory than visual cues (Roy and Raguso, 1997). For honey bees, colors as visual cues are only perceived from a short distance (Giurfa et al., 1996), so scent may be perceived before color information is available to the bees. In a butterfly-pollinated flower *Mussaenda frondosa*, the white, ultraviolet-absorbing bract played an important role in attracting the diurnal butterfly *Troides minos* (Papilionidae) at a long distance (Borges et al., 2003). In our study, solitary bees, hoverflies and flies were the main visitors to scent-manipulated individuals (Table 1); therefore, floral scent may have contributed a certain amount to attracting insects at a long distance (Giurfa et al., 1996; Roy and Raguso, 1997). However, flowers in each corner were similar in floral size including petal length and width and corolla opening length and width (Figure 3), possibly resulting in the lack of difference in visual signals when pollinators were close to the flowers and thus in visitation frequencies among these four scent-manipulated individuals. Repeating the experiment with different spatial arrangements of the four focal genotypes in the plots could be used to disentangle the distances over which olfactory cues act.

Fourth, the high pollen production in the pollen-donor genotype SC-*Cg* flowers may cause large numbers of pollen to be deposited on stigmas of scent-manipulated genotypes in just a few visits, diluting the potential difference in visitation frequency in the common-garden experiment and resulting in the lack of significant difference in outcrossing rates of seeds. Male siring success generally follows a diminishing curve and is not linearly correlated with stigmatic pollen deposition, usually with the increment decreasing gradually (Wilson et al., 1994). So, for an outcrossing rate analysis to duplicate the pattern of visitation frequency in a common-garden experiment, the SC-*Cg* pollen deposition (only ovules fertilized by pollen of SC-*Cg* with different background genotypes could be detected as outcrossing events) per visit should not be too large, which could slow down the diminishing of male siring success as mentioned above. Thus, compared with pollen transfer by intragenotype visits, higher pollen transfer from SC *C. grandiflora* (because of its high pollen production) per visit may interfere with the detection of differences in outcrossing rates, because a single visit could contribute to saturating deposition of hybrid pollen and dilute any differences in potential visitation frequencies in the common-garden experiment.

CONCLUSIONS

We were able to restore BAld and β-ocimene emissions to a plant with a selfing-syndrome phenotype to study the effects of the scent compounds in isolation; this reductionist system can be used to assay individual scent compounds while other floral signals are essentially constant. Most importantly, this system is transgene-free, making it compatible with common-garden experiments under largely natural environments. However, to better investigate the effect of the focal compounds on different aspects of the pollination process such as pollen flow, male and female fitness return, a pollen donor material with related genome background and similar BAld and β-ocimene emission levels is needed, which could be generated by screening the progeny of SC *C. grandiflora* hybridizing with selfing *C. rubella* in future. Also, adjustments to the spatial layout of the plots should be considered to address the distances over which different types of cues act.

AUTHOR CONTRIBUTIONS

M.L. and Y.-Z.X. devised this study. F.J., N.W., A.S., and Y.-Z.X. was responsible for the construction of scent-manipulated genotypes. Y.-Z.X. conducted the field experiments, scent collection, seed DNA extraction, and amplicon-based sequencing. L.H. analyzed the samples of floral scent. C.K. analyzed the high-throughput amplicon-based sequencing data. Y.-Z.X. conducted the statistical analyses. Y.-Z.X. drafted most of the manuscript and M.L. and S.-Q.H. revised the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

All data generated for this manuscript can be found in the online supplementary material (Appendices S1–S5).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Crossing scheme for construction of scent-manipulated genotypes (*CNL1*/*TPS2*, *CNL1*/*tps2*, *cnl1*/*TPS2* and *cnl1*/*tps2*).

Appendix S2. Primers for scent-manipulated individual genotyping and haplotype amplification.

Appendix S3. Peak area of BAld and β-ocimene in 3-h samples of floral scent and air headspace.

Appendix S4. Number of visits by different pollinator groups to flowers of the four scent-manipulated genotypes and SC *Capsella grandiflora* in 40 observations of 15 min each.

Appendix S5. Haplotype frequency for each amplicon.

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