FLOWER METABOLISM AND POLLINATORS

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FLOWER METABOLISM AND POLLINATORS

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EDITORIAL
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Editorial: Flower Metabolism and Pollinators

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Keywords: bio-communication, chemodiversity, florivory, floral volatiles, folivory, pollination

Editorial on the Research Topic

Flower Metabolism and Pollinators

Outcrossing species that use animals as vectors of pollen transfer lure pollinators by exhibiting floral chemical signals that stimulate their visual, olfactory, and gustatory apparatus. It is said that these traits undergo selective pressure exerted by pollinators according to their specific preferences. However, many of these specialized metabolites initially evolved to protect flowers toward abiotic and biotic stresses and were later co-opted to mediate plant–pollinator interactions. This Research Topic collects recent advances on the chemistry of floral traits, providing insights on the chemodiversity of scent composition and pigments, the intrinsic genetic factors controlling their phenotypical manifestation, as well as the climatic and biotic factors which influence them.

Coevolution between plants and insects is associated with increased biodiversity and both mutualistic and antagonistic interactions contribute to diversification of traits and species. Eilers et al. characterized a remarkable intraspecific variability in flower production, floral metabolic composition and pollen quality in *Tanacetum vulgare*, which affected floral attractiveness for florivorous beetles. Interestingly, not only the chemical phenotype of individuals determined the interaction frequency between the flowers and the antagonists, but also the chemotype of neighboring *T. vulgare* individuals contributed to the susceptibility to florivory. Powers et al. recorded the timing of floral scent emission of two endemic Hawaiian plant species, *Schiedea kaalae* and *S. hookeri*, that share pollination by an endemic Hawaiian moth. While the scent of both species bouquet differed in scent compounds, they shared the timing of peak emissions of floral scent, which coincided with the activity of the moth pollinator. Salzman et al. demonstrated that volatile compounds produced by closely related species of *Zamia* cycads are strikingly different from each other, and that two distantly related pollinating weevil species respond specifically to volatiles from their host Zamia species. The authors highlight chemical communication as a key mechanism of coevolution between cycads and their weevil pollinators. Braunschmid and Dötterl were interested in whether rarity in floral scent related to higher pollination success and fitness. They studied two populations of the deceptive orchid *Cypripedium calceolus* and tested whether flowers with rarer scent bouquets within these populations had more pollination success than flowers with more common scents. The authors found that rarity in floral scent was not correlated with the prospect of the plants setting fruits.

The studies of Sundaramoorthy et al. and Han et al. focus on the genetic and cellular mechanisms underlying the display of floral pigmentation and their spontaneous fading. Using soybean flowers as a model for anthocyanin biosynthesis, Sundaramoorthy et al. identified four recessive purple-blue EMS-induced mutants that all had increased pH in their petals compared to wild-type flowers. Via genetic mapping, the authors of this study showed that the MYB transcription factor GmPH4

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and the vacuolar P3A-ATPase GmPH5 gene concur to vacuolar pH regulation, therefore, control changes in petal pigmentation from light pink to dark blue-purple. A transcription factor of the MYB family (MhMYB10) was also associated with the petal pigmentation of *Malus halliana*, of which the color spontaneously fades after pollination had occurred. Interestingly, Han et al. observed a high level of DNA methylation in the promoter region of the MhMYB10 gene, which is associated with decreased expression of MhMYB10 and the downregulation of anthocyanin biosynthetic genes. Finally, Liu et al. by surveying twenty different *Brassicaceae* genotypes for the content of their major floral specialized metabolites via liquid chromatography-mass spectrometry (LC-MS), identified the metabolic features which better discriminate self-compatible (SC) from self-incompatible (SI) species. In particular, the authors identified phenylacylated-flavonoid, and five phenolamides were indicated as significant contributors to this discrimination providing new insights on floral specialized metabolism in relation to the environment and their divergent evolution under biotic/abiotic stresses.

Changes in land use alters habitat affecting the community composition of plants and their pests, along with beneficial insects such as pollinators and predators of herbivorous pests. Landscapes dominated by agriculture are frequently associated with lower diversity of pollinators and higher susceptibility to herbivore pests. Schroeder et al. synthesize evidence of changes in plant trait across land use gradients and discuss the potential for plant adaptation across agricultural landscapes. Their data from a common garden experiment on three wild *Brassicaceae* suggests variation in defensive and reproductive traits along an agricultural gradient. Climate change is also posing an increasing challenge for stability of plant-pollinator interaction. Höfer et al. investigated links between flower visitor behavior and floral traits in the context of increasing drought and temperature and found that the short-term water stress does not alter initial attraction of *Sinapis arvensis* flowers but negatively impacts bumblebees' visitation on flowers resulting in their inferior pollination services. Farré-Armengol et al. quantitatively summarize data on floral-scent emissions from more than 300 plant species exposed to adverse climatic conditions and further discussed implications for pollinators. Finally, Ruiz-Hernández et al. investigate the effect of human selection and breeding on floral traits of ornamental snapdragon plants discovering that human and bee preferences align well for color and scent of the parental lines.

This Research Topic covers various aspects of flower metabolism that are of relevance for the interaction with animal pollinators and provide insights on plant-pollinator communication mediated by floral chemical compounds, and the consequences for the evolution of floral traits, across natural and human-modified habitats.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Floral Scent Composition and Fine-Scale Timing in Two Moth-Pollinated Hawaiian *Schiedea* (Caryophyllaceae)

John M. Powers[1]*, *Roger Seco*[2,3], *Celia L. Faiola*[1], *Ann K. Sakai*[1], *Stephen G. Weller*[1], *Diane R. Campbell*[1] *and Alex Guenther*[4]

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Floral scent often intensifies during periods of pollinator activity, but the degree of this synchrony may vary among scent compounds depending on their function. Related plant species with the same pollinator may exhibit similar timing and composition of floral scent. We compared timing and composition of floral volatiles for two endemic Hawaiian plant species, *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae). For *S. kaalae*, we also compared the daily timing of emission of floral volatiles to evening visits of their shared pollinator, an endemic Hawaiian moth (*Pseudoschrankia brevipalpis*; Erebidae). The identity and amount of floral volatiles were measured in the greenhouse during day and evening periods with dynamic headspace sampling and GC-MS (gas chromatography – mass spectrometry). The timing of emissions (daily rise, peak, and fall) was measured by sampling continuously for multiple days in a growth chamber with PTR-MS (proton transfer reaction mass spectrometry). Nearly all volatiles detected underwent strong daily cycles in emission. Timings of floral volatile emissions were similar for *S. kaalae* and *S. hookeri*, as expected for two species sharing the same pollinator. For *S. kaalae*, many volatiles known to attract moths, including several linalool oxides and 2-phenylacetaldehyde, peaked within 2 h of the peak visitation time of the moth which pollinates both species. Floral volatiles of both species that peaked in the evening were also emitted several hours before and after the brief window of pollinator activity. Few volatiles followed a daytime emission pattern, consistent with increased apparency to visitors only at night. The scent blends of the two species differed in their major components and were most distinct from each other in the evening. The qualitative difference in evening scent composition between the two *Schiedea* species may reflect their distinct evolutionary history and may indicate that the moth species uses several different floral cues to locate rewards.

Keywords: *Schiedea kaalae*, *Schiedea hookeri*, *Pseudoschrankia*, floral volatiles, island flora, moth pollination, gas chromatography - mass spectrometry (GC-MS), proton transfer reaction - mass spectrometry (PTR-MS)

INTRODUCTION

In flowering plants, attraction of pollinators is often required for reproduction, but the multimodal signals that attract pollinators are costly and require both carbon and energy (Dicke and Sabelis, 1989; Grison-Pigé et al., 2001). Floral signals that attract pollinators may also attract visitors that reduce fitness such as herbivores (e.g. Theis and Adler, 2012; Schiestl, 2015; Nunes et al., 2016), nectar robbers (e.g. Kessler et al., 2008; Kessler and Halitschke, 2009), or generalist pollinators with high heterospecific pollen loads (Morales and Traveset, 2008). Selection on floral signals via pollinators is therefore expected to favor allocation of resources to traits that optimize pollen received or dispersed and minimize costs of apparency to other visitors. When pollinators are active only during a specific time period, temporal regulation of a floral signal is one way to increase efficiency in signaling (Hoballah et al., 2005). For example, the fitness of *Nicotiana attenuata* plants is affected if the timing of flower orientation or olfactory pollination cues is altered physically or genetically (Baldwin et al., 1997; Yon et al., 2017). Overlap between the window of pollinator activity and the timing of floral signals is common, whether the signals are related to physical access (Overland, 1960; Goldblatt et al., 2004), flower orientation (Yon et al., 2017), or scent production (Heath et al., 1992; Huber et al., 2004; Effmert et al., 2005; Kumano and Yamaoka, 2006; Okamoto et al., 2008; Prieto-Benítez et al., 2016; Chapurlat et al., 2018).

These and other previous studies have been useful in identifying the volatiles emitted during a known period of animal activity, for example during the foraging periods of diurnal versus nocturnal pollinators (Bischoff et al., 2014). Knowledge of how closely the time courses of volatile emissions match the activity of a pollinator is still limited, especially since pollinator activity can also change on very short time scales (Herrera, 1990; Knop et al., 2018). Here we generate continuous measurements of volatile emissions to observe the start and end of emissions, so that we can determine if volatiles are emitted outside of the period of pollinator activity and thus at times when costs might exceed benefits for a channel of information for the pollinator. Continuous measurements can also distinguish a volatile that is rising in emission, which might indicate a period of pollinator activity is starting, from a volatile that is declining at a given point in time.

Plant species with the same pollinator might be expected to display similar floral signals, but most tests of floral scent convergence within genera have been restricted to flowers that mimic a female insect (Cortis et al., 2009; Gögler et al., 2009) or oviposition site (Jürgens et al., 2013) or provide a fragrance reward (Nunes et al., 2017). These pollination systems require the presence of key compounds in precise ratios to produce a successful mimic or species-specific pheromone. Food-seeking pollinators may not require such highly specific floral chemical displays. Plant species that reward pollinators with food and have distinct scents might nevertheless attract shared pollinators if pollinators learn to associate the scent of each species with a reward. If heterospecific pollen transfer between related species reduces fitness (by clogging stigmas or producing infertile hybrids), plants would benefit from species-specific signals if distinct scents reduce heterospecific pollen transfer through floral constancy of pollinator individuals (Waelti et al., 2008).

We investigated the composition and timing of floral scent in *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae), two hermaphroditic species with specialized floral nectaries and similar floral morphology (Wagner et al., 2005b) which are pollinated by the endemic Hawaiian moth *Pseudoschrankia brevipalpis* (Erebidae; Weisenberger et al., 2014; Medeiros, 2015; Weller et al., 2017). In this plant genus, wind pollination evolved from biotic pollination (Sakai et al., 2006; Willyard et al., 2011). Reversals from wind to biotic pollination are also possible but cannot be currently verified given the poor resolution of the clade containing nearly all wind-pollinated species as well as several hermaphroditic species, including *S. hookeri* (Willyard et al., 2011). The clades containing *S. kaalae* and *S. hookeri* diverged c. 1.3 Mya (Willyard et al., 2011). Because these species share the same moth pollinator, which visits for a brief period of time in the early evening, we predicted that the two *Schiedea* species would share similar timing of maximum emissions of compounds known to attract moths, but differ in evening floral scent composition due to their separate evolutionary histories.

We first describe the patterns of volatile emissions in these two moth-pollinated species by asking how *S. kaalae* and *S. hookeri* differ in the composition (identity and amount) of evening floral volatile emissions. Next, we characterize how individual volatiles change throughout the day and night in each species. Finally, we quantify the degree of overlap of volatiles (in aggregate and individually) with pollinator activity for one of the species, *S. kaalae*.

MATERIALS AND METHODS

Study System

Schiedea kaalae Wawra (sect. *Mononeura*) and *S. hookeri* A. Gray (sect. *Schiedea*) are hermaphroditic, self-compatible, protandrous, perennial herbs native to O'ahu, Hawai'i, USA, where populations of the two species occur in sympatry in parts of the Wai'anae Mountains [*S. kaalae* (410–730 m above sea level, asl) and *S. hookeri* (260–870 m asl), Wagner et al., 2005b] and can flower at the same time. *Schiedea kaalae* also occurs in the Ko'olau Mountains (Wagner et al., 2005b). Both species are listed as endangered by the US Fish and Wildlife Service and critically endangered by the IUCN (Ellshoff et al., 1991; Bruegmann and Caraway, 2003; Wagner et al., 2005a; Bruegmann et al., 2016), and a total of only about 28 *S. kaalae* individuals in five populations remained in the wild before restoration efforts (Weisenberger et al., 2014), precluding studies of the remnant populations *in situ*. *Schiedea hookeri* is more common in nature than *S. kaalae*, and large populations also exist following restoration efforts (D. Sailer, personal communication). The species produce inflorescences with 20–300 (*S. kaalae*) or 20–150 (*S. hookeri*) flowers per inflorescence and both species possess similar floral morphology with reflexed

sepals 3–4 mm long, no petals, 10 stamens, 3 styles, and 5 nectaries (Wagner et al., 2005b).

Prior Studies of Pollination Biology

The shared moth pollinator of *Schiedea kaalae* and *S. hookeri, Pseudoschrankia brevipalpis* (Weller et al., 2017), perches on flowers (or more rarely, on other parts of the inflorescence) and feeds on nectar extruded from the tips of specialized tubular nectary extensions adjacent to the stamens (Harris et al., 2012; Weisenberger et al., 2014). At ʻĒkahanui Gulch (Waiʻanae Mountains) *P. brevipalpis* was the only visitor to flowers of *S. kaalae*, based on observations over three years (Weller et al., 2017). Fewer pollinator observations were made for *S. hookeri* because of the inaccessibility of the sites, although direct and indirect observations both indicated that *P. brevipalpis* was the primary pollinator at ʻĒkahanui Gulch (Weller et al., 2017). Very low numbers of other endemic moth species were observed visiting *S. hookeri* at a second site and a few carried *Schiedea* pollen, but pollen deposition was threefold lower than at ʻĒkahanui Gulch, and no correlation between moth scales and pollen deposition was observed, indicating the absence of effective pollination (Weller et al., 2017). No daytime floral visitors to either species have been observed (Weisenberger et al., 2014).

The elliptic flight patterns of the moths before they land on flowers suggest they rely little on visual targeting even before dark and are characteristic of moths seeking floral volatiles through anemotaxis (upwind flight; Cardé and Willis, 2008; Weller et al., 2017 and videos therein).

New Analyses of Field Data for Time of Moth Visits

For comparison with timing of volatile emissions, we determined the timing of flower visits by the moth *P. brevipalpis*. Our earlier studies (Weller et al., 2017) reported the duration of visits to flowers in male and female stages of anthesis but not arrival times. Here we analyzed arrival time of visits (landings on a flower) of *P. brevipalpis* to *S. kaalae* at ʻĒkahanui Gulch (n = 48 visits on three consecutive dates in March 2014 and one in July 2014; landings occurred from 17:49–19:28 HAST, 0.2–1.6 h after sunset). Observations of the field population always began at least a half hour in advance of any moth visit and continued until after moth activity ceased, so the entire spectrum of potential arrival times was included. We did not include *S. hookeri* in the analysis of timing of visits because we had too few direct observations of pollinator visits.

Because the timing of moth behavior and floral volatile emission patterns may be driven by light levels (Altenburger and Matile, 1990; Hansted et al., 1994; Hendel-Rahmanim et al., 2007) or circadian rhythms entrained by light cycles (Kolosova et al., 2001; Fenske et al., 2015; Yon et al., 2016; Fenske et al., 2018), we calculated the difference between the times of each moth visit to a flower and local sunset. The angle of elevation to the nearby ridge towards the median solar azimuth at sunset across observation dates was used to determine local sunset, using the *crepuscule* function of the R package *maptools* (Bivand et al., 2019). This technique corrects for the shadows cast by the mountainous terrain. We combined these relative times across dates to create a temporal distribution of moth visits to *S. kaalae*.

Plants Sampled

Volatile emissions were measured on plants of *Schiedea kaalae* and *S. hookeri* grown in the University of California, Irvine greenhouse. Plants were potted in UC mix (a soil mix developed by the University of California; 1:1:1 sand, peat, and redwood fiber) with added perlite and watered as needed with dilute liquid fertilizer (Grow More; 20-20-20 NPK plus micronutrients). Plants were grown from seeds or cuttings of six populations from the Waiʻanae Mountains (10 *S. kaalae* and 10 *S. hookeri* plants, **Supplementary Table S1**; all collections were made before species were listed as federally endangered in 1991 and 1996, for *S. kaalae* and *S. hookeri*, respectively). Plants also were grown from intraspecific (mostly interpopulation) crosses between cultivated plants from these populations (22 *S. kaalae* plants, 22 *S. hookeri* plants). Interpopulation crosses within species were used because most natural populations now consist of a single individual and are highly inbred (Weisenberger et al., 2014). For GC-MS measures, we sampled 32 plants of each species in the evening (see below). Four *Schiedea kaalae* and eight *S. hookeri* plants from this group were also sampled during the day. For continuous PTR-MS measurements of plants in a growth chamber over multiple days, we sampled five *S. kaalae* plants, two from Puʻumaialau (Takeuchi 3587) and three from Pahole Gulch (Weller and Sakai 904), and three *S. hookeri* plants, one from Kaluaʻa Gulch (Weller and Sakai 879, 400 m south of Puʻuhapapa) and two from Waiʻanae Kai (**Supplementary Table S1**). All plants chosen had ≥ 10 open flowers. The numbers of open male- and female-phase flowers, closed (post-anthesis) flowers, and floral buds were recorded immediately after sampling for both methods. Inflorescence age, as estimated by the ratio of closed to open flowers, did not vary between species in the sampled plants (ANOVA, P = 0.90, n = 64).

Scent Collections and Analysis by GC-MS

Scent Collections

Procedures for dynamic headspace sampling for GC-MS were modified from Campbell et al. (2019). Scent traps, consisting of a glass capillary tube filled with 5 mg of Tenax TA and held with plugs of silanized quartz wool, were cleaned before initial use by heating in helium carrier gas for 5 min at 250 °C. Scent samples were collected from November 2016 - April 2017 in the greenhouse during evening and daytime sampling periods. The natural day length varied from 10–12 h. For the evening period, samples were taken with pumping start times between 16:30–21:00 PST (2.5 h before sunset–3.9 h after sunset, mean ± SD relative to sunset 1.4 ± 1.3 h, with 86 % of samples taken after sunset). This wide sampling window was used to capture the potential gradient along the transition from light to dark, which was treated as a linear rather than discrete effect in the analysis (see below). For the day period, samples were taken from the same inflorescence earlier in the same day (start times 12:50–

13:50 PST, 0.8–2.0 h after solar noon). Each plant was used on one date only. Dynamic headspace samples of floral volatiles were taken by enclosing inflorescences in 19 x 10 cm nylon-6 oven bags (Reynolds, USA). Volatiles were allowed to equilibrate for 30 min at 22–32 °C (day) or 20–26 °C (evening) and pumped for 30 min through a scent trap using a pump (Supelco PAS-500, Spectrex, Redwood City, California, USA) set to a pre-trap flow rate of 200 mL/min. Ambient controls (n = 19) were taken from an empty oven bag sampled for the same duration to identify contaminants (see below). Samples were stored in capped glass vials at -20 °C until analysis.

GC-MS Analysis

Floral scent composition (the identity and emission rate of each volatile in the overall scent blend) was characterized and quantified by thermal desorption gas chromatography-mass spectrometry (TD-GC-MS). We employed an Agilent 6890N GC (Agilent Technologies, Palo Alto, California, USA), with a 30 m × 0.25 mm internal diameter x 0.25 μm film thickness HP-5ms column (Agilent). The flow of helium carrier gas was 1 mL/min. Scent traps were placed in the sample tube of a Markes UNITY 2 thermal desorption device, purged with helium for 1 min, heated to 200°C for 5 min while re-trapping on Tenax adsorbent at 25 °C, and desorbed at 200°C for 3 min. After a 2 min hold at 40 °C, the temperature of the GC oven was ramped to 210 °C at 10 °C/min, then to 275 °C at 30 °C/min and held for 2 min. A coupled Agilent 5973N MSD mass spectrometer was operated in electron-impact ionization mode at 70 eV and scanned in the range 50–500 m/z at 3 s^{-1}.

Peak deconvolution, integration, and tentative compound identification were performed in the Automated Mass Spectral Deconvolution and Identification System (AMDIS) using the NIST 2017 mass spectral library. Components were included if they had mass spectral match scores greater than 75%, had maximum abundances across samples greater than 120,000 counts (6.6% of the median sample), and occurred in more than one sample. After calibration with a C_7-C_{30} alkane ladder, compound identities were verified by comparing retention indices (RI) with those given in the NIST library. Volatile emission rates were calculated within each compound class from peak integrations by calibration across 4 orders of magnitude with 7 authentic standards ((Z)-hex-3-en-1-ol, α-pinene, indole, linalool, β-caryophyllene, benzaldehyde dimethyl acetate, (E,E)-farnesol) in hexane applied to scent traps. Compounds in floral samples that did not exceed the amounts in ambient controls or GC blanks were considered contaminants (using t-tests with alpha adjusted by the false discovery rate method) and excluded from analyses. Based on the PTR-MS data, oct-1-en-3-ol and (Z)-hex-3-en-1-ol were likely induced by handling the inflorescences because both sharply decreased in the first two hours after bagging. Both compounds can be induced by mechanical damage (Ozawa et al., 2000; Leitner et al., 2005; Kigathi et al., 2009; Boggia et al., 2015). We excluded (Z)-hex-3-en-1-ol from GC-MS analyses because its emissions remained low for days after the initial bagging, but because oct-1-en-3-ol resurged consistently at night (**Supplementary Figure S2**), likely indicating floral emission, we included it in analyses. Emission rates were standardized by the number of open flowers.

Statistical Analyses of Scent Composition

The total scent emissions per flower during the evening sampling period were compared between species with a Mann-Whitney test. To identify volatiles that differed between the two species and between times of day, we employed canonical analysis of principal coordinates (CAP; Anderson and Willis, 2003; Campbell et al., 2016) with Bray-Curtis dissimilarities, as implemented in the function *capscale* from the R package *vegan* (R Core Team, 2018; Oksanen et al., 2019). This constrained ordination method is suited to discover multivariate patterns among predefined predictors, in this case, species, time relative to sunset (as a continuous variable because sampling windows were wide), and their interaction. We used a permutation test (*anova.cca*) to test each term of the full model sequentially and determine whether there was a significant interaction after accounting for the main effects. For visualization and to improve interpretation of the time axis, CAP was repeated within each species with time of day as the constraining variable. The CAP method constructs metric multidimensional scaling (MDS) axes to summarize variation that is not explained by the predictors. Volatile emission rates were square-root transformed to reduce skew before analysis.

Scent Analysis in Real Time by PTR-MS

Advantages of Real-Time Sampling

To identify temporal patterns of scent emissions and pollinator activity, most studies have compared scent (all volatiles and their emission rates) and pollinator activity during two discrete daily sampling periods (e.g. Prieto-Benítez et al., 2015). More intensive sampling has yielded qualitative comparisons between selected scent compounds at 1 h resolution and pollinator visitation rates in three daily periods (Dötterl et al., 2012b), and between overall scent intensity at 10 min resolution and a time range of pollinator visits (Dötterl et al., 2012a). To make fine-scale comparisons that quantify scent-pollinator overlap, we take advantage of proton transfer reaction mass spectrometry (PTR-MS) to generate continuous measurements of volatile emissions for multiple days, rather than the average emissions across a sampling period generated by trapping followed by GC-MS. We then compare those time courses with information on timing of pollinator visits at the scale of quarter hours using an overlap statistic to quantify the degree of synchrony. Prior studies with PTR-MS have revealed the daily emission profiles of individual volatiles (Abel et al., 2009), and overlap between thermogenesis and scent signals (Marotz-Clausen et al., 2018), but have not previously been paired with fine scale information on timing of pollinator visits.

Proton transfer reaction time-of-flight mass spectrometry (PTR-MS) allows extremely sensitive, real time quantitation of plant volatile emissions by using hydronium ions for chemical ionization (Lindinger et al., 1998; Jordan et al., 2009). Through direct ionization of the sample gas, PTR-MS can measure small molecules that are not efficiently trapped on adsorbents. Identification of individual components of complex mixtures

with PTR-MS is difficult due to fragmentation and overlap of ions at unit mass resolution, but the technique has been used successfully on complex biological samples when paired with GC-MS to positively identify the volatiles expected in the mixture (Eugenio et al., 2007; Cappellin et al., 2012; Masi et al., 2015; Schuhfried et al., 2017). Identifications can be made for ions not found in the GC-MS spectra from standards reported in the literature.

PTR-MS Experiment

Emission rates of volatiles are often highly sensitive to the environment (Farré-Armengol et al., 2014; Burkle and Runyon, 2016; Campbell et al., 2019) and thus could differ between the growth chamber and field sites where the moths were studied. To minimize these variations, we sampled floral volatiles for 2–4 days with PTR-MS under environmental conditions similar to the sites where pollinator observations were conducted. Unlike emission rates, timings of volatile emissions are known to be driven by either direct light cues or the circadian clock calibrated by light cues and are not expected to differ relative to those light cues (Fenske et al., 2018). We lined up temporal patterns of volatiles to those that occur under field conditions by expressing time courses relative to the time of sunset or the light-to-dark transition in the photochamber and using light conditions (intensity and photoperiod) and temperature conditions typical of the field. The remaining differences between the field and the photochamber were that temperature was kept constant to observe changes in emission rates not driven directly by heating, and the light transitions were abrupt rather than gradual so that volatiles that respond to light could be distinguished from those with slower regulation. The detailed methods for sampling, data processing, verification with reference standards, and identification are reported in **Supplementary Methods S1**.

Statistical Analysis of Volatile Time Courses

To visualize patterns of multivariate change in scent through time and between the species, we performed a principal components analysis of the PTR-MS ion time series with maxima over 0.001 counts·s^{-1}·$flower^{-1}$ for all plants at all time points (van Ruth and de Visser, 2015). All ions that met this criterion were analyzed, including those not identified by GC-MS or comparison to reference spectra. Time points were connected with lines to show the progression of each plant through scent space over multiple days. To identify volatiles with similar patterns of emission over time, we constructed WPGMA hierarchical clusterings of Pearson distances (Liao, 2005) among ion time series (with each ion signal scaled to its maximum). The resulting clustering of volatiles, visualized in a clustered heatmap, reflects similarity in both temporal patterns and presence or absence in each species.

To model the temporal peaks of individual volatile emissions, we fit Weibull functions to each ion time series for each plant and each day using the R package *cardidates* (Rolinski et al., 2007). These functions allow different slopes in the rising and falling periods, and different baseline levels before and after the peak. From these fits we extracted the times of the beginning of exponential increase (0.5% of the modelled peak area), maximum, and end of exponential decrease (99.5% of the modelled peak area). For each species, we calculated the median time of maxima for each ion across days and plants.

To quantify the degree of scent-pollinator synchrony in *S. kaalae*, we compared the 24 h distributions of *P. brevipalpis* visits across all dates to both a) the modelled times of maximum emission (from the fitted Weibull function) aggregated across all PTR-MS ions, days, and plants (which provides a single metric of synchronization between pollination and the timing of peaks across all scent compounds) and b) the actual time courses of emissions for each PTR-MS ion across days and plants (which shows which volatiles are the most or least synchronized with pollination). After aligning the sunset time to the dark transition in the growth chamber, we placed times of moth visits into bins that were 16 min in duration, centered on the 4-min sampling blocks for each plant. We normalized each distribution to have an area of one, and then calculated the areal overlap between the two distributions (defined as the integral of the minimum of the two distributions; Miller-Rushing et al., 2010). This statistic is affected by the position of the two distributions relative to each other, and the match in their width. We define the null expectation as the overlap between the moth visit distribution and a flat line, where the flat line represents either a) a uniform distribution of times of maxima or b) a hypothetical volatile holding a constant emission rate throughout the day.

Compounds Attractive to Moths

Selection for overlap between emission of a specific compound and moth visitation might be more likely if the compound is one that moths respond to behaviorally. The behavioral responses of *Pseudoschrankia brevipalpis* to individual floral volatiles are unknown, so we surveyed the literature for information on the detectability (search terms: moth + {antenna, EAD, EAG}) and attractiveness (search terms: moth + {attraction, behavior}) of the volatiles produced by *Schiedea* inflorescences. Electroantennographic detection (EAD) studies were used to determine whether a compound can be detected by moth antennae. Evidence of moth attraction is presented from behavioral tests. In these studies, the volatile was considered attractive if it induced more interactions than the control. Volatiles were applied to either an open trap with a scent emitter, a scent emitter within a wind tunnel, or a flower spiked with additional scent. From the literature, we recorded the number of moth species, their families, and the apparatus used (**Supplementary Table S2**).

RESULTS

Species Differences in Floral Scent

Using GC-MS we detected 32 floral volatiles produced by *S. kaalae* and 36 produced by *S. hookeri*, for a total of 40 volatiles present in > 20% of samples of either species. These included 19 aliphatics, 7 benzenoids, 5 irregular terpenes, and 9 monoterpenes (**Table 1**). Of the 40 compounds, 28 were produced by both species. The literature survey of

TABLE 1 | Evening floral volatile emissions from *Schiedea kaalae* and *S. hookeri* detected by GC-MS in > 20% of samples of either species (40 of 76 compounds, *n* = 32 plants for each species).

Class	RI [1]	Mean match score	CAS [2]	Name	Proportion of evening samples[3]		Mean nonzero emission rate (ng/flower/hr)		Mean emission rate (ng/flower/hr)		Mean relative emission rate [4]	
					S.kaalae	*S.hookeri*	*S.kaalae*	*S.hookeri*	*S.kaalae*	*S.hookeri*	*S.kaalae*	*S.hookeri*
Aliphatic	796	87%	4440-65-7	(E)-hex-3-enal	0%	50%		0.56		0.28		3.4%
	797	94%	66-25-1	hexanal	**100%**	**100%**	0.56	0.80	0.56	0.80	3.4%	9.5%
	830	90%	96-04-8	heptane-2,3-dione	8%	**86%**	0.05	0.54	0.00	0.46	0.0%	5.6%
	840	91%	6728-26-3	(E)-hex-2-enal	0%	**81%**		0.43		0.35		4.2%
	848	94%	928-96-1	(Z)-hex-3-en-1-ol	21%	**89%**	0.24	6.64			excl.	excl.
	855	78%	7642-10-6	hept-3-ene	5%	22%	0.24	0.26	0.01	0.06	0.1%	0.7%
	855	84%	4412-91-3	furan-3-ylmethanol	8%	25%	0.10	0.95	0.01	0.24	0.0%	2.8%
	855	89%	2415-72-7	propylcyclopropane	32%	25%	0.08	0.33	0.03	0.08	0.2%	1.0%
	882	84%	2216-34-4	4-methyloctane	21%	3%	0.03	0.01	0.01	0.00	0.0%	0.0%
	901	92%	13129-23-2	methyl furan-3-carboxylate	0%	33%		0.03		0.01		0.1%
	905	92%	3008-40-0	cyclopentane-1,2-dione	21%	22%	0.78	1.85	0.16	0.41	1.0%	4.9%
	933	83%	18829-55-5	hept-2-enal	37%	0%	0.06		0.02		0.1%	
	949	79%	26456-76-8	3,5,5-trimethylhex-2-ene	5%	47%	0.05	0.40	0.00	0.19	0.0%	2.3%
	960	92%	3391-86-4	oct-1-en-3-ol	**100%**	**100%**	3.17	3.38	3.17	3.38	19.3%	40.6%
	961	92%	106-68-3	octan-3-one	**89%**	**97%**	0.49	0.72	0.44	0.70	2.7%	8.4%
	971	84%	111-13-7	octan-2-one	21%	0%	0.04		0.01		0.0%	
	981	92%	72237-36-6	hex-4-enyl acetate	0%	25%		0.35		0.09		1.1%
	1152	87%	53398-84-8	[(E)-hex-3-enyl] butanoate	0%	28%		0.29		0.08		1.0%
	1347	90%	31501-11-8	[(Z)-hex-3-enyl] hexanoate	0%	28%		0.27		0.07		0.9%
Benzenoid	896	91%	100-66-3	anisole	3%	39%	0.01	0.02	0.00	0.01	0.0%	0.1%
	937	95%	100-52-7	benzaldehyde	**100%**	**100%**	0.07	0.30	0.07	0.30	0.5%	3.7%
	1017	92%	122-78-1	2-phenylacetaldehyde	**95%**	50%	0.29	0.01	0.27	0.01	1.7%	0.1%
	1179			unknown benzenoid [5]	11%	**64%**	0.02	0.42	0.00	0.27	0.0%	3.2%
	1198	86%	103-70-8	N-phenylformamide	0%	25%		0.09		0.02		0.3%
	1268	92%	120-72-9	indole	13%	**89%**	0.02	0.16	0.00	0.15	0.0%	1.8%
	1316	94%	134-20-3	methyl 2-aminobenzoate	5%	**61%**	0.00	0.14	0.00	0.08	0.0%	1.0%
Irregular terpene	1086	82%	19945-61-0	(3E)-4,8-dimethylnona-1,3,7-triene	3%	**64%**	0.05	0.15	0.00	0.09	0.0%	1.1%
	1115	90%	1125-21-9	4-oxoisophorone	**100%**	3%	0.13	0.00	0.13	0.00	0.8%	0.0%
	1120	87%	28564-83-2	3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one	16%	22%	0.14	0.13	0.02	0.03	0.1%	0.3%
	1139	87%	20547-99-3	2,2,6-trimethylcyclohexane-1,4-dione	**84%**	0%	0.06		0.05		0.3%	
	1322	81%	141891-14-7	4-hydroxy-2,6,6-trimethyl-3-oxocyclohexene-1-carbaldehyde	**71%**	3%	0.04	0.04	0.03	0.00	0.2%	0.0%
Mono-terpene	964	84%	123-35-3	β-myrcene	47%	3%	0.02	0.02	0.01	0.00	0.1%	0.0%
	978	90%	99-83-2	α-phellandrene	**76%**	42%	0.46	0.04	0.35	0.02	2.2%	0.2%
	997	89%	99-87-6	p-cymene	**55%**	22%	0.12	0.06	0.07	0.01	0.4%	0.2%
	1013	85%	3779-61-1	(E)-β-ocimene	26%	0%	0.02		0.00		0.0%	
	1061	94%	5989-33-3	linalool oxide (furanoid)	**100%**	22%	2.13	0.08	2.13	0.02	13.0%	0.2%
	1071	82%	78-70-6	linalool	34%	47%	0.11	0.05	0.04	0.03	0.2%	0.3%
	1087	93%	33933-72-1	linalool oxide (pyranoid) ketone	**100%**	**75%**	4.82	0.11	4.82	0.08	29.4%	1.0%
	1152	93%	39028-58-5	linalool oxide (pyranoid)	**100%**	22%	3.98	0.10	3.98	0.02	24.2%	0.3%
	1245	78%	EPA-7965	epoxy-linalooloxide	39%	0%	0.04		0.02		0.1%	

Evidence of EAD (electroantennographic detection) responses or attraction of moths for these compounds is presented in ***Supplementary Table S2****.*

[1] Kovats retention index (RI). [2] CAS registry number or NIST library number. [3] Percentage of all evening samples in which the compound was detected, with entries > 50% in bold. [4] The mean of emission rates scaled to 100%. [5] Fragment ions relative to m/z 91 (100%): 65 (20%), 119 (19%), 162 (11%), 92 (9%), 63 (8%), 89 (6%), 51 (5%). NIST MS Search "Substructure Information" analysis indicates molecular mass of 162, probable disubstituted phenyl with a carbonyl group.

electrophysiological and behavioral studies in other moth species showed 9 are EAD-active (with no behavioral data available), 12 are EAD-active and attractive, one is not attractive, and no data are available for the others (**Supplementary Table S2**). Including rarer volatiles and excluding two putative wound volatiles, a total of 74 volatiles were detected and used for analysis.

Schiedea kaalae produced more total scent per flower than *S. hookeri* in the evening in the GC-MS measurements (median ± median absolute deviation 23 ± 12 ng·flower^{-1}·h^{-1} compared to 5.0 ± 3.9 ng·flower^{-1}·h^{-1} for *S. hookeri*, Mann-Whitney test, U = 179, $P < 10^{-10}$). Major components of the scent blends differed (CAP species effect, **Table 2A**). For *S. kaalae* in the evening, three cyclic linalool oxides (the pyranoid oxide ketone, pyranoid oxide, and furanoid oxide) made up 67% of the average scent blend, followed by five volatiles each making up more than 1.5% of the blend: oct-1-en-3-ol, hexanal, octan-3-one, α-phellandrene, and 2-phenylacetaldehyde (**Table 1**, **Figure 1A**). The evening blend was more complex for *S. hookeri* than *S. kaalae* (Shannon diversity index of 2.1 ± 0.3 [mean ± SD] versus 1.6 ± 0.2 for *S. kaalae*), and composed of oct-1-en-3-ol (41%), followed by 11 volatiles each making up 1.5–10% of the blend: hexanal, octan-3-one, heptane-2,3-dione, cyclopentane-1,2-dione, two hexenal isomers, benzaldehyde, an unknown benzenoid, furan-3-ylmethanol, 3,5,5-trimethylhex-2-ene, and indole (**Table 1**, **Figure 1B**). The first CAP axis that separated the floral scents of the species reflects these major differences (**Table 2B**).

These differences in evening scent between the two species were supported by PTR-MS measurements (**Figure 2**). The two species produced distinct scent blends at all times of day (principal components analysis of ions in the PTR-MS spectrum across all timepoints, **Figure 3**). The scent compositions of the two species were most distinct from each other during the evening (**Figure 3**) and this was verified by the full CAP analysis of GC-MS volatile compositions (**Table 2A**, ordination not shown). Individuals from the two *S. kaalae* populations differed from each other in their evening scent composition (**Figure 3**), primarily by the emission of indole by the two plants from Pu'umaialau (Takeuchi 3587) which was absent in the three plants from Pahole Gulch (Weller & Sakai 904; both in the Wai'anae range, **Figure 2**, **Supplementary Table S1**).

TABLE 2 | Canonical analysis of principal coordinates (CAP) of the effects of species (*Schiedea kaalae* or *S. hookeri*) and time of day on floral scent composition. (A) ANOVA-like permutation test (n = 99999 iterations) of each term. (B) Compound scores on the first CAP axis, which discriminated between the species. Absolute scores ≥ 0.02 are included. Negative values indicate compounds associated with *S. hookeri*, and positive values indicate compounds associated with *S. kaalae*.

(A) Test of CAP model

	df	SS	F	P
Species	1	6.74	52.7	0.00001
Time	1	0.61	4.8	0.00146
Species : Time	1	0.37	2.9	0.02012
Residual	72	9.20		

(B) Compounds separating species

Name	CAP1 Score
	S. hookeri
unknown benzenoid	-0.09
indole	-0.08
(E)-hex-2-enal	-0.07
(E)-hex-3-enal	-0.06
methyl 2-aminobenzoate	-0.06
heptane-2,3-dione	-0.06
1,3-dihydro-2-benzofuran	-0.06
benzaldehyde	-0.05
(3E)-4,8-dimethylnona-1,3,7-triene	-0.05
3,5,5-trimethylhex-2-ene	-0.04
anisole	-0.02
N-phenylformamide	-0.02
furan-3-ylmethanol	-0.02
2,2,6-trimethylcyclohexane-1,4-dione	0.06
oct-1-en-3-ol	0.06
4-oxoisophorone	0.09
α-phellandrene	0.12
2-phenylacetaldehyde	0.12
linalool oxide (furanoid)	0.43
linalool oxide (pyranoid)	0.59
linalool oxide (pyranoid) ketone	0.62
	S. kaalae

Daily Patterns in Floral Scent

Comparisons Between Day and Night Using GC-MS

In both *Schiedea kaalae* and *S. hookeri*, total floral scent emissions increased and scent composition changed markedly in the evening. Median evening scent emissions measured by GC-MS for *S. kaalae* were 1.5 times higher than daytime emissions and 1.8 times higher for *S. hookeri*. Scent composition varied by species, time of day, and their interaction (full canonical analysis of principal coordinates, **Table 2A**). The scent composition of individual plants changed between the day and evening within both species (time effects in separate CAP analyses: $F_{1,38}$ = 6.17, P = 0.0001 for *S. hookeri* and $F_{1,34}$ = 3.11, P = 0.0024 for *S. kaalae*). For *S. kaalae*, the volatiles with the highest evening loadings were linalool oxide (pyranoid), linalool oxide (furanoid), and 2-phenylacetaldehyde (**Figure 1A**), all of which are EAD-active in moths (**Supplementary Table S2**). In *S. hookeri*, volatiles with the highest evening loadings were the unknown benzenoid, 1-3-dihydro-2-benzofuran, and indole (**Figure 1B**; indole attracts hawkmoths, **Supplementary Table S2**).

Fine Scale Timing Using PTR-MS

The floral scents of both species intensified in the evening in the PTR-MS measurements (**Supplementary Table S3**, **Figure 4**) as they did with GC-MS. This daily modulation was driven by pulses of individual volatiles from diverse biochemical pathways with periodicity of approximately 24 h (**Supplementary Figure S2**). Each volatile had a distinctly-shaped time course (**Figure 2**) but the times of maximum emission among the evening volatiles fell within a 4 h period (**Figure 4**). The volatile emission patterns formed three main groups based on their starting times relative to the light and dark transitions (**Supplementary Table S3**). Morning volatiles, such as acetaldehyde (m/z 45), started to rise from their baseline emission rates when plants are exposed to light, plateaued near their maximum within 1 h, began to fall at dark, and returned to baseline 1–5 h after dark. Afternoon

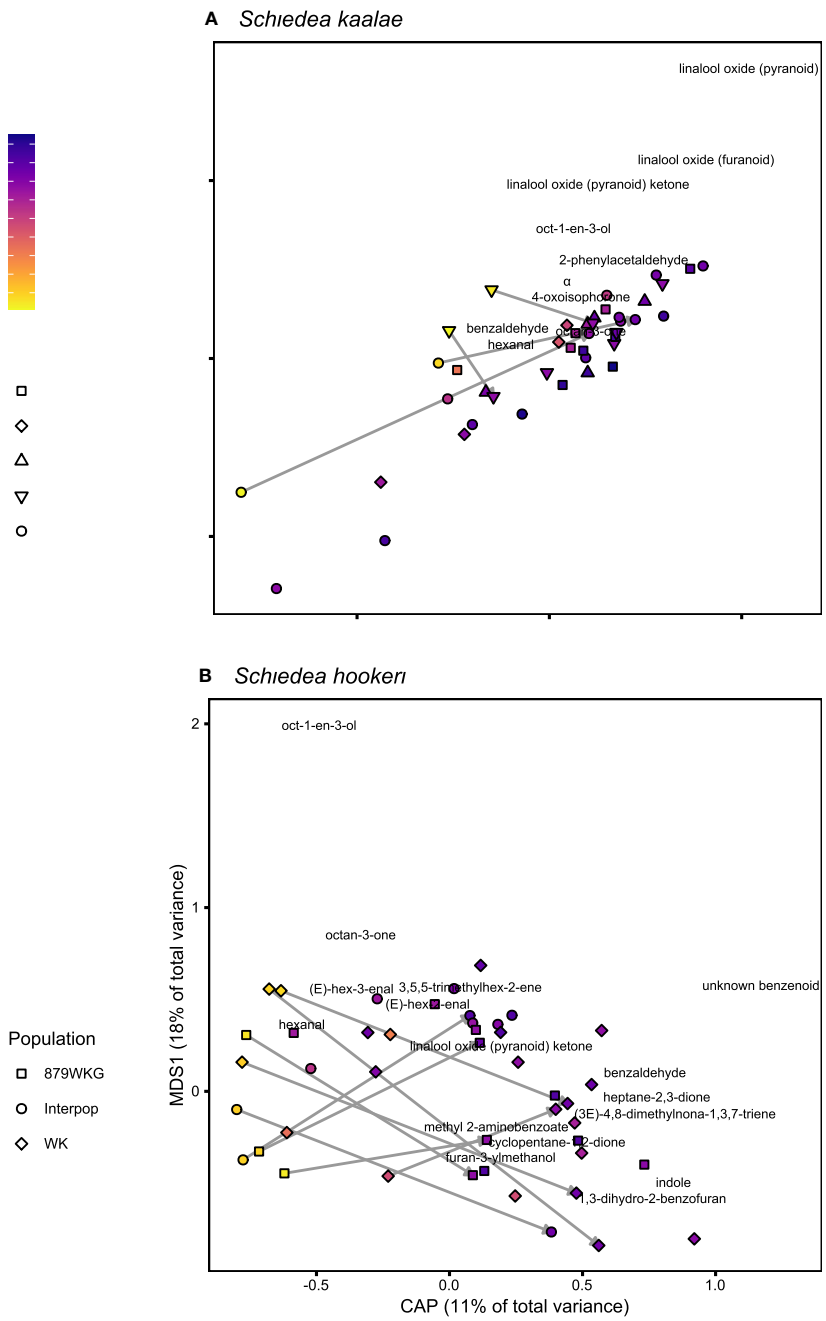

FIGURE 1 | Floral scent composition determined by GC-MS of **(A)** *Schiedea kaalae* and **(B)** *S. hookeri* plants sampled at different times of day, visualized by canonical analyses of principal coordinates (CAP). The CAP axis shows scent variation explained by time of day (evening on the right), and the first multidimensional scaling (MDS) axis shows additional unconstrained scent variation. The shape of the points indicates the source population number (collection locations in **Supplementary Table S1**) or a plant from a cross between populations ("Interpop"). Color indicates the time of collection, with zero indicating sunset, positive values indicating time after sunset, and negative values indicating time before sunset. Arrows connect samples of the same inflorescence during the day and following night. The names of volatiles are positioned by their CAP and MDS scores and labelled if they are > 0.05 units from the origin.

volatiles, such as linalool ketone (pyranoid) (m/z 169), rose 0–6 h before dark, peaked 0–2.5 h after dark, and returned to baseline 4–10 h after dark. Some of the afternoon volatiles that started rising slowly in the afternoon showed an inflection point at the dark transition and began rising more quickly (**Figure 2**, e.g. indole). Dark volatiles, such as benzaldehyde (m/z 107), rose at dark, peaked 1–3 h after dark, and returned to baseline 3–8 h after dark.

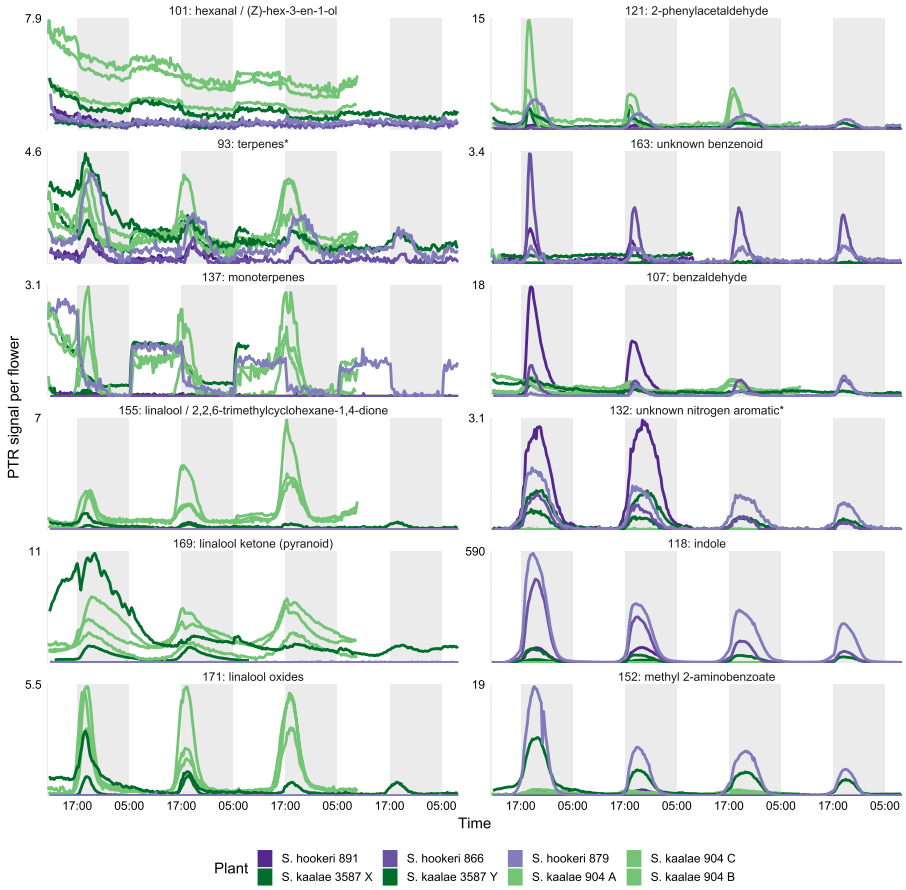

FIGURE 2 | PTR-MS signals per flower (arbitrary units) for floral volatile emissions from five *Schiedea kaalae* plants (green shades) and three *S. hookeri* plants (purple shades) across 2–4 d. Periods of darkness in the growth chamber are indicated by darker gray shading. Plants are named with their population number and a letter (collection locations in **Supplementary Table S1**), and colored by population. Panels present those PTR-MS ion signals (*m/z* value given in the label) that correspond to molecular or fragment ions of volatiles identified by GC-MS in evening scent emissions. Scales vary according to the maximum signal per flower, displayed next to each panel.

Both species started emitting more volatiles in the afternoon or after dark than in the morning (**Supplementary Table S3**). Production of all of the known moth attractants started in the afternoon or after dark (**Figure 4**). Daytime emission rates for many evening-peaking volatiles were generally very low, on the order of tens to hundreds of times less than emission rates in the evening (**Figure 4**, **Supplementary Table S3**), although some daily changes were more subtle [e.g. linalool oxide (pyranoid) ketone in *S. kaalae*; **Figure 2**]. The magnitude of the diel ratio (the emission rate 2–3 h after dark relative to the rate 5–6 h before dark) varied between species for the same volatile (**Figure 4**, **Supplementary Table S3**); for example, *S. hookeri* showed more extreme increases at night than *S. kaalae* in methyl 2-aminobenzoate, indole, and the unknown nitrogen aromatic and unknown benzenoid. The temporal patterns were consistent across days, plants, and in some cases between species, although the volatile emissions of *S. hookeri* often started and peaked later compared to the same compound in *S. kaalae* (**Figure 4**). In some plants, maximum emissions of some volatiles varied over consecutive days and generally decreased over time, perhaps due to aging of the inflorescence (**Figure 2**, **Supplementary Figure S2**).

Overlap With Moth Visitation

Pseudoschrankia brevipalpis visited *S. kaalae* in ʻĒkahanui Gulch from 0.2–1.6 h after sunset (mean ± SD 1.1 ± 0.4 h after sunset, n = 48). For *S. kaalae*, most volatiles began emission 1–5 h before the first *P. brevipalpis* visit to any flowers, peaked 1.5 h before–1 h after the mean time of moth visits, and returned to baseline 1–4 h after the last visit (times relative to sunset or the dark transition in the growth chamber, **Figure 4**).

The areal overlap between the time of moth visitation to *S. kaalae* and the times of maxima across PTR-MS ions, days, and plants was 49%, much greater than the null expectation of 14% for a uniform distribution of maxima, given these moth observations. The median time of ion maxima was 1.6 h after dark for *S. kaalae* and 2.4 h after dark for *S. hookeri*. The time courses of individual *S. kaalae* volatiles varied in their degree of overlap with moth

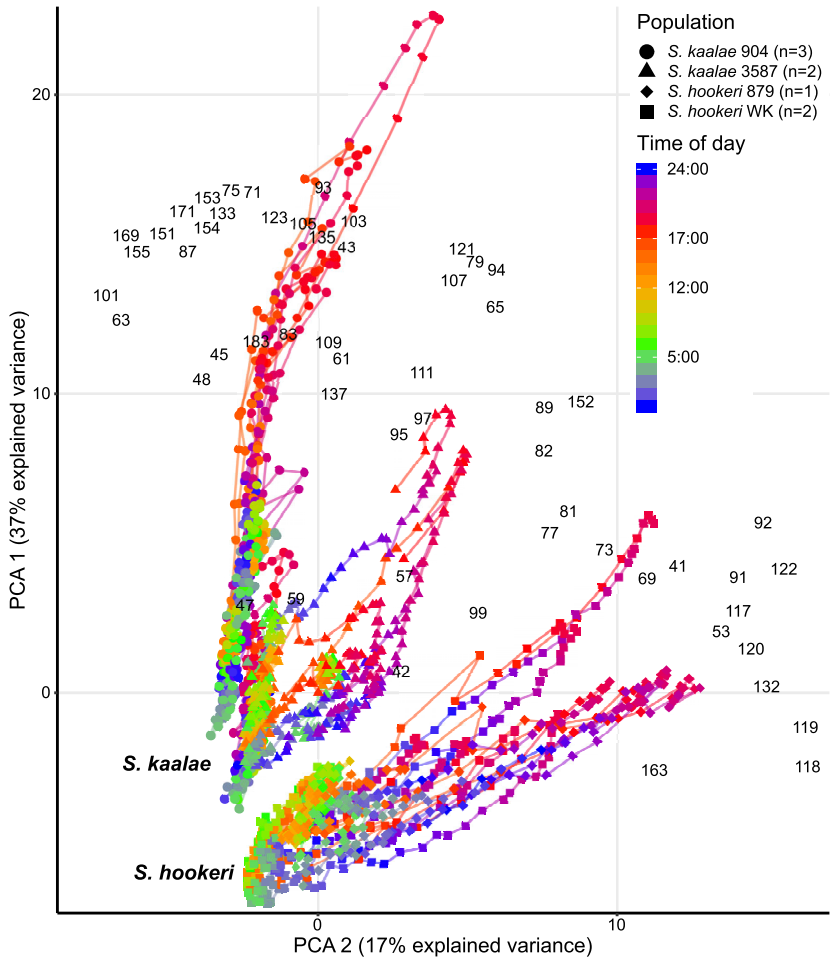

FIGURE 3 | Daily patterns of floral scent in the same five *Schiedea kaalae* plants and three *S. hookeri* plants as in Figure 2 mapped by principal components analysis (PCA) of PTR-MS ion signals with maxima over 0.001 counts·s^{-1}·flower^{-1}, including unidentified ions. The first and second principal components are shown on the vertical and horizontal axes. Loadings for each ion are indicated by the black *m/z numbers* (**Supplementary Table S3**). Lines connect adjacent time points for each plant. Time of day is represented by different colors on the line, with transitions from dark to light at 5:00 (cyan-green) and light to dark at 17:00 (orange-red) marked on the scale. The source population (904, 3587, 879, WK) is indicated by the shape of the points (collection locations in **Supplementary Table S1**). Each plant was sampled for 2–4 d.

visitation (**Figure 4**), with an unknown cyclohexane, the linalool oxides, 2-phenylacetaldehyde, and methyl 2-aminobenzoate having the highest overlap (both 2-phenylacetaldehyde and methyl 2-aminobenzoate are moth attractants; **Supplementary Table S2**). The mean overlap for the individual time courses of *S. kaalae* volatiles and moth visits was 25 ± 16%, 25 ± 15% for EAD-active volatiles, and 30 ± 16% for moth attractants that were EAD-active (mean ± SD), compared to a null expectation of 14% overlap for volatiles emitted at a constant rate. Volatiles that rose in the morning and peaked during the day (such as acetaldehyde) had low overlap with moth visitation, and of this group only the green leaf volatile (Z)-hex-3-en-1-ol (m/z 101, PTR-MS ion signal shared with hexanal) was an attractant, for moths that feed on leaves (**Supplementary Table S2**). The degree of overlap also varied across nights and plants, driven primarily by variation in the diel ratio and secondarily by changes in the timing of the maximum (**Figure 4**).

DISCUSSION

In two *Schiedea* species pollinated by the same moth, the timing of emission of floral volatiles was more similar than the identity of the major compounds released by those species in the evening. The floral scents produced by *S. kaalae* and *S. hookeri* were notable for the biochemical diversity of compounds that oscillate between day and night. The timings of peak pollinator activity for *S. kaalae* and of peak emissions of known moth attractants was similar, although volatile emissions started prior to pollinator activity and continued after cessation of pollinator activity.

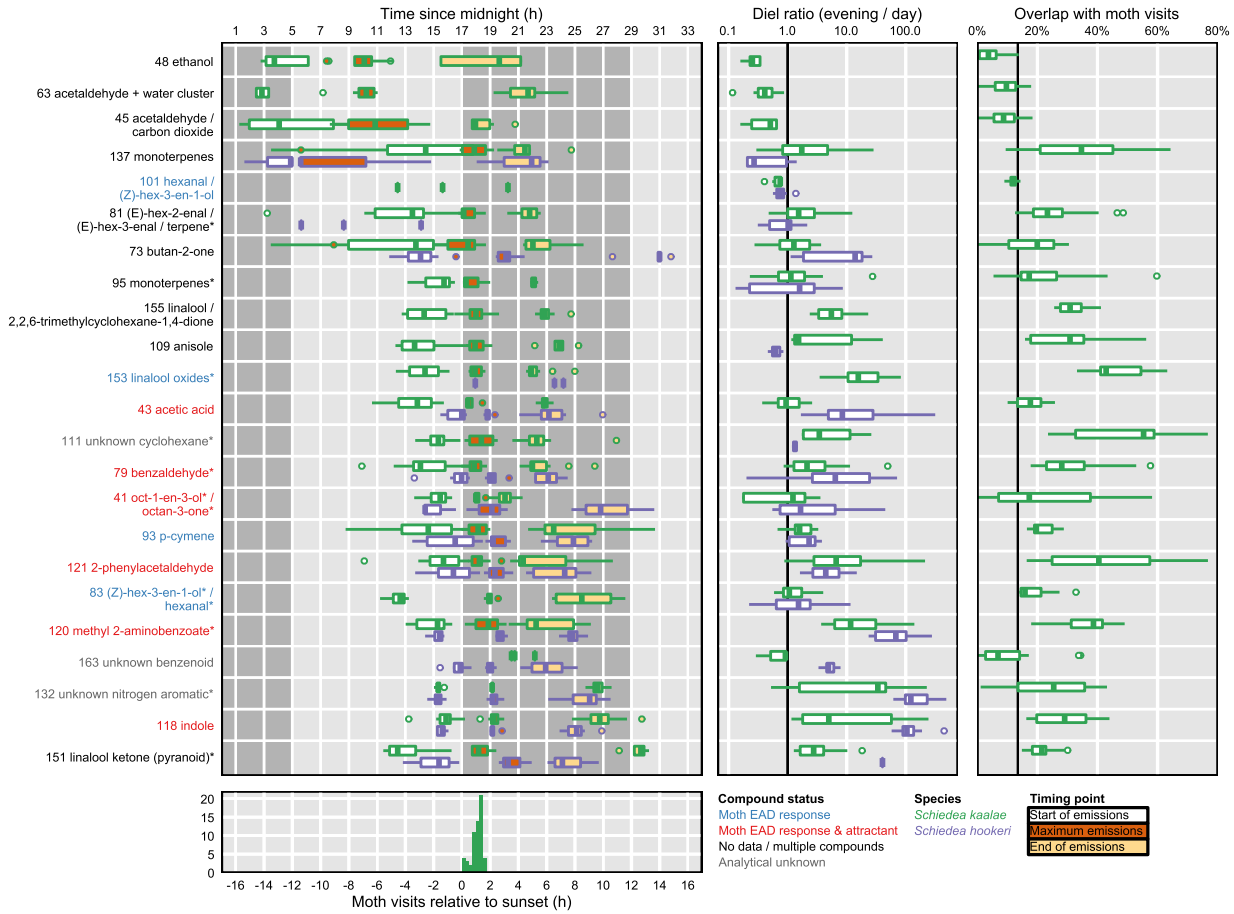

FIGURE 4 | Moth foraging activity and summaries of temporal peaks of floral volatile emissions of *Schiedea kaalae* (green) and *S. hookeri* (purple). Boxplots contain the median, first and third quartiles, range, and outliers (beyond 1.5 times the interquartile range from the first or third quartile). *Left:* The timing of volatiles emissions. Tentative identifications for each ion are given after their protonated *m/z* value. Fragment ions are indicated by an asterisk by the name. One PTR-MS ion per compound is shown (**Supplementary Table S3**), for ions with maxima over 0.001 counts·s^{-1}·flower^{-1} for all plants at all time points. For each ion and species, three boxplots summarize (across all plants and days) the start (white), maximum (maroon), and end (light orange) of emissions. Timing points were inferred by fitting Weibull functions to ion signals and trimming to 99% of the fitted peak area. Ions are arranged vertically by the mean starting time relative to the dark transition in the growth chamber. Light and dark periods in the growth chamber are indicated by background shading. The dark transition in the growth chamber was approximately coincident with the ambient greenhouse sunset time. Ion labels are colored by whether they elicit a moth antennal EAD response (blue), elicit an EAD response and attraction (red), are not reported in the literature (black, labelled 'no data'), contain signals from multiple compounds (black), or are analytical unknowns (gray, all references in (**Supplementary Table S2**) except acetic acid, Knight et al., 2011). *Middle:* The magnitude of daily changes in emission of floral volatiles. Boxplots show the diel ratio in emissions (evening/day) for evening (19:00–20:00 PST, 2–3 h after dark) and day (12:00–13:00 PST, 5–4 h before dark) for each plant and date. A ratio > 1 (right of vertical bar) indicates that emissions increased in the evening. *Right*: The overlap of *S. kaalae* volatile emissions with moth activity. Boxplots show the areal overlap value between two curves: the time course of each volatile relative to dark, and the distribution of *P. brevipalpis* visit times relative to sunset. Overlap values vary among plants and days. The vertical bar indicates the null overlap expectation for a hypothetical volatile that does not change in emission over the course of a 24 h period (14% overlap). *Bottom:* Visits of *Pseudoschrankia brevipalpis* to *S. kaalae* flowers relative to sunset at the field site over four dates.

Moth Attractants

Many volatiles that peak in the evening in *S. kaalae* and *S. hookeri* are typical benzenoid, oxygenated terpene, and nitrogen-containing floral attractants of crepuscular noctuid and sphingid moths (**Supplementary Table S2**), such as those found in the nocturnal floral emissions of moth-pollinated orchids (Kaiser, 1993), *Nicotiana* (Loughrin et al., 1991), *Petunia* (Hoballah et al., 2005), and other diverse taxa (Knudsen and Tollsten, 1993; Dobson et al., 1997; Miyake et al., 1998). In other studies, the hawkmoth *Hyles lineata* shows antennal responses to many volatiles emitted in the evening by the two *Schiedea* species (**Supplementary Table S2**).

The potential attractive role of these nocturnally-emitted compounds in *Schiedea* is highlighted by their increase in production with evolutionary transitions to moth pollination in several other genera. In *Clarkia*, production of linalool and linalool oxides (the pyranoid and furanoid forms produced by *S. kaalae*) evolved in a transition from bee to nocturnal moth pollination (Raguso and Pichersky, 1995). In *Ipomopsis*, indole (in our study produced primarily by *S. hookeri*) attracts

hawkmoths to *I. tenuituba* but is not emitted by its hummingbird-pollinated sister species *I. aggregata* (Bischoff et al., 2015). *Nicotiana bonariensis* produces the apocarotenoid 4-oxoisophorone and its variant 2,2,6-trimethylcyclohexane-1,4-dione (both produced by *S. kaalae*) from flowers that open at dusk and are pollinated by small crepuscular moths (Noctuidae) rather than the hawkmoths and hummingbirds attracted to close relatives of *N. bonariensis* that lack these compounds (Raguso et al., 2003; Clarkson et al., 2004; Kaczorowski et al., 2005). None of the evening-peaking volatiles in *Schiedea hookeri* and *S. kaalae* were present in the wind-pollinated *Schiedea* species (*S. globosa* and *S. kealiae*, Jürgens et al., 2012) that *P. brevipalpis* largely avoided in field choice tests (Weller et al., 2017).

Species Differences in Floral Scent

In this study, *S. kaalae* and *S. hookeri* share a sole pollinator in an area of sympatry, but have different evolutionary histories, leading us to predict distinct floral volatile compositions. In sympatric species from different lineages of sexually-deceptive and oil-secreting orchids, similar selection pressures imposed by the same pollinator have driven convergence in overall floral scent, or in the subset of compounds that have antennal activity (Cortis et al., 2009; Gögler et al., 2009; Nunes et al., 2017). We found instead that the evening floral scents of the two *Schiedea* species pollinated by *P. brevipalpis* differ qualitatively in composition. Scent differences between the species are more accentuated during the evening than during the day, echoing the same pattern found in nine *Nicotiana* species, some of which are nocturnally pollinated by hawkmoths (Raguso et al., 2003). The overall composition and major compounds of each species are unique: *Schiedea kaalae* produces a set of three linalool oxides and 2-phenylacetaldehyde, which are produced in relatively minute amounts by *S. hookeri*, and *S. hookeri* uniquely produces an unknown benzenoid and heptane-2,3-dione (**Table 1**). These qualitative differences could result from the evolutionary history of *S. hookeri*, which is in a clade of wind-pollinated species (*Schiedea* sect. *Schiedea*) and may represent a reversal to moth pollination from ancestral wind pollination (the current phylogenetic hypothesis does not fully resolve the direction of this shift, Willyard et al., 2011). However, both *S. kaalae* and *S. hookeri* produce the moth attractant benzaldehyde (Hoballah et al., 2005) and the insect attractant oct-1-en-3-ol (Hall et al., 1984), and *S. hookeri* emits the moth attractants indole (Bischoff et al., 2015) and methyl 2-aminobenzoate (Bisch-Knaden et al., 2018) which are emitted at lower rates by *S. kaalae* (**Figure 2**). Experiments that test moth preferences in the field at sites of both species (as in Bischoff et al., 2015) are needed to elucidate whether one critical volatile, a blend of the shared volatiles, or other factors are important for attraction of pollinators. Given the observed differences in scent between these related species that share the same moth species as a pollinator, future community studies should not always assume strict similarity in scent composition across unrelated plant taxa visited by the same pollinator or pollinator guild. Instead, distinct sets of compounds may be perceived by those pollinators.

Overlap With Moth Visitation

Our work builds on diverse examples of synchrony in floral signals and pollinator activity during the day (Matile and Altenburger, 1988; Kite and Smith, 1997; Dötterl et al., 2012a; Nunes et al., 2016) and night (e.g. Nilsson, 1983; Dötterl et al., 2005; Hoballah et al., 2005; Dötterl et al., 2012b; Steen et al., 2019) and enhances temporal resolution to characterize the overlap of pollinator activity and floral volatile production. In both *Schiedea* species, the emissions of many floral volatiles were restricted to the afternoon and evening hours and in *S. kaalae* peaked within 2 h of the mean time of *P. brevipalpis* visits in the field (**Figure 4**). In *S. kaalae*, the distribution of timings of maximum emissions across all volatiles, days, and plants indicated a good but imperfect temporal match between potential signals and the insect receiver. The volatiles that peak during the day and fall at dark would not be perceived by crepuscular moths after sunset, and their patterns of emission were all consistent with induction by light. The daytime volatiles could be related to photosynthesis (in the bracts of *S. hookeri*) or transpiration, rather than pollinator attraction (as is the case for both the daytime-peaking acetaldehyde and ethanol, its precursor; Graus et al., 2004). The maximum emissions of *S. hookeri* evening volatiles were shifted about 1 h later on average than their counterparts in *S. kaalae*, and many *S. hookeri* volatiles continued to be emitted until the early morning. These differences could stem from alternate temporal selection pressures (perhaps moths visit *S. hookeri* at a later time than they visit *S. kaalae*), or differences in evolutionary history of the plant species.

In *S. kaalae*, many volatile emissions spanned a much broader time range than the period of moth visitation. This could indicate constraints on how fast volatile emissions can be modulated, low ecological costs (e.g. apparency to herbivores) or low energetic costs of volatiles at those times, or a marginal benefit of attracting any moths that may be active at those times. Early initiation of volatile emission (i.e., for the volatiles that rose in the afternoon) could create a long downwind scent plume for long-distance attraction of moths (**Supplementary Table S3**, Cardé and Willis, 2008). Conversely, the volatiles that rise after dark just as moths are beginning to forage could be important for short-distance attraction. The peaks of individual *S. kaalae* evening volatiles differed in their degree of overlap with the distribution of moth visitation (20–55%; **Figure 4**). Known moth attractants, but not EAD-active volatiles, had slightly higher areal overlap in time with moth visits than the mean across all volatiles. This areal overlap statistic captured temporal differences from both early or late shifts in the time course of emissions and differences in peak width (narrow or broad), the two types of differences that are characterized in studies of phenology (Miller-Rushing et al., 2010). These two components were also examined separately by calculating times of maxima and diel ratios. Either type of difference could affect how and when pollinators or other visitors could perceive these volatiles.

Daily regulation of attractants may increase the fitness of plants by reducing energetic costs, and it may also serve to reduce the attraction of plant antagonists that use the same floral cues as

pollinators (Baldwin et al., 1997; Nunes et al., 2016). No native florivores or herbivores have been reported on outplanted or natural populations of these or any other *Schiedea* species. Though the fitness costs of emitting the evening volatiles during the day are unknown, the high level of daytime and before-dawn suppression indicates they could be substantial.

Floral scent is a complex trait in both synthesis and perception, and identification of volatiles or suites of volatiles that serve different functional roles (defense, attraction, metabolism) within diverse scent blends is challenging. However, categorizing volatiles by their pattern of temporal regulation (Nielsen et al., 1995; Marotz-Clausen et al., 2018) narrows the set of compounds that potentially influence the behavior of pollinators with constrained windows of activity. Follow-up behavioral studies might be able to test these candidate volatiles to confirm a function. In this case, volatiles could be classified by whether they increased immediately with light (e.g. monoterpenes), increased in the afternoon without a light cue (e.g. pyranoid linalool ketone), or increased after dark (e.g. benzaldehyde; **Figures 2** and **4**). Volatiles could also be ranked by their relative change in emission rate when the pollinator is active vs. not active, and by their overlap with pollinator visitation. Future studies could investigate the proximate causes of regulation of these volatiles (e.g. by the circadian clock, reviewed in Fenske and Imaizumi, 2016), and identify which class is most attractive to pollinators. We predict that the afternoon-rising volatiles are long-range attractants because they would diffuse a great distance by the time moths are active, allowing moths to detect the population. Volatiles that increase after dark may be short-range attractants because they would not establish a long scent plume by the time moths are active.

CONCLUSIONS

Almost all volatiles released from inflorescences of *Schiedea kaalae* and *S. hookeri* displayed strongly time-specific modulations. Most *S. kaalae* volatiles peaked during or several hours after the brief time of evening visitation of *Pseudoschrankia brevipalpis*, a pollinator of both species. This pattern is generally consistent with selection that maximizes the attraction of pollinators by producing volatiles when pollinators are active, but the emission of most evening volatiles extended hours before the period of pollinator activity, when they could be active in long-range attraction. Additionally, some volatiles, perhaps unrelated to pollinator attraction, followed a daytime cycle. The composition of volatiles differed markedly between species, especially in the evening, and yet the timings of peak emissions were similar between the species. Knowing when emissions of each volatile begin, peak, and end will help to focus studies on the ecological functions of volatile compounds based on their temporal overlap with the activity of mutualists and antagonists.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.7280/D12H4M. The data repository is Dryad.

AUTHOR CONTRIBUTIONS

All authors participated in the design of the experiment. DC, RS, and CF provided advice on volatile analysis, and AS and SW provided advice on the study system. AG and RS designed and provided equipment. JP and RS collected and analyzed the data. JP wrote the manuscript, and all authors contributed substantially to revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01116/full#supplementary-material

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SYSTEMATIC REVIEW
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Deciphering the Biotic and Climatic Factors That Influence Floral Scents: A Systematic Review of Floral Volatile Emissions

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Currently, a global analysis of the information available on the relative composition of the floral scents of a very diverse variety of plant species is missing. Such analysis may reveal general patterns on the distribution and dominance of the volatile compounds that form these mixtures, and may also allow measuring the effects of factors such as the phylogeny, pollination vectors, and climatic conditions on the floral scents of the species. To fill this gap, we compiled published data on the relative compositions and emission rates of volatile organic compounds (VOCs) in the floral scents of 305 plant species from 66 families. We also gathered information on the groups of pollinators that visited the flowers and the climatic conditions in the areas of distribution of these species. This information allowed us to characterize the occurrence and relative abundances of individual volatiles in floral scents and the effects of biotic and climatic factors on floral scent. The monoterpenes trans-β-ocimene and linalool and the benzenoid benzaldehyde were the most abundant floral VOCs, in both ubiquity and predominance in the floral blends. Floral VOC richness and relative composition were moderately preserved traits across the phylogeny. The reliance on different pollinator groups and the climate also had important effects on floral VOC richness, composition, and emission rates of the species. Our results support the hypothesis that key compounds or compounds originating from specific biosynthetic pathways mediate the attraction of the main pollinators. Our results also indicate a prevalence of monoterpenes in the floral blends of plants that grow in drier conditions, which could link with the fact that monoterpene emissions protect plants against oxidative stresses throughout drought periods and their emissions are enhanced under moderate drought stress. Sesquiterpenes, in turn, were positively correlated with mean annual temperature, supporting that sesquiterpene emissions are dominated mainly by ambient temperature. This study is the first to quantitatively summarise data on floral-scent emissions and provides new insights into the biotic and climatic factors that influence floral scents.

Keywords: climate, floral volatiles, phylogeny, pollination syndromes, terpenoids, VOC composition, VOC richness

INTRODUCTION

Floral scent is an important trait of flowering plants and plays major roles in the interactions of plants with other organisms, including the attraction of pollinators (Raguso, 2004; Schiestl, 2010; Farré-Armengol et al., 2013; Junker and Parachnowitsch, 2015; Kantsa et al., 2018). Effective pollinators (those that carry pollen from the anthers to the stigmas of conspecific plants) are either specialist floral visitors of a limited spectrum of plant species or generalist floral visitors with a short-term specialization known as flower constancy (Chittka et al., 1999). Both pollinators with specialized innate flower preferences and those temporarily specialized *via* associative learning depend on cues or signals to distinguish amongst plant species (Chittka and Thomson, 2001; Kunze and Gumbert, 2001; Chittka and Raine, 2006; Majetic et al., 2008; Burger et al., 2010; Leonard et al., 2011a; Leonard et al., 2011b). Floral volatiles are key floral traits that mediate flower–visitor interactions by attracting pollinators, structuring flower–visitor communities, and defending against plant and flower antagonists (Junker and Blüthgen, 2010; Junker et al., 2010; Galen et al., 2011; Schiestl et al., 2014; Junker and Parachnowitsch, 2015). In addition to pollinator attraction, floral scents play major roles in the interactions with herbivores, parasitoids, and floral larcenists (Junker and Blüthgen, 2008; Raguso, 2008a; Kessler et al., 2008; Junker and Blüthgen, 2010; Galen et al., 2011; Farré-Armengol et al., 2013; Junker, 2016), and they also have important effects on the growth and composition of floral microbial communities (Heil, 2011; Junker et al., 2011; Huang et al., 2012; Junker and Tholl, 2013; Farré-Armengol et al., 2016a).

Pollinators play a major role in the reproduction of most angiosperms (Ollerton et al., 2011) and exert important selection pressures on plant and floral phenotypes, including floral scents (Wright and Schiestl, 2009; Parachnowitsch et al., 2012; Parachnowitsch et al., 2013; Schiestl and Johnson, 2013). The pollination syndrome hypothesis postulates that the floral traits of unrelated plants pollinated by the same pollinators tend to converge, including advertising signals (Faegri and van der Pijl, 1979; Fenster et al., 2004). Researchers have long discussed pollination syndromes, arguing in favour or against their reliability as effective classifiers of floral phenotypes that can be used to predict the plant's most efficient pollinators (Herrera, 1996; Ollerton, 1996; Waser et al., 1996; Armbruster et al., 2000; Fenster et al., 2004; Lázaro et al., 2008; Raguso, 2008b; Ollerton et al., 2009; Rosas-Guerrero et al., 2014; Ollerton et al., 2015). Many studies have described cases of floral-trait convergence by mono- and polyphyletic groups of plant species that share their main pollinators (Thomson et al., 2000; Stuurman et al., 2004; Wilson et al., 2004; Rosas-Guerrero et al., 2014). Some studies have reported convergent evolution in floral-scent composition driven by a shared reliance on the same pollinator group (Knudsen and Tollsten, 1993; Knudsen and Tollsten, 1995; Miyake et al., 1998; Andersson et al., 2002; Knudsen et al., 2004). Notable evidence also suggests that pollinators have strong evolutionary impacts on the intensity and composition of floral scents emitted by plants (Parachnowitsch et al., 2012; Parachnowitsch et al., 2013).

Plant emissions of volatile organic compounds (VOCs), including floral scents, can be affected by climatic variables such as temperature and humidity and by other environmental abiotic variables such as light, CO_2 concentration, wind speed, or the concentration of diverse oxidative pollutants such as ozone and nitrogen oxides. The effects of all these environmental abiotic variables and stresses on foliar VOC emissions and on VOC emissions from vegetation at a global scale are well characterized (Kesselmeier and Staudt, 1999; Peñuelas and Llusià, 2001; Owen et al., 2002; Niinemets et al., 2004; Duhl et al., 2008; Niinemets et al., 2010; Holopainen and Gershenzon, 2010; Niinemets, 2010; Peñuelas and Staudt, 2010), as well as those of endogenous variables that are partially controlled by the environment, such as plant nutrient contents (Fernández-Martínez et al., 2018 and references therein). Few studies, though, have addressed the effects of climatic variables on floral-scent emissions. Some of these studies have shed some light on the responses of floral volatile emissions to temperature (Jacobsen and Olsen, 1994; Sagae et al., 2008; Hu et al., 2013; Farré-Armengol et al., 2014; Farré-Armengol et al., 2015a), drought (Burkle and Runyon, 2016; Glenny et al., 2018), light (Jacobsen and Olsen, 1994; Hu et al., 2013), and pollution (Girling et al., 2013; Lusebrink et al., 2015; Farré-Armengol et al., 2016b; Saunier and Blande, 2019). Environmental variables have such effects on floral-scent emissions, so we hypothesize that climate can potentially select floral scents with properties that are most suited to the environmental conditions that plants and their flowers experience.

A global analysis of the currently available information on the floral scents of various species from many families is needed to shed light on how factors such as phylogeny, pollinators, and climate determine floral VOC emissions of the species. Previous studies by Knudsen et al. (1993) and Knudsen et al. (2006) qualitatively described the occurrence of >1,700 compounds in the flowers of 991 species and discussed whether the occurrence and richness of particular volatiles had phylogenetic signals and whether the compounds depended on the pollination biology of the species, i.e. their main pollinator type. The available data on the quantitative compositions and emission rates of floral VOCs, however, have not yet been compiled and analyzed. We aimed to fill this gap by searching published studies for data on the complete composition of floral scents and the emission rates of each compound or alternatively describing the relative percentage of contribution of each compound to the blend. We aimed to identify the effects of biological (pollinators) and climatic factors on the floral scents of the species by combining the data on the floral scents with the available data on the pollinators and climatic conditions in the regions where the plant species were sampled.

We compiled the available information on floral-VOC emission rates and/or relative VOC compositions for 305 plant species from 66 families. The database we compiled contained >800 compounds classified into nine groups: fatty acid derivatives, amino acid derivatives, benzenoids, monoterpenes, sesquiterpenes, irregular terpenes, nitrogen-containing compounds, sulphur-containing compounds, and miscellaneous compounds. We also

obtained information about the location and the climatic conditions where the populations from the different species that were measured grew and about the type of pollinators that visited the flowers, as described by the original studies. The information contained in our database allowed us to identify the most ubiquitous and dominant VOCs in floral scents (those that more frequently had the highest relative abundances in floral VOC blends). We further determined whether phylogeny, reliance on different types of pollinators, and climate were correlated with floral VOC richness, scent composition, and rate of emission. We hypothesized that the compositions and rates of emission of floral scents have been preserved throughout evolutionary history, and we aimed to differentiate between the effects of phylogeny on floral scents and the effects of biotic and climatic factors. We expected that the pollination syndrome would be correlated with VOC richness, composition, and emission rate of floral scents. Finally, we hypothesized that climate would exert some selective pressures on the production and emission of floral scents, thus positively or negatively stimulating the emissions of all or some compounds under the environmental conditions where each species grew and flowered.

METHODS

Search Criteria

We exhaustively searched the Web of Knowledge and Google Scholar for studies of floral scent using combinations of the keywords "floral"/"flower" and "volatiles"/"VOCs"/"scent". We chose studies that provided complete data on the emission rates and/or relative percentages of all floral VOCs emitted. We discarded studies that did not report the complete bouquet of VOCs of the floral scents but focused only on particular compounds, thus omitting other compounds that were emitted but were not the focus of the study. We finally selected 58 studies that provided information on the complete compositions of floral scents of one or more species. The references for all the studies from which we used data to make our database can be found in **Table S1**, where all plant species included in the database are found classified by families.

Data Entry

Each case in our database corresponded to a description of the floral scent of one plant species in one study. The name of the species (and subspecies when appropriate) was entered as provided by the source study, and equivalent synonyms following the Angiosperm Phylogeny Group III classification system, the genera, and the families were also recorded in our database. We obtained the longitudes and latitudes of the populations from which individuals of each species were sampled according to the methods described in the papers, and several climatic variables were obtained from the *WorldClim* database: Mean Annual Temperature, Max Temperature of Warmest Month and Min Temperature of Coldest Month (K), Mean Annual Precipitation, Precipitation of Wettest Month, and Precipitation of Driest Month (L/m^2). We calculated the Gaussen index of aridity using the climatic data as: *Gaussen index* = *annual precipitation*/(*2 * mean annual temperature*). We further obtained information on the pollinators that visited the flowers of each plant species as described by the source studies.

We classified floral VOCs based on their biosynthetic pathways, which was the predominant classification in all our data sources and enabled comparisons with phylogenetic hypotheses. We thus divided floral VOCs into the nine major classes: fatty acid derivatives, amino acid derivatives, benzenoids, monoterpenes, sesquiterpenes, irregular terpenes, nitrogen-containing compounds, sulphur-containing compounds, and miscellaneous compounds. We entered the data on the floral scent of each species as "presence/absence" (1/0), "relative percentages of the total blend" (%), and "emission rates" for all individual compounds and for the nine major groups of floral volatiles identified above. The source studies did not always provide emission rates, but all studies provided percentages of the total floral VOC blend. Emission rates were provided in various units ($\mu g\, h^{-1}\, flower^{-1}$, $\mu g\, h^{-1}\, inflorescence^{-1}$, $\mu g\, h^{-1}\, g\, DW^{-1}$, and $\mu g\ h^{-1}\ g\ FW^{-1}$), depending on the methods used in each study. Some studies specified the isomer(s) of various isomeric compounds in the floral scent of the species, but others did not. Several compounds thus appear in our database as repeated variables with and without a specified isomer.

Classification of the Plant Species Into Pollination Syndromes

We obtained information from the source studies of the pollinators that visited each plant species. Several species were pollinated or visited by different pollinator groups, so we created several binary variables in our database to indicate whether a particular plant species was or was not pollinated by a particular pollinator group (*wind*, *animals*, *insects*, *Lepidoptera*, *Coleoptera*, *Diptera*, *Hymenoptera*, *bats*, and *birds*). The description of pollinators differed across classification levels amongst the studies, so we created different binary variables to characterize the spectrum of pollinators, some of which were included in other groups (e.g. *Lepidoptera<insects<animals*) and provided information that was redundant to some extent. Both higher and lower classification levels, however, were useful for conducting different comparisons to answer different questions.

We further classified as many plant species as possible (239 from a total of 305) as predominantly pollinated by *wind*, *Lepidoptera*, *Coleoptera*, *Hymenoptera*, *Diptera*, *bats*, or *birds*. Plant species for which no information on main pollinator type was provided or those that were generalists (pollinated by different pollinator groups) to important degrees could not be classified into these groups and were therefore not included in the classification figures or analyses that required this classification.

Statistical and Phylogenetic Analyses

We prepared a phylogenetic tree containing the species in our database to test whether emission traits were phylogenetically preserved using R statistical software (R Core Team, 2017). We thereby obtained a phylogenetic tree containing a selection of 193 species from PhytoPhylo, an available megaphylogeny of

vascular plants (Qian and Jin, 2016). We used the *phylosig* function from the R package *phytools* (Revell, 2012) to test for phylogenetic signals for floral VOC richness, composition, and emission rate for the species. The *phylosig* function calculates statistics of a phylogenetic signal (Pagel's λ and Blomberg's K) and *P* values based on the variance of phylogenetically independent contrasts relative to tip shuffling randomisation (Blomberg et al., 2003).

We also used the phylogenetic tree to reconstruct the ancestral states of floral VOC emissions and pollination syndromes. We used stochastic character mapping (Nielsen, 2002; Huelsenbeck et al., 2003) to reconstruct ancestral transitions amongst the emission types and the pollination syndromes across the phylogeny. This method reconstructs the state of the ancestors of a phylogeny based on its structure and the observed traits of the current species. The ancestral reconstructions were prepared using the *make.simmaps* function of the R package *phytools* (Revell, 2012), simulating 1,000 stochastic ancestral reconstructions using the "mcmc" method (Markov chain Monte Carlo) and specifying equal rates of transition amongst the character states. The trees were simulated with a discrete-character map, with the states representing the dominant groups of floral VOCs (*fatty acid derivatives*, *benzenoids*, or *terpenoids*) and the pollination syndromes (*wind*, *bats*, *birds*, *Coleoptera*, *Lepidoptera*, *Hymenoptera*, or *Diptera*).

We used the Kruskal-Wallis (K-W) test for non-parametric data to test for differences in floral VOC richness and in the percentages of the classes of volatiles between plants pollinated by different pollinator groups. We further used the K-W test to compare the percentages of the most common floral volatiles, i.e. benzaldehyde, limonene, linalool, trans-β-ocimene, and benzyl alcohol, in floral blends amongst plants pollinated by different pollinators. K-W tests were conducted with R software using the *kruskal* function of the *agricolae* package (De Mendiburu, 2009). We performed multiple comparisons with the same predictor (pollination syndrome), so we used Bonferroni correction for multiple comparisons ($\alpha = 0.05/number\ of\ comparisons$).

We analyzed the effects of the pollinator types and climatic variables on VOC richness, relative percentages, and emission rates of each chemical class using phylogenetic linear regression models with R software. We used the *phylolm* function of the *phylolm* package, which fits phylogenetic linear models, allowing us to exclude the effect of phylogenetic distance (Ho and Ane, 2014). We tested for the effects of pollinator types using the binary variables describing whether the species were pollinated/visited by *wind*, *Lepidoptera*, *Coleoptera*, *Diptera*, *Hymenoptera*, *bats*, or *birds*. For emission rates, we conducted the phylogenetic linear regression models only with the data from species whose emissions were in units of $\mu g\ h^{-1}\ flower^{-1}$.

We tested whether plant species that shared the main group of pollinators had similar compositions of floral-scent bouquets using non-metric multidimensional scaling (NMDS) based on two distance measures and then fitted the pollination system onto the ordination using the *envfit* function in the R package *vegan* (Dixon, 2003; Oksanen et al., 2018). We used Bray-Curtis distances implemented in *vegan* that considers each compound as an independent variable and measures the similarities in the percentages of emission of individual compounds. We also applied the biosynthetically informed distance measure, $d_{A,B}$ (Junker, 2018), that considers the shared biosynthesis of compounds. Each compound was assigned to one of the nine major classes of compounds described above. $d_{A,B}$ informs the proportion of shared biosynthetic pathways leading to the floral-scent emissions of the plant species. Finally, we merged Bray-Curtis and $d_{A,B}$ distances in different ratios using weight w to calculate the weighted mean of both distance measures (see Junker (2018) for details). These merged distances compensate for the lack of information of the enzymes involved in the biosynthesis of the compounds.

RESULTS AND DISCUSSION

Diversity and Distribution of Floral Scent

We compiled 851 VOCs in the floral scents of 305 plant species belonging to 66 families (**Table S1**). Terpenoids were the most common floral volatiles (in the floral scents of 88.2% of the species), followed by benzenoids (80.7%), fatty acid derivatives (77.4%), nitrogen-containing compounds (30.8%), amino acid derivatives (9.2%), and sulphur-containing compounds (3.6%; **Table S2**). Phylogenetic signals were detected for floral VOC richness, relative composition and emission rates for some groups of compounds (**Table 1**), thus supporting that to some extent the compositions and rates of emission of floral scents have been preserved throughout evolutionary history.

Terpenoids, fatty acid derivatives, and benzenoids were the most diversified chemical groups of floral volatiles, with the highest richness of compounds. Terpenoids and benzenoids were the most predominant in the floral scents, followed by fatty acid derivatives (**Table S2**, **Figure S1**). These three groups of volatiles are the most important constituents of floral scents (Dobson, 2006). Other chemical groups were much rarer, sometimes only present or dominant in a small group of species. Sulphur-containing compounds were a special case; their occurrence and higher relative abundance in floral scents was strictly associated with bat pollination. These results are in accordance with findings that bat-pollinated plants attract pollinators by emitting floral scents rich in sulphur-containing compounds (Knudsen and Tollsten, 1995; Bestmann et al., 1997; von Helversen et al., 2000; Knudsen et al., 2006).

Our database of floral VOC emissions identified the benzenoid benzaldehyde and the monoterpenes limonene, trans-β-ocimene, and linalool as the most ubiquitous volatiles in floral scents (**Figure 1A**), coinciding with previous studies by Knudsen et al. (1993; 2006). We further found that benzaldehyde, trans-β-ocimene, and linalool were the most common predominant floral VOCs (**Figure 1B**). This finding strongly supports the important ecological role in floral scents of β-ocimene, which is a common floral volatile emitted by plants pollinated by different groups of pollinators (Dobson, 2006; Knudsen et al., 2006; Filella et al., 2013; Farré-Armengol et al.,

TABLE 1 | Results of the phylogenetic signal tests for richness (N = 197), relative percentage (N = 197), and emission rate (N = 67) of fatty acid derivatives (FADs), amino acid derivatives (AADs), benzenoids, monoterpenes, sesquiterpenes, irregular terpenes, terpenes, nitrogen-containing compounds (NCCs), and sulphur-containing compounds (SCCs).

	Richness				Relative percentage				Emission rate			
	λ	*P*	K	*P*	λ	*P*	K	*P*	λ	*P*	K	*P*
FADs	**0.40**	**0.015**	**0.08**	**<0.001**	0.42	0.05	**0.07**	**0.003**	**0.22**	**0.018**	0.07	0.37
AADs	**0.27**	**<0.001**	**0.08**	**0.04**	0.06	0.24	0.04	0.625	0.11	0.298	0.07	0.292
Benzenoids	**0.52**	**<0.001**	**0.05**	**0.022**	0.18	0.204	0.03	0.359	**0.47**	**0.012**	0.05	0.457
Monoterpenes	<0.01	1	0.03	0.351	**0.39**	**0.026**	0.03	0.102	**0.75**	**0.010**	0.07	0.427
Sesquiterpenes	0.04	0.538	0.03	0.4	<0.01	1	**0.13**	**0.004**	<0.01	1	0.04	0.594
Irr. terpenes	<0.01	1	**0.08**	**0.011**	0.8	1	**0.08**	**0.047**	**0.33**	**<0.001**	0.06	0.261
Terpenes	0.09	0.191	0.03	0.332	0.39	0.068	**0.04**	**0.025**	**0.75**	**0.008**	0.07	0.427
NCCs	**0.26**	**0.028**	0.02	0.781	0.14	0.753	0.02	0.697	<0.01	1	0.07	0.445
SCCs	**0.96**	**<0.001**	0.07	0.36	**0.97**	**<0.001**	0.19	0.151	<0.01	1	0.02	0.821

Pagel's λ, Bloomberg's K, and their associated P values are provided for each variable. Significant values are depicted in bold type.

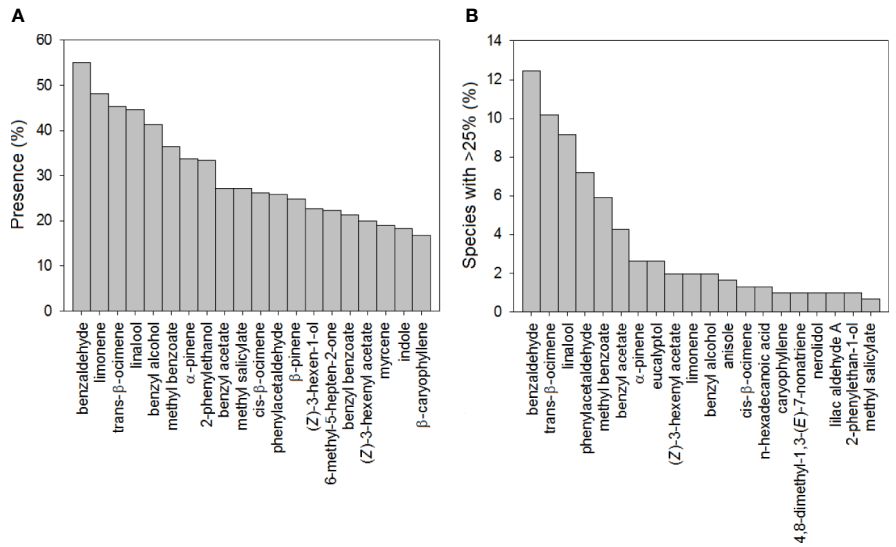

FIGURE 1 | Bar chart showing **(A)** the percentage of plant species with the most common floral volatiles and **(B)** the percentage of plant species where the most abundant floral volatiles represented more than 25% of the total floral scent (N = 305).

2017). Benzaldehyde and linalool are also good attractants of Lepidoptera (Dobson, 2006), which are a predominant group of pollinators of many angiosperms (Andersson et al., 2002; Dötterl et al., 2006). Linalool also has various other functions in floral ecology, ranging from repellent properties to effects in interactions with bacteria (Raguso and Pichersky, 1999a; Junker and Blüthgen, 2008; Raguso, 2016; Burdon et al., 2018).

Pollination Syndromes and the Composition and Emission Rates of Floral Scents

Pollination syndromes consist of particular combinations of floral traits that attract particular groups of pollinators with shared floral preferences (Faegri and van der Pijl, 1979; Fenster et al., 2004). Plants pollinated by different pollination vectors are thus expected to emit floral VOC blends dominated by different types of compounds (Dobson, 2006). The plant species included in our floral-scent database represented the different main pollination vectors relatively well, although some of them were more represented than others, with Lepidoptera pollination the most frequent pollination vector in our data set (47.68%) (**Figure S2A**). We phylogenetically reconstructed the trait "main pollination vector" from the plants included in our database, which indicated how the main pollination vectors were distributed in the phylogeny and how the species switched from one pollination vector to another within the evolution of different plant lineages (**Figure S2B**). We also found that all the pollination vectors were distributed in different branches in the phylogeny, despite some phylogenetic clustering.

We found that mean total floral VOC richness was higher in zoophilous species (pollinated by animals) as a group than in anemophilous species (pollinated by wind), although the differences were not significant (H = 4.06, P = 0.044, **Figure 2A**). Some studies have demonstrated that plants

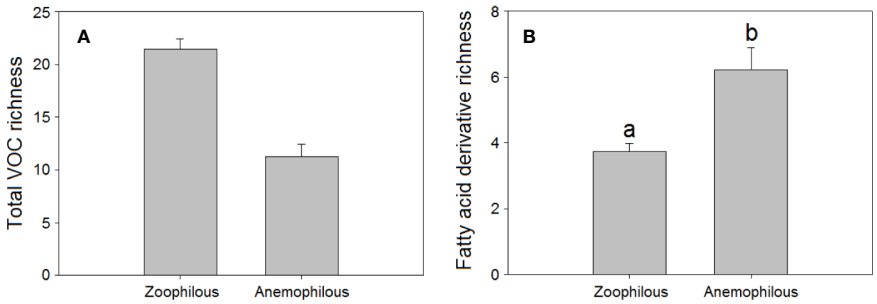

FIGURE 2 | Richness of VOCs in the floral scents of zoophilous (N = 254) and anemophilous plant species (N = 9): **(A)** total VOC richness, **(B)** fatty acid derivative richness. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0056$).

pollinated by wind tend to emit fewer floral volatiles and in lower amounts than do entomophilous plants (Magalhães et al., 2005; Wragg and Johnson, 2011; Farré-Armengol et al., 2015b). Zoophilous plants need to attract pollinators to their flowers to cross-pollinate them, using VOCs, visual signals, and floral rewards (Raguso, 2004; Whitehead and Peakall, 2009; Schiestl, 2010; Kantsa et al., 2017). Anemophilous plants, though, do not need to attract pollinators to their flowers to be pollinated and tend to emit weak floral scents, although they can emit some VOCs that may have functions other than pollinator attraction, such as defence. We found that anemophilous flowers emitted a significantly higher diversity (H = 8.75, $P = 0.003$, **Figure 2B**) and higher proportions of fatty acid derivatives than did entomophilous flowers (H = 13.7, $P < 0.001$, **Figure 3**). We hypothesize that VOC emissions of anemophilous flowers were dominated by fatty acid derivatives because some of the most common compounds in this group, the green leaf volatiles (GLV), develop defensive functions in vegetative as well as in other plant tissues (Scala et al., 2013; Naeem ul Hassan et al., 2015), and anemophilous plants are not negatively affected by presenting defensive (toxic or deterrent) compounds in their flowers, as zoophilous plants do (Lucas-Barbosa et al., 2011; Schiestl et al., 2011; Farré-Armengol et al., 2013).

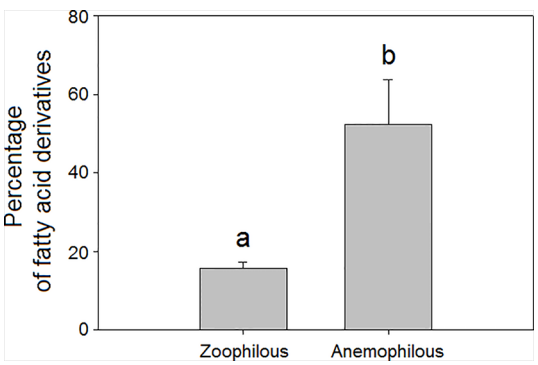

FIGURE 3 | Relative percentage composition of VOCs in the floral scents of zoophilous (N = 9) and anemophilous plant species (N = 254): percentages of fatty acid derivatives. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0063$).

Ornithophilous flowers (pollinated by birds) are almost scentless (Knudsen et al., 2004; Magalhães et al., 2005; Klahre et al., 2011), likely because birds rely more on vision than olfaction for floral location (Faegri and van der Pijl, 1979; Knudsen et al., 2004; Cronk and Ojeda, 2008). The lower VOC richness in species pollinated by birds (H = 38.11, $P < 0.001$, **Figure 4A**) and the negative correlation detected between bird pollination and total VOC richness (**Table 2**) supported this proposal.

The higher richness (H = 91.95, $P < 0.001$, **Figure 4G**) and relative percentage of sulphur-containing compounds in floral scents from bat-pollinated plants (H = 91.73, $P < 0.001$, **Figure 5C**) and the strongly significant positive correlations of bat pollination with both sulphur-containing compound richness (**Table 2**) and relative percentage (**Table 3**) supported a close relationship between the emission of sulphur-containing compounds and pollination mediated by bats. These results were in agreement with studies demonstrating convergent evolution of bat-pollinated plant species from different families to emit sulphur-containing volatiles such as dimethyl disulphide, dimethyl trisulphide, and dimethyl tetrasulphide (Knudsen and Tollsten, 1995; Bestmann et al., 1997). However, this pattern is not universal and seems to be restricted to bat-pollinated plants from the neotropics (Carter and Stewart, 2015). The emission of sulphur-containing compounds by neotropical bat-pollinated plants is an adaptation to attract flower-visiting bats that share an innate preference for this group of volatiles (von Helversen et al., 2000).

The differences in floral VOC richness (**Figures 4** and **6**), floral-scent composition (**Figures 5** and **7**), and relative abundance of individual compounds (**Figure S3**) amongst plant species pollinated by different animal groups may also support the existence of different pollination syndromes for floral scent. The higher richness of benzenoids and the monoterpene linalool in the scent of flowers pollinated by butterflies and moths strongly suggest a preference of Lepidoptera for these compounds (Raguso and Pichersky, 1999b; Andersson et al., 2002; Dötterl et al., 2006). Our results supported this preference:

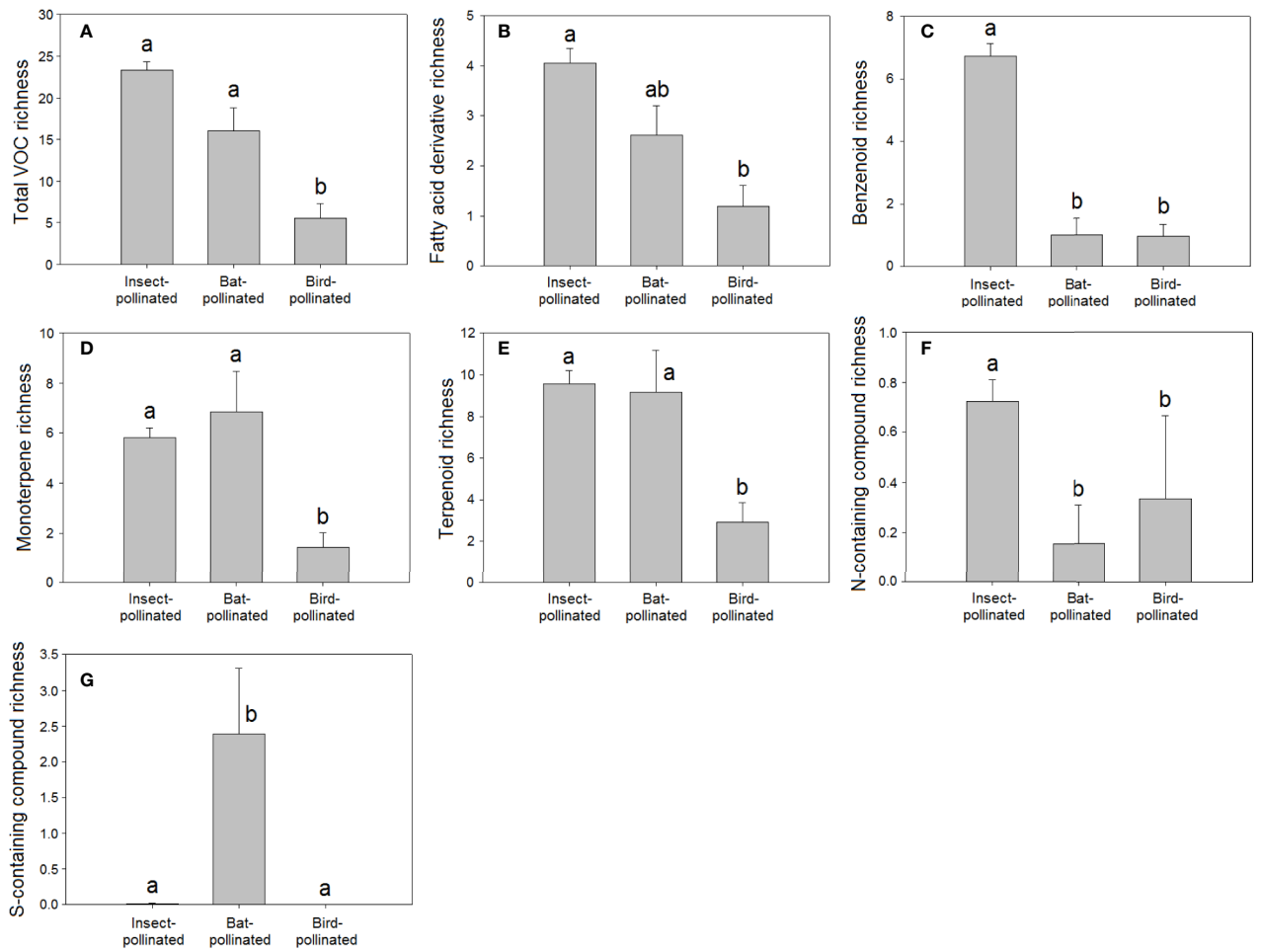

FIGURE 4 | Richness of VOCs in the floral scents of plant species pollinated by insects (N = 221), birds (N = 21), and bats (N = 13): **(A)** total VOC richness, **(B)** fatty acid derivative richness, **(C)** benzenoid richness, **(D)** monoterpene richness, **(E)** terpenoid richness, **(F)** nitrogen-containing compound richness, and **(G)** sulphur-containing compound richness. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0056$).

benzenoids were more diversified in the floral scents of Lepidoptera-pollinated plants (**Figure 6C**; **Table 2**), and linalool was also more dominant in Lepidoptera-pollinated species (**Figure S3C**). On the other hand, benzaldehyde is a common floral volatile that has been measured in important proportions in the floral scents of some plant species pollinated mainly by Diptera, such as *Leontopodium alpinum*, *Crataegus* sp., and *Filipendula ulmaria* (Dobson, 2006). Our results confirm that benzaldehyde was more abundantly represented in the floral scents of plant species that are pollinated by Diptera (**Figure S3A**). On the contrary, β-ocimene was recognized to be widely distributed in the floral scents of plants that belong to different pollination syndromes and has been proposed to play a key role as a generalist pollinator attractant (Filella et al., 2013; Farré-Armengol et al., 2017). Actually, trans-β-ocimene is a ubiquitous floral-scent constituent with high levels of occurrence and high relative abundances in floral scents (**Figure 1**) (Dobson, 2006; Knudsen et al., 2006), which usually co-occurs with its less abundant isomer, cis-β-ocimene (**Table S2**).

We found some support for the pollination syndromes in the composition of scent bouquets. The main pollinators significantly fitted onto the ordinations representing similarities in floral-scent composition between species, but no clear clusters were detected (**Figure 8**). Fitting the main pollinators onto the ordination resulted in lower r^2 values when NMDS was based on Bray-Curtis distances ($r^2 = 0.1568$, $P = 0.001$, mean of 1,000 permutations, **Figure 8A**) than on biosynthetically informed distances $d_{A,B}$ merged with Bray-Curtis distances in a ratio 36:964 ($r^2 = 0.1917$, $P = 0.001$, **Figure 8B**) (see Junker, 2018 for information on methodological details). Bray-Curtis distances consider each compound individually, whereas biosynthetically informed distances consider the proportion of shared major classes of compounds and therefore the proportion of shared biosynthetic pathways. This result indicates that often not specific

TABLE 2 | Significant results of the phylogenetic linear models (*phylolm*) testing for the effects of pollination vectors and climatic variables on the richness of total VOCs, fatty acid derivatives (FADs), amino acid derivatives (AADs), benzenoids, terpenoids, monoterpenes, sesquiterpenes, nitrogen-containing compounds (NCCs), and sulphur-containing compounds (SCCs) (N = 142).

	β	*P*
Total VOCs vs. Lepidoptera pollination	0.341	<0.001
Total VOCs vs. Bird Pollination	-0.24	0.036
FADs vs. Mean annual precipitation	13.763	0.014
FADs vs. Gaussen index	-13.347	0.016
Benzenoids vs. Lepidoptera pollination	0.253	0.009
Benzenoids vs. Bird pollination	-0.316	0.003
Benzenoids vs. Maximum temperature of warmest month	-0.617	0.045
Terpenoids vs. Lepidoptera pollination	0.236	0.023
Monoterpenes vs. Bat pollination	0.265	0.003
Sesquiterpenes vs. Lepidoptera pollination	0.228	0.033
Sesquiterpenes vs. Mean annual temperature	1.849	0.004
Sesquiterpenes vs. Maximum temperature of warmest month	-0.922	0.004
Sesquiterpenes vs. Minimum temperature of coldest month	-1.303	0.006
NCCs vs. Lepidoptera pollination	0.488	<0.001
NCCs vs. Coleoptera pollination	0.319	0.003
NCCs vs. Diptera pollination	0.245	0.004
NCCs vs. Hymenoptera pollination	0.231	0.017
NCCs vs. Bird pollination	0.33	0.004
SCCs vs. Bat pollination	0.514	<0.001
SCCs vs. Precipitation of wettest month	-1.073	<0.001
SCCs vs. Precipitation of driest month	-0.779	<0.001

The function phylolm fits phylogenetic linear models that allow the exclusion of the effect of phylogenetic distance. The standardized coefficients and P values are provided for each variable.

compounds mediated the attraction of the main pollinators, but the presence/absence or abundance of compounds sharing the same biochemical pathway. Several case studies have highlighted the importance of key compounds in flower–pollinator interactions (Riffell et al., 2009; Svensson et al., 2010; Schäffler et al., 2015; summarized in Junker, 2016); our results suggest that compounds from the same biosynthetical pathway may have redundant functions—at least in the context of higher taxonomic levels as in pollination syndromes.

Floral-scent bouquets are accordingly less integrated than bouquets emitted by leaves, so the proportional composition of floral-scent bouquets is much more variable than that of foliar volatiles (Junker et al., 2017). Individual compounds (or representatives of chemical classes) may thus be sufficient to mediate functions such as pollinator attraction, regardless of the presence/absence or emission rate of other compounds in the bouquet (Junker et al., 2017). These findings in combination with the finding that some compounds are over-represented in some of the pollination systems (see above) support the concept that key compounds mediate interactions of flowers with their pollinators, not ratios of compounds or entire compound classes (Junker, 2016; Junker et al., 2017). Our results, combined with earlier findings, thus suggest that pollination syndromes that consider floral-scent emissions should not be defined based on the composition of the bouquets. The presence of individual key compounds or the presence of compounds originating from specific biosynthetical pathways may instead be indicative of pollination by a

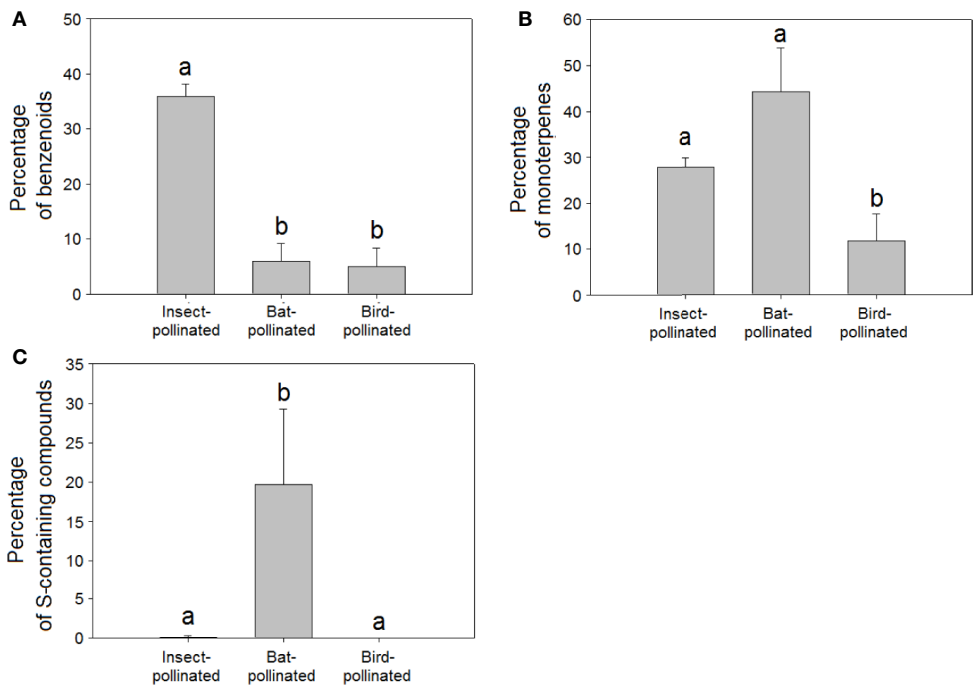

FIGURE 5 | Relative percentage composition of VOCs in the floral scents of plant species pollinated by insects (N = 221), birds (N = 21), and bats (N = 13): percentages of **(A)** benzenoids, **(B)** monoterpenes, **(C)** sulphur-containing compounds. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0063$).

TABLE 3 | Significant results of the phylogenetic linear models (*phylolm*) testing for the effects of pollination vectors and climatic variables on the relative percentage of fatty acid derivatives (FADs), amino acid derivatives (AADs), benzenoids, terpenoids, monoterpenes, sesquiterpenes, nitrogen-containing compounds (NCCs), and sulphur-containing compounds (SCCs) (N = 142).

	β	*P*
FADs vs. Wind pollination	0.289	0.001
FADs vs. Mean annual precipitation	0.194	<0.001
FADs vs. Gaussen index	-0.184	<0,001
Benzenoids vs. Bird pollination	-0.338	0.004
Terpenoids vs. Minimum temperature of coldest month	0.929	0.042
Terpenoids vs. Mean annual precipitation	-0.125	0.025
Terpenoids vs. Gaussen index	0.114	0.038
Monoterpenes vs. Mean annual temperature	-1.717	0.003
Monoterpenes vs. Maximum temperature of warmest month	1.081	<0.001
Monoterpenes vs. Minimum temperature of coldest month	1.844	<0,001
Monoterpenes vs. Mean annual precipitation	-0.148	0.005
Monoterpenes vs. Gaussen index	0.137	0.008
Sesquiterpenes vs. Mean annual temperature	1.4	0.03
Sesquiterpenes vs. Minimum temperature of coldest month	-0.966	0.042
SCCs vs. Bat pollination	0.402	<0.001
SCCs vs. Precipitation of wettest month	-1.824	<0.001
SCCs vs. Precipitation of driest month	-1.098	<0.001

The function phylolm fits phylogenetic linear models that allow the exclusion of the effect of phylogenetic distance. The standardized coefficients and P values are provided for each variable.

pollinator taxon. Note, however, that several compounds are over-proportionally found in the scent bouquets of plants pollinated by different taxa (the present study; Dobson, 2006), questioning the universal validity of these findings. The current data on floral-scent emission are generally strongly biased towards specialized plant-pollinator systems, and thus towards plant species that can be clearly assigned to a syndrome, which is also evident in our data set. Most plant species are visited and pollinated by several taxa, preventing the assignment of a plant species to a syndrome (Waser et al., 1996). Considering all pollinator assemblages and assessing the relative efficiency of all floral visitors are thus important for a better understanding of the role of plant-pollinator interactions in floral-trait evolution.

Secondary pollinators can play an important role in plant reproduction and floral-trait selection, potentially shifting evolutionary trends in pollination syndromes (Rosas-Guerrero et al., 2014). Plant fitness can significantly benefit from visits by pollinators that do not belong to the main functional group of pollinators (Fishbein and Venable, 1996; Miyake and Yahara, 1998; Kandori, 2002; Sahli and Conner, 2007). The suitability of attracting secondary or occasional pollinators to flowers can therefore also exert important selection pressures on floral traits (Aigner, 2001). Ollerton et al. (2009) proposed that selecting only the most effective pollinator failed to identify the range of logical possibilities that could account for the evolution of a floral trait. Some authors have suggested that the large temporal and spatial variation in the spectra of pollinators and effectiveness across years and locations may mitigate or dilute the relative impact of any specific pollinator as a selective agent on heritable floral variation (Herrera, 1996; Ollerton, 1996; Waser et al., 1996; Raguso, 2008b). This variation may also favour generalised

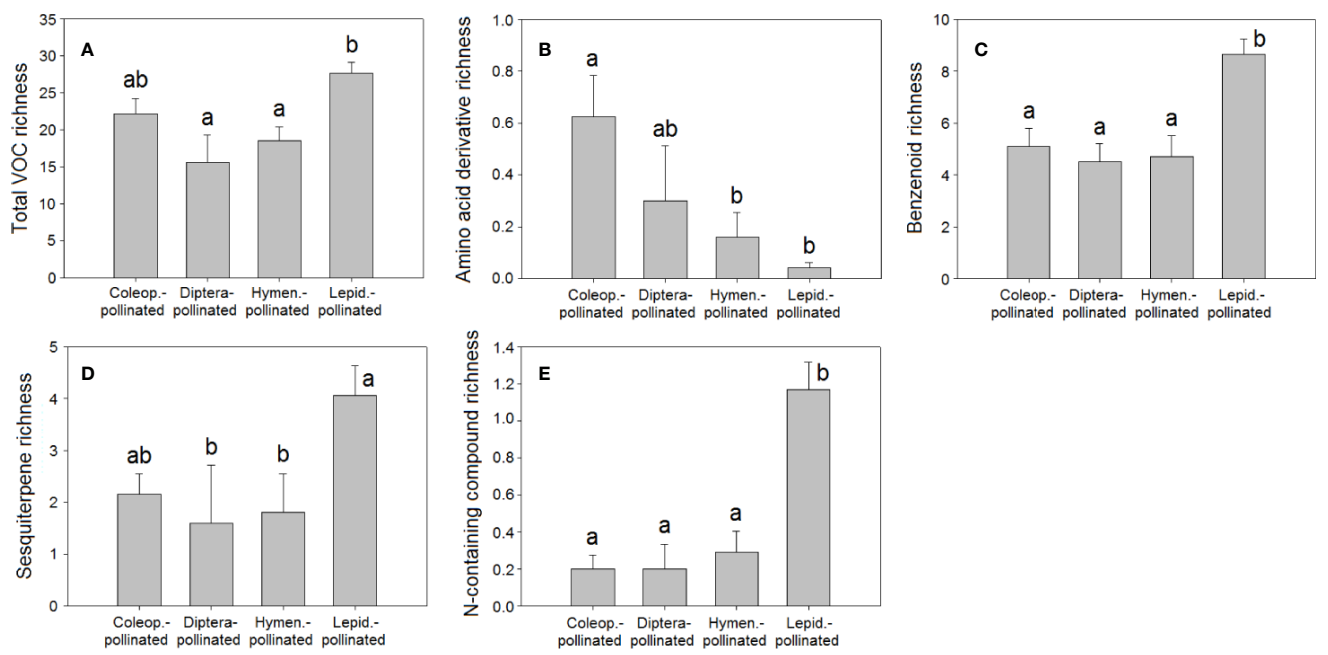

FIGURE 6 | Richness of VOCs in the floral scents of plant species pollinated by Coleoptera (N = 40), Diptera (N = 10), Hymenoptera (N = 31), and Lepidoptera (N = 113): **(A)** total VOC richness, **(B)** amino acid derivative richness, **(C)** benzenoid richness, **(D)** sesquiterpene richness, **(E)** nitrogen-containing compound richness. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0056$).

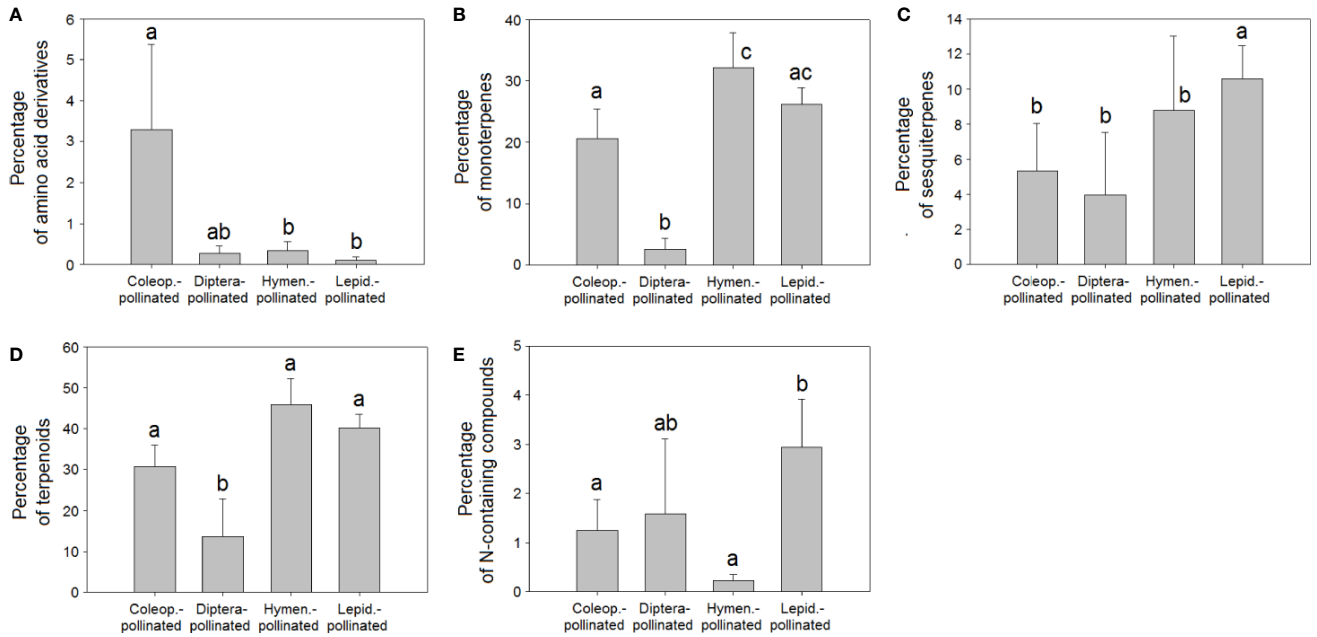

FIGURE 7 | Relative percentage composition of VOCs in the floral scents of plant species pollinated by Coleoptera (N = 40), Diptera (N = 10), Hymenoptera (N = 31), and Lepidoptera (N = 113): percentages of **(A)** amino acid derivatives, **(B)** monoterpenes, **(C)** sesquiterpenes, **(D)** terpenoids, **(E)** nitrogen-containing compounds. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.0063$).

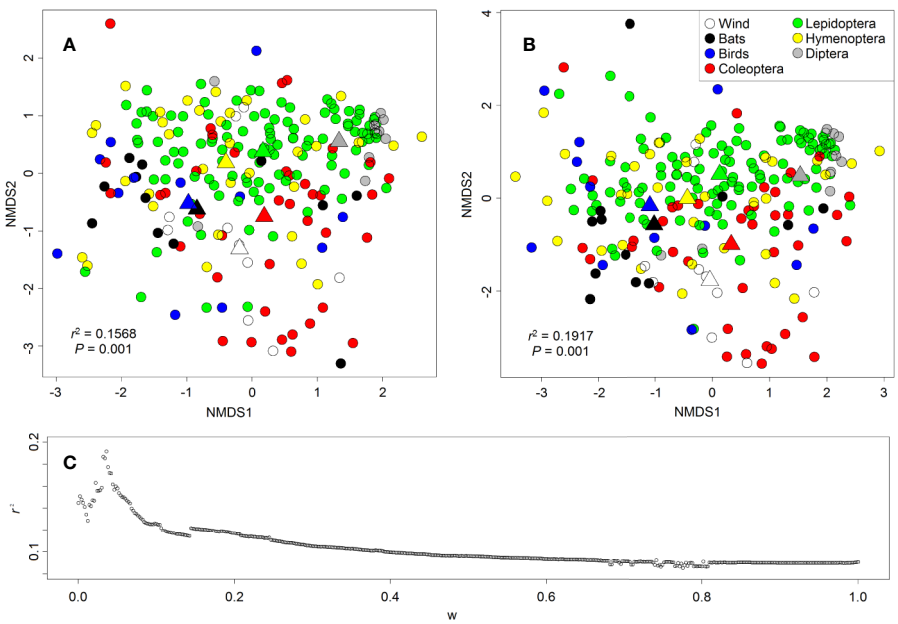

FIGURE 8 | Ordination (NMDS) of floral-scent bouquets based on **(A)** Bray-Curtis distances and **(B)** biosynthetically informed distances $d_{A,B}$ merged with Bray-Curtis distances in a ratio 36:964. Main pollinators are colour-coded as shown in the legend in **(B)**. Each circle represents a plant species, and triangles are the centroids of scent bouquets of flowers pollinated by the same pollinators. Although no clear clusters of pollination systems are visible, the mean position of pollination systems in the ordination are still significantly different from each other (see centroids). **(C)** r^2 of fitting of pollination systems onto the ordination as a function of weight w to calculate merged distances (*0, Bray-Curtis distance; 1, biosynthetically informed distance measure* $d_{A,B}$).

reproductive strategies and phenotypes that attract multiple pollinators. Future research should therefore focus on plant species that are not involved in specialized pollination mutualisms but are visited by many taxa. Studying scent bouquets in plant communities may help us to find more universal patterns (Junker, 2016; Larue et al., 2016; Junker et al., 2017; Kantsa et al., 2017; Kantsa et al., 2018).

Other biological agents not yet mentioned, such as herbivores, floral larcenists, and other floral visitors with negative impacts on plant fitness, and floral microbial communities and pathogens may also have important effects on floral-trait evolution, including floral scent (Strauss and Armbruster, 1997; Frey, 2004; Lau and Galloway, 2004; Parachnowitsch and Caruso, 2008; Junker et al., 2011; Junker, 2016). Scent blends are generally composed of many volatiles, so different components of the blend may play different roles and be under different forms of selection (Kessler et al., 2008; Schiestl et al., 2011). All these multiple agents of selection can exert different or even opposite selection pressures on the same floral traits (Cariveau et al., 2004; Kessler and Halitschke, 2009) and can have varying impacts across time and space (Brody, 1997; Kandori, 2002; Dupont et al., 2009; Schlumpberger et al., 2009).

Links Between Climatic Variables and Floral Scent

We found several significant relationships between climatic variables and the richness, relative composition, and emission rate of floral VOCs (**Tables 2**–**4**). Richness and relative percentage of fatty acid derivatives in the floral scents were positively correlated with annual precipitation (**Tables 2** and **3**). The relative percentage of monoterpenes showed negative correlations with mean annual temperature and also with annual precipitation (**Table 3**); the later would indicate a prevalence of monoterpenes in drier conditions, which could link with the fact that monoterpene emissions, rather than sesquiterpene emissions, seem to protect plants against oxidative stresses throughout drought periods (Ormeño et al., 2007) and their emissions are enhanced under moderate drought stress (Vallat et al., 2005; Yani et al., 2006; Ormeño et al., 2007). The richness, relative percentage, and emission rates of sesquiterpenes were positively correlated with mean annual temperature (**Tables 2**–**4**); these results strongly support the observations from previous studies that indicated that sesquiterpene emissions from vegetation are dominated mainly by ambient temperature, with a positive effect of temperature on them (reviewed by Duhl et al., 2008). The emission rates of nitrogen-containing compounds were negatively correlated with mean annual precipitation (**Table 4**). All these results suggest that climate is a relevant factor determining the compositions and emission rates of floral scents, in addition to the strong and well-known selective pressures exerted by biotic agents such as pollinators and other floral visitors (Jacobsen and Olsen, 1994; Yua et al., 2009; Farré-Armengol et al., 2013). The maximum temperatures that plant species can experience in their region during flowering, for example, have been positively correlated with the species-specific temperature thresholds that decrease floral-scent emissions, i.e. the maximum temperature tolerance of floral-scent emissions (Farré-Armengol et al., 2015a). Plants can thus adapt their physiology to optimise floral-scent emissions under the climatic conditions where they grow and flower. The responses of VOC emissions under particular environmental conditions are determined not only by plant physiology, but also by the temperature responses of the enzymes involved in their biosynthesis, the temperature responses of the membrane transporters and the cuticle composition and thickness, and also the particular physicochemical properties of the compounds (Niinemets et al., 2004; Copolovici and Niinemets, 2005; Noe et al., 2006; Harley, 2013; Farré-Armengol et al., 2014). We therefore hypothesize that climate can also select for floral scents that contain compounds with different physicochemical properties and increase the suitability of floral scents to the environmental conditions that plants and their flowers experience.

TABLE 4 | Significant results of the phylogenetic linear models (*phylolm*) testing for the effects of pollination vectors and climatic variables on the emission rates of total VOCs, fatty acid derivatives (FADs), amino acid derivatives (AADs), benzenoids, terpenoids, monoterpenes, sesquiterpenes, nitrogen-containing compounds (NCCs), and sulphur-containing compounds (SCCs) (N = 43).

	Estimate	*P*
Sesquiterpenes vs. Mean annual temperature	3.804	0.006
Sesquiterpenes vs. Maximum temperature of warmest month	-1.621	0.012
Sesquiterpenes vs. Minimum temperature of coldest month	-3.038	0.002
Sesquiterpenes vs. Precipitation of wettest month	3.368	0.006
NCCs vs. Maximum temperature of warmest month	1.622	0.027
NCCs vs. Mean annual precipitation	-0.289	0.024
NCCs vs. Gaussen index	0.306	0.022

The function phylolm fits phylogenetic linear models that allow the exclusion of the effect of phylogenetic distance. The standardized coefficients and P values are provided for each variable.

Future Prospects

Research bias from the non-random sampling of the natural world is an important problem in any review (Gurevitch and Hedges, 1999), and the authors cannot correct for it. Identifying gaps in the literature where more research is needed, however, is an important contribution of any review. The available information on floral scents that we compiled was collected for many families representing a broad phylogenetic range, which allowed us to characterize general trends in the distribution of floral emissions. The species also belonged to different pollination syndromes and had different geographical distributions, which allowed us to explore the relationships between floral scents and biotic and climatic factors. The floral scents for some pollination syndromes, however, are poorly represented in our database, especially those from plant species pollinated by wind, birds, bats, and Diptera. Most angiosperms rely on animals for pollination, and fewer species rely on abiotic vectors such as wind (Ollerton et al., 2011). Floral scents are especially associated with biotic pollination, so ecological studies of floral-scent chemistry tend to focus on the floral scents of animal-pollinated plants and their role in the attraction of the

pollinators. The smaller proportion of wind-pollinated species, and the smaller biological and ecological interest in characterizing their floral scents, may have therefore strongly biased what we know about the floral scents of wind-pollinated plants compared to the floral scents from other pollination syndromes. The same is true for bird-pollinated plant species. Studies of the pollination ecology of bird-pollinated species often focus on visual and morphological floral traits rather than scent, because birds rely more on vision than olfaction for floral location (Cronk and Ojeda, 2008), and bird-pollinated species emit weak or no floral scents (Knudsen et al., 2004; Magalhães et al., 2005).

The most important gap in our knowledge of floral scents is probably the scents of plant species with generalist spectra of pollinators. Species with generalist spectra of pollinators are under-represented in the floral-scent literature, because most studies of floral scent have focused on species with specific pollinator interactions. However, not all floral visitors are effective pollinators, and only some generate selection on plant and floral traits, despite receiving visits by two or more groups of floral visitors (Armbruster et al., 2000). We consider that this fact is important for our analyses, something that could be improved if the studies provided more accurate and detailed data and a greater certainty identifying the effective pollinators. Most studies analysing and describing the floral scents of animal-pollinated plant species that are not strict specialists unfortunately do not describe the relative importance of their pollinators in much detail. We thus focused on plants that most clearly belonged to the "specialist" syndromes when analysing the pollination syndromes. New studies of floral-scent biology and chemistry should continue to expand our current knowledge of the distribution of floral scents, taking special care to also characterize the less well represented groups of plants, including species from all families, pollination syndromes, and climatic regions.

Some studies of the composition of floral scents provided emission rates for all floral compounds, but many studies provided only the relative percentages. The relative composition of floral scents is very relevant information, but actual emission rates could contribute more to our understanding of floral scents. We therefore encourage authors to quantify and describe the emission rates for each compound when possible. We also encourage authors to use the same reference units when providing emission rates, which would simplify the inclusion of their results in combined analyses. We noticed that $\mu g\ h^{-1}$ flower^{-1} was the most commonly used unit of emission rate. We strongly recommend, however, the use of $\mu mol\ h^{-1}\ g\ DW^{-1}$ (instead of, or in addition to, any other units), because it is a more standardized unit for describing emissions from flowers or any other plant organs/tissues.

The responses of floral scents to different environmental climatic factors such as temperature or drought are highly plastic (Farré-Armengol et al., 2013; Farré-Armengol et al., 2014; Glenny et al., 2018), as are the responses to biotic interactions (Huber et al., 2005; Lucas-Barbosa et al., 2011; Schiestl et al., 2011; Schiestl et al., 2014; Junker, 2016; Hoffmeister and Junker, 2017). The high plasticity of floral-scent emissions within individual plants is usually not considered in sufficient detail (Majetic et al., 2009). The current literature, however, highlights the great potential of analyzing intraspecific floral-scent variation, which occurs within and amongst populations and within individuals (Delle-Vedove et al., 2017).

Phyllospheric microorganisms have important effects on the composition of floral volatile emissions (Peñuelas et al., 2014; Helletsgruber et al., 2017). Microorganisms living on flowers can produce and emit VOCs, transform or degrade the VOCs emitted by floral tissues, and affect plant physiology, causing multiple changes to floral emissions (Junker and Tholl, 2013; Farré-Armengol et al., 2016a). Future research will verify the importance of microorganisms for defining the chemical phenotype of flowers and the implications for the biological interactions that these olfactory signals mediate.

CONCLUSIONS

Floral scents are subject to many evolutionary pressures exerted by biotic and abiotic environmental factors. The need to attract pollinators is a major reason why animal-pollinated angiosperms have evolved complex and diverse floral scents. Plants have thus evolved various sets and mixtures of floral volatiles that help promote flower constancy, which in some cases stimulate the attraction of specific groups of pollinators. We identified some patterns indicating that particular VOCs were associated with particular pollination syndromes. Our results support the concept that key compounds or compounds originating from specific biosynthetic pathways play a significant role in mediating the interactions of flowers with their pollinators. Other floral visitors (e.g. herbivores and larcenists) and floral inhabitants (e.g. nectar yeasts and floral microbial communities and pathogens) also exert important selection pressures on plant secondary metabolism and floral scents. All these selection pressures act in different ways on floral phenotypes and they may difficult the appearance of patterns established across the entire phylogeny of flowering plants (or even within major plant clades) of shared emissions of complex VOC mixtures associated with the attraction of a particular pollinator group. We identified several significant relationships between climatic variables and the richness, relative composition, and emission rate of floral VOCs. Our results suggest that climate is a relevant factor determining the composition and emission rate of floral scents, in addition to the strong and well-known selective pressures exerted by biotic agents such as pollinators and other floral visitors. We therefore hypothesize that climate can also select for floral scents that contain compounds with different physicochemical properties, increasing the suitability of floral scents to the environmental conditions that plants experience during flowering.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

GF-A and JP conceived and designed the study. GF-A compiled all the information contained in the database used in the study. MF-M and RJ significantly contributed to the statistical analysis of data by conducting some of the analyses and advising GF-A on the suitability of all the analyses employed. GF-A wrote the manuscript, and JP, RJ, MF-M, and IF supervised and contributed to the writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01154/full#supplementary-material

FIGURE S1 | Phylogenetic tree showing the dominant class of floral VOCs for the species included in the phylogeny of reference *PhytoPhylo* (small circles; N = 197) and the probability of emission type of ancestor nodes (large circles) as pie charts. The ancestral reconstruction was performed using 1,000 stochastic character-mapped trees (see the Methods section for further information).

FIGURE S2 | (A) Pie chart showing the number of species in our data set that were pollinated by bats, birds, Coleoptera, Diptera, Hymenoptera, Lepidoptera, and wind (and in brackets the proportions they represent relative to the total number of N = 239 species that could be classified into the main pollination vector groups). **(B)** Phylogenetic tree showing the main pollination vector of the species included in the phylogeny of reference *PhytoPhylo* (small circles; N = 152) and the probability of main pollination vector types of ancestor nodes (large circles) as pie charts. The ancestral reconstruction was performed using 1,000 stochastic character-mapped trees (see the Methods section for further information).

FIGURE S3 | Relative percentages of abundance of the most common floral volatiles in the floral scents of plants pollinated by bats (N = 12), birds (N = 21), Coleoptera (N = 37), Diptera (N = 9), Hymenoptera (N = 37), Lepidoptera (N = 114) and wind (N = 9): **(A)** benzaldehyde, **(B)** limonene, **(C)** linalool, **(D)** trans-β-ocimene, and **(E)** benzyl alcohol. Error bars indicate standard errors of the means. Different letters indicate significant differences between groups (Kruskal-Wallis test, $\alpha = 0.01$).

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How the Color Fades From *Malus halliana* Flowers: Transcriptome Sequencing and DNA Methylation Analysis

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The flower color of many horticultural plants fades from red to white during the development stages, affecting ornamental value. We selected *Malus halliana*, a popular ornamental species, and analyzed the mechanisms of flower color fading using RNA sequencing. Forty-seven genes related to anthocyanin biosynthesis and two genes related to anthocyanin transport were identified; the expression of most of these genes declined dramatically with flower color fading, consistent with the change in the anthocyanin content. A number of transcription factors that might participate in anthocyanin biosynthesis were selected and analyzed. A phylogenetic tree was used to identify the key transcription factor. Using this approach, we identified *MhMYB10* as directly regulating anthocyanin biosynthesis. *MhMYB10* expression was strongly downregulated during flower development and was significantly positively related to the expression of anthocyanin biosynthetic genes and anthocyanin content in diverse varieties of *Malus*. To analyze the methylation level during flower development, the *MhMYB10* promoter sequence was divided into 12 regions. The methylation levels of the R2 and R8 increased significantly as flower color faded and were inversely related to *MhMYB10* expression and anthocyanin content. Therefore, we deduce that the increasing methylation activities of these two regions repressed *MhMYB10* expression.

Keywords: flower color, anthocyanin, methylation, MYB10, transcriptome sequencing, *Malus halliana*

INTRODUCTION

Malus halliana is a traditional and important ornamental plant in the Rosaceae family because of its elegant flower shape and gorgeous flower color. *M. halliana* is also valued for its profound cultural heritage and broad pharmaceutical value in China (Cui et al., 2018). Flower color is a major characteristic of ornamental value, but the petal color of *M. halliana* clearly changes from red to pale pink during flower development (**Figure 1A**), which affects its ornamental value. This fading of flower color is widespread in *Malus* plants and other ornamental plants (Jiang et al., 2014; Yue et al., 2019). The phenotype change of flower color of *M. halliana* presents distinct visible phases, so *M. halliana* is considered as an excellent model plant for research on flower color.

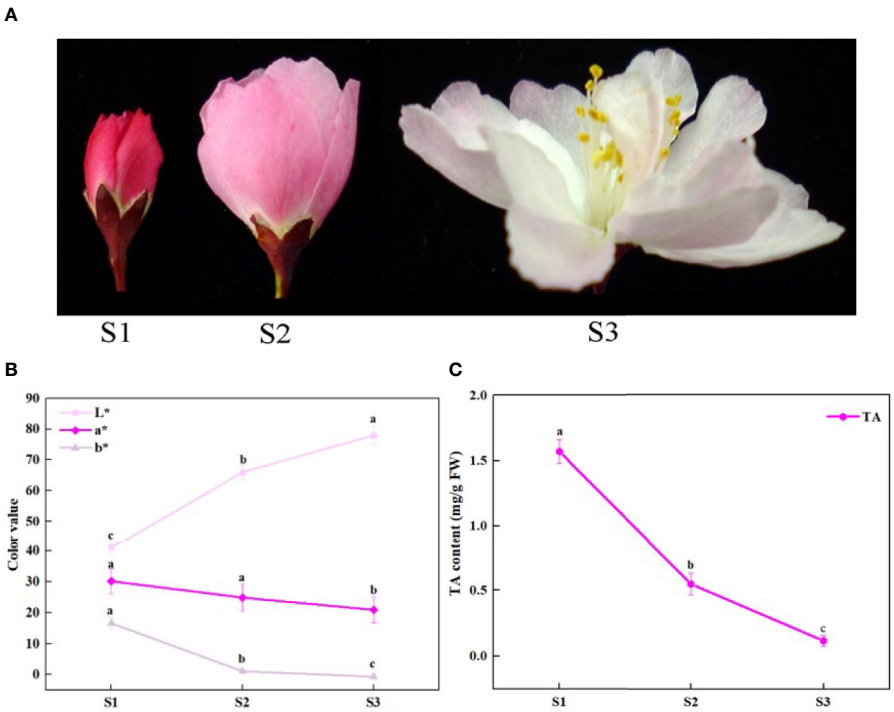


FIGURE 1 | Schematic of phenotypic and total anthocyanin content at different developmental stages of *Malus halliana*. **(A)** The flower color change at three stages. **(B)** L*a*b* values of petals at three stages. **(C)** The total anthocyanin (TA) content at three stages. S1, small bud stage; S2, initial-flowering stage; S3, late-flowering stage. Error bars indicate standard deviation (SD) obtained from four biological replicates. Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$).

A number of authors have reported that the contents and concentrations of anthocyanins play crucial roles in flower color formation (Alessandra and Luca et al., 2013; Qin et al., 2018). Anthocyanins, a class of plant flavonoid metabolites, are synthesized through a number of enzymes, which catalyze sequential reactions for anthocyanin biosynthesis within the cytosol compartment (Xie et al., 2014). Firstly, phenylalanine is converted to coumarate-CoA by phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). Secondly, coumarate-CoA is catalyzed to flavanone by chalcone synthase (CHS) and chalcone isomerase (CHI). The formation of various dihydroflavonols is catalyzed by flavanone 3-hydroxylase (F3H) and flavonoid-3'-monooxygenase (F3'H). Thirdly, the anthocyanidins are synthesized by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS). Finally, the synthesized anthocyanidins are unstable and modified through a series of glycosylation to form stable anthocyanins catalyzed by UDP-glucose transferase enzymes (UGT) (Zhao and Tao, 2015). The expression of most genes encoding for these enzymes is positively associated with anthocyanin content in *Malus* spp. (Honda et al., 2002; Xie et al., 2014; Tian et al., 2015). Comparative transcriptomic of *Malus* spp. reveals that the *ANS*, *4CL*, and *FGT* genes in the anthocyanin biosynthetic pathway are highly expressed in red petals (Huang et al., 2020).

The anthocyanin biosynthetic pathway is controlled by a transcription complex composed of three transcription factors (TFs): R2R3-MYB, bHLH, and WD40 protein. The MYB family is the important TFs in anthocyanin biosynthetic pathway; MYBs are divided into 28 subgroups (Sg, from Sg1 to Sg28) based on conserved amino acid sequence domains in C terminal, and Sg6 was identified as directly regulating anthocyanin biosynthesis (Stracke et al., 2001; Hong et al., 2015). MYB10, which belongs to Sg6, performs a key regulating function in anthocyanin biosynthesis through binding to the promoter of anthocyanin biosynthetic structural genes in *Malus domestica*, *Pyrus* spp., and *Amygdalus persica* (Espley et al., 2007; Feng et al., 2010; Zhou et al., 2019). Moreover, researchers have reported that other TFs, such as WRKY, ethylene response factors (ERF), NAC, zinc finger, and MADS-box proteins, are involved in the anthocyanin biosynthetic pathway in *Pyrus* spp., *Litchi chinensis*, *Arabidopsis*, and *Brassica napus* (Duan et al., 2018; Ni et al., 2018; Jiang et al., 2019). However, there has been little research addressing how genetic information and transcription contribute to the regulation of flower color in *Malus* spp.

Recent studies have demonstrated that the anthocyanin biosynthetic pathway is controlled by epigenetic mechanisms. A major and conserved epigenetic mechanism in regulating gene expression is DNA methylation of the gene promoter (Huang et al., 2019). Liu et al. (2012) reported that the failure of anthocyanin accumulation in floral tissues of *Oncidium* spp. might be attributed to expression inhibition of *CHS* because of the methylation of the promoter sequence. Diverse methylation

on the *MYB10* promoter was found to be the key reason for fruit color formation (Telias et al., 2011; Wang et al., 2017). However, research on the effect of promoter methylation of the transcriptional factor on flower color formation during development remains unclear.

In addition to biosynthesis, the process of anthocyanin transport from the endoplasmic reticulum and accumulation of pigmentation in the vacuole is necessary for tissue coloring and is catalyzed by corresponding enzymes: glutathione S-transferase (GST) family, the ATP-binding cassette (ABC) and multidrug and toxic compound extrusion (MATE) families (Mueller et al., 2000; Klein et al., 2006; Gomez et al., 2009). Recently, much attention has also been directed to the relationship of color fading and anthocyanin degradation involved in three common enzyme families, including polyphenol oxidases (PPOs), class III peroxidases, and intracellular laccase (Oren-Shamir, 2009; Fang et al., 2015). However, in contrast to the detailed knowledge regarding anthocyanin biosynthesis, little is known about degradation of these pigments.

To understand the mechanism of flower color fading in *M. halliana* in this study, the petals at different flowering stages were analyzed using RNA sequencing. A set of differentially expressed genes (DEGs) potentially involved in flower coloration was identified. The methylation in the *MhMYB10* promoter was also analyzed, which revealed the dynamic mechanism of methylation during flower color formation. This study will help to advance knowledge of the temporal and spatial regulation of anthocyanin biosynthesis in *M. halliana*, which underlies the diverse flower color intensities and patterning in *Malus* spp.

MATERIALS AND METHODS

Plant Materials

Malus spp., including *M. halliana*, *M.* 'Pinkspires', *M.* 'Strawberry Parfait', and *M.* 'Snowdrift', grown in the crabapple resource nursery of Northwest A&F University, Yangling, China, were used as plant materials. The flowers were collected from healthy and approximately uniform trees from March to April 2018. Sepals, pistils, and stamens were quickly removed, and only petals were collected, immediately frozen in liquid nitrogen, and stored at −80°C for further analysis. The flowers were divided into three stages according to color and size: S1, bud stage; S2, initial-flowering stage; and S3, full-flowering stage.

Flower Color Measurement

The colors of fresh petals were measured and described according to the Royal Horticultural Society Color Chart with a CR-400 chroma meter (Konica Minolta, Tokyo, Japan). Three parameters, L^*, a^*, and b^* were determined: L^* (ranging from 0 to 100) indicates lightness. Positive and negative values of a^* standard for red and green, respectively, and those of b^* represent yellow and blue, respectively (McGuire, 1992; Liu et al., 2016). Five independent biological replicates were obtained to calculate the mean.

Pigment Extraction and Analysis of Total Anthocyanin Content

To extract pigment, plant samples (0.5 g) were ground into powder in liquid nitrogen and added to 10 mL of methanol. The extraction was incubated for 48 h at 4°C away from light with vortexing every 8 h. The supernatant was removed after 10 min centrifugation (6,000 rpm) and stored at −20°C for further analysis. Three biological replicates were prepared for analysis.

The total anthocyanin (TA) content was determined according to literatures (Li et al., 2012) with a slight change. Hydrochloric acid (24 μL of 12 M) was added to 800 μL of the extraction, and the mixture was incubated for 15 min at room temperature. The absorbance of the reaction mixture was measured immediately at 530, 620, and 650 nm. The content of total anthocyanin was calculated using a standard curve of cyanidin-3-galactoside. The results are presented as milligrams per gram fresh weight (mg/g FW). Three biological replicates and three technical replicates were analyzed.

HPLC-Diode Array Detector Analysis

The samples were filtered through 0.45 μm filters and the phenolic compounds were analyzed using HPLC coupled with DAD; HPLC-diode array detector (HPLC-DAD) analysis was carried out using a Shimadzu LC-2030C liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Inertsil C-18 column (5.0 μm particle size, 4.6 mm × 250 mm). The HPLC-DAD separation was performed as previously described by Han et al. (2020). The compound varieties were determined by comparing their retention times and UV spectral data with those of the known standards according to reported LC-MC and NMR spectroscopic results of *Malus* spp. (Li et al., 2007; Hu et al., 2017). The concentration of each compound was calculated using corresponding standard calibration curves with three biological replicates.

RNA Isolation and Library Construction for Transcriptomic Analysis

Total RNA was extracted from petals using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Three biological replicates were analyzed to conducted RNA sequencing experiments. The RNA quality was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase free agarose gel electrophoresis. After total RNA was extracted, eukaryotic mRNA was enriched using Oligo (dT) beads. The enriched mRNA was then fragmented into short fragments using fragmentation buffer and reverse transcripted into cDNA with random primers. Second-strand cDNA was synthesized by DNA polymerase I, RNase H, dNTP, and buffer. Then, the cDNA fragments were purified using a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end-repaired, poly(A) tails added, and ligated using Illumina sequencing adapters.

The RNA sequencing and assembly were performed using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China). Pair ends were used for the sequencing approach, and the read length was 150 nt. Clean reads were obtained by removing reads containing adapter and more than

10% of unknown nucleotides (N), as well as low-quality sequence containing more than 50% low-quality ($Q \leq 20$) bases from raw data, and were aligned to *Malus* × *domestica* HFTH1 Whole Genome version 1.0 (https://www.rosaceae.org/species/malus_×_domestica_HFTH1/genome_v1.0) using Bowtie (Langmead and Salzberg, 2012) and HISAT2 (Kim et al., 2015). The "-RNA- strandness RF" was set to 2.4, and the other parameters were set as default. The mapped reads of each sample were assembled using StringTie v1.3.1 in a reference-based approach (reference sequence: *Malus* × *domestica* HFTH1). For each transcription region, an FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations using StringTie software.

DEG Analysis

DEG analysis of three stages of flowers was performed using the DEGseq R package; $q < 0.005$, FDR<0.05, and |log2(foldchange (FC))| >1 were set as the thresholds for significantly differential expression. If a gene was only expressed in one treatment, then the expression in other treatments was calculated at the minimum value of 0.01. The gene function of DEGs was annotated according to the following databases: NCBI nonredundant protein sequences (Nr), NCBI nonredundant nucleotide sequences (Nt), Pfam (Protein family); clusters of orthologous groups of proteins (KOG/COG), Swiss-Protein (a manually annotated and reviewed protein sequence database), Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog database (KO), and Gene Ontology (GO). GO includes three ontologies: molecular function, cellular component, and biological process. The basic unit of GO is the GO-term. First, all DEGs were mapped to GO terms in the GO database (http://www.geneontology.org/), gene numbers were calculated for every term, and significantly enriched GO terms in DEGs, compared with the genome background, were defined by hypergeometric testing. The KEGG is the major public pathway-related database. Pathway enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in DEGs relative to the whole genome background.

Homolog Search and Phylogenetic Tree Construction

A total of 32 MYB genes were isolated from DEGs of *M. halliana* and translated into protein using the NCBI Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf). Sequences of *Arabidopsis* AtMYB proteins were retrieved from the UniProt Database (http://www.uniprot.org). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were computed using the *p*-distance method (Nei and Kumar, 2000) and expressed in the units of the number of amino acid differences per site. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Quantitative Real-time PCR Analysis

Total RNA was isolated from the frozen sample following the method described previously. Approximately 1 μg of total RNA was used for first-strand cDNA synthesis using a PrimeScriptTM RT reagent kit with a gDNA Eraser (TaKaRa BioInc., Shiga, Japan) kit, following the manufacturer's instructions. Quantitative RT-PCR was performed based on the 2 × plus SYBR real-time PCR mixture (Bioteke Corporation, Beijing, China). Samples for qRT-PCR were run in three biological replicates and three technical replicates; 18s RNA was used as the reference gene (Lu et al., 2017). The mean expression levels of the genes of interest were normalized to the relative expression level. Specific primers were designed from the selected gene sequences using Primer 5.0 and the primer sequences are given in **Supplementary Table S1**.

DNA Isolation and *MhMYB10* Sequence Clone

Total DNA was extracted from petals using a DNA extraction kit (Bioteke, Beijing, China) according to the manufacturer's protocol. Full-length coding region DNA and the 2,129 bp promoter sequence of *MhMYB10* were isolated from petals according to the EU518249.2 sequence in NCBI (https://www.ncbi.nlm.nih.gov/) and using primers listed in **Supplementary Table S1**. The PCR reactions were performed using TransStart Fastpfu DNA Polymerase, following the manufacturer's instructions (Transgen Biotech, Beijing, China). The PCR products were purified following the manufacturer's instructions (Bioteke Corporation, Beijing, China). The DNA fragments from three independent biological replicates were cloned using the pMD19-Teasy vector (Takara Bio Tech Co. Ltd., Beijing, China), and sequenced by Augct Co (Augct, Xi'an, China).

Methylation Assay of *MhMYB10* Promoter

Bisulfite sequencing analysis was used to measure the methylation levels of the *MhMYB10* promoter as described by Telias et al. (2011), with three biological replicates. Briefly, 1 μg of gDNA was treated using the DNA bisulfite conversion kit (Tiangen Biotech Co. Ltd., Beijing, China). Using the treated DNA as a template, the targeted *MhMYB10* promoter fragments were amplified using 2 × Taq Master Mix (Novoprotein Co., Shanghai, China) with degenerate primers (**Supplementary Table S1**), ligated into the pMD19-T vector (Takara Bio Tech Co. LTD, Beijing, China), and then sequenced by Augct company (Augct, Xi'an, China). Sequences of 10 independent clones per biological replicate were obtained and analyzed using the Kismeth online software (Gruntman et al., 2008), and the methylation level of the targeted fragments was calculated based on the percentage of detected cytosines in methylated DNA sequence relative to the reference sequence.

RESULTS

Analysis of Flower Phenotype and Phenolic Compounds

The petal color of *M. halliana* was significantly faded from red to pale pink during developmental stages from S1 to S3 (**Figure 1A**).

Consistently, the chromatic parameters of petals showed that L^* values increased significantly, and a^* and b^* values decreased during flower development (**Figure 1B**). The total anthocyanin (TA) contents dropped from S1 to S3 (**Figure 1C**).

Furthermore, the HPLC-DAD results demonstrated that 13 types of phenolic compounds were detected in petals; these includes anthocyanins, flavonols, flavanols, flavones, and phloridzin. Of four kinds of cyanin glycoside detected (**Table 1**), cyanidin-3-galactoside was the major anthocyanin, and its concentrations were the highest compared with other anthocyanins, flavonols, flavanols, and flavones in petals. The highest concentration of cyanidin-3-galactoside (S1) was 3.4 times greater than the smallest (S3). In addition, the abundant phloridzin was determined in petals, and the concentration decreased from S1 to S3. The concentrations of other compounds remained low and stable during flower development.

Transcriptomic Assembly and Expression Analysis of DEGs

The RNA sequencing results of *M. halliana* petals showed that, of the total clean reads from the samples, 87.69–89.48% matched the *Malus* domestica genome (HFTH1 Whole Genome v1.0) with 84.85–87.13% unique mapped reads and 2.53–2.84% multiple mapped reads (**Supplementary Table S2**). The total number of sequenced genes was 33,819, including 31,660 sequenced reference genes and 2,159 new genes (**Supplementary Table S3**).

DEGs were identified between samples of each treatment group. Totals of 7,111, 11,269, and 9,085 DEGs were detected in S1 vs. S2, S1 vs. S3, and S2 vs. S3, respectively. Overall, 2,316 genes were detected in all three comparisons (**Figure 2A**). The KEGG pathway enrichment analyses were used to identify the functions enriched in DEGs (**Supplementary Figure S1**). The most heavily enriched KEGG pathways were related to the metabolic pathway and biosynthesis of secondary metabolites. Of these pathways, the phenylpropanoid biosynthetic pathway included 77, 115, and 108 DEGs, and the flavonoid biosynthetic pathway included 33, 41, and 33 DEGs for S1 vs. S2, S1 vs. S3, and S2 vs. S3, respectively. These pathways are involved in anthocyanin biosynthesis, suggesting that these DEGs had important effects on flower color formation of *M. halliana*.

A trend analysis was carried out to explore the expression patterns of all DEGs in detail (**Figure 2B**). All DEGs were clustered into eight profiles, of which four trend profiles (0, 4, 3, and 7) showed high enrichment ($p < 0.01$) with the colored block, and four profiles represented the enrichment of significant trends without color. The expression of 2,587 genes displayed a reducing trend during the whole flower development in Profile 0, and the expression of 1,466 genes demonstrated an opposite trend in Profile 7. The expression of 2,677 genes showed no difference in S1 vs. S2, but subsequent extreme reduction in S2 vs. S3 in Profile 3. However, the expression of 2,488 genes exhibited no change in S1 vs. S2 but subsequent increased in S2 vs. S3 in Profile 4. Based on the pattern of the flower color fading, we focused on Profile 0 and Profile 7 in the subsequent analyses.

Dynamic Changes of DEGs are Related to Anthocyanin Metabolism

In this work, genes participating in the anthocyanin metabolic pathway were screened and expression heat maps were constructed based on FPKM (**Table S4**; **Figure 3**). A total of 47 genes were involved in anthocyanin biosynthesis. The predicted proteins encoded by upstream genes included five PAL, six 4CL, four C4H, five CHS, six CHI, and two F3H. The predicted proteins encoded by downstream genes contained one DFR, three ANS, and three anthocyanidin 3-O-glucosyltransferase (AGT). Almost all genes previously described were significantly downregulated during flower fading, suggesting that they play important roles in anthocyanin biosynthesis. In particular, *MhPAL* (HF01560), *MhCHS* (HF00720 and HF00721), *MhCHI* (HF23861 and HF25490), *MhDFR* (HF13503), and *MhANS* (HF39612 and HF08300) had especially high expression levels at S1, which decreased dramatically along with the fading of flower color. These genes were considered the key candidate genes affecting the anthocyanin biosynthesis of *M. halliana* flowers. Some branch genes in the anthocyanin biosynthetic pathway were also studied, including six flavonol synthases (*FLS*), three leucoanthocyanidin reductases (*LAR*), and three anthocyanidin reductase (*ANR*) genes, which did not show a consistent expression pattern.

In addition, 10 predicted coding genes involved in anthocyanin transport were identified, including five *GST*s and

TABLE 1 | The HPLC-DAD results of petal extraction of *Malus halliana*.

Classification	Polyphenol (mg/g)	S1	S2	S3
Anthocyanin	Cyanidin-3-O-galactoside	1.752 ± 0.052^{a}	1.075 ± 0.742^{ab}	0.474 ± 0.107^{b}
	Cyanidin-3,5-O-diglucoside	0.062 ± 0.001^{a}	0.013 ± 0.001^{b}	0.005 ± 0.001^{c}
	Cyanidin-3-O-arabinoside	0.185 ± 0.0005^{a}	0.175 ± 0.020^{a}	0.160 ± 0.022^{a}
	Cyanidin-3-O-rutinoside	0.368 ± 0.008^{a}	0.382 ± 0.007^{a}	0.421 ± 0.056^{a}
Flavonol	Quercetin-3-O-glucuronide	0.632 ± 0.015^{a}	0.535 ± 0.011^{ab}	0.543 ± 0.076^{b}
	Hyperoside	0.270 ± 0.005^{a}	0.277 ± 0.004^{a}	0.301 ± 0.039^{a}
	Taxifolin	1.361 ± 0.012^{a}	0.868 ± 0.040^{b}	0.741 ± 0.124^{b}
	Kaempferol-3-glucoside	0.192 ± 0.003^{b}	0.194 ± 0.0002^{b}	0.437 ± 0.079^{a}
Flavanol	Catechin	0.022 ± 0.002^{c}	0.045 ± 0.006^{b}	0.032 ± 0.008^{a}
	Epicatechin	0.104 ± 0.010^{a}	$H0.091 \pm 0.011^{ab}$	0.069 ± 0.016^{b}
Flavone	Naringenin	0.019 ± 0.002^{b}	0.021 ± 0.006^{b}	0.056 ± 0.001^{a}
	Eriodictyol	0.278 ± 0.005	ND	ND
Dihydrochalcone	Phloridzin	34.819 ± 1.133^{a}	20.648 ± 0.711^{b}	7.952 ± 1.317^{c}

The results are presented as mean ± SD. Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$).

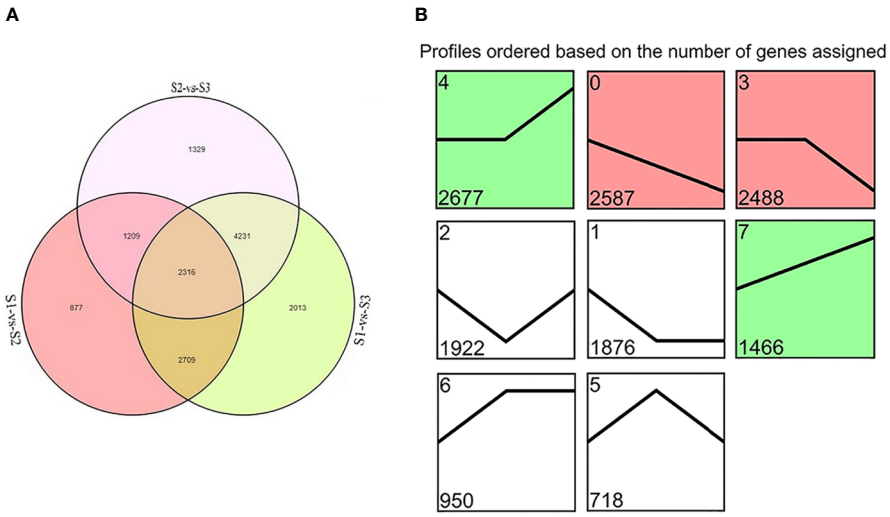


FIGURE 2 | The differentially expressed genes (DEGs) screened by RNA-Seq analysis during flower developmental stages of *Malus halliana*. **(A)** Venn diagram of DEGs in three comparisons (S1-vs-S2, S1-vs-S3, and S2-vs-S3, respectively). **(B)** Trend analysis of DEG expression during flower developmental stage (from S1 to S3). Colored block trend: significant enrichment trend ($Q \leq 0.05$). Green color indicated upregulation trend and red color indicated downregulation trend. Without color trend: the enrichment of trends.

five *ABCC*s. The expression levels of two *MhGST* genes (HF07438 and HF32109) were very high and the differences at different stages were highly significant. Moreover, we analyzed the anthocyanin degradation-related genes, including *POD*, *PPO*, and *Lac* genes. Most of the degradation genes were expressed at low levels, except HF17954 (*MhPPO*) whose expressions increased significantly as flower color fading (**Figure 3**).

Expression Profiling of Transcription Factors Associated with Anthocyanin Biosynthesis

In this study, to identify the putative TFs that regulate anthocyanin biosynthesis, all MYBs (MYB domain proteins), bHLHs (basic helix-loop-helix), WD40s, ERFs (ethylene-responsive transcription), bZIPs (basic region-leucine zipper), NACs (NAM/ATAF/CUC), WRKYs (WRKY proteins), and MADS-boxes (AGL and SOC) were selected and analyzed in detail in DEGs (**Table 2**). Of these TFs, MYBs were the largest family with 111 genes, followed by NACs (94), ERF (88), and bHLH (79), while WD40s, MADS-boxes, and bZIPs had fewer genes. The expression heat maps of these TFs were constructed based on FPKM, and the complex expression patterns were present in TFs (**Supplementary Figure S3**). Further, we also screened TFs belonging to Profile 0 and Profile 7 that were consistent with and the opposite to the changing pattern of flower color, respectively. There were 27 MYBs in Profile 0 (such as HF36879, HF02692, and HF33298) and only five in Profile 7 (such as HF38722, and HF17885), indicating that most MYBs activated anthocyanin biosynthesis. Similarly, 18 bHLHs, five WD40s, nine ERFs, and three MADS-boxes were identified in Profile 0, while eight bHLHs, four ERFs, and one WD40 were identified in Profile 7. However, more NACs, WRKYs, and bZIPs were present in Profile 7 than those in Profile 0 (**Table 2** and **Supplementary Figure S3**).

MYB Family Phylogenetic Tree Construction

The MYBs are the largest TF family related to anthocyanin biosynthesis. In this study, 32 MYBs were identified from RNA sequencing data and 99 *Arabidopsis* MYBs from the UniProt Database (http://www.uniprot.org) were used to conduct a phylogenetic tree (**Figure 4**). The MYBs were integrated with AtMYBs in clustered phylogenetic clades or subclades and divided into 23 MYB subgroups based on the available literature (Stracke et al., 2001), and there were no genes in Sg8 and Sg17. The MYBs in Sg6 were reported to be regulators related to anthocyanin biosynthesis (Stracke et al., 2001). In the present study, only one MYB (HF36879, renamed *MhMYB10*) belonged to Sg6.

Expression Profile of the Selected Genes in Diverse *Malus* Spp.

We used qRT-PCR to validate whether the difference in RNA sequencing levels of DEGs in the anthocyanin biosynthetic pathway truly reflects the actual transcription level (**Figure 5**). The expression patterns of nine genes (*MhPAL* (HF01560), *MhCHS* (HF00720), *MhCHI* (HF23861), *MhFHT* (HF19324), *MhDFR* (HF13503), *MhANS* (HF39612), *MhFLS* (HF44548), *MhGSTU17* (HF07438), and *MhMYB10* (HF36879) obtained by qRT-PCR were consistent with the RNA sequencing data, confirming the validity of our results. It is worth noting that the reduction in most of these gene expressions was greater from S1 to S2 than that from S2 to S3.

Meanwhile, these gene expression levels were analyzed in other *Malus* spp. with varying flower phenotypes to confirm

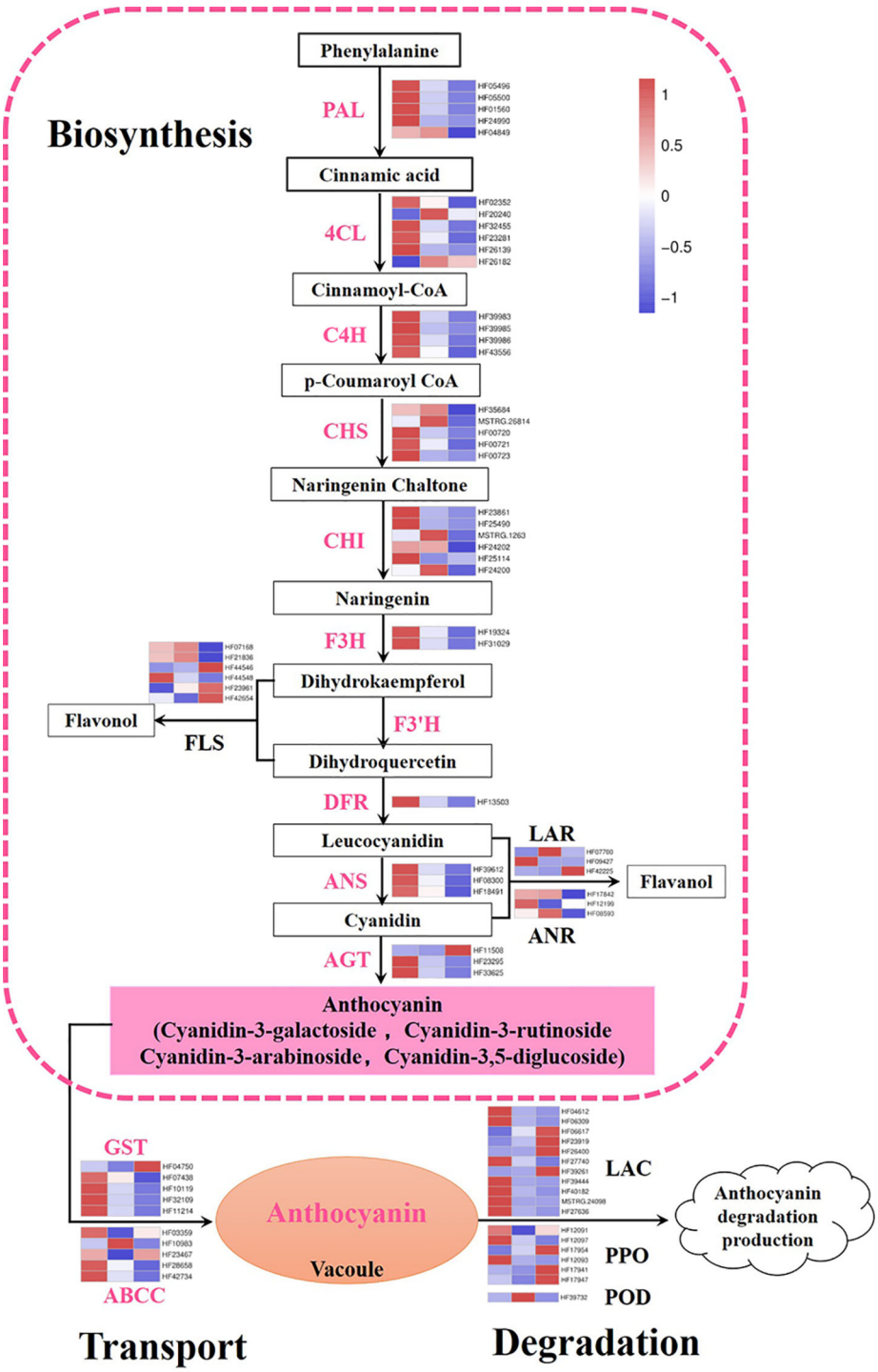


FIGURE 3 | Expression pattern of genes involved in anthocyanin biosynthetic, degradation, and transport pathway. Color boxes from left to right represent genes expression level at S1, S2 and S3. PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate coenzyme A ligase; C4H, cinnamate 4-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H: flavonoid 3'-monooxygenase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; AGT, anthocyanidin 3-O-glucosyltransferase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; GST, glutathione S-transferase; ABCC, ATP-binding cassette; LAC, laccase; PPO, polyphenol oxidases; POD, class III peroxidases. Color saturation represents the normalize expression level of genes at S2 and S3 with S1 based on fold change, the color gradient on the right, ranging from blue to white to red represents low, middle, and high values of gene expression.

TABLE 2 | Candidate anthocyanin regulatory genes in *Malus halliana*.

Gene family	NO. all[a]	S1 vs. S2		S1 vs. S3		S2 vs. S3		S1 vs. S2 vs. S3	
		NO. up[b]	NO. down[c]	NO. up	NO. down	NO. up	NO. down	Profle 0	Profile 7
MYB	111	16	53	31	61	29	31	27	5
NAC	94	18	34	47	32	44	17	10	19
ERF	88	13	36	24	25	40	18	9	4
bHLH	79	9	35	26	36	41	19	18	8
WRKY	57	6	19	39	6	43	2	1	8
WD40	25	4	12	4	13	4	4	5	1
MADS-box	23	3	4	5	15	8	9	3	0
bZIP	20	7	1	6	10	2	12	0	3
Total	497	76	194	182	198	211	112	73	48

[a]*indicates the total number of regulatory genes in DEGs,* [b]*indicates the number of upregulated genes in each comparison, and* [c]*indicates the number of downregulated genes in each comparison. All candidate anthocyanin regulatory genes in this table are screened from DEGs.*

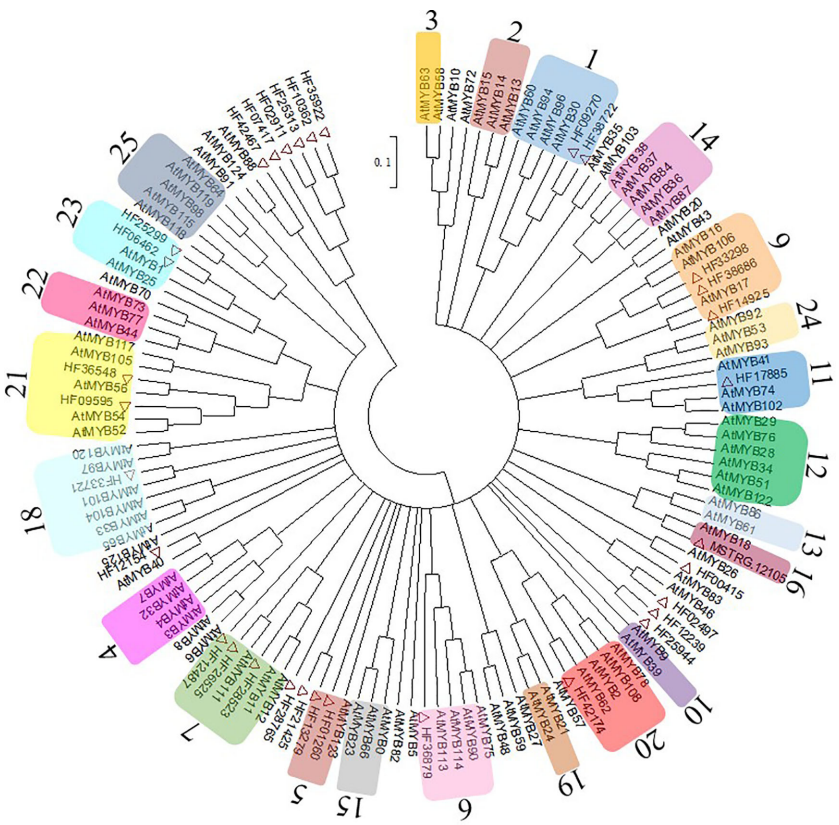


FIGURE 4 | Evolutionary relationships of *MhMYBs*. Full-length amino acid sequences of R2R3-MYBs from the chrysanthemum transcriptome dataset and arabidopsis genome were first aligned using ClustalW in MEGA6. The phylogenetic tree was constructed according to the neighbor-joining method. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. The evolutionary distances were computed using the p-distance method. Thirty-two MhMYBs marked red triangle were identified from the RNA sequencing data of *M. halliana*. The arabic numerals on the cycle indicated gene families.

their wide function in flower color formation (**Figure 6**). The flower of *M.* 'Strawberry Parfait' is rose at S1 and fades to light-pink at the flower opening, and *M.* 'Pinkspires' has a pink flower at S1 and fades to white at S3. The flowers of *M.* 'Snowdrift' are pink at S1 and quickly change to white at S2. Consistent with flower color characteristics, TA in petals declined dramatically during flower development; at the same time, *M.* 'Strawberry Parfait' and *M.* 'Pinkspires' had higher TA than *M.* 'Snowdrift'.

Of the nine genes selected, the expressions of *MhPAL*, *MhCHI*, *MhDFR*, and *MhANS* decreased remarkably in three *Malus* spp. during flower fading. Correlation analysis showed an extremely significant correlation between the expressions of *MhPAL*, *MhCHS*, *MhCHI*, *MhDFR*, *MhANS*, *MhMYB10*, and

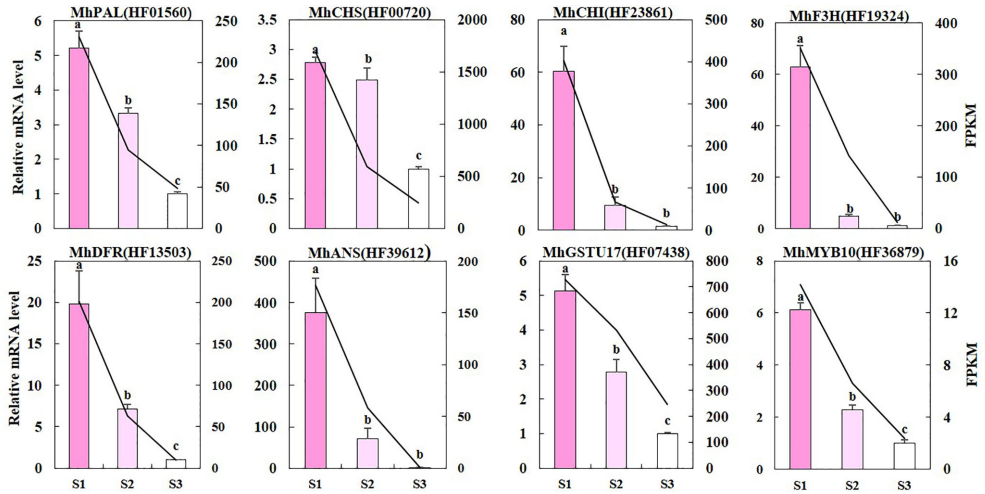


FIGURE 5 | The qRT-PCR validation of DEGs. The left y-axis denotes the RNA relative expression obtained by qRT-PCR. Error bars indicate the standard errors. Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$). The right y-axis represents the fragments per kilobase per million fragments (FPKM) value of each gene using RNA sequencing analysis.

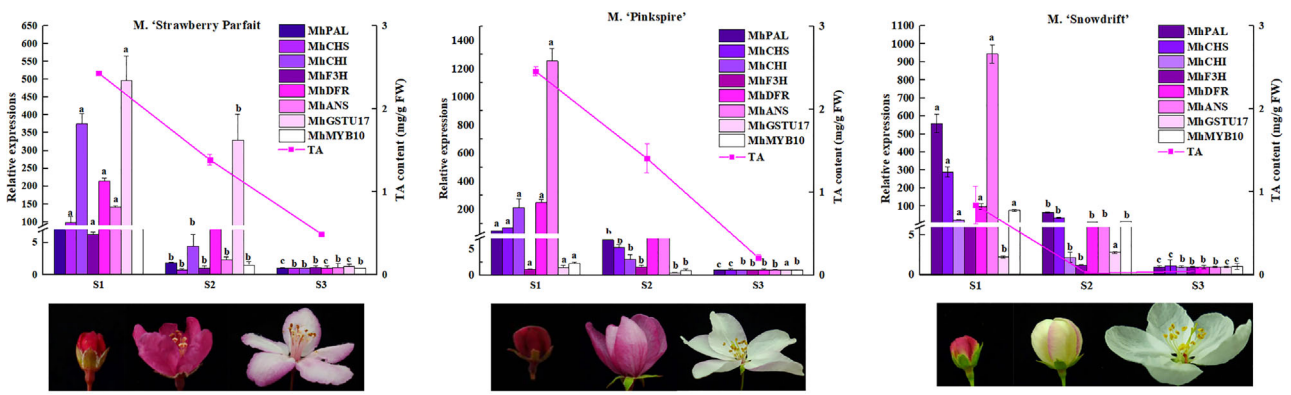


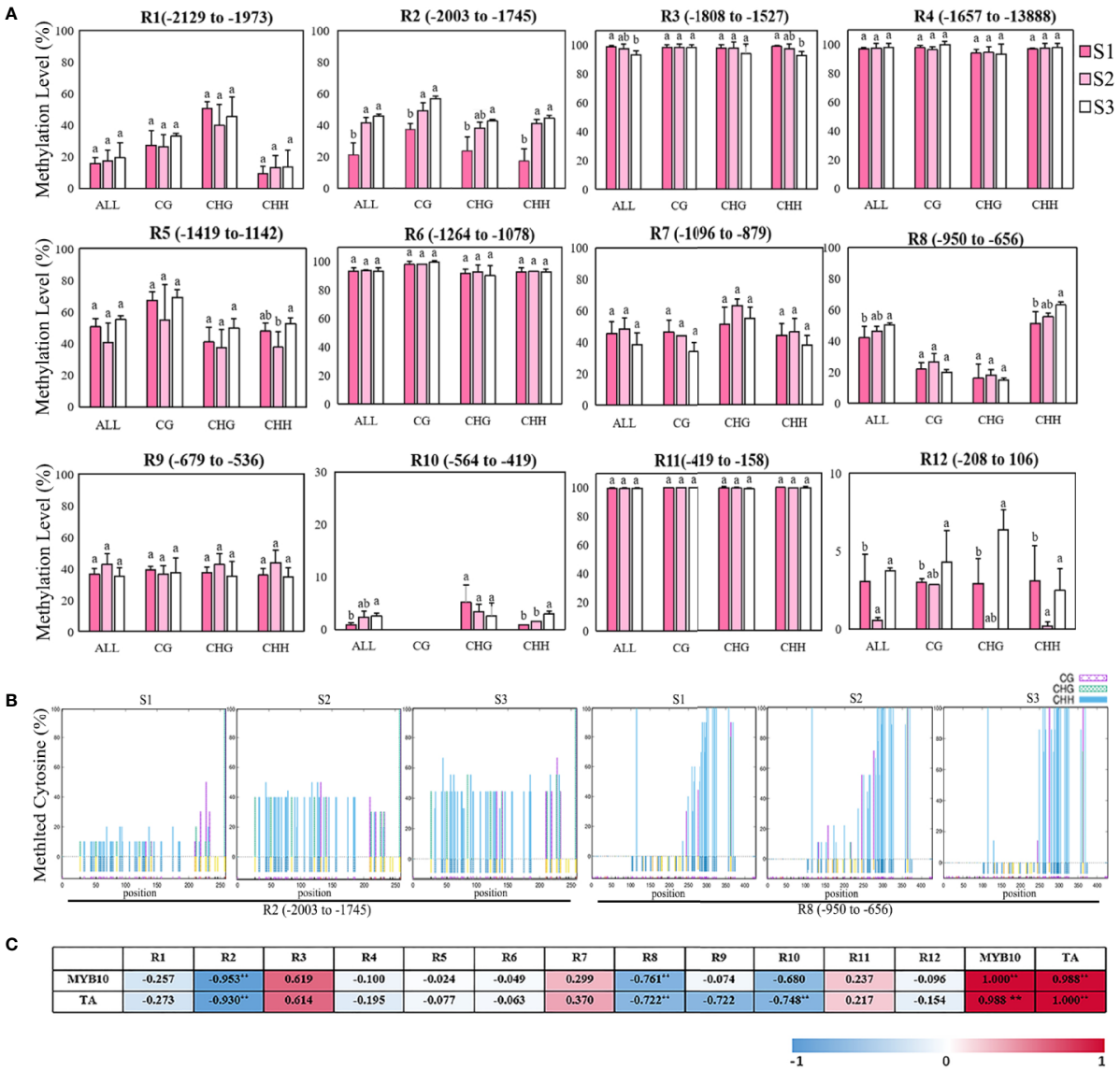
FIGURE 6 | Anthocyanin contents and the key DEGs transcript levels in anthocyanin metabolism in three *Malus* flower in varying colors. The relative expressions of genes are exhibited as column diagram. Anthocyanin levels are indicated by lines. Correlation coefficient values between gene expression level and anthocyanin content are presented above each gene legend correspondingly. Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$).

TA contents in all three *Malus* spp. For *M.* 'Strawberry Parfait' and *M.* 'Snowdrift', *MhF3H* expression was closely related to TA content.

Methylation of *MhMYB10* Promoter at Flower Fading

The previous results show that the repression of *MhMYB10* expression probably plays a key role in the flower color fading of *Malus* spp. during development. To examine whether there is an alteration in the dynamics of methylation in the *MhMYB10* promoter during flower fading, 2,129 bp upstream sequences from the translation initiation site were isolated (**Supplementary Figure S2**) and the methylation levels of 12 regions were analyzed using bisulfite sequencing (**Figure 7A**). The highest methylation activities of *MhMYB10* occurred in R3 (−1,808 to −1,527 bp), R4 (−1,657 to −1,388 bp), R6 (−1,264 to −1,078 bp), and R11 (−419 to −158 bp) with more than 90% in all cytosine contexts (CG, CHG, and CHH) at three stages. The methylation level of R3 decreased somewhat during flower development. The methylation levels of R5 (−1,419 to −1,973 bp), R7 (−1,096 to −879 bp), and R9 (−679 to −536 bp) did not significantly differ during flower development. Although the methylation levels of R10 (−546 to −419 bp) and R12 (−208 to 106 bp) were low during flower opening, the methylation activity of R10 increased significantly and was detected only in CHG and CHH context.

Interestingly, the methylation activities in R1 (−2,129 to −1,973 bp), R2 (−2,003 to −1,745 bp) and R8 (−1,419 to −1,142 bp) visibly increased during flower opening. The total methylation levels were 21.25%, 41.67%, and 45.64% for R2, and 39.38%, 46.25%, and 50.14% for R8 at S1, S2, and S3, respectively.



	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	MYB10	TA
MYB10	-0.257	-0.953**	0.619	-0.100	-0.024	-0.049	0.299	-0.761**	-0.074	-0.680	0.237	-0.096	1.000**	0.988**
TA	-0.273	-0.930**	0.614	-0.195	-0.077	-0.063	0.370	-0.722**	-0.722	-0.748**	0.217	-0.154	0.988 **	1.000**

FIGURE 7 | Bisulfite sequencing analysis of cytosine methylation levels of different regions in *MhMYB10* promoter at the different flower stages. **(A)** The methylation levels of *MhMYB10* promoter at the different stages. Each data point represents a mean ± SD of three independent DNA extractions with three independent technical replicates. Ten independent clones from each reaction were sequenced and analyzed. In the x axis, "All" refers to overall methylated cytosines, while CG, CHG and CHH refer to the three different contexts of cytosines, in which H represents nucleotide A, C, or T. Different letters between cultivars denote significant differences (Duncan test, $p < 0.05$). **(B)** The methylation levels of different cytosine contexts (CG, CHG, CHH, H represents nucleotide A, C, or T) in R2 and R8 regions at the different stages. **(C)** The correlation analysis between the methylation level of *MhMYB10* and *MhMYB10* expression and anthocyanin content.

The methylation activity at S1 was less (24.39%) than that at S3 (**Figure 7A**). The methylation activity in R2 and R8 could be detected in all three cytosine contexts. By contrast, the highest methylation activity of R2 occurred in the CG context, but R8 in the CHH context was much higher than that in the CG and CHG contexts (**Figure 7B**).

The correlation analysis results showed that *MhMYB10* expression and TA content were significantly negatively related to the methylation level of R2 and R8 among 12 regions. The correlations between the R2 methylation level with *MhMYB10* expression and TA content were 0.953 and 0.930 ($p < 0.01$), respectively (**Figure 7C**).

DISCUSSION

Anthocyanin Biosynthesis and Degradation Control Flower Color

It is a common phenomenon that the flower color often changes during development, acting as a signal for pollinators. In most

cases, color changes during flower development are due to the induction of anthocyanin biosynthesis (Oren-Shamir, 2009). In this study, the concentration of cyanidin-3-O-galactoside, which is the most important anthocyanin in the *M. halliana* flower, decreased dramatically along with flower color fading, which is the main reason for floral color fading. In addition, other pigments, such as flavonols and flavanols, may act co-pigmentations of anthocyanin to participate in the flower color formation (Iwashina, 2015).

The decline of anthocyanin content is the result of the complex mechanics involved in anthocyanin biosynthesis, transport, and degradation (Oren-Shamir, 2009). In this study, DEG analysis results showed that 47 candidate genes were involved in anthocyanin biosynthesis, and the expression of almost all of these genes (especially *MhPAL*, *MhCHS*, *MhCHI*, *MhDFR*, *MhANS*) were downregulated during flower fading of *Malus* spp., and positively correlated to anthocyanin content. The repressions of these genes were closely related to the color fading of the flower. In addition, the expression levels of *MhGSTU17* and *MhGSTF12* were extremely high at S1 and decreased sharply after S1, suggesting that these two genes could be key players in anthocyanin transport. One *MhPPO* gene (HF17954) was upregulated as flower color faded, indicating that HF17954 might play a primary function in anthocyanin degradation of *M. halliana*. Wang et al. (2017) reported that three families (GST, ABCC, and MATE) involved in anthocyanin transport and that two families (Lac and POD) involved in degradation were identified in transcriptome analysis of pear color fading, which is different from *M. halliana*, whose *MhPPO* was responsible for anthocyanin degradation. Moreover, the flower development process of *M. halliana* occurs during a period of increasing temperature (from late March to late April in Shannxi Chinese). Anthocyanin biosynthesis is repressed, and catabolism is activated by high temperature (Oren-Shamir, 2009). Therefore, a change in environmental conditions may result in a decrease of anthocycanin content to promote flower fading of *M. halliana.*

Considering that a greater number of genes and higher expression intensities were identified in anthocyanin biosynthesis than those in transport and degradation during flower development, similar results were reported by Huang et al. (2020) for a study on the flower color variation of *Malus* spp., suggesting that the expression repression of anthocyanin biosynthetic genes is the major reason for the decline of anthocyanin content during flower development.

Transcription Factors Involved in the Anthocyanin Biosynthetic Pathway

The anthocyanin biosynthetic pathway is regulated by the highly conserved MYB (Xu et al., 2015). In our study, MYBs were identified as the largest TF family, containing 111 genes. Among these MYBs, the expressions profiles of 27 genes were consistent with the changing trend of flower color, and only five genes had an opposite expression trend. These results indicated that more MYBs could promote anthocyanin biosynthesis. Furthermore, phylogenetic tree analysis revealed that, of 32 genes, only *MhMYB10* (HF36879) was clustered in Sg6, in which MYBs in *Arabidopsis* have been proven to directly regulate the anthocyanin biosynthetic pathway (Stracke et al., 2001). Recently, Huang et al. (2020) found, by transcriptome sequencing of different flower color *Malus* spp., that *MYB113*-like (HF36879, namely, *MYB10*) expressions in red petals were several thousand times greater than those in white flowers. Moreover, *MhMYB10* expression significantly reduced during color fading, and was positively correlated with anthocyanin content not only in *M. halliana* but also in other *Malus* spp. These results strongly suggested that *MhMYB10* played important roles not only in flower color variation but also in flower color fading of *Malus* spp. Furthermore, *MdMYB10* is thought to play an important role in the anthocyanin biosynthetic pathway of *M. domestica* by regulating *DFR* and *ANS* expression (Espley et al., 2007). Taken together, this evidence demonstrates that *MhMYB10* expression is downregulated during color fading to induce decreases in anthocyanin content.

In addition, many authors have reported other TFs that participate in anthocyanin biosynthesis. For example, when *Brassica oleracea NAC019* is overexpressed in *Arabidopsis*, anthocyanin content decreased (Wang et al., 2018), while *AtERF4* and *AtERF8* are associated with changes in the transcript levels of anthocyanin biosynthetic genes and mediate the production of anthocyanin biosynthetic genes (Koyama and Sato, 2018). When *Brassica napus WRKY41-1* is overexpressed in *Arabidopsis*, there is a significant increase in anthocyanin content (Duan et al., 2018). In the present study, a number of transcription factors were screened as candidates for participation in regulating the anthocyanin biosynthetic pathway, including NACs, ERFs, WRKYs, MADS-boxes, and bZIPs, with 28 found to be downregulated and 35 upregulated as the flower color faded. These results suggest that, unlike the function of the MBW complex, most of these families likely act as transcription repressors in anthocyanin biosynthesis to promote color fading, especially NAC and WRKY families. These results suggest directions for further study on the flower coloration of *Malus* spp.

Epigenetic Regulation of *MhMYB10* Play an Important Role in Flower Color Fading

DNA methylation, a conserved epigenetic modification, plays an important role during plant growth and development, such as the processes of tomato and orange fruit ripening and coloration (Lang et al., 2017; Huang et al., 2019). Methylation in the promoter can directly inhibit gene transcription and expression (Zhang et al., 2018). Deng et al. (2015) reported on the methylation level of the *ANS* promoter in two *Nelumbo nucifera* cultivars resulting in different color phenotypes. In the present study, the methylation analyses results demonstrate that, of 12 regions in the *MhMYB10* promoter, the methylation activity of R2 (−2,003 to −1,745 bp) and R8 (−950 to −656 bp) significantly increased along with petal color fading in *M. halliana*, and was negatively related to the TA content and *MhMYB10* expression. These results suggest that the increasing methylation level of R2 and R8 could repress *MhMYB10* expression, thereby slowing the anthocyanin accumulation.

How the methylation level of the *MYB10* promoter affects fruit pigment has also been studied. Research on red and green

striped apple skin showed that higher methylation activity of the *MdMYB10* promoter occurred in the region from −1,400 to −651 bp (Telias et al., 2011); moreover, the methylation levels of the fragments (−604 to −911 bp and −1,218 to −1,649 bp) in *PcMYB10* promoter were much greater in green pear skin than those in red pear skin (Wang et al., 2013). In the present study, however, high methylation activities (>90%) were detected in R3 (-1,808 to −1,527 bp), R4 (−1,657 to −1,388 bp), R6 (−1,264 to −1,078 bp), and R11 (−419 to −158 bp), and no significant differences were detected at the different stages. However, significant differences were observed in R2 (−2,003 to −1,745 bp) and R8 (−950 to −656 bp) during flower fading, which were distinct from the methylation function location during fruit coloration. The methylation activities of these two fragments were tissue-specific and might play important roles in the regulation of the *MhMYB10* promoter in flowers. The methylation level of R2 increased sharply from S1 to S2 (20.42%), consistent with the expression of *MhMYB10* and anthocyanin biosynthetic structure genes, demonstrating that the period from S1 to S2 is important for flower fading.

In all three cytosine contexts, the highest methylation levels of R2 were in CG, followed by CHG and CHH. Meanwhile, the methylation levels of all three cytosine contexts increased with flower fading. However, for R8, much greater methylation levels were detected in CHH than those in CG and CHG. A consistent trend with flower color fading was exhibited in CHH, but not in CG and CHG, indicating that hypermethylation of the CHH context in R8 has an important effect on *MhMYB10* expression. Similar results were found in other fragments from −2,585 to −2,117 bp of *MdMYB10* promoter of apple (El-Sharkawy et al., 2015) and −1,218 to −1,649 bp of *PcMYB10* of pear (Wang et al., 2013). However, in maize, DNA methylation related to color formation largely occurred in CG and CHG contexts (Sekhon and Chopra, 2009). This evidence reveals that ligneous plants might have a more complex methylation regulation mechanism.

The petal color fading of *M. halliana* is accompanied by aging of the flower. The natural process of flower senescence, broadly the combination of events that lead to the death of cells, tissues, or organs, can regulate flower color formation, gene expressions and DNA methylation (Teixeira da Silva et al., 2014; Zhang et al., 2018). In this study, most anthocyanin metabolism gene expression was regulated significantly, and methylation activities of R2 and R8 were gradually increased with flower development. During apple maturation, the methylation in the promoter of *MdMYB10* is developmentally controlled (El-Sharkawy et al., 2015). These results suggest that gene expression and *MhMYB10* methylation are regulated at the genetic and development levels.

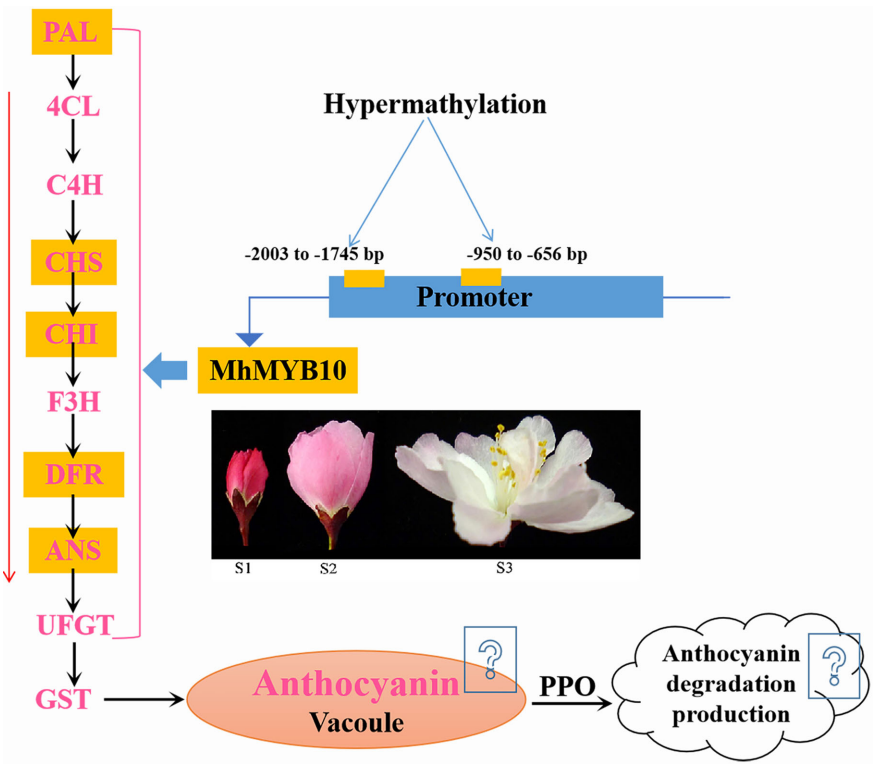


FIGURE 8 | A simplified model of flower color fading of *M. halliana*. During flower development, hypermethylation in the *MhMYB10* promoter, especially –2003 to –1745 bp and –950 to –656 bp, repressed the expression of *MhMYB10*. *MhMYB10* influenced the transcription of structural genes (e.g., *MhANS*, *MhDFR*, and *MhCHS*), which decreased the anthocyanin biosynthesis. In additionally, genes expressions in anthocyanin transport and degradation were downregulated or upregulated, which may influence anthocyanin content. Finally, these complex process resulted in decline of anthocyanin content, so the flower color faded.

CONCLUSION

Through complete transcriptomic analysis of petals at the different development stages, we describe how anthocyanin biosynthetic, transport, and degradation pathway control flower color fading in *M. halliana* (**Figure 8**). Multiple genes were identified as the key genes that lead to a decrease of anthocyanin content, including *MhPAL*, *MhCHS*, *MhCHI*, *MhF3H*, *MhDFR*, *MhANS*, *MhGSTU17*, and *MhPPO*. Many transcription factors were down or upregulated during flower development, including MYBs, bHLHs, WD40s, WRKYs, NACa, ERFs, bIZPs, and MADS-boxes. The phylogenetic tree analysis found that, of the five MYBs that regulate anthocyanin biosynthesis, *MhMYB10* is a key transcription factor. Therefore, we next analyzed the methylation activity of the *MhMYB10* promoter. Methylation levels of R2 (−2,003 to −1,745 bp) and R8 (−950 to −656 bp) of the *MhMYB10* promoter increased as the flower color faded, suggesting that methylation of the *MhMYB10* promoter might play a critical role in the downregulation of anthocyanin biosynthetic genes, resulting in decreasing anthocyanin content and color fading.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA) with bioproject No. PRJNA658719, and under GenBank accession numbers of SAMN15887511, SAMN15887512, SAMN15887513, SAMN15887514, 538 SAMN15887515, SAMN15887516, SAMN15887517, SAMN15887518, 539 SAMN15887519.

AUTHOR CONTRIBUTIONS

M-LH and JY performed most of the experiments and data analysis. Y-HZ, X-WS, J-XM, and FZ carried out material collection and pigment extraction. Y-HZ and X-WS conducted pigment analysis. J-XM conducted a part of RNA extraction. M-LH, JY, and JZ participated in the preparation of the manuscript. H-HL conceived, designed and coordinated the studies. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.576054/full#supplementary-material

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A P_{3A}-Type ATPase and an R2R3-MYB Transcription Factor Are Involved in Vacuolar Acidification and Flower Coloration in Soybean

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The determination of flower color mainly depends on the anthocyanin biosynthesis pathway and vacuolar pH; however, unlike the former, the mechanism of vacuolar acidification in soybean remains uncharacterized at the molecular level. To investigate this mechanism, we isolated four recessive purple–blue EMS-induced flower mutants from the purple flower soybean cultivar, Pungsannamul. The petals of all the mutants had increased pH compared with those of wild Pungsannamul. One of the mutants had a single nucleotide substitution in *GmPH4*, a regulator gene encoding an MYB transcription factor, and the substitution resulted in a premature stop codon in its first exon. The other three mutants had nucleotide substitutions in *GmPH5*, a single new gene that we identified by physical mapping. It corresponds to *Glyma.03G262600* in chromosome 3 and encodes a proton pump that belongs to the P_{3A}-ATPase family. The substitutions resulted in a premature stop codon, which may be a defect in the ATP-binding capacity of GmPH5 and possibly a catalytic inefficiency of GmPH5. The result is consistent with their genetic recessiveness as well as the high pH of mutant petals, suggesting that GmPH5 is directly involved in vacuolar acidification. We also found that the expression of *GmPH5* and several putative "acidifying" genes in the *gmph4* mutant was remarkably reduced, indicating that GmPH4 may regulate the genes involved in determining the vacuolar pH of soybean petals.

Keywords: flower color, vacuolar acidification, H^+ P-ATPase, GmPH5, GmPH4, soybean (*Glycine max*)

INTRODUCTION

Flower color is manifested by three main classes of pigments: anthocyanins, carotenoids, and betalains (Brockington et al., 2015). Anthocyanins and carotenoids can be abundantly found in angiosperms, whereas betalains are only found in Caryophyllales (Grotewold, 2006). Carotenoids are hydrophobic compounds that are produced and stored in plastids (Ng and Smith, 2016), and they are responsible for the yellow and orange colors in ornamentals such as marigold, daffodil, *Lilium*, and *Rosa* (Grotewold, 2006). In contrast, anthocyanins are water-soluble flavonoids stored in vacuoles; they contribute to various flower colors, such as red, pink, blue, and purple (Tanaka et al., 2008; Ng and Smith, 2016). Of these pigments, anthocyanins are the most extensively

studied owing to their wide distribution among angiosperms (Grotewold, 2006). Furthermore, anthocyanins are the predominant pigments in ornamentals such as petunia, *Ipomoea*, and snapdragon as well as soybean (Grotewold, 2006; Iwashina et al., 2008).

The early steps in the anthocyanin biosynthesis pathway are catalyzed by chalcone synthase, chalcone isomerase, and flavanone 3-hydroxylase, whereas the later steps are mediated by dihydroflavonol-4-reductase, anthocyanin synthase, and flavonoid 3-*O*-glucosyltransferase (3GT) (Morita and Hoshino, 2018). Anthocyanidin 3-glucosides are the first stable anthocyanins synthesized by 3GT-catalyzed glycosylation at the third carbon position of anthocyanidin aglycones (Morita and Hoshino, 2018). In most higher plants, the anthocyanin biosynthesis pathway is reportedly regulated by a MBW complex comprising transcription factors that contain MYB, basic-helix-loop-helix (bHLH), and WD-repeat (WD40) domains (Hartmann et al., 2005; Lepiniec et al., 2006; Quattrocchio et al., 2006a; Solfanelli et al., 2006; Gonzalez et al., 2008; Albert et al., 2014).

Plant MYB proteins contain a single or multiple repeat of structurally conserved MYB DNA-binding domain(s). Among the MYB protein families, the largest is the two-repeat class (R2R3), which is associated with the anthocyanin pathway (Allan et al., 2008). In several plant species, such as petunia, *Phalaenopsis* orchids, *Antirrhinum*, and soybean, the activity of MYB proteins influences differential pigmentation in different parts of the petal (Quattrocchio et al., 1998; Schwinn et al., 2006; Takahashi et al., 2013; Hsu et al., 2015). R2R3-MYB transcription factors of the MBW complex are considered as a key determinant controlling distinct pigmentation patterns throughout the plant, whereas WD40 and bHLH transcription factors are shared between floral and vegetative pigmentation regulation (Albert et al., 2011). In petunia, ANTHOCYANIN2 (AN2) and AN4 encode members of the R2R3-MYB transcription factor family that regulates anthocyanin synthesis in floral tissues (Quattrocchio et al., 1999; Albert et al., 2011), whereas PH4 is another MYB transcription factor that regulates vacuolar acidification (Quattrocchio et al., 2006b).

Vacuolar pH plays an important role in hueing anthocyanin pigments, providing varying degrees of flower color. In all cells, the vacuolar lumen has a lower pH than the surrounding cytoplasm. In petunia, the hyperacidity of the vacuoles of flower petals results in red-colored flowers (Faraco et al., 2014). Mutations affecting vacuolar pH regulation lead to bluish flower color and increased pH of petal homogenates (Verweij et al., 2008; Faraco et al., 2014). In most plant cells, the pH gradient across the tonoplast is generated by vacuolar ATPases (V-ATPase) or H^+-pyrophosphatases (Eisenach et al., 2014). However, recent studies involving flower color mutants of petunia and other ornamental plants revealed that phosphorylated ATPases (P-ATPases) also reside in the tonoplast and play a key role in determining the vacuolar pH of petals (Verweij et al., 2008; Faraco et al., 2014).

Petunia has seven distinct loci (*PH1* to *PH7*) controlling flower coloration. Wild-type (WT) petunia petals with accumulated cyanidins display red color, and vacuolar pH is ∼5.5 when all *PH* genes are functional (Faraco et al., 2014). However, mutations in any of the seven loci lead to an increase in the vacuolar pH of petals, up to ∼6.0, thereby exhibiting blue color (Spelt et al., 2002; Faraco et al., 2014). In petunia, the activity of the MBW complex is sometimes enhanced by another transcription factor that contains the WRKY domain (Verweij et al., 2016). The genes involved in vacuolar acidification are activated by MBWW complex proteins, including PH4 (an MYB protein), AN1 (a bHLH protein), AN11 (a WD40 protein), and PH3 (a WRKY protein) (Spelt et al., 2002; Koes et al., 2005; Quattrocchio et al., 2006b). Moreover, *PH5* and *PH1* are the most important downstream structural genes involved in vacuolar acidification (Verweij et al., 2008; Faraco et al., 2014). PH5 is a P_{3A}-ATPase-type proton pump, whereas PH1 is a P_{3B}-ATPase-like bacterial Mg^{2+} transporter, and these P-type ATPases are located in the tonoplast (Verweij et al., 2016). Although PH1 has no H^+ transport activity on its own, it can physically interact with PH5, thereby promoting the proton-pumping activity of PH5 (Li et al., 2016).

In recent years, flower color variations in soybean (*Glycine max*) have been extensively studied. To date, six genes have been identified: five structural genes (*W1*, *W3*, *W4*, *Wm*, and *Wp*) encoding enzymes involved in flavonoid biosynthesis and one transcriptional regulator gene (*W2*; hereafter referred to as *GmPH4*) (Sundaramoorthy et al., 2015). The purple–blue coloration of the flowers of a soybean landrace, Nezumisaya, was identified to be controlled by the *GmPH4* locus (Takahashi et al., 2008). Unlike mutations in soybean structural genes, mutation in the *GmPH4* locus did not change the flavonoid content in the purple–blue flower but increased the pH of petal saps (Iwashina et al., 2008; Takahashi et al., 2008, 2011). Other factors involved in pH determination of the vacuolar sap in soybean have not been determined thus far. In the present study, we characterized two factors that are involved in soybean flower coloration: one is the MYB transcription factor encoded by the regulator gene *GmPH4,* and the other is the putative vacuolar P_{3A}-ATPase encoded by *GmPH5*, a new gene identified in this study. Here we have discussed the possible roles of these genes in vacuolar pH regulation of soybean petals.

MATERIALS AND METHODS

Plant Materials

Four mutant lines (PE704, PE282, PE734, and PE971) with purple–blue flowers (**Figure 1A**) were isolated from an EMS-induced population of a soybean cultivar [*Glycine max* (L.) Merr], Pungsannamul (Chae et al., 2013). Cultivars such as Pungsannamul, Harosoy, and Jinpung were used in this study. A soybean mutant that previously reported as a purple-blue mutant, Nezumisaya (Iwashina et al., 2008; Takahashi et al., 2008) was used as a mutant control. All the mutant lines were crossed with either Pungsannamul or Jinpung (**Table 1**), and then segregation analysis was performed. Next, physical mapping was performed using the F_2 population derived from the crosses of mutant lines PE704 and PE282 with Harosoy, a contrasting cultivar. To conduct the allelism test, breeding crosses were made between mutant lines, and the data are

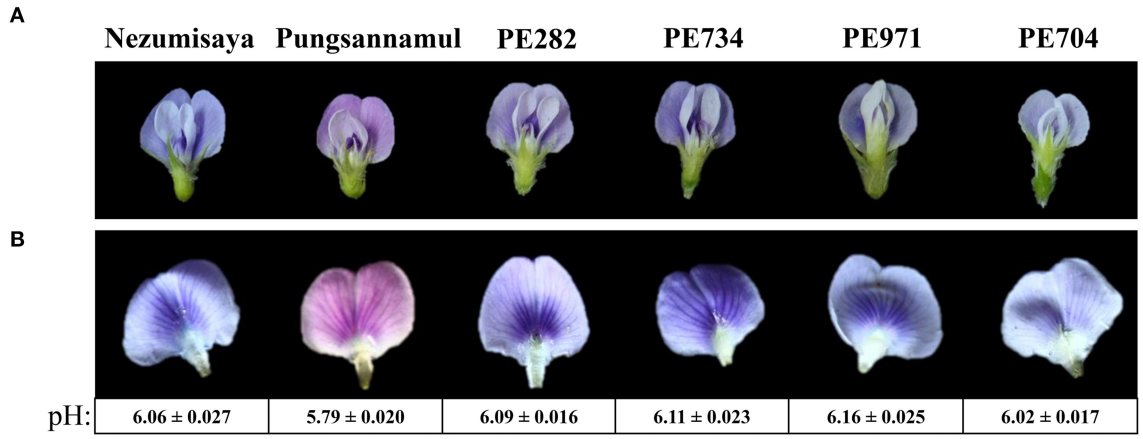

FIGURE 1 | Photographic images showing the flower color of the plant specimens. Images displaying a whole flower **(A)** and a standard petal **(B)** of soybean cultivars Nezumisaya (purple-blue), Pungsannamul (purple), and EMS mutant lines (PE282, PE734, PE971, and PE704; purple-blue). The pH values of the petal homogenates are shown below the standard petals of purple and purple–blue flower lines. Values are presented as mean ± standard error. The result of the *t*-test supports a highly significant difference in pH values between the purple flowers and the purple–blue-flowered lines from WT ($p < 0.00001$).

detailed in **Table 2**. All the experimental populations were grown in the experimental fields at Kyungpook National University (Gunwi, 36°07′N, 128°38′E, Korea).

Measurement of pH of the Petal Homogenates

The whole petal limb of an open flower was ground in 1 ml of distilled water, and its pH was measured immediately using a portable pH electrode (Compact pH meter LAQUAtwin-pH-33, Horiba Scientific, Japan). For each line, 10 whole petal limbs were analyzed individually in three replicates. The pH values are presented as mean ± standard error from three independent replications for each line. A comparison between Pungsannamul and the mutant lines was performed using an online *t*-test analysis portal (https://www.usablestats.com/calcs/2samplet). Statistical significance was set as $p \leq 0.0001$.

Physical Mapping and Sequence Analysis of *GmPH4* and *GmPH5*

Genomic DNAs were isolated from trifoliate leaves using the cetyltrimethylammonium bromide extraction method (Doyle, 1987). A physical map was constructed from the cross between Harosoy and PE704 using an Affymetrix 180K Axiom® single-nucleotide polymorphism (SNP) array (Affymetrix USA). We selected a total of 14 F_2 individuals with purple–blue flowers. The region containing the *GmPH4* locus was demarcated by detecting recombinants among the F_2 individuals using Microsoft Excel. The coding sequences of these candidate genes were amplified using the following PCR conditions: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 20 s, annealing at 55–58°C for 40 s, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were sequenced (SolGent, Korea) using the sequencing primers listed in **Supplementary Table 1**.

Another physical map for PE282 was constructed from the cross between Harosoy and PE282. Here, 20 F_2 individuals with purple–blue flowers were used. The region containing the *GmPH5* locus was demarcated by detecting recombinants among the F_2 individuals using Microsoft Excel. Fine mapping was performed using specific SNP markers that were developed in this study (data not shown). For next-generation sequencing (NGS) analysis, genomic DNA isolated from Pungsannamul (a wild type) and two mutant lines (PE282 and PE734) were sequenced on an Illumina Hiseq™ 2500 platform (Macrogen, Korea) to construct a paired-end NGS library. The clean reads were rechecked using the FASTQC program for quality control after trimming the adaptor. The clean reads were aligned and mapped to the *G. max* reference genome assembly version 2.0 (Wm82.a2.v1) derived from Phytozome using a Burrows-Wheeler Aligner (BWA) tool with default parameters. The homozygous SNPs between the mutant lines and Pungsannamul cultivar were used for further analysis. The coding sequence of *GmPH5* (*Glyma.03G262600*) was determined using PCR as described in the previous section.

Multiple Alignment and Phylogenetic Analysis of GmPH5 and P_{3A}-ATPase Proteins

P_{3A}-ATPase proteins retrieved from the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and proteomics databases (https://www.proteomicsdb.org/) were used for multiple alignment analysis that was performed using ClustalW (http://www.genome.jp/tools-bin/clustalw). The P_{3A}-ATPase proteins used in the tree construction was derived from the PANTHER classification system for proteins (http://pantherdb.org/) and NCBI database when "GmPH5 protein" was used as a query. The phylogenetic tree was constructed using the MEGA 7.0 software (Kumar et al., 2016).

TABLE 1 | Segregation and co-segregation of F_2 individuals for flower color phenotypes.

♀	♂	Total number of plants	Phenotype		Segre-gation ratio	χ^2-value	*P*-value	Genotype			Segre-gation ratio	χ^2-value	*P*-value
			Purple	Purple-blue				W†	H†	M†			
Pungsannamul	PE704 (*gmph4-p1*)	189	142	47	3:1	0.002	0.96	50	92	47	1:2:1	0.228	0.89
Harosoy	PE704 (*gmph4-p1*)	72	58	14	3:1	1.185	0.27	–	–	–	–	–	–
Pungsannamul	PE282 (*gmph5-a*)	112	85	27	3:1	0.048	0.82	28	57	27	1:2:1	0.054	0.97
Harosoy	PE282 (*gmph5-a*)	154	116	38	3:1	0.009	0.92	–	–	–	–	–	–
Jinpung	PE734 (*gmph5-b*)	137	105	32	3:1	0.197	0.65	37	68	32	1:2:1	0.370	0.83
Pungsannamul	PE971 (*gmph5-c*)	183	136	47	3:1	0.046	0.83	44	92	47	1:2:1	0.104	0.94

†*W, wild homozygote; H, heterozygote; M, mutant homozygote.*

TABLE 2 | Allelism analysis of F_2 individuals for purple-blue flower phenotypes.

P1 ♀	P2 ♂	Total number of plants	Phenotype		Segre-gation ratio	χ^2-value	*P*-value	Genotype			Segre-gation ratio	χ^2-value	*P*-value
			Purple	Purple-blue				A†	C†	B†			
Nezumisaya (*gmph4*)	PE704 (*gmph4-p1*)	95	–	95	–	–	–	–	–	–	–	–	–
Nezumisaya (*gmph4*)	PE282 (*gmph5-a*)	85	47	38	9:7	0.048	0.82	–	–	–	–	–	–
Nezumisaya (*gmph4*)	PE734 (*gmph5-b*)	101	51	50	9:7	0.062	0.92	–	–	–	–	–	–
PE704 (*gmph4-p1*)	PE971 (*gmph5-c*)	71	39	32	9:7	0.057	0.81	–	–	–	–	–	–
PE282 (*gmph5-a*)	PE734 (*gmph5-b*)	71	–	71	–	–	–	21	35	15	–	1.021	0.60
PE282 (*gmph5-a*)	PE971 (*gmph5-c*)	118	–	118	–	–	–	–	–	–	–	–	–

†*A, P1 mutant homozygote; B, P2 mutant homozygote; C, mutant heterozygote.*

Isolation of RNA and qRT-PCR Analysis

Total RNA was isolated from freeze-dried samples using the phenol–chloroform and lithium chloride precipitation methods (McCarty, 1986). The RNA samples were treated with DNase I to remove DNA contaminants (Takara, Japan). First-strand cDNA was synthesized by reverse-transcribing the total RNA with an oligo-$dT_{(20)}$ primer and Superscript III according to the manufacturer's instructions (Invitrogen, USA). Three replicates of relative gene expression quantification was performed according to Park et al. (2015) using the LightCycler® 480 Real-Time PCR System (Roche, Germany). The primers used in this analysis are listed in **Supplementary Table 1**.

CAPS and dCAPS Analysis

The genomic DNA isolated from the F_2 individuals derived from different crosses was used for the cleaved amplified polymorphic sequence (dCAPS) and CAPS analyses. The dCAPS PCR primers (**Supplementary Table 1**) were designed to detect SNPs in the PE282, PE734, and PE704 mutant lines, together with the Nezumisaya *gmph4* mutant. In Nezumisaya, PE704, and PE282, the nucleotide substitutions (G to A; C to T; and G to A) generated *Hpa*II (CCGG), *Hha*I (GCGC), and *Hha*I (GCGC) sites, respectively, in the PCR products that were amplified from the WT parent. In PE734, the nucleotide substitution (G to A) generated a *Hin*dIII site (AAGCTT) in the PCR product that was amplified from the mutant parent. In PE971, the CAPS PCR primer set was designed to detect the SNP in the mutant line. The nucleotide substitution (C to T) generated a *Bcl*I (TGATCA) site in the PCR products that were amplified from the mutant parent. The PCR conditions were the same as those mentioned in the previous sections. The amplified products were digested and separated on 1.2% agarose gel.

RESULTS

Isolation of Soybean Mutants With Purple–Blue Flowers

To identify the genes involved in vacuolar acidification of soybean flowers, we screened an EMS-induced mutant population developed from the Pungsannamul soybean cultivar. We identified four mutant lines (PE704, PE282, PE734, and PE971) with purple–blue flowers (**Figure 1A**). We measured the pH of petal homogenates of the mutants and the WT cultivar to investigate the physiologic basis of flower color in the mutants (**Figure 1B**), which may serve as evidence for vacuolar acidification (Faraco et al., 2014). The Pungsannamul cultivar's purple flower had a pH of 5.79, whereas the purple–blue flower of the mutants had a pH value of 6.02–6.16 (**Figure 1B**). The *t*-test showed that there was a significant difference between the purple and purple-blue flower lines, suggesting that the increased pH in mutant petal homogenates is responsible for the purple–blue coloration. This result is consistent with the increased pH of petal homogenates in petunia blue flower mutants (*ph1–ph7*) and Nezumisaya, a soybean purple–blue flower mutant (Iwashina et al., 2008; Takahashi et al., 2013; Faraco et al., 2014).

Genetic Inheritance Patterns of the New Mutations

The flower colors of the F_2 individuals derived from the crosses of cultivars (Pungsannamul, Jinpung, and Harosoy) along with those of the mutants (PE282, PE704, PE734, and PE971) were analyzed to investigate the inheritance of mutant alleles in the presence or absence of a purple–blue flower color (**Table 1**). The segregation patterns of the four F_2 populations were statistically consistent with a 3:1 ratio (purple:purple–blue). These results indicate that the purple–blue flowers in each mutant are because of a single recessive allele.

Allelism Test for the *gmph4-p1* and *gmph5* Mutant Alleles

All four mutant lines showed the same phenotype, i.e., purple–blue flower, which led us to perform an allelism test. First, we made crosses between the three mutant lines (PE704, PE282, and PE734) and Nezumisaya, having been previously reported as a *gmph4* mutant line (Takahashi et al., 2008). The resulting flower colors of 95 F_2 individuals were all identical (purple–blue) in the cross between Nezumisaya and PE704 (**Table 2**). The result indicated that the purple–blue flower in the PE704 mutant may have been caused by either the same or different alleles of *GmPH4*, hereafter designated as the *gmph4-p1* allele. In contrast, the F_2 individuals obtained from the populations of PE282 × Nezumisaya and PE734 × Nezumisaya showed a segregation of flower colors to purple and purple–blue in a ratio of 9:7. In addition, we made three more crosses, i.e., PE704 × PE971, PE282 × PE734, and PE282 × PE971. The F_2 individuals from PE971 × PE704 showed a segregation of flower colors to purple and purple–blue at a ratio of 9:7. Moreover, all F_2 individuals derived from PE282 × PE734 and PE282 × PE971 crosses showed an identical phenotype, i.e., the purple–blue color. These results suggested that the purple–blue flowers in the PE282, PE734, and PE971 mutants are caused by either the same or different allele of a new gene, which we are to describe as *GmPH5* and hereafter designate as *gmph5-a* in PE282; *gmph5-b* in PE734; and *gmph5-c* in PE971.

Physical Mapping of the *GmPH4* Locus and Molecular Analysis of the *gmph4-p1* Allele

We performed physical mapping analysis using an Affymetrix Axiom® SNP array to identify the gene involved in the mutant phenotype of PE704 (*gmph4-p1*). A physical map was constructed using 10 F_2 individual lines with purple–blue flowers derived from Harosoy × PE704 (**Table 1**). The locus was mapped to the 18.3 Mb region between Affx-89126782 and Affx-89127681 SNP arrays on chromosome 14 (**Figure 2A**). In a previous study, the *GmPH4* locus involved in purple–blue soybean flower coloration was genetically mapped in chromosome 14 and was shown to be flanked by the Satt318 marker at a distance of 1.1 cM (Takahashi et al., 2008). Takahashi et al. (2011) previously characterized *GmPH4* (*Glyma.14G154400*) that was located in the region that we mapped. *GmPH4* encodes an MYB

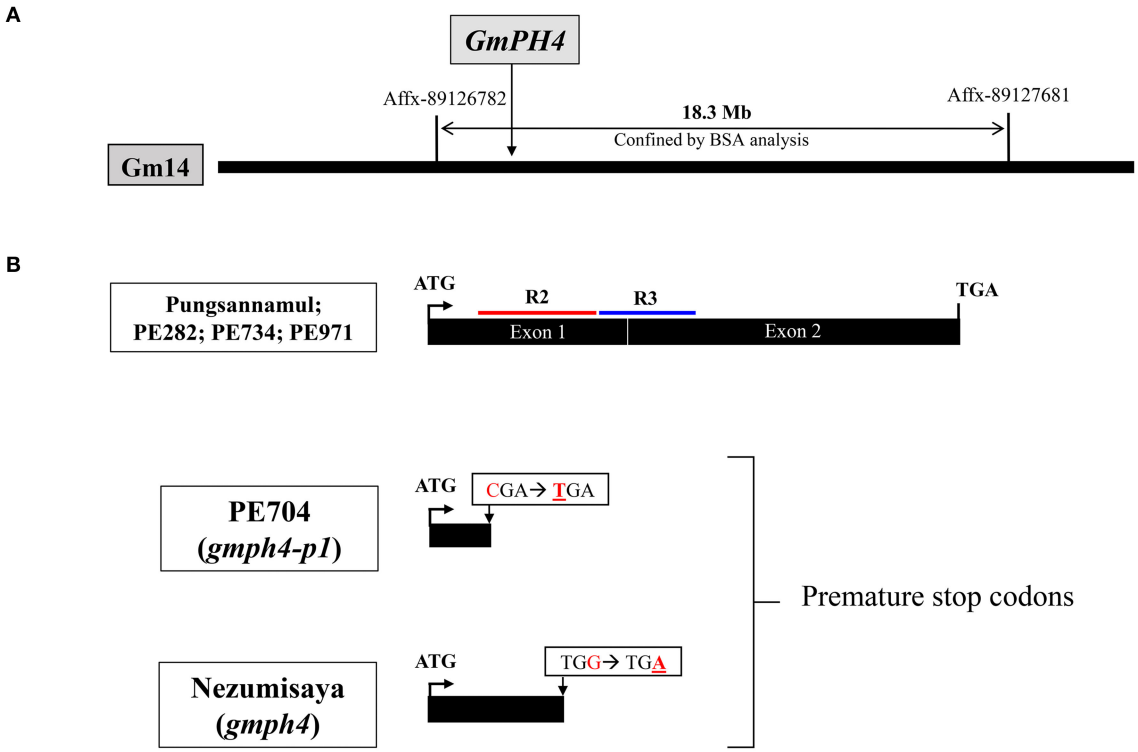

FIGURE 2 | Physical map and gene structure of *GmPH4* in soybean. **(A)** Physical map construction of the *GmPH4* locus. **(B)** Gene structure of *GmPH4* showing the sequence polymorphisms among *GmPH4*, *gmph4* (Nezumisaya), and *gmph4-p1* (PE704) alleles. Nucleotide substitutions in the mutant alleles are indicated. The regions corresponding to R2 and R3 MYB domains are marked in red and blue, respectively.

transcription factor containing two MYB repeats (R2 and R3) (**Figure 2B**).

We analyzed the coding sequence of *Glyma.14G154400* (position +1 to 1086) to determine whether *GmPH4* is responsible for the purple–blue flower coloration in the PE704 mutant (**Figure 2B**). The sequence analysis revealed that PE704 had an SNP (C to T) at nucleotide position 133 of *Glyma.14G154400*. This SNP resulted in a premature stop codon at amino acid position 45. The result indicated that the mutation produced a GmPH4 truncated protein, thereby leading to a complete loss of function, which strongly agrees with the previously reported purple–blue flower mutant line, Nezumisaya, in which its SNP introduced a premature stop codon (TGA) at amino acid position 88 (Takahashi et al., 2011).

Physical Mapping of the *GmPH5* Locus and Molecular Analysis of the *gmph5* Alleles

To identify the gene responsible for the purple–blue flowers of PE282 (*gmph5-a*), PE734 (*gmph5-b*), and PE971 (*gmph5-c*) mutants, we first used an Affymetrix Axiom® SNP array of 20 F_2 individuals with purple–blue phenotypes derived from Harosoy × PE282 (**Table 1**). The *GmPH5* locus was mapped to a 4.1 Mb-spanning region between the Affx-89050767 and Affx-89052254 SNP arrays on chromosome 3 (**Figure 3A**) in the initial mapping. The mapped region was further narrowed down by the specific SNP markers that were developed in this study. The *GmPH5* locus was mapped to a 0.5 Mb-spanning region between the GM03-D3 marker (**Supplementary Table 1**) and Affx-89052254.

We performed NGS analysis and obtained data for the PE282 and PE734 mutants to identify the candidate gene. In PE282, each of the five genes in the mapped region had an SNP in their exons, leading to amino acid changes in their respective proteins (**Table 3**). In PE734, we found that out of the five genes, *Glyma.03G262600* was the only gene with an SNP in the exon, which led to amino acid substitution. We designated it as a candidate gene for *GmPH5* based on the premises that the allelism test indicated that all the mutant alleles (*gmph5-a*, *gmph5-b,* and *gmph5-c*) corresponded to the same gene, the *Glyma.03G262600* of both *gmph5-a* and *gmph5-b* had SNPs, and *Glyma.03G262600* (position +1 to 2805) was predicted to encode an H^+ P-ATPase in the Phytozome soybean genome database (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Gmax). The sequence analysis of *Glyma.03G262600* from PE971 (*gmph5-c*) also revealed an SNP (C to T) in the seventh exon and introduced a premature stop codon at the amino acid position 276 (**Figure 3B**). The SNPs detected in the PE282 (*gmph5-a*) and PE734 (*gmph5-b*) mutant alleles led to amino acid substitutions; the former substituted Thr for Ala (A373T) and the latter substituted Glu for Gly (G432E) as compared with the corresponding sequences of Pungsannamul and Williams 82 cultivars (**Table 3**, **Figure 3B**).

P-ATPases are widely distributed in plants and are involved in transporting diverse small cations and phospholipids (Axelsen

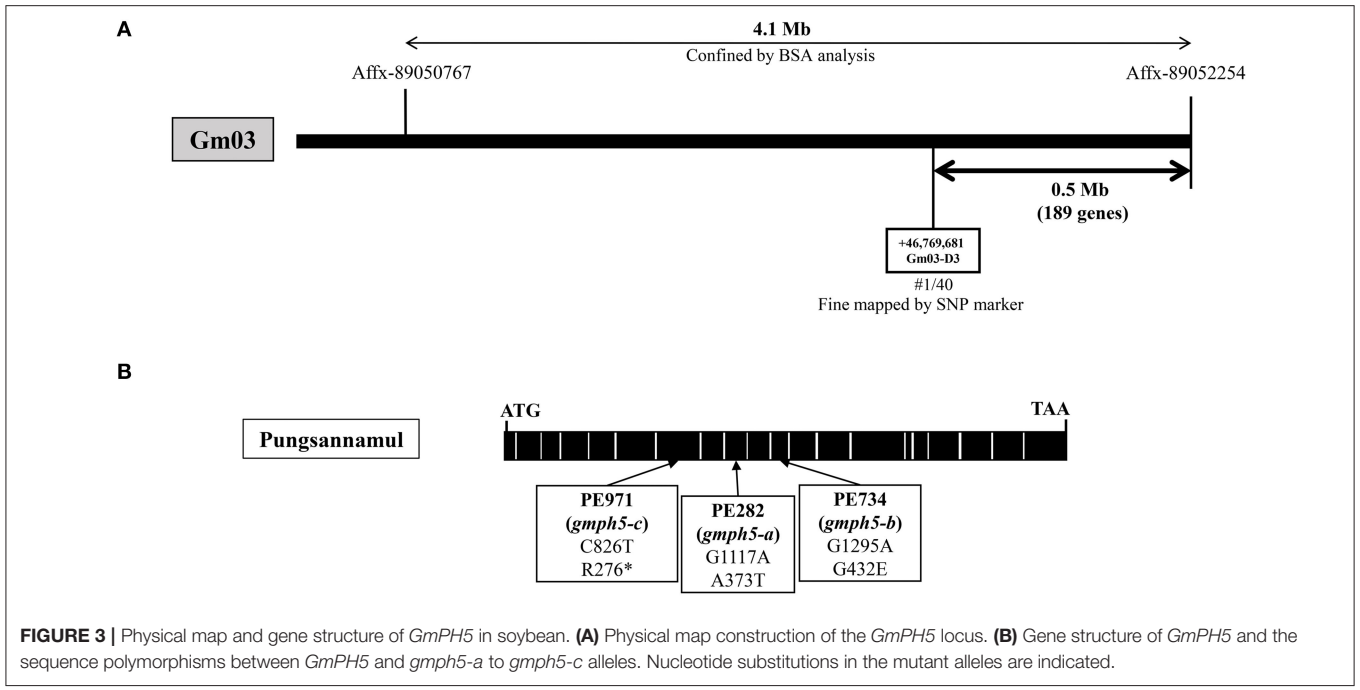

FIGURE 3 | Physical map and gene structure of *GmPH5* in soybean. **(A)** Physical map construction of the *GmPH5* locus. **(B)** Gene structure of *GmPH5* and the sequence polymorphisms between *GmPH5* and *gmph5-a* to *gmph5-c* alleles. Nucleotide substitutions in the mutant alleles are indicated.

TABLE 3 | List of candidate genes with point mutations identified in PE282 and PE734 mutant lines.

Mutant line	Gene	Annotation	Physical position	SNP	Amino acid change
PE282 (*gmph5-a*)	*Glyma.03G248400*	Perakine reductase	46420245	G→ A	L→ F
	Glyma.03G251500	Serine/threonine-protein phosphatase PP1 isozyme 2-related	46612239	G→ A	L→ F
	Glyma.03G255700	Protein of unknown function (DUF1070)	46986507	G→ A	A→ V
	Glyma.03G256700	WRKY DNA -binding domain (WRKY)	47074609	G→ A	P→ S
	Glyma.03G262600	H^+ P-ATPase	47535452	G→ A	A→ T
PE734 (*gmph5-b*)	*Glyma.03G262600*	H^+ P-ATPase	47535630	G→ A	G→ E

and Palmgren, 1998). The P-ATPase transporters have been classified into five major subfamilies (P_1-P_5) with subgroups (P_{1A-B}, P_{2A-D}, P_{3A-B}, P_4, and P_{5A-B}) (Sørensen et al., 2019). In the P_3-ATPase subgroups, P_{3A}- and P_{3B}-ATPases transport H^+ and Mg^{2+} ions, respectively. Judging from its homology with petunia PH5 (Verweij et al., 2008; Faraco et al., 2014), *GmPH5* encodes an H^+ P-ATPase belonging to the P_{3A}-type proton pump subfamily. We performed multiple sequence alignment with 26 P_{3A}-type ATPases from different plant species along with those from fungus, human, and pig (**Figure 4**). The resulting alignment showed that the amino acid changes in *gmph5-a* and *gmph5-b* were located at the positions that were highly conserved among all the P_{3A}-type ATPases that were analyzed. The PE282 (*gmph5-a*) mutant showed an amino acid change in the catalytically active and cytosolic domain C loop (Novoa-Aponte et al., 2012). The PE734 (*gmph5-b*) mutant showed an amino acid change in the KGAPE motif, which functions in ATP binding (Novoa-Aponte et al., 2012). Based on these findings, we suggest that those SNPs could partially nullify GmPH5 H^+ P_{3A}-ATPase function, which is consistent with the recessive nature of *gmph5-a* and *gmph5-b* as well as the elevated pH values in their petal saps.

A phylogenetic tree was constructed to infer the evolution and relationship of GmPH5 with 46 P_{3A}-ATPase proteins of different plant species showing above 80% sequence similarities (**Supplementary Figure 1**). The phylogenetic tree was split into two major clades; one of which contained species from only three families of angiosperms (Fabaceae, Poaceae, and Brassicaceae) and the other contained the rest of angiosperm species. In both the clades the species were clustered together with the species under their respective families, suggesting that most of the P_{3A}-ATPase protein sequences are specific at the family level. The P_{3A}-ATPases of all species sharing high homology with the petunia PH5 (**Supplementary Figure 1**). A previous study (Li et al., 2016) has also revealed the phylogenetic relationship between GmPH5 (GenBank number, XP_003521833) and petunia PH5.

Co-segregation Analysis Using dCAPS Markers

We developed dCAPS markers for the *gmph4-p1* allele and determined the co-segregation patterns of these markers and the purple–blue phenotypes in PE704 mutant populations

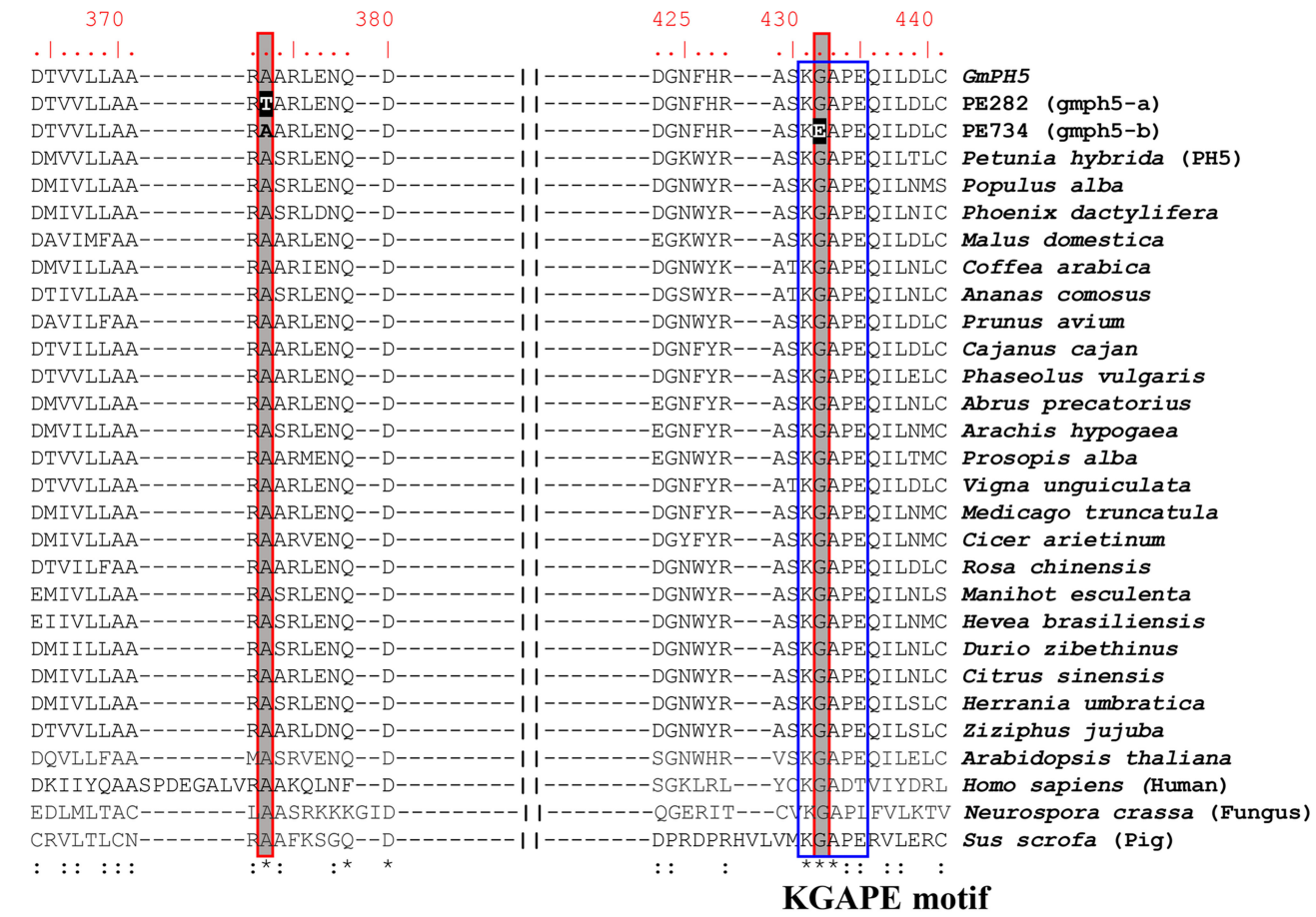

FIGURE 4 | Amino acid sequence alignment of P_{3A}-ATPase proteins. Amino acid sequences of P_{3A}-ATPase proteins from different species were compared with WT GmPH5 and mutant proteins. SNPs detected in mutants are highlighted in black. The conserved KGAPE motif is highlighted in a blue box. Asterisks indicate identical residues; colons, conserved, or semi-conserved substitutions. GenBank accession numbers of P-ATPase proteins are as follows: *Petunia hybrida*, DQ888719; *Populus alba*, TKR85646; *Phoenix dactylifera*, XP_008783452; *Malus domestica*, XP_008342517; *Coffea arabica*, XP_027124527; *Ananas comosus*, XP_020104345; *Prunus avium*, XP_021819909; *Cajanus cajan*, XP_020211004; *Phaseolus vulgaris*, XP_007139050; *Abrus precatorius*, XP_027348813; *Arachis hypogaea*, RYR52881; *Prosopis alba*, XP_028796327; *Vigna unguiculata*, XP_027902873; *Medicago truncatula*, XP_013450626; *Cicer arietinum*, XP_004494890; *Rosa chinensis*, XP_024181824; *Manihot esculenta*, XP_021610990; *Hevea brasiliensis*, XP_021654241; *Durio zibethinus*, XP_022723470; *Citrus sinensis*, XP_006465725; *Herrania umbratica*, XP_021294688; *Ziziphus jujuba*, XP_015901337; *Arabidopsis thaliana*, 5KSD; *Homo sapiens*, 6K7G; *Neurospora crassa*, 1MHS; and *Sus scrofa*, 5Y0B.

(**Supplementary Figure 2A**). dCAPS analysis showed that the genotype segregation fits a 1:2:1 ratio (**Table 1**, **Supplementary Figure 3A**), confirming that the *gmph4-p1* allele is *GmPH4*-recessive. We also performed dCAPS analysis using a *gmph4* marker developed for the Nezumisaya mutant and a *gmph4-p1* marker for the PE704 mutant. Our results showed that the *gmph4* marker was not complemented by the *gmph4-p1* marker, confirming that *gmph4* and *gmph4-p1* are allelic to each other (**Table 2**).

Because *Glyma.03G262600* was identified as the *GmPH5* gene, we developed dCAPS markers for the *gmph5-a* and *gmph5-b* alleles, as well as a CAPS maker for the *gmph5-c* allele, and determined the co-segregation patterns of the markers and the purple–blue flower color phenotypes (**Supplementary Figures 2B–D**). All the dCAPS and CAPS markers consistently co-segregated with the flower color phenotypes of F_2 plants derived from all the segregation crosses (**Table 2**), confirming that these mutant alleles are strongly associated with the purple–blue flower. The analysis also showed that the genotype segregation fits a 1:2:1 ratio (**Table 1**, **Supplementary Figures 3B–D**), confirming that the *gmph5-a*, *gmph5-b*, and *gmph5-c* alleles are all *GmPH5*-recessive. We also performed dCAPS and CAPS analyses using 71 F_2 individuals derived from PE282 × PE971. The result showed that the *gmph5-a* marker was not complemented by the *gmph5-c* marker, confirming that *gmph5-a* and *gmph5-c* are allelic to each other.

Expression Profiles of Genes Involved in Vacuolar Acidification

PH4, AN1, AN11, and PH3 proteins in petunia are MBWW complex components that control the expression of downstream genes, including *PH1* and *PH5*, which are involved in

vacuolar acidification (Verweij et al., 2008; Faraco et al., 2014). GmPH4 and GmPH5 showed 60.39 and 83.26% amino acid sequence similarity with petunia PH4 and PH5, respectively (**Supplementary Figure 4**). With the exceptions of GmPH4 and GmPH5, no other soybean factors that correspond to these petunia proteins have been recently identified, and this prompted us to search for the soybean homologs of PH3, AN1, and AN11 through BLASTP analysis using the "highest densities" criterion, thereby identifying Glyma.19G177400, Glyma.02G147800, and Glyma.06G136900 as GmPH3, GmAN1, and GmAN11, respectively. Their amino acid sequence similarities with the corresponding proteins of petunia were 47.92, 57.86, and 76.70%, respectively (**Supplementary Figure 4**). As petunia PH1 was reported to enhance PH5 activity (Li et al., 2016), we also identified a soybean homolog of PH1 as GmPH1 (Glyma.05G175300.2) by BLASTP analysis, and the results showed that they shared 67.89% identity (**Supplementary Figure 4**).

The expression of the aforementioned MBWW complex genes, along with *GmPH1* and *GmPH5,* were analyzed using quantitative real-time (qRT)-PCR analysis using fully bloomed banner petals of the Pungsannamul cultivar and mutant lines (**Figure 5**). In addition, the *F3′5′H* (*W1*) gene involved in anthocyanin biosynthesis was analyzed (Sundaramoorthy et al., 2015). PE704 (*gmph4-p1*) and the *gmph4*-mutated Nezumisaya showed a lower level of *GmPH4* transcripts compared with WT Pungsannamul. Interestingly, the transcript levels of *GmPH5*, *GmPH1*, *GmPH3*, *GmAN1*, and *GmAN11* in the *gmph4* mutants were also lower compared with those of the WT. Similar results have been observed in petunia (Faraco et al., 2014). The result indicated that the loss of function of *GmPH4* downregulated all the genes that encode MBWW transcriptional complex components and, consequently, their downstream genes, i.e., *GmPH1* and *GmPH5*. The level of *GmPH1* transcripts was noticeably lower than that of *GmPH5* in both *gmph4* lines. The results are consistent with the findings of Faraco et al. (2014), in which *PH1,* rather than *PH5,* was more prominently influenced by the PH4 MYB transcription factor. All the three *gmph5* mutants showed significant reductions in the transcript levels of *GmPH1* and *GmAN1,* although the reductions in the transcript levels of *GmPH5, GmPH4,* and *GmAN11* were less remarkable. The significant transcript level reductions of the *gmph4-p1* and *gmph5-c* alleles for their respective genes may be caused by the nonsense-mediated mRNA decay to prevent truncated protein expression (Chang et al., 2007; Takahashi et al., 2011). In addition, the low *GmPH5* expression in the *gmph5-a* missense mutant may have been caused by abnormal folding or instability of the GmPH5 protein (Antonarakis and Cooper, 2013). However, neither *gmph4* nor *gmph5* mutations affected *F3′5′H* expression, which is another gene involved in anthocyanin biosynthesis.

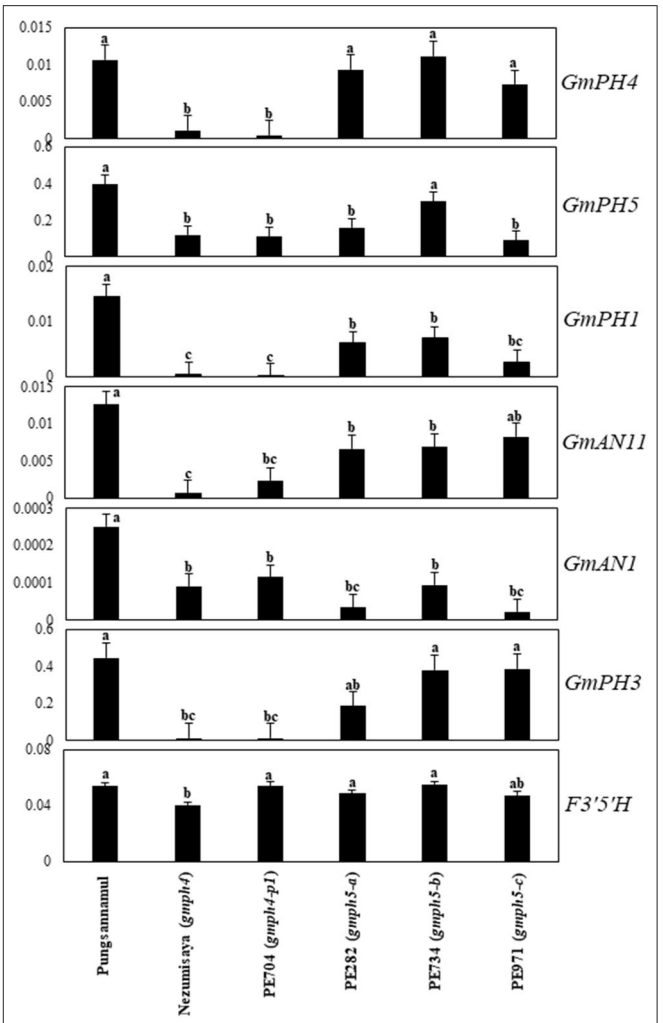

FIGURE 5 | Expression profiles of regulatory and structural genes involved in flower coloration in soybean. qRT-PCR analyses of the regulatory genes (*GmPH4*, *GmAN11*, *GmAN1*, and *GmPH3*), and structural genes (*GmPH1* and *GmPH5*) were performed with mRNAs extracted from fully bloomed petals. *F3′5′H* expression levels are shown for comparison. Error bars represent standard deviation of three biological replicates. The expression of each gene was normalized using constitutive gene 7 (*Cons7*) as the reference gene.

DISCUSSION

In soybean, the regulatory components involved in vacuolar acidification have remained largely unexplored. In the present study, we explored two of these components to understand the process of vacuolar acidification. Moreover, we isolated four soybean mutants with purple–blue flowers. Among them, one mutant had an allelic *GmPH4* variation that encoded an MYB transcription factor, and its petunia homolog, PH4, was reportedly involved in regulating vacuolar pH (Faraco et al., 2014). The other three mutants had allelic variations in *GmPH5*, which was newly identified as a petunia homolog of *PH5*. Segregation analysis showed that the purple–blue phenotype of *gmph5-a* to *gmph5-c* is controlled by recessive alleles at the *GmPH5* locus. Physical mapping confirmed that *GmPH5* corresponded to *Glyma.03G262600*, which encoded a member of the P_{3A}-ATPase family. Finally, the co-segregation study indicated that the *GmPH5* locus is tightly linked to purple–blue flower phenotypes.

The *GmPH4* mutations downregulated *GmPH1* and *GmPH5* expression, which is consistent with the results that showed the increased pH of petal homogenates and the purple–blue hue of petals (**Figures 1**, **5**). This explanation strongly agrees with the petunia system, in which PH1 and PH5 were necessary for vacuolar acidification (Faraco et al., 2014). PH5 proteins can homodimerize to engage in proton-pumping activity and can also form a heterodimeric complex with PH1, thereby enhancing the proton-pumping activity (Faraco et al., 2014). The outcome of PH1–PH5 interaction is involved in vacuolar acidification, thereby modifying flower color (Faraco et al., 2014; Verweij et al., 2016). Notably, *GmPH1* expression was downregulated in the *gmph5* mutant, indicating that the loss of function of the GmPH5 P-ATPase exerted a negative effect on *GmPH1* expression (**Figure 5**). This phenomenon may be explicable if a feed-forward regulation of GmPH1 by GmPH5 existed.

In petunia, the MBWW (PH4-AN1-AN11-PH3) complex proteins jointly induce *PH1* and *PH5* expression (Faraco et al., 2014). Similarly, the mutation in *GmPH4* downregulated *GmPH1* and *GmPH5* expression, as well as that of *GmPH3*, *GmAN1*, and *GmAN11,* for the putative components of the MBWW complex in soybean (**Figure 5**). These results are similar to those of previous studies that evaluated the *ph4* mutant in petunia (Verweij et al., 2008; Faraco et al., 2014). In conclusion, *GmPH5* may encode a new soybean P_{3A}-type ATPase gene, whose expression might be regulated by the GmPH4 MYB transcription factor. However, there is a need for further study to prove the interactions between MBWW complex proteins and/or interaction between GmPH1 and GmPH5 in soybean.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JS and GTP performed most experiments and wrote the manuscript. J-DL provided the plant materials. GHK, J-DL, and HSS helped in the manuscript writing and discussion. JTS designed the study and supervised all of work. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.580085/full#supplementary-material

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Does the Rarity of a Flower's Scent Phenotype in a Deceptive Orchid Explain Its Pollination Success?

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Floral scent, a key mediator in plant–pollinator interactions, varies not only among plant species, but also within species. In deceptive plants, it is assumed that variation in floral scents and other traits involved in pollinator attraction is maintained by negative frequency-dependent selection, i.e., rare phenotypes are more attractive to pollinators and hence, have a higher fitness than common phenotypes. So far, it is unknown whether the rarity of multivariate and/or continuous floral scent traits influences the pollination success of flowers. Here, we tested in the deceptive orchid *Cypripedium calceolus*, whether flowers with rarer scent bouquets within a population have a higher chance to getting pollinated than flowers with more common scents. We collected the scent of more than 100 flowers in two populations by dynamic headspace and analyzed the samples by gas chromatography coupled to mass spectrometry (GC/MS). From the same flowers we also recorded whether they set a fruit or not. We introduced rarity measures of uni- and multivariate floral scent traits for single flowers, which allowed us to finally test for frequency-dependent pollination, a prerequisite for negative frequency-dependent selection. Our results do not show rarity has an effect on the likelihood to set fruits in neither of the two populations and in none of the scent characteristics analyzed. Hence, there is no evidence of negative frequency-dependent pollination mediated by the floral scent of *C. calceolus*. We discuss that our approach to determine rarity of a scent is applicable to any univariate or multivariate (semi)quantitative trait.

Keywords: evolution, floral volatiles, orchids, variability, frequency-dependent selection, pollinator-mediated selection, *Cypripedium calceolus*, quantitative traits

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INTRODUCTION

Most angiosperm species are pollinated by animals, mainly insects (Ollerton et al., 2011), and flowers of animal-pollinated plants typically advertise their presence by visual and olfactory cues (Chittka and Raine, 2006). These cues vary among and within species (Schiestl, 2005). The variation in floral traits is believed to be especially high in deceptive plants, which signal the presence of a reward without providing it (e.g., Dafni, 1984; Renner, 2006). This is because the variability in such signaling traits increases the difficulty for pollinators to recognize and learn to avoid cheating plants (Schiestl, 2005). Hence, it is expected that common phenotypes have a lower reproductive success than rare phenotypes. The evolutionary process by which the fitness of a phenotype depends on its frequency relative to other phenotypes in a population, is called frequency-dependent selection

(Futuyma and Kirkpatrick, 2017). In rewarding plants, positive frequency-dependent selection is expected, in deceptive plants, negative frequency-dependent selection.

Negative frequency-dependent selection has been studied in many groups of living beings (reviews in Ayala and Campbel, 1974; Delph and Kelly, 2014; Brisson, 2018). For plants, these include studies of self-incompatibility, heterostyly, host-pathogen systems, and flower color. So far, negative frequency-dependent selection exerted by pollinators has mostly been investigated in plants that are dimorphic in flower color. The bumblebee-pollinated orchid *Dactylorhiza sambucina* maintains a color dimorphism of yellow and purple flowers with the reproductive success being negatively correlated with relative morph frequencies in an experimental setting (Gigord et al., 2001). Subsequent studies have compared morph frequencies with reproductive success, but hardly found any correlation (Pellegrino et al., 2005; Jersáková et al., 2006). Also, preferred switching of pollinators to rare phenotypes in natural populations could not be confirmed (Groiß et al., 2017), as Smithson and Macnair (1997) observed in a study with artificial flowers. Studies in two other color dimorphic deceptive orchid species did not show evidence of frequency-dependent selection either (Aragón and Ackerman, 2004; Ackerman and Carromero, 2005). One study on pollinator-mediated frequency-dependent selection investigated the deceptive orchid *Psychilis monensis*, which shows a continuous variation in flower color (Aragón and Ackerman, 2004). However, the authors reduced the variation by painting the labellum to obtain three uniform flower color categories. They also did not find significant effects. Hence, the study of Gigord et al. (2001) stands out as the only one showing negative frequency-dependent selection on floral colors. They worked with artificial plots at sites that were similar to natural habitats of *D. sambucina*. Though likely present, the authors did not record co-flowering species that might have influenced the behavior of pollinators. Thus, negative frequency-dependent selection was detected despite other processes were potentially active, such as facilitation (Peter and Johnson, 2008) and competition (Jersáková et al., 2006), that might have overwritten negative frequency-dependent selection in other studies.

The only study we know on frequency-dependent selection acting on floral scent was conducted with the epiphytic orchid *Tolumnia variegata*, a deceptive plant with two scent morphs: fragrant and odorless flowers (Ackerman et al., 1997). The authors built artificial plots with different frequencies of fragrant and odorless flowers and did not find a pattern of negative frequency-dependent selection. Flower scent is a complex communication medium between plants and their pollinators, and several qualitative and (semi-)quantitative continuous characteristics are relevant for pollinator attraction: the strength of the overall scent (total quantity), the presence or absence of individual components (qualitative composition), and the ratios of the scent components to each other (relative amounts; semi-quantitative composition) (reviewed in Raguso, 2008). The phenotype of a flower/inflorescence is univariate in its total quantity, but multivariate in its qualitative and semi-quantitative composition, because the scent of a single flower/inflorescence consists of up to several dozen components (e.g., 48 in this study; see section "Results"). Intraspecific differences in these traits determine the individuality of the scent of a flower or inflorescence.

A prerequisite for negative frequency-dependent selection acting on floral scent is that individuals with rare scent phenotypes in a population are more attractive to pollinators and have a higher reproductive success than individuals with more common phenotypes. So far, however, there is no study, which quantified the rarity of specific scent phenotypes within a population, and tested, whether the rarity of scent phenotypes correlates with the likelihood of a plant individual/flower to set fruit(s).

Here, we introduce rarity measures of floral scent for single flowers, and test whether rarity of a flower's scent phenotype in the deceptive orchid *Cypripedium calceolus* L. explains its pollination success. Rarity was calculated for uni- and multivariate scent traits to be used for the tests on frequency-dependent pollination. *C. calceolus* is pollen limited, dependent on insects, mainly bees, for pollination, and emits a strong and variable scent (Nilsson, 1979; Bergström et al., 1992; Braunschmid et al., 2017). Various of its components are detectable by pollinating insects (Braunschmid et al., 2017). In this study, we collected floral scents in two natural populations by non-invasive dynamic headspace, and later in the season determined whether these same flowers set fruits. The scent was analyzed by gas chromatography and mass spectrometry. Based on pairwise resemblance measures of all different scent characteristics, chemical rarity values for all individual flowers were calculated. We then calculated logistic regressions to test for an effect of rarity in each floral scent characteristic on the likelihood to set fruits.

MATERIALS AND METHODS

Plant Species

Cypripedium calceolus L. is a pollen-limited perennial orchid distributed in boreal and temperate forests of Europe and Asia (Cribb, 1997; Kull, 1999). It is one of the largest European orchids with a stem height of 20–60 cm, and a large and conspicuous flower with a labellum shaped like a shoe. The pollen consists of individual pollen grains aggregated in a sticky smear (Nilsson, 1979). The successful pollination of *C. calceolus* depends on insects, which are temporarily trapped in the labellum. They can only leave the slippery cave through a rear, narrow exit. Thereby, they first pass the stigma, where the pollen they might have collected from another flower is stripped off. Then, they squeeze past one of the two anthers, where pollen smear is loaded onto their backs (Nilsson, 1979, and references therein). This limits self-pollinations, although the plant is self-fertile. The plant grows vegetatively with horizontal rhizomes and forms patches with up to several dozen shoots.

Study Sites

The investigations were carried out in the Austrian Limestone Alps near the city of Salzburg. Population Faistenau (FAI) is situated at 800 m above sea level on the eastern side of a mountain

range in a forest characterized by *Picea abies* (L.) Karst., *Fagus sylvatica* L., and *Pinus mugo* Turra. Somewhat more than 300 shoots grow along a gravel stream within a 200 m radius. They are patchily distributed within this area. Groups consist of single plants and patches of up to 50 shoots. The Maria Alm population (MAA) is on the southern slope of a limestone mountain at 1,200 m above sea level, in an open mountain pine forest. More than 400 shoots are quite evenly distributed within a radius of approximately 300 m, but are still grouped in patches of up to 20 shoots. The flowering season of *C. calceolus* in the study populations was in May (FAI) and June (MAA). The populations flowered for about 3 weeks.

Scent Collection and Analysis

Dynamic headspace samples of floral volatiles were collected *in situ* during daytime (10 a.m. to 3 p.m.) from individual flowers, using dynamic headspace methods (Dötterl et al., 2005). Flowers were enclosed in polyester oven bags and volatiles were trapped by pulling the air from the bag through small quartz adsorbent tubes (length: 15 mm, inner diameter: 2 mm; Hilgenberg GmbH, Malsfeld, Germany) for 8 min, using a rotary vane pump (G12/01 EB, Gardner Denver Thomas GmbH, Fürstenfeldbruck, Germany; flow rate: 200 ml/min). The tubes contained roughly 1.5 mg Tenax-TA (mesh 60–80) and 1.5 mg Carbotrap B (mesh 20–40; both Supelco) fixed by glass wool plugs (Heiduk et al., 2015). Samples collected from leaves and ambient air served as negative controls.

In population FAI the scents of 70 flowers available were sampled, of which 48 set a fruit and 22 did not set a fruit. In population MAA the scents of 210 flowers (all flowers available in sampling area) were sampled, of which only 18 set a fruit. Of the samples collected from flowers that did not set a fruit, we randomly selected a same number of flowers (18; Microsoft Excel, function RAND) to represent the frequency of phenotypes available among the non-pollinated flowers in the population. To verify that this sampling approach was representative, we performed two simulations (based on relative amounts of scent), one with samples of population FAI, and the second one with all samples included in the study (106; both populations, flowers with and without fruit set). In population FAI, we selected from the 48 flowers that set fruit 10,000 times 22 samples randomly, and every time tested whether rarity is a predictor of fruit set. In 99.5% of cases we found that rarity does not predict fruit set, which is in agreement with the analysis that included all data of population FAI (see section "Results"). A slightly different approach was taken for the simulations with all the 106 scent samples: out of these samples, we randomly took (again 10,000 times) 18 samples (as we did in the present study for population MAA) and calculated the mean rarity to see whether the mean rarity of the subsamples was the same as the mean rarity of the complete data set. Our simulations showed that 92% of our subsamples had the same mean rarity as the complete sample (single sample *t*-test $p < 0.05$).

The adsorbent tubes with the trapped volatiles were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) using an automatic thermal desorption (TD) system (TD-20, Shimadzu, Japan) coupled to a Shimadzu GC/MS-QP2010 Ultra equipped with a ZB-5 fused silica column (5% phenyl polysiloxane; 60 m, i.d. 0.25 mm, film thickness 0.25 μm, Phenomenex), the same as used by Braunschmid et al. (2017). The samples were run with a split ratio of 1:1 and a consistent helium carrier gas flow of 1.5 ml/min. The GC oven temperature started at 40°C, then increased by 6°C/min to 250°C and was held for 1 min. The MS interface worked at 260°C. Mass spectra were taken at 70 eV (EI mode) from m/z 34 to 350. GC/MS data were processed using the GCMSsolution package, Version 4.11 (Shimadzu Corporation 1999–2013).

The compounds were (tentatively) identified with the databases ADAMS, ESSENTIALOILS-23P, FFNSC 2, and W9N11 as well as a database generated from synthetic standards available in the Plant Ecology Laboratory of the University of Salzburg. Total scent emission was estimated by injecting known amounts of monoterpenoids, aromatics, and aliphatics (Etl et al., 2016). Based on the compounds detected in *C. calceolus* (Braunschmid et al., 2017, present work), an own library was created and used for the semi-automatic analyses of samples. Compounds were included in the study if they were only present in flower samples or if the peak areas in flower samples were at least five times larger than in green leaf and ambient air controls.

To check whether the scent phenotype of single flowers is distinct, we sampled 12 flowers more than once (10 min to 5 days in between the replicate samples) and found that scent was indeed flower specific (ANOSIM: $R = 0.80$; $p < 0.01$). Thus, a single sample collected from a specific flower well characterizes the phenotype of this flower.

Definition of Rarity

To measure how rare a scent phenotype is within a population, we use a formula that was originally developed by Violle et al. (2017) to define rarity in the context of biodiversity and ecosystem functioning. As a plant with a rare scent pattern is on average more dissimilar to the scent pattern of other plants within a population than a plant with a common scent pattern, pairwise dissimilarity/distance measures were used to learn about the rarity of and individual's scent blend/characteristic. We determined the rarity of a specific scent sample as the mean of dissimilarities/distances of this sample with all other scent samples of a population. The higher the values in a specific data set, the rarer is a sample. Thus, we define rarity R_i of sample i within n samples as

$$R_i = \frac{1}{n-1} \sum_{k=1;k\neq i}^{n} \bar{D}_{ik}$$ with $\bar{D}_{ik}$ as dissimilarity or distance between *i* and *k*.

We used Euclidean distances for the univariate (total quantity), and Sørensen and Bray-Curtis dissimilarities for the multivariate qualitative (presence/absence of compounds) and semi-quantitative (percentage amounts of scent components within a flower in relation to the total amount) data sets, respectively. Bray-Curtis dissimilarities were calculated using the original percentage contribution of a single compound to the total scent, as well as on square-root and fourth-root transformed data to manipulate the importance of the main and minor compounds on the resemblance. These different

scent characteristics were used for our analyses to meet possible olfactory search images of pollinators, which might be (more) based on total absolute amounts, qualitative, and/or semi-quantitative scent traits.

Statistical Analysis

Similarities and dissimilarities in scent patterns among the samples were visualized using non-metric multidimensional scaling (NMDS), based on the Bray–Curtis dissimilarities calculated on untransformed relative amounts of compounds. A SIMPER analysis was used to determine the compounds most responsible for the similarity among samples. We used permutational multivariate ANOVA (PERMANOVA, 9,999 permutations, crossed design with fixed factors *fruit set* and *population*), to test for differences in scent among flowers which did or did not produce a fruit, also considering population effects. Multivariate dispersion as a measure for variability of traits (Anderson et al., 2006) was calculated using PERMDISP (Anderson et al., 2008; results are in **Supplementary Table S1**). The resulting *z*-values can be interpreted on the scale of the originally chosen dissimilarities (Anderson et al., 2008). All multivariate statistics were performed with Primer 6.1.16 (Clarke and Gorley, 2006).

We used logistic regressions with the fixed factors *population* and *fruit set* to determine whether rarity values of the different floral scent characteristics (total scent quantity, semi-quantitative and qualitative scent patterns) are significant predictors of whether the flowers set fruits [R (The R Core Team, 2019), Packages stats version 3.6.1 and vegan 2.5-6]. In addition, we calculated a model that includes the interaction of rarity and population to test whether the impact of rarity on setting fruit is the same in both populations. A *U*-test (two-sample Wilcoxon test) was used, also in R, to test for differences in rarity between the populations.

RESULTS

Composition and Variation of the Floral Scent

The floral scent samples of *C. calceolus* in the two investigated populations varied in absolute quantities from 3.2 to 420 ng/min, and included in total 57 compounds (10–48 per sample, **Supplementary Table S2**). Terpenoids (25 substances), aliphatic (13), and aromatic (10) compounds were the most numerous, completed by one nitrogen-containing and eight unknown substances. Seven substances were found in more than 99% of the samples: 4-oxoisophorone, heptyl acetate, (*Z*)-3-nonenyl acetate, 1-hexanol, geranylacetone, benzaldehyde, and hexyl acetate (**Supplementary Table S2**).

The three main compounds, linalool, octyl- and decyl acetate, together contributed 76% to the total scent sampled (linalool: 0–69%, on average 24%; octyl acetate: 0–74%, on average 36%; and decyl acetate: 0–33%, on average 11%) and were, according to a SIMPER analysis, most responsible for the similarity (in sum 79%) in relative amounts of compounds among samples. Half of the other substances were found in traces and contributed in sum for just about 1% of the total scent quantity.

The graphic representation of the scent samples indicated at least some variation in relative scent patterns among populations, independent of whether the flowers set a fruit or did not set a fruit (**Figure 1A**). This was confirmed by permutational multivariate analysis based on relative amounts of scent, which found significant population effects [PERMANOVA: Pseudo-$F_{(1,102)}$ = 16.6; $P < 0.01$], and non-significant effects of fruit set [PERMANOVA: Pseudo-$F_{(1,102)}$ = 1.3; P = 0.26] and of the interaction between population and fruit set [PERMANOVA: Pseudo-$F_{(1,102)}$ = 1.2; P = 0.29].

Rarity and Analysis of Negative Frequency-Dependent Pollination

About two thirds of the rarity values, based on relative amounts, in population FAI were in the range between 30 and 40, and the other third spread between 40 and 88. In MAA, 56% of the rarity values were between 30 and 40, the others between 40 and 72 (**Figure 1B** and **Table 1**). The graphical representation of rarity in MDS showed that those samples with a larger distance to the majority of samples consistently had larger rarity values than samples with more common scent phenotypes (**Figure 1B**). The rarity values became consistently smaller the more the data were transformed, i.e., they were largest for untransformed data and smallest for qualitative data (presence/absence-transformation; **Table 1**). Rarity values differed among populations for relative amounts and their square and fourth root transformations, but not for qualitative data and total quantity of scent (**Table 1**).

Logistic regressions indicated that rarity is not a predictor of whether a flower sets a fruit, independent of the scent characteristic analyzed (total scent quantity, semi-quantitative and qualitative scent patterns, **Figure 2** and **Table 1**). As indicated by a non-significant interaction effect of *rarity* and *population*, the influence of rarity on fruit set is the same in both populations (**Table 1**).

DISCUSSION

We used a recently developed measure to determine uni- and multivariate rarity of floral scent characteristics and found that the rarity of floral scent phenotypes is not a predictor of whether flowers of *Cypripedium calceolus* set fruits. This finding is in line with results of studies on pollinator-mediated negative frequency-dependent selection on floral colors (Pellegrino et al., 2005; Jersáková et al., 2006; Groiß et al., 2017; but see on the contrary Gigord et al., 2001) and on floral scents (frequency of fragrant vs. odorless flowers; Ackerman et al., 1997).

A prerequisite for pollinator-mediated negative frequency-dependent selection, and thus, also for negative frequency-dependent pollination is that pollinators can discriminate among flowers within a population and are capable of learning (Ackerman et al., 1997; Schiestl, 2005). Both conditions seem to be fulfilled in our study system. This is because pollinators

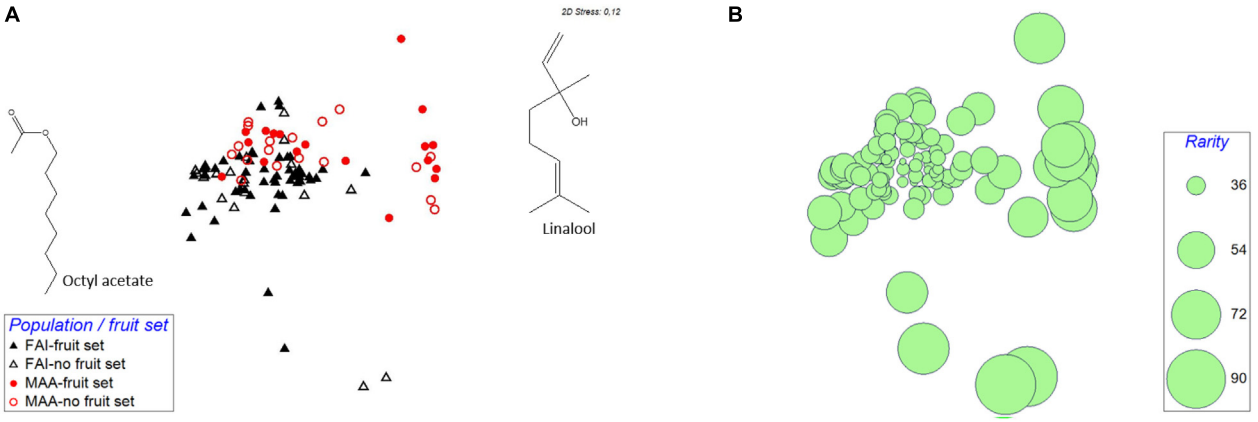

FIGURE 1 | Non-metric multidimensional scaling (NMDS) to visualize semi-quantitative dissimilarities between the individual scent samples taken in the two investigated populations (FAI, Faistenau; MAA, Maria Alm). This ordination is based on pairwise Bray-Curtis similarities. In **(A)**, symbols of scent samples whose flowers have been pollinated are filled, while those of unpollinated flowers are unfilled. **(B)** is the same ordination as **(A)**, here, however, the calculated rarity of the single samples is indicated, with the sizes of the circles being proportional to the rarity of the samples.

TABLE 1 | Mean (± *SD*) rarities in various scent properties (non-transformed, square and fourth root transformed relative scent bouquet; presence-absence of compounds; total absolute amounts of scent trapped) of individuals that set fruits and individuals that did not set fruits of two different populations (FAI, Faistenau; MAA, Maria Alm). Rarity values differ between the populations for relative amounts and their square and fourth root transformations, but not for qualitative data and total quantity of scent. Results of logistic regressions (logit) indicate that rarity does not influence fruit set in any of the scent trait analyzed. Since there is no interaction effect with population, influence of rarity on fruit set is not different among the two populations.

Scent	Transformation	Rarity				Population effect on rarity (*U*-Test)	Effect of rarity on fruit set (logit)	Effect of rarity × population on fruit set (logit)
		Population FAI (*n* = 70)		Population MAA (*n* = 36)				
		Flowers with fruit set	Flowers w/o fruit set	Flowers with fruit set	Flowers w/o fruit set			
		Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	Mean ± *SD*	*W*/*p*-value	*z*/*p*-value	*z*/*p*-value
Bouquet	Not transformed	38.3 ± 7.7	42.5 ± 15.1	48.4 ± 14.0	43.0 ± 11.4	875/0.01*	−0.3/0.79	1.9/0.06
Bouquet	Square root	34.0 ± 6.9	36.6 ± 12.0	39.4 ± 9.1	36.5 ± 7.7	956/0.04*	−0.3/0.79	1.5/0.13
Bouquet	Fourth root	20.7 ± 6.8	32.6 10.3	33.4 ± 6.0	31.9 ± 5.2	933/0.03*	−0.4/0.66	1.2/0.24
Bouquet	Presence-absence	26.3 ± 7.5	27.9 ± 10.2	26.7 ± 4.0	26.1 ± 3.5	987/0.07	−0.5/0.58	0.7/0.49
Total quantity	Not transformed	153.6 ± 9.7	152.3 ± 10.5	171.7 ± 40.0	164.4 ± 30.0	1017/0.11	0.8/0.44	−0.27/0.78

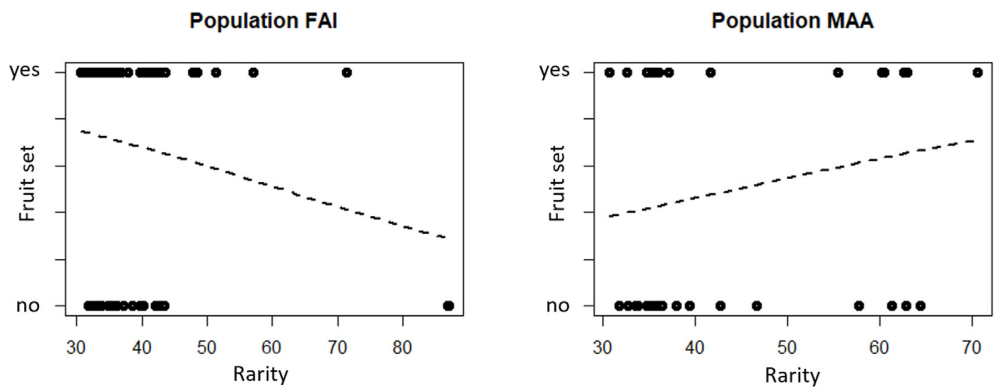

FIGURE 2 | Rarity of scent in flowers with and without fruit set of the two study populations FAI (Faistenau) and MAA (Maria Alm). Rarity values are the mean Bray-Curtis dissimilarities of a sample to all other samples in the population, based on relative amounts of the scent bouquets. Logistic regression results indicate that the rarity of the scent of a flower is not a significant predictors of whether a flower sets a fruit (see also **Table 1**).

of *C. calceolus* have the olfactory capability to detect most of the compounds, among them compounds strongly varying in relative amounts among the samples (Braunschmid et al., 2017) and, although learning abilities in bees have been shown mainly for *Apis melifera* and *Bombus* species (review in Jones and Agrawal, 2017), there is also indication for the learning capability of solitary bees (Amaya-Márquez et al., 2008; Dukas and Real, 1991; Dötterl et al., 2011). Learning implies that pollinators forage (and hence, select flowers) differently depending on their experience. Deceptive plants often benefit from the inexperience of their pollinators, whether they are generally flower-naive or naive regarding the deceptive species. *C. calceolus* is pollinated by more than 30 species of insects (Braunschmid et al., 2017), which emerge and are present at different times. It can therefore be assumed that there is only a limited overlap in phenology of some of these pollinators with the flowering period of *C. calceolus*, making it not possible for them to gain enough experience regarding the different scent phenotypes, and to preferably visit rare ones.

There is evidence that pollinators of deceptive plants only respond to very pronounced unbalancing of the relevant traits. In their study, Jersáková et al. (2006) suspected that negative frequency-dependent selection occurs in natural populations only when color morph frequencies are strongly different. They had no signals for negative frequency-dependent selection in their investigated 22 populations of *D. sambucina*, except for the most unbalanced one with just 7% for one of the two colors. The Gigord et al. (2001) study gives further indications for this hypothesis: fruit set and pollinia deposition (in contrast to pollinia removal) were actually not different between the two color morphs when the frequencies of one of the morphs were 30, 50, and 70% (Figure 3 in Gigord et al., 2001), but were different at frequencies of 10 and 90%. These results suggest that negative frequency-dependent pollination/selection is more likely to occur when some of the phenotypes are particularly rare. In traits like scent, where there often are no distinct chemotypes, but differences among individuals are on a continuous scale, it can be assumed that negative frequency-dependent pollination only acts in populations with both, strongly pronounced differences in scents among individuals and strongly different frequencies of the phenotypes. It appears that at least the latter condition is met in both of our studied populations, with more than half of the samples having similar scents and small rarity values, and about one third of the samples having different scents and higher rarity values (**Figure 1**). However, we do not know whether the differences in scent are large enough to be recognized by pollinators and whether they are also behaviorally relevant for them.

The frequency of traits attractive to pollinators is not the only aspect influencing the behavior of pollinators. Factors such as the co-flowering community (Jersáková et al., 2006), the spatial variation in pollinator interactions (Chapurlat et al., 2015), and floral abundance (Sabat and Ackerman, 1996) might have significant influence. In consequence, frequency-dependent pollination/selection might no longer be detectable in the behavior of pollinators, and therefore, have no effect on fruit set.

The term rarity has already been used in some previous pollination studies on frequency-dependent selection, but rather informatively, always for categorical traits, and never for comparison of individuals within a population (e.g., Gigord et al., 2001; Iserbyt et al., 2013; Janif et al., 2014). In order to carry out regression analyses with the factor rarity, groups of individuals with trait ratios of $\frac{1}{5}$, $\frac{2}{5}$, $\frac{3}{5}$, $\frac{4}{5}$ etc. were formed and the ratio values used as rarity values. In artificial plots of $\frac{1}{5}$ red flowers and $\frac{4}{5}$ blue flowers, the red flowers were denoted as rarer. Each treatment (e.g., artificial plot of red and blue-flowered plants) served as a replicate with a mean value of fitness measure for a specific phenotype. Mean values of replicate samples were finally used for statistical analysis to compare the fitness of a specific morph in artificial plots that differed in the frequency of this morph. Our approach exceeds such previous studies from a methodical point of view in three aspects: it allows to study (a) multivariate and (b) continuous traits, and (c) assigns the rarity measure to individual phenotypes within a population. The multivariate aspect of data sets that consist of various continuous (e.g., relative amount of scent compounds) or categorical variables (presence/absence of a specific compound) is dealt with the proven means of resemblance measures (e.g., Bray-Curtis dissimilarity, Sørensen dissimilarity). By then calculating the average dissimilarity/distance of a specific subject to all other subjects in the study population, we obtain a measure of rarity for each subject in a population. We used Bray-Curtis, Sørensen, and Euclidean resemblances, but any other dissimilarity/distance measure can be used.

CONCLUSION

We have shown how to calculate rarity of multivariate and continuous floral traits, and how to apply the term *rarity* to individual phenotypes in a population. This approach allowed us to demonstrate that the rarity of a flower's scent does not explain its pollination success in the deceptive orchid *C. calceolus*. Our approach is not restricted to determine rarity of scent phenotypes, but is also applicable to other univariate and multivariate traits.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

HB and SD designed the research and obtained funds for the research. HB collected and analyzed the data, and wrote the first draft of the manuscript. SD revised the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.584081/full#supplementary-material

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Bumblebee Behavior on Flowers, but Not Initial Attraction, Is Altered by Short-Term Drought Stress

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Climate change is leading to increasing drought and higher temperatures, both of which reduce soil water levels and consequently water availability for plants. This reduction often induces physiological stress in plants, which in turn can affect floral development and production inducing phenotypic alterations in flowers. Because flower visitors notice and respond to small differences in floral phenotypes, changes in trait expression can alter trait-mediated flower visitor behavior. Temperature is also known to affect floral scent emission and foraging behavior and, therefore, might modulate trait-mediated flower visitor behavior. However, the link between changes in flower visitor behavior and floral traits in the context of increasing drought and temperature is still not fully understood. In a wind-tunnel experiment, we tested the behavior of 66 *Bombus terrestris* individuals in response to watered and drought-stressed *Sinapis arvensis* plants and determined whether these responses were modulated by air temperature. Further, we explored whether floral traits and drought treatment were correlated with bumblebee behavior. The initial attractiveness of drought and watered plants did not differ, as the time to first visit was similar. However, bumblebees visited watered plants more often, their visitation rate to flowers was higher on watered plants, and bumblebees stayed for longer, indicating that watered plants were more attractive for foraging. Bumblebee behavior differed between floral trait expressions, mostly independently of treatment, with larger inflorescences and flowers leading to a decrease in the time until the first flower visit and an increase in the number of visits and the flower visitation rate. Temperature modulated bumblebee activity, which was highest at 25°C; the interaction of drought/water treatment and temperature led to higher visitation rate on watered plants at 20°C, possibly as a result of higher nectar production. Thus, bumblebee behavior is influenced by the watered status of plants, and bumblebees can recognize differences in intraspecific phenotypes involving morphological traits and scent emission, despite overall morphological traits and scent emission not being clearly separated between treatments. Our results indicate that plants are able to buffer floral trait expressions against short-term drought events, potentially to maintain pollinator attraction.

Keywords: soil water availability, temperature, climate change, floral traits, pollinator behavior, Brassicaceae

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INTRODUCTION

With changing climate, drought periods and temperatures will increase (Intergovernmental Panel on Climate Change [IPCC], 2014), leading to reduced soil water levels, and might lead to physiological stress in plants (Beier et al., 2012). Abiotic stress is well-known to induce phenotypic changes in vegetative traits (Cornwell and Ackerly, 2009; Jung et al., 2014; La Rosa et al., 2019) but can also affect floral development and production, resulting in phenotypic alterations in flowers (Galen, 2000; Strauss and Witthall, 2006; Descamps et al., 2018). Such changes in floral trait expression can alter trait-mediated flower-visitor interactions and behavior, as flower visitors are able to notice and respond to small intraspecific differences in floral phenotypes within one species (Thomson et al., 1982; Conner and Rush, 1996; Mothershead and Marquis, 2000; Kuppler et al., 2016). However, the linkage between intraspecific responses to abiotic stress and different behaviors, e.g., number of visited flowers, remain unclear.

Drought is a complex term that can be defined in various ways, e.g., meteorological drought can be defined as "the number of days with precipitation less than some specified threshold" or to reduced groundwater or reservoir levels as characteristics of agricultural drought (National Drought Mitigation Center [NDMC], 2020). Generally speaking, drought is considered as a time span of unusual dry weather long enough to cause a severe hydrological imbalance (Intergovernmental Panel on Climate Change [IPCC], 2014) that depends on the amount, intensity, and timing of precipitation (National Drought Mitigation Center [NDMC], 2020) and the relationship of these parameters to historical data (Slette et al., 2019). Here, we use and define drought in the broad sense of "prolonged absence or marked deficiency of precipitation" (Slette et al., 2019) and as a synonym for reduced water availability. Short- and long-term drought periods are employed here to describe time spans that can last from several days or weeks for short-term to many months or even years for long-term drought.

Drought is known to cause intraspecific changes in trait expression (Jung et al., 2014) that can increase the stability of communities against environmental changes (Oney et al., 2013; Kuppler et al., 2016; Moran et al., 2016). Drought responses are known to modulate vegetative plant traits, e.g., reducing specific leaf area (Quiroga et al., 2013), to increase water-use efficiency and reduce water loss and prevent dehydration (Ludlow, 1989; Kooyers, 2015). Such responses are not limited to vegetative traits but can also be present in floral traits. For example, a decrease in flower size, in the number of flowers and/or in floral height under drought conditions can reduce transpirational water loss through flowers (Feild et al., 2009; Teixido and Valladares, 2014; Lambrecht et al., 2017) and can decrease water consumption for flower maintenance (Galen et al., 1999). However, these changes are often species-specific (Burkle and Runyon, 2016; Descamps et al., 2018, 2020; Glenny et al., 2018).

Phenotypic changes in floral traits often exert an influence on floral visitors, e.g., a reduced corolla length or reduced floral size leads to a reduced visitation rate (Burkle and Runyon, 2016; Gallagher and Campbell, 2017). So far, most studies have focused on observed visitation rates. However, the connection between visitation rate and pollination is not always straight forward but is dependent on pollinator behavior on plants and flowers (Engel and Irwin, 2003; Ne'eman et al., 2010). For example, differences in visit durations or number of flowers per plant visited can affect the amount of pollen deposited (Ohara and Higashi, 1994; King et al., 2013) and the amount of pollen removal (Sahli and Conner, 2007). Therefore, an understanding of the possible changes brought about in pollinator behavior, e.g., flower visit duration, by drought stress in plants should provide greater insights into the effect of drought on pollination.

In addition to the modulation of morphological floral traits under drought conditions (Carroll et al., 2001; Caruso, 2006; Halpern et al., 2010; Burkle and Runyon, 2016; Glenny et al., 2018; Descamps et al., 2020; Walter, 2020), plasticity can also occur in scent emission. Drought stress increases floral scent emission and causes a shift in the composition of floral VOCs as some compound pathways might be up- or down-regulated. Studies showed an increase under drought for some compounds such as: (Z)-3-hexenol, 6-methyl-5-hepten-2-one, benzaldehyde, α- and β-pinene, and (E)-β-ocimene, (E,E)-α-farnesene (Burkle and Runyon, 2016; Glenny et al., 2018; Campbell et al., 2019; Rering et al., 2020).

Further, drought is usually associated with temperature increases, which can also affect floral scent. Under increasing temperature, floral scent emission can increase within minutes (Farré-Armengol et al., 2014), and a positive effect of warming on scent emission is generally assumed (Jamieson et al., 2017). However, temperature can also affect the relative contribution of the various compounds within the scent bouquet, e.g., an increase in aromatic hydrocarbons (Jamieson et al., 2017) and terpenes (Farré-Armengol et al., 2014). In combination with the direct effect of temperature on pollinator activity (Kühsel and Blüthgen, 2015), these changes are likely to alter the visitation patterns and behavior of pollinators that rely on such information for the detection of suitable flowers (Junker and Parachnowitsch, 2015). Therefore, temperature and drought can independently act upon floral traits, flower-visitor interactions, and pollinator behavior but may also have interacting effects (Jamieson et al., 2017). An exploration of their combined effects should elucidate these linkages.

In this study, we have examined (1) the effects of drought on floral morphology, phenology, and scent emission; (2) the way in which such phenotypic changes influence bumblebee behavior; and (3) possible interactions between temperature and drought on floral traits and trait-mediated bumblebee behavior. Bumblebee – or other flower visitor – behavior in response to plants and their phenotypic expression under various conditions can conveniently be observed in a wind tunnel. First, in a climate-controlled room ambient conditions such as airflow, humidity and temperature can be readily controlled in this set-up, and second, due to a constant airflow, flower visitor behavior in response to scent plums can be explored (Dötterl et al., 2006; Klahre et al., 2011). We have tested in a wind tunnel whether a combination of short-term drought stress and changes in temperature result in altered pollinator behavior. Additionally, we have measured floral traits to determine

whether altered bumblebee behavior is linked with possible phenotypic changes in floral traits induced by drought stress and altered temperatures.

MATERIALS AND METHODS

Plants and Drought Treatment

Sinapis arvensis L. (wild mustard, Brassicaceae) is an annual, self-incompatible, cruciferous plant native to southern and middle Europe. It attracts a broad range of flower visitors, mostly bees, and syrphid flies (Kunin, 1993). *S. arvensis* and other Brassicaceae generally grow in meadows and agricultural landscapes and provide important resources for multiple pollinator species. Therefore, they can be seen as a representative of a common widespread plant with a generalist pollination system. We obtained seeds of *S. arvensis* from wild populations in southern Germany (purchased from Rieger-Hofmann GmbH, Blaufelden, Germany). The seeds were treated with aqueous gibberellic acid solution (1000 ppm; Sigma, St. Louis, MO, United States) and left on wet filter paper in darkness at room temperature until germination. Subsequently the seeds were transferred into 0.6-liter-pots containing a soil mixture of 3:1 peat:sand. Once the cotyledons had emerged (∼3 days), we transplanted the seedlings individually into 0.6-liter pots containing a soil mixture of 3:2:1 TKS 2:compost:sand (TKS2, Floragard Vertriebs-GmbH, 26135 Oldenburg, Germany). We reared 20 plants per batch per week (in total, six batches). Plants were kept in a phytochamber (Phytotron 1, Vötsch Industrietechnik GmbH, Balingen, Germany) in the Botanical Garden of Ulm University at 20°C and 66% relative humidity with a 12:12 day:night cycle at a light intensity of 500 μmol m^{-2} s^{-1}. Plants were randomly grouped into pairs consisting of a control and a drought-stressed plant (in total, 20 plant pairs were used). Control plants were watered daily once with 100 ml water. Drought-stressed plants were watered once every other day with the same amount of water. This pulsed drought treatment, which started 2–3 days before flowering and lasted for 18 days (Burkle and Runyon, 2016), resembles a short-term drought period similar to that prevailing in the field and has often been used in drought stress studies. We also tested longer periods of drought, but after 2 days without water, mortality, or signs of severe drought stress were observed. Soil humidity was controlled using a self-made soil humidity sensor with an Arduino system (Iduino ME110, Arduino software version 1.8.8, board: Genuino Uno). The applied drought stress significantly reduced the soil humidity of the drought-stressed plants [mean (SD)%: **watered:** 34.8 (5.2)%, **drought-stressed**: 24.7 (9.2)%; and Wilcoxon Rank-Sum test: $W = 356180$, $p < 0.001$].

Bumblebees

As a common visitor of *S. arvensis*, we used *Bombus terrestris* L. (Apidae) from self-reared colonies at the Institute of Evolutionary Ecology and Conservation Genomics at Ulm University (Rottler et al., 2013; Rottler-Hoermann et al., 2016) for our behavioral assays. The founding queens were descendants of commercial colonies (Koppert Biological Systems, Netherlands). The colonies were kept in wooden boxes (39 cm × 16.5 cm × 16 cm) in constant darkness at a temperature of 27°C and a relative humidity of 60%. The bumblebees were provided *ad libitum* with a 55% sugar solution of API-Invert® (Südzucker AG, Mannheim, Germany) and fresh pollen (Koppert Biological Systems, Netherlands).

Flower-Visitor Interactions

For the behavioral tests, we used two-month-old colonies with about 30 workers (in total, six colonies were used). Bumblebee behavior on watered and drought-stressed plants was investigated by conducting a two-choice bioassay in a wind tunnel (200 cm × 80 cm × 80 cm). 2 days before the experiments, the colony was connected via a tube (length 30 cm, diameter 1.5 cm) to the wind tunnel to allow the bumblebees to acclimatize to and to forage within the wind tunnel. The bumblebees were provided *ad libitum* with the above sugar solution and fresh pollen within the wind tunnel. After 2 days, the colony was removed from the wind tunnel and connected to a flight cage via a tube (60 cm × 60 cm × 60 cm, BugDorm, MegaView Science Co., Ltd., Taiwan) with the same food provision. On the morning of the experiments, individual bumblebees were caught, marked individually, and starved until they were used.

A watered and a drought-stressed plant were placed next to each other at a distance of 30 cm in the middle of the wind tunnel. A fan (D440/E15 with an FDR 32 speed controller; Fischbach, Neunkirchen, Germany) blew charcoal-filtered air through the tunnel. A single bumblebee was placed in the tunnel at the end opposite to the fan. The bumblebee was allowed to acclimatize for at least 5 min. After this time, any bumblebees that did not start to fly or were otherwise active with regard to the plants were removed and excluded from the analysis (excluded individuals, $N = 49$). Bumblebees ($N = 66$) were observed for a maximum of 10 min (Hoover et al., 2012) with the following behavior types being recorded: (1) time to first visit [sec], (2) number of approaches (≤5 cm distance to flower), (3) number of landings, (4) number of all visits (sum of approaches and landings), (5) number of visited flowers per landing, (6) duration of landings [sec], (7) visitation rate, which was calculated as the number of visited flowers during a landing divided by the total flower number of the plant individual per min, and (8) relative duration, which was calculated by dividing duration with the total active time during the observation. Between the replicates, the wind tunnel was cleaned with unscented soap, and the position of plants and plant pairs were switched regularly.

Temperature

To test whether air temperature affected bumblebee behavior, we performed the wind tunnel experiment at three different air temperatures. The day before the trial, the air temperature in the wind tunnel room was set to the relevant temperature: 20, 25, or 30°C. If possible, we observed the same bumblebees with their corresponding plant pairs at all three temperatures (bumblebees: **20°C** $N = 35$; **25°C** $N = 33$; **30°C** $N = 37$; and plant pairs: 12 pairs per temperature).

Trait Measurements

To test differences in plant phenotype between treatments, we measured eight morphological floral traits and nectar volume, which are all known to mediate flower-visitor interactions (Thomson et al., 1982; Stang et al., 2006; Junker et al., 2013; Kuppler et al., 2016). Flower height (height of the highest flower) [cm], display size of flowers (greatest expansion of flower) [mm], display size of inflorescences (greatest expansion of inflorescences, either vertical or horizontal) [mm], number of flowers and inflorescences, calyx length [mm], style length [mm], and longest stamen (filament plus anther) [mm] were measured directly on the plant by using a digital caliper (Traceable® Digital Caliper 6-inches, VWR International LLC, Leuven, Belgium), except for flower height (measured with a folding yardstick). Nectar volume per flower [μl] was measured via a glass capillary (0.5 μl). All measurements were taken on three freshly open flowers or inflorescences from a low, middle, and high position to avoid position and age effects; means were used for statistical analyses. Trait measurements took place in the morning on the same day that we observed bumblebee behavior.

Scent Collection and Analysis

We used dynamic headspace to examine the effect of drought and temperature on the quality and quantity of scent emission. All inflorescences of each plant were enclosed within an oven bag (Toppits® , Minden, Germany) from which the air was pumped for 10 min to remove ambient air. After 20 min of scent enrichment, the emitted volatiles were trapped for 3 min on 1.5 mg Tenax (mesh 60–80; Supelco, Bellefonte, PA, United States) and 1.5 mg Carbotrap B (mesh 20–40; Supelco) in a quartz vial (length 20 mm, inner diameter 2 mm) by using a membrane pump (G12/01 EB; ASF Rietschle-Thomas, Puchheim, Germany) with a flow rate of 200 ml min^{-1}. Scent enrichment and trapping were repeated; thus, the total sample time was 46 min for each plant. All samples were collected between 08:00 and 12:00 h. The temperature in the room with the wind tunnel was controlled by a thermostat. Plant pairs spent at least 15 h at the respective temperature before scent collection. Scent samples were analyzed using an automatic thermal desorption system (TDU, Gerstel, Mühlheim a. d. Ruhr, Germany) and a cold-injection apparatus (CIS 4C, Gerstel) coupled with a GC-MS (7890B GC–5977A MSD, Agilent Technologies, Germany). The GC-MS was equipped with a DB-5MS silica column (5% diphenyl, 95% dimethyl polysiloxane; 30 m long, inner diameter 0.25 mm, film thickness 0.25 μm), and the column flow (carrier gas: helium) was set to 1.5 ml min^{-1}. The GC oven temperature was initially at 40°C, was then increased by 6°C per minute to 250°C, and subsequently held constant for 1 min. The MS interface was set at 250°C. Mass spectra were taken at 70 eV (in EI mode) from m/z 30 to 350. The GC/MS data were analyzed using the GCMSolution package (Version 2.72, Shimadzu Corporation, Kyoto, Japan). Compounds were identified using the mass spectral libraries Wiley 9, Nist 2011, FFNSC 2, and Adams, 2007. The compounds found in flowers were compared with those found in the blanks (empty oven bags) to determine which compounds were emitted in particular by flowers. The amount of each compound emitted was standardized by the number of flowers. Compounds were considered as being most common when they appeared in more than four plants per treatment.

Data Analysis

We tested the effects of plant drought stress on bumblebee behavior. For all models, we used treatment and temperature as fixed factors and each bee nested in nest ID as random factors to account for differences between individuals and colonies. The effect of drought stress and temperature on time until the first visit, visitation rate, and total number of visits was analyzed using the *lmer*-function with restricted maximum likelihood (REML). The visitation rate was log10(x + 0.001)-transformed to achieve normal distribution. If no model convergence was reached after the default 10,000 iterations, we restarted the model from the previous fit with a maximum of 100,000 iterations. To investigate the effect of treatment and temperature on the relative duration, we used the *glmmTMB*-function from the *glmmTMB*-package (Brooks et al., 2017) with beta-family distribution. Therefore, the relative duration was transformed as suggested by Brooks et al., 2017: $\frac{\text{dependent variable} \times (n-1)+0.5}{n}$ with n being the sample size of the dependent variable.

Further, in order to test the association between bumblebee behavior and floral phenotype in dependence on treatment, we correlated bumblebee behavior with floral traits and drought stress with trait and treatment as fixed factors by using the same models as above. Time to first visit, the number of visits and visitation rate as the dependent variables were log-transformed or log10(x + 1)-transformed. We also tested the effect of floral scent emission on time until the first visit, number of visits, visitation rate, or relative duration as dependent variables and each bee nested in nest ID as random factors. All model fits were validated using the *DHARMa*-package (Hartig, 2020) and were adequate. Number of visits depending on flower height and number of inflorescences, and the visitation rate depending on flower height, the number of inflorescences and the number of flowers as a model fit showed an imperfect fit of error distribution. All data were analyzed and plotted using R (version 3.5.0, R Core Team, 2018), except for MDS of floral scent bouquet, which was analyzed and plotted with PRIMER-E (version 6.1.15, with PERMANOVA+, version 1.0.5; PRIMER-E Ltd., 2012).

In order to test whether differences in floral traits between drought-stressed and watered plants occurred, we performed linear mixed-effect models (LMMs) by using the *lmer*-function from the *lme4*-package (Bates et al., 2015) with treatment as the fixed factor, and the mean of flower height, number or size of inflorescences, number or size of flowers, calyx, style or stamen length, nectar volume, or total floral scent emission as dependent variables and the plant as the random factor by using REML. To achieve normal distribution, scent emission variable was log10 + 1-transformed. We used the Kruskal–Wallis test to analyze the effect of drought stress on the amount of emitted scent compounds per flower. The effect of drought treatment and temperature on floral scent bouquet was assessed by permutational multivariate ANOVA (PERMANOVA, 9999 permutations, Bray–Curtis similarity distance matrix). One

sample from a plant in the drought treatment was empty and was therefore excluded.

RESULTS

Bumblebee Behavior and Drought Stress

Initial attractiveness (= time to the first visit) did not differ between the two treatments (LMM: $\chi^2 = 0.94$, $p = 0.332$). However, the behavior of the bumblebees after the initial visit differed between treatments. Bumblebees visited watered plants more often than drought-stressed plants [mean (SD): **watered:** 10.9 (4.8); **drought-stressed**: 9.1 (4.5); and LMM: $\chi^2 = 7.72$, $p < 0.01$] and visited twice as much flowers per min (= visitation rate) of watered plants compared with drought-stressed plants [mean (SD) flowers/min: **watered:** 0.09 (0.12); **drought-stressed**: 0.04 (0.08); and LMM: $\chi^2 = 9.12$, $p < 0.01$]. Moreover, bumblebees spent on average more time on watered plants [mean (SD), **watered:** 0.7 (0.3); **drought-stressed**: 0.6 (0.4); and GLMM: $\chi^2 = 4.25$, $p < 0.05$].

Effects of Drought Stress and Temperature on Behavior

Temperature had no effect on time to first visit (LMM: $\chi^2 = 1$, $p = 0.607$, **Figure 1A**). Number of all visits per plant were significantly affected by temperature (LMM: $\chi^2 = 29.37$, $p < 0.0001$, **Figure 1B**). At 25°C, the number of visits was one and a half times higher than at the other temperatures [mean (SD): **20°C**: 8.7 (5.6); **25°C**: 13 (2.7); **30°C**: 8.5 (4.3); *Post hoc* Tukey-test: **20–25°C** $p < 0.001$, **20–30°C** $p = 0.73$, **25–30°C** $p < 0.0001$]. The visitation rate [mean (SD) flowers/min: **20°C**: 0.09 (0.15), **25°C**: 0.06 (0.1), **30°C**: 0.04 (0.06), LMM: $\chi^2 = 0.6$, $p = 0.741$] and the duration of landings [mean (SD): **20°C**: 0.68 (0.39), **25°C**: 0.58 (0.36), **30°C**: 0.7 (0.35), LMM: $\chi^2 = 3.26$, $p = 0.596$] showed a tendency to be higher at 20°C. The interaction of treatment and temperature had no significant effects on the behavior of the bumblebees. However, visitation rate and relative duration tend to be highest at 20°C on watered plants (**Figures 1C,D**).

Additionally, the percentage of bumblebees that participated in the experiments were higher at 25°C and 30°C (82.5% and 66.1%, respectively). Only 60.3% of the bumblebees participated at 20°C. However, this difference was not significant (GLM with binomial error: $\chi^2 = 0.46$, $p = 0.499$).

Effects of Floral Traits and Drought Stress on Behavior

In addition, we tested whether floral traits and drought treatment correlated with time to first visit, number of visits per plant, visitation rate of flowers, and relative duration of landings (**Figures 2A–D**).

The time to first visit was not influenced by treatment (**Figure 2A** and **Supplementary Table 3**), but was significantly affected by floral traits. Bumblebees needed less time until the first visit when plants had larger inflorescences (LMM: $\chi^2 = 4.44$, $p = 0.035$) and flowers (LMM: $\chi^2 = 3.39$, $p = 0.066$) and more nectar per flower (LMM: $\chi^2 = 2.74$, $p = 0.098$). Further, the decreases in time to first visit with increasing floral size was stronger for drought-stressed plants (LMM: $\chi^2 = 4.61$, $p = 0.032$). Increasing scent emission significantly decreased the time to first visit (LMM: $\chi^2 = 3.20$, $p = 0.073$; **Figure 3A**). For the other floral traits, neither trait, *nor* treatment, nor the interaction of the two factors had an effect on time to the first visit.

Number of visits per plant was also affected by treatment as bumblebees visited watered plants more often than drought-stressed plants for all correlated floral traits (**Figure 2B** and **Supplementary Table 3**). Measured floral traits also had significant effects on visits. The visits significantly increased for plants with larger inflorescences (LMM: $\chi^2 = 23.23$, $p < 0.001$) and more inflorescences (LMM: $\chi^2 = 23.18$, $p < 0.001$) and flowers (LMM: $\chi^2 = 26.76$, $p < 0.001$) and decreased with higher nectar volume per flower (LMM: $\chi^2 = 7.54$, $p = 0.006$). Flower size and height had no influence on the number of visits. However, the interaction of floral traits and treatment significantly influenced the number of visits as, with increasing floral size, the visits decreased for watered and increased for drought-stressed plants (LMM: $\chi^2 = 7.63$, $p = 0.006$). Higher scent emission (LMM: $\chi^2 = 22.61$, $p < 0.001$; **Figure 3B**) and the interaction of scent and treatment significantly affected the number of visits, as the visits increased more strongly for watered plants than for drought-stressed plants with increasing scent emission (LMM: $\chi^2 = 6.01$, $p = 0.014$, **Figure 3B**).

Visitation rate was significantly influenced by treatment, as the rate was higher on watered plants for all measured floral traits (**Figure 2C** and **Supplementary Table 3**). Furthermore, the rate was significantly affected by floral traits as it increased with increasing flower size (LMM: $\chi^2 = 5.48$, $p = 0.019$) and inflorescence size (LMM: $\chi^2 = 6.08$, $p = 0.014$). The other traits had no effect on visitation rate; however, a tendency was noted that visitation rate increased with nectar volume (**Figure 2C** and **Supplementary Table 3**). Scent emission positively affected the visitation rate (LMM: $\chi^2 = 3.67$, $p = 0.055$; **Figure 3C**).

Relative duration of landings was significantly affected by treatment, as bumblebees visited watered plants for longer periods (**Figure 2D** and **Supplementary Table 3**). However, duration was not significantly affected by floral traits, although tendencies were observed indicating that plants were visited for longer when they had more flowers (LMM: $\chi^2 = 2.81$, $p = 0.094$) or inflorescences (LMM: $\chi^2 = 2.74$, $p = 0.098$); landings were shorter with increasing nectar volume (LMM: $\chi^2 = 1.25$, $p = 0.264$). The interaction of floral trait and treatment had no effect on duration for all measured traits. Scent emission (LMM: $\chi^2 = 0.1$, $p = 0.754$, **Figure 3D**) and the interaction of scent and treatment had no effect on relative duration.

Floral Traits and Drought Stress

Drought-stressed plants were 10 cm smaller than watered plants [mean (SD) cm: **watered:** 47.5 (11.5) cm, **drought-stressed**: 37.2 (13.1) cm; LMM: $\chi^2 = 4.03$, $p = 0.045$, **Figure 4** and **Supplementary Table 1**]. We found no significant effect of

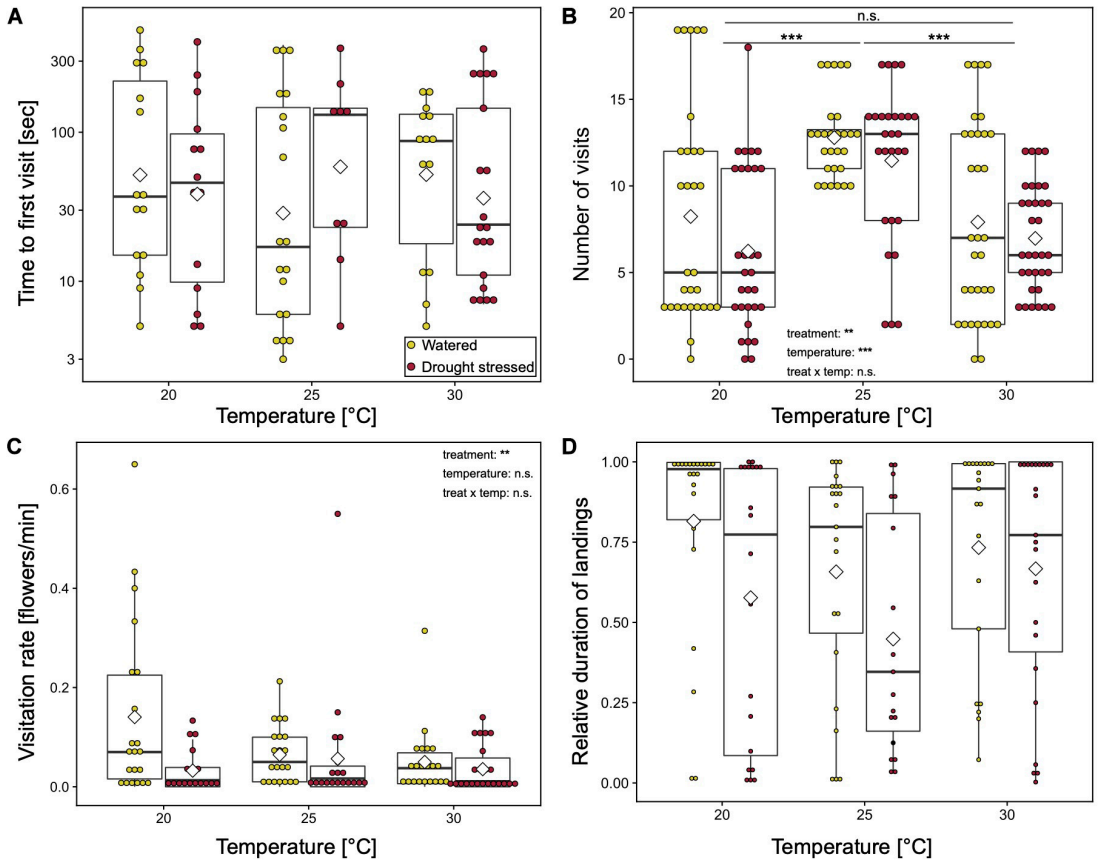

FIGURE 1 | Behavior of bumblebees at various temperatures with respect to watered and drought-stressed plants. **(A)** Time to first plant visit [sec] (approach or landing); **(B)** number of visits per plant (approaches plus landings); **(C)** flower visitation rate per landing [min]; and **(D)** relative duration of landings. Boxplots show median range, interquartile range, and minimum/maximum range. White diamonds show mean value. Means of each bumblebee were compared using general linear mixed effect models. ***$p < 0.001$; **$p < 0.01$; and n.s., non-significant.

drought treatment for the other morphological traits, nectar volume and floral scent (**Supplementary Table 1**). Nevertheless, nectar volume per flower was twice as high in watered plants [mean (SD) µl: **watered:** 0.006 (0.012) µl, **drought-stressed:** 0.003 (0.005) µl; LMM: $\chi^2 = 2.60$, $p = 0.107$, **Supplementary Table 1**], and the highest amount was found in watered plants at 20°C. The flowers emitted the same 25 compounds in both treatments (**Supplementary Table 2**). Mean total scent emission per flower tended to be higher in drought-stressed plants (LMM: $\chi^2 = 0.06$, $p = 0.814$; **Supplementary Figure 1**). Temperature (LMM: $\chi^2 = 2.64$, $p = 0.267$) and the interaction of treatment and temperature (LMM: $\chi^2 = 0.69$, $p = 0.707$) had no significant effect on the total scent emission. The emission rate for single compounds did not differ between the two treatments (**Supplementary Figure 2**). The scent bouquet also did not differ between the two treatment groups (PERMANOVA: *Pseudo-*$F_{1,65} = 0.81$, $p = 0.557$; **Supplementary Figure 3A**). Temperature and the interaction of treatment and temperature also had no effect on the composition of scent bouquet (PERMANOVA: temperature*:* Pseudo-$F_{2,65} = 1.06$, $p = 0.385$; treatment × temperature*:* Pseudo-$F_{2,65} = 1.07$, $p = 0.363$; **Supplementary Figure 3B**). Additionally, we found that total scent emission was positively correlated with nectar amount per flower (LMM: $\chi^2 = 5.01$, $p = 0.025$).

DISCUSSION

Drought stress can alter a number of flower-visitor interactions, although little has been documented about the effects of drought stress on pollinator behavior, the way that this might be linked with induced phenotypic changes in flower morphology and floral scent emission, and the possible influences of a combination of temperature and drought on such behavior. Our results show that drought-stress treatment negatively affects bumblebee behavior. Drought-stressed plants are visited less frequent, and bumblebees stay for shorter periods on their flowers, but no difference has been found in the initial attraction, as measured by the time to the first visit. Bumblebees are more active under increased temperature, although we have not detected a clear interaction of treatment and temperature, despite the visitation rate tending to be highest on watered plants at 20°C. Further, we have shown that bumblebees respond to differences in plant phenotypes, being able to find those plants

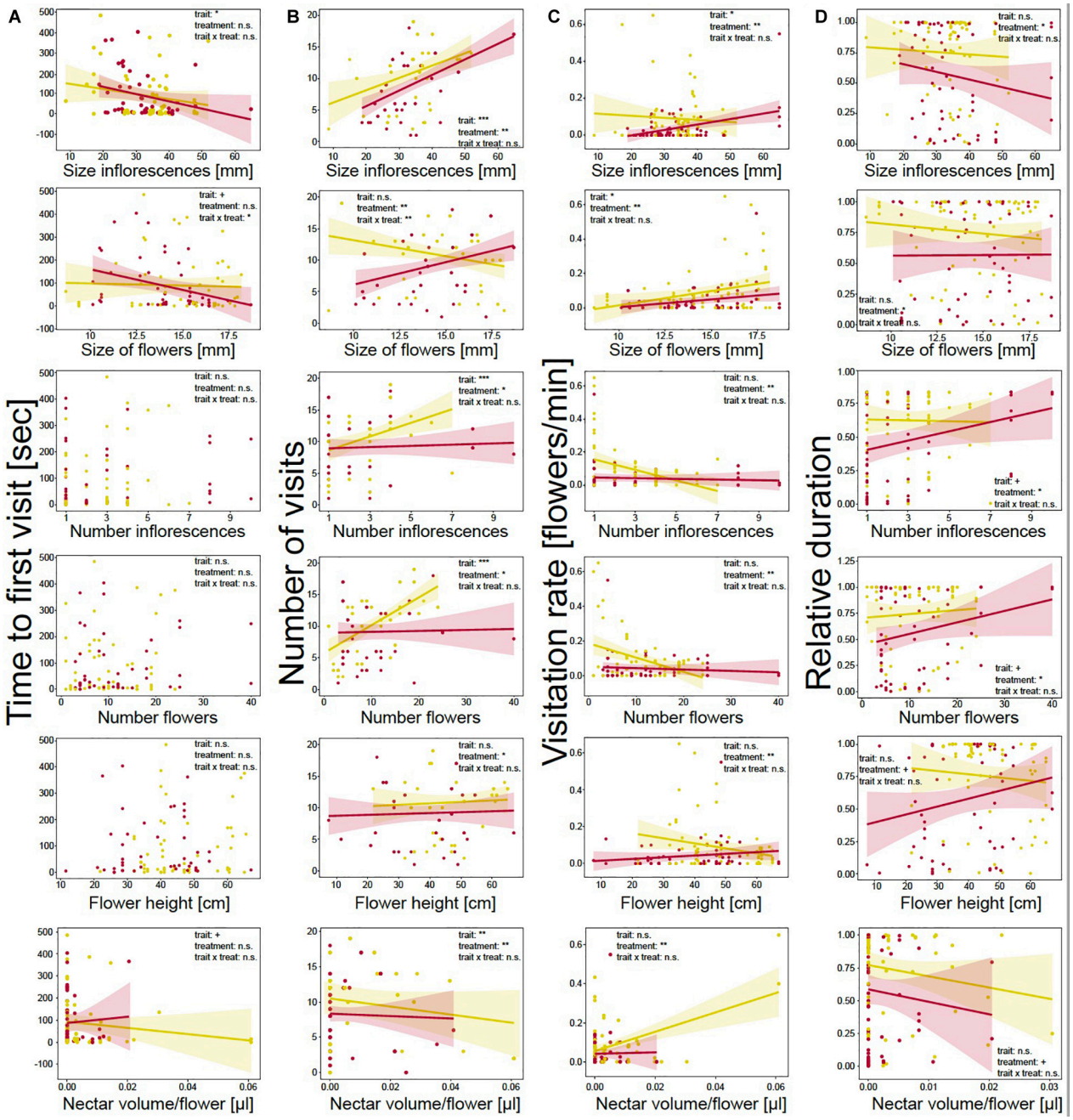

FIGURE 2 | Behavior of bumblebees in correlation with measured morphological traits and drought treatment. **(A)** Time to first plant visit [sec] (approach or landing); **(B)** number of plant visits (approaches plus landings); **(C)** visitation rate of flowers [min]; and **(D)** relative duration of landings. Plant trait values are mean values. Each dot represents one event on a plant. Colored lines show trend lines for significant correlations of behavior and plant trait; colored areas show confidence interval. Correlations were measured using linear mixed-effect models. Significance levels are given as asterisks: $^{***}p < 0.001$; $^{**}p < 0.01$;$^{*}p < 0.05$; $^{+}p < 0.10$; and n.s., non-significant.

with larger inflorescences and/or higher scent emission much faster. However, variability in floral traits is generally large with drought-stressed plants growing smaller and tending to produce less nectar per flower. Overall, our study reveals that plants are at least partially able to compensate for induced drought stress by reducing plant growth in order to maintain reproductive traits for pollinator attraction. However, we have found trait-mediated differences in behavior between watered and drought-stressed plants, indicating that plants are not fully able to compensate for drought stress to maintain bumblebee behavior. Thus, if stress levels increase, they will have even greater impacts on plants and trait-mediated bumblebee behavior.

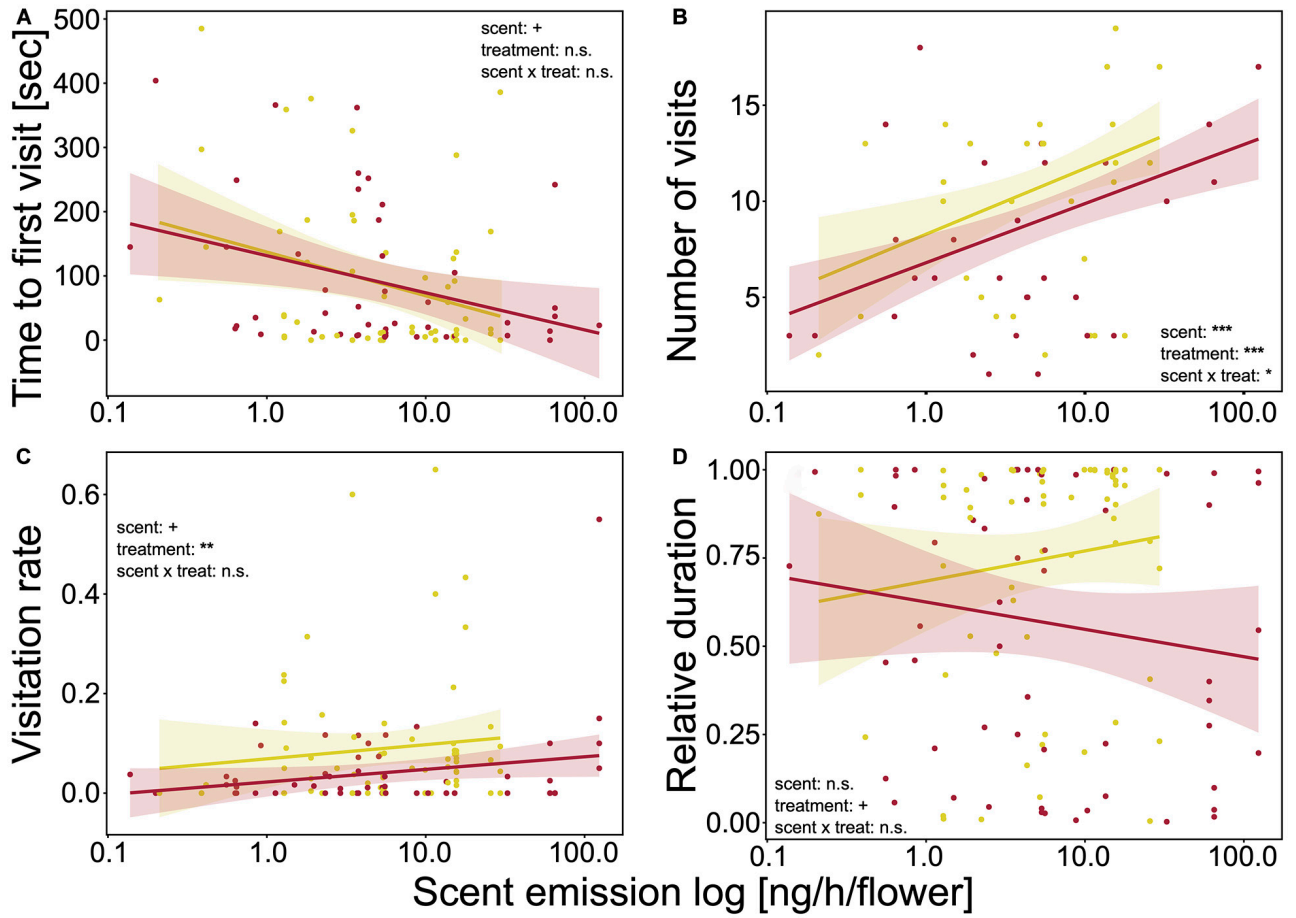

FIGURE 3 | Behaviors of bumblebees in correlation with total scent emission [ng/h/flower] (logarithmic scale). **(A)** Time to first plant visit [sec] (approach or landing); **(B)** number of visits (approaches plus landings); **(C)** flower visitation rate per landing [min]; and **(D)** duration of landings, relative to active time of bumblebees. Colored lines show trend lines for significant correlations of behavior and plant trait; colored areas show confidence interval. Correlations were measured using linear mixed-effect models. Significance levels are given as asterisks: $^{***}p < 0.001$; $^{**}p < 0.01$;$^{*}p < 0.05$; $^{+}p < 0.10$; and n.s., non-significant.

Whereas the initial attractiveness of plants in both treatments was similar for bumblebees, watered plants were visited more often than drought-stressed plants (see also Burkle and Runyon, 2016; Descamps et al., 2018). Additionally, the visitation rate of flowers and the number of visits per plant were higher on watered plants, and bumblebees remained for longer on these plants. As we have determined no differences in number of flowers or morphology between watered and drought-stressed plants, this behavior might be explained by the reduced nectar production per flower in the drought-stressed plants, as these plants are less attractive for foraging (e.g., Carroll et al., 2001; Waser and Price, 2016; Descamps et al., 2018). Indeed, nectar production is the highest in watered plants at 20°C, reflecting the pattern for the visitation rate. Bumblebees are able to optimize their foraging behavior by choosing flowers with higher nectar amounts thereby collecting more nectar in a shorter time (Chittka et al., 1997; Blarer et al., 2002; Cartar, 2004; Dreisig, 2012). This is the reason that the landing duration tends to decrease with higher nectar volume. Nectar production under normal circumstances is costly in terms of energy consumption (Southwick, 1984; Pyke, 1991). When plants are exposed to stressors such as drought or heat, resources may be not sufficient to compensate fully for the drought stress and for the maintenance of the reproductive organs and normal nectar production, leading to decreased nectar secretion in drought-stressed plants.

For successful pollination, not only pollinator attraction and visitation rate are important, but also the duration of visits. If bumblebees stay for shorter periods of time on each flower, pollen is less likely to be received by the stigma (Ohara and Higashi, 1994; King et al., 2013) or will be transferred from the anthers to the body of the pollinator (Sahli and Conner, 2007), potentially impacting female and male reproductive success. Further, the reduced number of flowers visited per plant suggest that not all flowers will be efficiently pollinated. In *Fagopyrum esculentum* (Polygonaceae), Rering et al. (2020) have shown that drought stress leads to reduced visits, decreased pollination success, and consequently lower seed set (see also Gallagher and Campbell, 2017). Therefore, increasing drought events and longer drought periods will influence bumblebee foraging behavior leading to

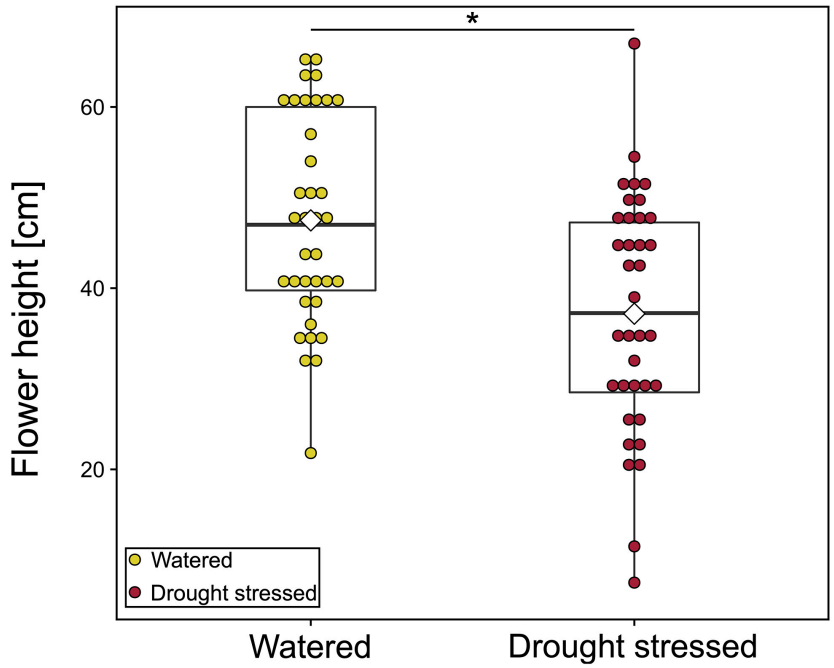

FIGURE 4 | Flower height [cm] of watered and drought-stressed plants. Each colored dot shows mean value of one plant for all observation days. Watered: $N = 36$ [mean (SD) 47.5 (11.5) cm]; drought stressed: $N = 36$ [mean (SD) 37.2 (13.1) cm]. Boxplots show median range, interquartile range, and minimum/maximum range. White diamonds show mean height. Means of each plant were compared using linear mixed-effect models. $^*p < 0.05$.

fewer and shorter visits on drought-stressed plants and hence to reduced pollination success (Chagnon et al., 1989).

Bumblebee behavior differed between floral trait expressions independently of treatment in our experiments. Larger inflorescences and flowers decreased the time until the first visit and increased the number of visits and the flower visitation rate. This indicates that bumblebees are able to differentiate between the phenotypes of plants (Thomson et al., 1982; Conner and Rush, 1996; Hoffmeister and Junker, 2017). Additionally, with higher scent emission the number of visits increased. As the emission is positively correlated with nectar volume per flower, this may suggests that it is an honest signal for reward, namely that plants with a higher scent emission provide more food resources for bumblebees (Wright and Schiestl, 2009; Knauer and Schiestl, 2015). However, under drought stress, other plant species also emit more scent (Burkle and Runyon, 2016; Gallagher and Campbell, 2017) possibly to simulate the presence of nectar.

Temperature did neither affect the total amount of scent emission nor the composition of the scent compounds of watered and drought-stressed plant; this finding might be the reason that, under the two treatments, the flowers have a similar initial attractiveness to the bumblebees. Thus, *S. arvensis* is able to emit a stable scent bouquet even under drought stress and across various temperatures, potentially in order to maintain function in pollinator attraction. However, other studies have revealed that total scent emission increases because of higher vaporization up to a maximum of 30°C (Sagae et al., 2008; Scaven and Rafferty, 2013; Farré-Armengol et al., 2014), and that scent emission can change within 2 h (Hu et al., 2013). Such higher floral scent emissions attributable to increasing temperatures and drought (Burkle and Runyon, 2016) might also have negative effects. An increase of, for example, terpene emissions in floral parts and other tissues may involve higher metabolic costs by pathways producing these compounds (Farré-Armengol et al., 2014). Higher metabolic costs might then lower the plastic response of plants to drought stress. Furthermore, qualitative changes in floral scent bouquets brought about by drought stress and increasing temperatures (Llusià and Peñuelas, 2000; Farré-Armengol et al., 2014) possibly disturbs flower-visitor communication. The actual visitor species is no longer able to find its host plant (Vereecken et al., 2010), which in turn would lower visitation rate and pollination success.

In our study, we have shown that temperature in combination with drought stress in plants plays no significant role in bumblebee behavior. However, the pattern for visitation rate follows that of nectar production, with highest values occurring at 20°C for watered plants, indicating the interacting effects of drought and temperature on trait-mediated flower-bumblebee behavior.

Temperature on its own significantly influences bumblebee behavior, as the number of visits per plant was highest and most bumblebees were active and participating in the experiments at the medium temperature of 25°C. This corresponds to the reported temperature of the highest foraging activity at 25°C, whereas at 32.7°C foraging activity significantly decreased by 69.7%, indicating that 25°C is the optimal foraging temperature for *B. terrestris* and possibly supports their thermoregulation

(Kwon and Saeed, 2003). Other studies have shown that, at lower ambient temperatures, bees prefer warmer flowers with warmer nectar to maintain body temperature (Dyer et al., 2006; Norgate et al., 2010; Shrestha et al., 2018). Similarly, at high temperatures bumblebees might adjust their behavior to avoid overheating by changing foraging patterns and floral preferences and handling. Thus, interacting effects between temperature and drought might especially occur under severe drought and temperature conditions.

Sinapis arvensis plants are able to grow and flower under our drought treatment. In congruence with other studies (Chaves et al., 2003; Burkle and Runyon, 2016; Kahl et al., 2019; La Rosa et al., 2019), our drought-stressed plants grow less than the daily watered control plants. However, we have found that flower size did not decrease as has commonly been observed (Carroll et al., 2001; Halpern et al., 2010; Burkle and Runyon, 2016) for plant species with similar moisture value to ours (Ellenberg et al., 1992). These differences might be explained by different growing conditions, because our plants were reared and kept in a phytochamber with constant temperature and light conditions. Under field or semi-natural conditions, such as in a greenhouse with fluctuating light and temperatures and the potential impact of herbivores or pathogens (Rusman et al., 2019), it may be more difficult for plants to compensate for water deficits. Herbivory mediates the effects of drought on floral size for certain plant species (Burkle and Runyon, 2016) and limits the plastic responses to herbivore damage during low water treatment (Halpern et al., 2010). However, several species have been shown to maintain floral trait expression under drought treatment (Caruso, 2006; Glenny et al., 2018; Walter, 2020). Thus, plants exposed only to one stressor may be more likely to compensate for drought stress by reducing growth to invest resources in floral parts for the maintenance of pollinator attraction based on visual information. Therefore, the determination of ranges of drought exposure in which plant species are still able to compensate for this stress in order to maintain their normal floral phenotype, in combination with other stressors, might represent an important step for predicting impacts of drought on the floral phenotype of plants.

Overall, our study has revealed that *S. arvensis* plants are able to maintain pollinator attraction under drought stress, but that bumblebee behavior changes during flower handling. Floral trait expression, largely independent of treatment, mediates bumblebee behavior. However, the response of bumblebees to certain floral trait expression, e.g., floral size and nectar amount, differs between drought and watered treatments. Thus, our results indicate that plants are able to buffer floral trait expressions against short-term drought, potentially maintaining the attractiveness of their flowers to ensure at least a few visits by pollinators. Nevertheless, we have found indications that the quality and quantity of pollinator visits are impaired by drought stress. Therefore, plants are able to withstand reduced water availability within a certain range. These findings highlight the need for a comprehensive understanding of the impacts of various drought intensities on plants for the planning of future drought management. Moreover, the impact of drought on possible changes of behavior of pollinators on flowers and the consequences for female and male reproductive success should be assessed in future studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JK conceived the study. RH, MA, and JK designed the study. RH collected the data. RH and JK analyzed the data. RH drafted the first version of the manuscript. RH, JK, and MA wrote the final version. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.564802/full#supplementary-material

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Human-Mediated Land Use Change Drives Intraspecific Plant Trait Variation

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In the Anthropocene, more than three quarters of ice-free land has experienced some form of human-driven habitat modification, with agriculture dominating 40% of the Earth's surface. This land use change alters the quality, availability, and configuration of habitat resources, affecting the community composition of plants and insects, as well as their interactions with each other. Landscapes dominated by agriculture are known to support a lower abundance and diversity of pollinators and frequently larger populations of key herbivore pests. In turn, insect communities subsidized by agriculture may spill into remaining natural habitats with consequences for wild plants persisting in (semi) natural habitats. Adaptive responses by wild plants may allow them to persist in highly modified landscapes; yet how landscape-mediated variation in insect communities affects wild plant traits related to reproduction and defense remains largely unknown. We synthesize the evidence for plant trait changes across land use gradients and propose potential mechanisms by which landscape-mediated changes in insect communities may be driving these trait changes. Further, we present results from a common garden experiment on three wild *Brassica* species demonstrating variation in both defensive and reproductive traits along an agricultural land use gradient. Our framework illustrates the potential for plant adaptation under land use change and predicts how defense and reproduction trait expression may shift in low diversity landscapes. We highlight areas of future research into plant population and community effects of land use change.

Keywords: microevolution, natural selection, plant secondary metabolites, floral traits, plant fitness, plant defense, landscape simplification, plant–insect interactions

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INTRODUCTION

Agriculture now represents the largest anthropogenic biome on the planet, occupying over a third of the earth's ice-free land (Foley et al., 2005; Ellis and Ramankutty, 2008; Holzschuh et al., 2011; Seto et al., 2011). Loss of natural habitats is linked to rapid declines in biodiversity worldwide, driving many species locally extinct and homogenizing species composition (Brooks et al., 2002; Keil et al., 2015). Precipitous declines in insect abundance and diversity documented in the last century are largely attributed to habitat loss, fragmentation, and degradation associated with land use change (Hallmann et al., 2017; Jauker et al., 2019; Seibold et al., 2019). Though insect abundance and diversity is generally declining, insects that can utilize the modified habitat

(e.g., crop pests) often undergo population surges (Chaplin-Kramer et al., 2011; Veres et al., 2013). The consequences of insect population shifts are not limited to modified landscapes. Because of their frequently high mobility, changes in insect abundance and composition within modified habitat matrices also affect species interactions in surrounding habitats as insects track resources across the landscape. Due to their central role in ecosystem functioning and species interactions, changes in insect communities therefore can have cascading effects on ecological processes within and around modified landscapes. However, the mechanisms driving the directionality and magnitude of the effects of landscape-mediated changes in insect communities on wild plants remain unclear (Irwin et al., 2018). Whether wild non-crop plants in landscapes dominated by agriculture are adapting to compensate for declines in native pollinators and natural enemies and outbreaks of insect herbivores could have important implications for wild plant population persistence (Thomann et al., 2013).

The coevolution of plants and insects has resulted in both an evolutionary arms race of defense and counter defense as well as a sweeping reliance on insects for reproduction. Given that both antagonistic and beneficial insects are major agents of selection, frequently on similar plant traits, it is likely that changes in the insect community driven by land use change alter plant trait evolution (Schiestl and Johnson, 2013). Additionally, antagonistic and beneficial insect populations may impose conflicting selection on the limited resources plants can allocate to defense and reproduction (Knauer and Schiestl, 2017). Because the combined effects of herbivores and pollinators can be reinforcing or conflicting, changes in either community could alter the outcomes of selection on morphological and phenological traits (Gómez, 2003; Kessler and Halitschke, 2009; Sletvold et al., 2015).

Ultimately, there is evidence that landscape-mediated changes in insect communities affect fitness in wild plants (Holzschuh et al., 2011). For example, wild plants in natural habitats surrounded by landscapes dominated by natural land cover produce more and heavier seeds than those surrounded by landscapes dominated by agriculture, matching with metrics of pollinator availability within each landscape (Albrecht et al., 2007; Diekötter et al., 2010; Jakobsson and Ågren, 2014). Similarly, our data from a greenhouse common garden experiment in which shepherd's purse plants (*Capsella bursa-pastoris*) were allowed to self-pollinate, we found that plants sourced from populations surrounded by natural land cover, produce more and/or heavier seeds than populations surrounded by more agricultural land (see **Supplementary Methods**). These results suggest that the self-pollination success is reduced in landscapes with increased agricultural cover, indicating either (1) a reduced ability to autonomously self-pollinate, or (2) a higher level of self-incompatibility (**Figure 1**). The evidence that seed number and size varies with landscape context motivates this review to examine the potential for landscape mediated adaptation in wild plants.

Here we hypothesize that landscape-mediated changes in insect community composition result in predictable variation in plant traits (**Figure 2**). While land use change can take many forms, we focus largely on agriculture in our predictive framework because it represents the largest land area of human-mediated land use change (Ellis and Ramankutty, 2008). However, when available, we also include examples of the effects of urbanization. Further, although agriculture and urbanization can encompass many levels of intensification and a large variety of management practices, we center our work on the replacement of natural areas by agricultural or urban areas *per se*. To define underlying assumptions, we first provide evidence that herbivores, natural enemies, and pollinators are agents of selection on plant traits. Next, we outline how land use change affects each insect functional group, highlighting the potential mechanisms by which landscapes could mediate these changes and developing predictions for the outcomes of insect utilization of the habitat. Finally, we synthesize the current evidence that landscape-mediated effects on insect communities result in evolutionary changes in plant populations and supplement these data with a case study in three wild Brassicaceae species. Through this review we provide a framework to inform predictions for plant trait evolution under land use change scenarios, focusing on agriculturally driven habitat conversion. We propose that land use change gradients can serve as a model for studying microevolutionary dynamics and the evolution of species interactions by simulating the timeline of land use change experienced by plants and insects in recent history. The functional traits and natural history of the interactors will dictate the scale at which the landscape is experienced while the rate of adaptation will be dependent on the strength of selection and genetic constraints of the plant.

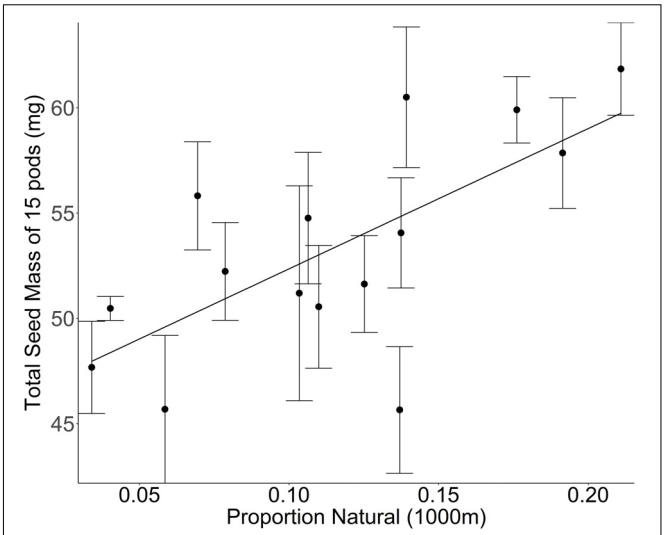

FIGURE 1 | Mass of seeds produced by the first 15 self-fertilized pods on the central inflorescence of *Capsella bursa-pastoris* plants sourced from 15 sites along a gradient of increasing proportion of agricultural land cover and grown together in a greenhouse common garden [$F_{(1,13)} = 9.4862$, $p = 0.0088$]. The proportion of natural cover at a 1000 m radius around the collection site is a measure of land use composition but does not provide any information regarding the intensity of the management practices within any given land use. Mean ± 1 SE values per site are calculated based on the measurement of 201 different *C. bursa-pastoris* plants (2–25 plants per site).

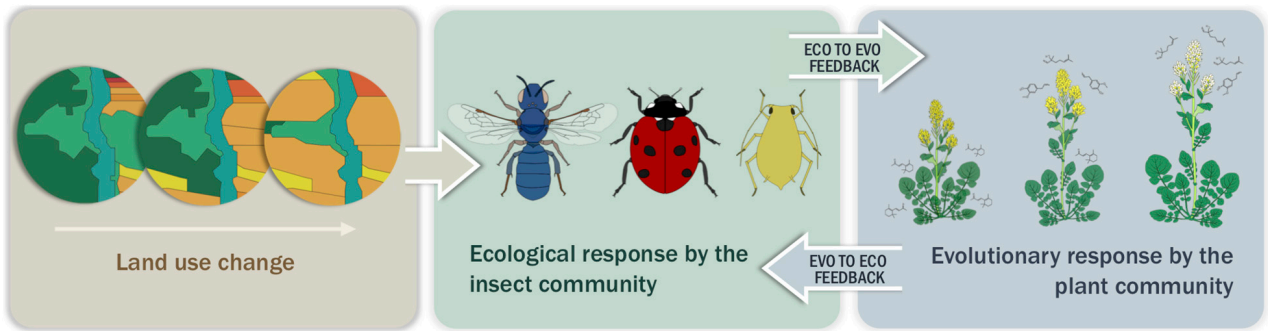

FIGURE 2 | Simple conceptual model with one potential output demonstrating how land use change, through habitat loss, species introduction and/or management practices, affects the insect community, which in turn selects for certain plant traits (depicted here are plant size, flower color, and linalool production) at the population level. These changes in traits lead to an eco-evolutionary feedback loop between the insect community and the plant population.

LAND USE CHANGE AFFECTS PLANT DIRECT DEFENSES, MEDIATED BY HERBIVORE COMMUNITIES' RESPONSES TO LAND USE CHANGE

Herbivores Affect Plant Defenses

Plants and herbivores are thought to be locked in a co-evolutionary arms race where plant defenses counter herbivore resistance to plant defenses (herbivore counter defenses) and *vice versa* (Ehrlich and Raven, 1964). Although the major prediction of this hypothesis, the reciprocal natural selection, is rarely tested, it is increasingly clear that herbivores frequently function as major agents of selection on plant chemistry and defenses (Agrawal et al., 2012; Uesugi and Kessler, 2013; Gervasi and Schiestl, 2017; Kalske et al., 2019). Increased selection by herbivores usually results in higher mean values for constitutive plant resistance in a population (Agrawal et al., 2012; Bode and Kessler, 2012). Relief from herbivore pressure usually selects for decreased herbivore resistance and higher competitive ability (Uesugi and Kessler, 2013; Uesugi et al., 2017).

The evolution of novel defense traits is predicted to result in the plants' escape from a significant proportion of potential herbivores available in an environment (Kessler, 2015). Similarly, the occurrence of a novel herbivore species (or genotypes) can have strong negative effects on plant communities and populations (Moxley et al., 2017). Plant traits that mediate resistance against herbivores can function directly on their attacker or indirectly mediate interactions with third party organisms [e.g., natural enemies of herbivores (Dicke and Baldwin, 2010; Kessler and Heil, 2011), associational resistance (Barbosa et al., 2009), keystone herbivores (Poelman and Kessler, 2016)] to the plants' advantage. Direct defenses can be physical structures (e.g., thorns, spines, prickles, trichomes, silica, and thickened cell walls), chemical or combinations of the two (e.g., exudating trichomes, latex, adhesives). In theory, these traits can occur simultaneously in a plant but may differentially affect different attackers. Consequently, the way in which plant defenses are altered in response to natural selection strongly depends on the major agent of selection as well as the ecological context in which the interactions are played out. For example, general relief from herbivory in tall goldenrod, *Solidago altissima*, populations resulted in lower mean resistance to a major specialist herbivore, the goldenrod leaf beetle, *Trirhabda virgata*, but had no effect on the resistance to an herbivore uncommon in this system, the fall armyworm (*Spodoptera frugiperda)* (Bode and Kessler, 2012). Nevertheless, if land use changes are associated with significant shifts in the herbivore community (diversity, abundance, and composition), plant population genetic changes and shifts in the mean defense phenotypes can be predicted.

Plants respond to environmental stressors, such as herbivory, with an alteration of their metabolism (Karban, 2011). Such plant induced responses to herbivory can function in multiple different ways: (I) Induced changes to primary and secondary metabolism allow the plant to adjust to exposure to stress and thus maintain structural integrity and reproduction (tolerance). (II) Some metabolic responses to herbivory include the increased production of toxic, antidigestive or antinutritive compounds and so confer increased resistance when needed, in case of an actual attack (induced defenses) (Kessler and Baldwin, 2002). (III) The simple change in metabolism *per se*, rather than the increased production of defensive compounds can provide a moving target for foraging herbivores (moving target hypothesis) (Adler and Karban, 1994). (IV) Herbivore-induced changes in secondary metabolism can provide information about the physiological, attack and defense status of the plant and so mediate interactions with natural enemies of their herbivores (indirect defenses) (Dicke and Baldwin, 2010) or neighboring plants (associational resistance) (Heil and Karban, 2010). The plant's ability to respond to herbivory is a heritable trait and thus expected to be subject to selection by herbivory much like constitutive defensive traits discussed above.

Overall, changes in the herbivore community (abundance, diversity, composition) can have short-term ecological effects due to the inducibility of metabolic changes in response to herbivory or they can have intermediate-term microevolutionary effects on the population genetic structure that feeds back to the ecological interactions (**Figure 2**). Correlative studies that target the relationship between land use change and

plant–organismal interactions need to consider both potential causes when studying population-wide effects.

Land Use Change Affects Herbivores

The response of herbivore communities and populations to land use change varies dramatically. Greater proportions of agriculture in the surrounding landscape can lead to augmentation, suppression or no change in the herbivore communities or populations (**Table 1**). Increasing agriculture in the surrounding landscape has been shown to decrease herbivore abundance on wild plants in a series of studies (**Table 1**). For example, seed predators and folivores were more common on wild sunflowers (*Helianthus annuus texanus*) that were farther away from cultivated sunflowers (*H. annuus*), than those growing next to cultivated sunflowers (Chamberlain et al., 2013). Domesticated crops are often considered less defended than their wild relatives, resulting in increased herbivore damage and herbivore preference and performance on crops in comparison to a wild conspecific (Whitehead et al., 2017). In those cases where wild plants are better defended than domesticated plants, it is likely that at the landscape scale, herbivores would prefer crop-host over non-crop-hosts, this way reducing herbivore pressure on the wild plants. Additionally, pest control methods ranging from intercropping (Tamburini et al., 2020) to the use of pesticides in cultivated crops could decrease the overall abundance of herbivores, leading to reduced herbivory in adjacent wild relatives. A long term, broad scale study in China found that the increased spatial and temporal use of Bt cotton did not just reduce the pest pressure of cotton bollworm (*Helicoverpa armigera*) on cotton, but also on other crops such as corn, peanuts and other vegetables attacked by this pest (Wu et al., 2008). This suggests that management practices aimed at decreasing pest pressure in one crop can not only affect herbivore pressure on other crops, but could also decrease pest pressure on wild plants, in those cases where herbivores are shared.

Conversely, there is evidence that herbivore populations can be subsidized by agriculture and spillover to natural plant communities (Blitzer et al., 2012). The introduction of novel herbivores to a system through the use of non-native crops (Kaiser et al., 2008; Squires et al., 2009) may drive increases in herbivore pressure on wild plants. For example, the introduced coffee berry moth (*Prophantis smaragdina*), which is a known pest of coffee was found to be feeding on the wild rubiaceae, *Bertiera zaluzania*, with negative effects on the plant's fitness (Kaiser et al., 2008). Additionally, agricultural systems provide alternative resources for existing herbivores increasing their populations (Gladbach et al., 2011). In a study done on wild sunflowers in prairie remnants, McKone et al. (2001) found that close proximity to maize fields increased the amount of corn rootworm beetles (*Diabrotica barberi* and *D. virgifera*) in wild sunflowers. The wild sunflowers had more floral damage and decreased seed set when they were close to the edges of the corn fields, due to a combination of the life history of the pest and the timing of the crop. When the adults emerge in July they feed on corn silks and immature ears, but once the crop is mature and dry they no longer prefer it and will shift to the wild sunflowers that are blooming at that time. Similarly, wild mustard (*Sinapis arvensis*) experiences higher herbivory from the pollen beetle (*Meligethes aeneus*), a pest of canola (*Brassica napus*), immediately following canola bloom in areas with higher canola cover (Gladbach et al., 2011).

Overall, land use change has a significant impact on herbivores attacking wild plants, through changes in the composition (richness, evenness, and abundance) but also very likely through changes in herbivore traits, that have not been as clearly documented (but see Gámez-Virués et al., 2015). However, the direction and severity of the impact is dependent on the management practices used in the modified habitats, the resistance traits of crops and wild plants alike and the ability of invasive herbivores that come with novel crops to attack native plants. Further, resistance in the crops and wild plants

TABLE 1 | Effect of land use change on herbivore communities and populations of natural plant systems.

Mechanism	Outcome and Prediction	References
Management practices	↓ When management practices such as pesticide use and tilling reduce herbivore communities and populations and result in lower herbivore pressure on wild plants.	Wu et al., 2008; Chamberlain et al., 2013
Habitat loss	↓ When herbivores of wild plants do not use the landscape matrix causing herbivore populations to decrease in the whole landscape due to habitat loss.	
	↑ Herbivores of wild plants do not use the landscape matrix causing the herbivore populations to concentrate on the wild plants and transiently increase herbivore pressure.	
Crops as alternative resources	↓ When crops offer a higher plant quantity and/or quality than wild plants due to domestication, management practices or mass resources, herbivores are more attracted to crops reducing herbivore pressure.	Chamberlain et al., 2013
	↑ Herbivores of wild plants use the surrounding crop matrix, that allows them to increase population size and spill back to the natural environment.	Moxley et al., 2017
Introduced pests	↑ Novel herbivores are introduced in the environment when novel crops are planted, increasing herbivore pressure on wild plants.	Kaiser et al., 2008; Squires et al., 2009

Here we present the direction of the response (↑ = positive effects on herbivores, ↓ = negative effects on herbivores) and the potential mechanisms for that response. When examples from the literature are available that suggest those mechanisms, we cite them under references. Rows without references reflect areas of future work.

can feedback to select for resistance in the pest species. Also, indirect effects mediated by changes in the natural communities (discussed further down) could affect herbivore pressure in wild plants. Either direction, the consequences of herbivore shifts due to land use change have been shown to affect plant fitness and therefore should have consequences on plant defense traits that will ultimately allow plants to adapt to the new environment they are experiencing.

Land Use Change Affects Wild Plant Direct Defenses

We are not aware of any studies looking at the effect of land use change on wild plant defenses. However, here we present results from our own work that has explored the importance of land use change on plant defenses. In the summer of 2017, we collected seeds of the Brassicaceae species *Barbarea vulgaris* from sites of varying landscape composition across the Finger Lakes region in New York State, United States. Plants were grown in a greenhouse common garden and bioassays were conducted with 3-day old *Trichoplusia ni* (Noctuidae) caterpillars to evaluate resistance on 3-week-old plants (see **Supplementary Methods**). Caterpillars gained less weight on plants sourced from sites with a higher proportion of agriculture in the surrounding landscape than those feeding on plants sourced from low agriculture sites (**Figure 3**). This pattern suggests that wild *B. vulgaris* plants growing in high agriculture landscapes invest relatively more in plant defenses, providing evidence for landscape mediated trait adaptation either through genetic changes in plant defense trait expression or maternal effects. More studies like this are needed to determine if this is a generalizable pattern, if wild plant adaptive responses are highly variable by species and region, and to evaluate the role of maternal effects.

LAND USE CHANGE AFFECTS FLORAL TRAITS, MEDIATED BY POLLINATOR COMMUNITIES' RESPONSES TO LAND USE CHANGE

Pollinators Affect Plant Traits

Flowering plants and insect pollinators share a long coevolutionary history in which plant fitness is highly dependent on the ability to attract effective pollinators. As mediators of plant reproduction, pollinators are important in shaping floral morphology, scent, and outcrossing potential. Pollinator declines can intensify selection on floral traits like flower number, size and selfing rate (Brys and Jacquemyn, 2012; Panique and Caruso, 2020). In a study that compared floral traits across time from a site with known pollinator declines, contemporary populations had larger floral displays and longer receptivity compared to their ancestor population (Thomann et al., 2015). Similarly, Castellanos et al. (2019) demonstrate that stable pollinator environments can stifle current evolutionary change while maintaining heritable variation necessary for future adaptation.

The preferences and morphology of the local pollinator communities can also drive changes in plant reproductive traits. Pollinators often prefer larger flowers (Galen, 1989; Benitez-Vieyra et al., 2006; Sandring and Ågren, 2009; Parachnowitsch and Kessler, 2010) and floral scents that are distinct and reliable within species (Parachnowitsch et al., 2012; Ramos and Schiestl, 2019). In *Erysimum mediohispanicum*, spatial variation in pollinator community assemblage resulted in divergent selection on corolla shape and tube width (Gómez et al., 2009). Nagano et al. (2014) demonstrated that floral size variation across mountain ecosystems was positively correlated with changes in the average pollinator size rather than altitude.

Changes in pollinator communities can also drive plants away from investment in pollinator attraction and toward greater self-compatibility. For example, *Brassica rapa* plants became shorter with reduced floral volatiles and increased selfing under fly pollination compared to bumble bee pollination (Gervasi and Schiestl, 2017). Under low pollinator conditions, selection favors traits that promote autonomous self-fertilization, such as inward facing anthers and a reduced anther-stigma distance (Toräng et al., 2017). The shift from outcrossing to selfing is also associated with reduction in corolla size and an increase in pistil length (Carleial et al., 2017). Collectively, this work demonstrates that shifting insect communities are sufficient to drive evolution in plant traits.

Land Use Change Affects Pollinators

The impact of land use change on plant–pollinator interactions in habitat fragments can have diverse outcomes (**Table 2**) and will depend on the traits of the pollinators as well as the surrounding habitat matrix. Human modification of the landscape alters resource availability and at the same time presents additional stressors (pesticides, novel pathogens, altered microclimate; see Goulson et al., 2015). Consequently, reduced pollinator abundance and diversity within natural habitat fragments has frequently been documented in human-dominated landscapes (Jennersten, 1988; Aizen and Feinsinger, 1994a; Steffan-Dewenter and Tscharntke, 1999; Steffan-Dewenter et al., 2001; Kormann et al., 2015; Wenzel et al., 2020). However, given that landscape composition affects pollinators through two potential mechanisms: (1) changes in population size and (2) distribution of pollinators among habitats, we expect that pollinator interactions with wild plants respond differently depending on the mechanism at play. For example, agricultural covers such as mass flowering crops may provide complementary or supplementary resources which subsidize pollinator populations (Westphal et al., 2003; Rundlöf et al., 2014) and bee visitation to wild plants in natural habitats (Holzschuh et al., 2016). During bloom, however, the cover of mass flowering crops is generally associated with lower pollinator abundance in semi-natural habitats (Holzschuh et al., 2016; Montero-Castaño et al., 2016).

Often the impact of land use change is not evenly distributed across pollinator communities. Instead, the traits of some pollinator groups allow them to persist in human-modified landscapes while other traits make some groups more vulnerable (Gámez-Virués et al., 2015; Wenzel et al., 2020). For example, Steffan-Dewenter et al. (2001) found no change in bumble bee visitation to a native plant across a semi-natural habitat gradient

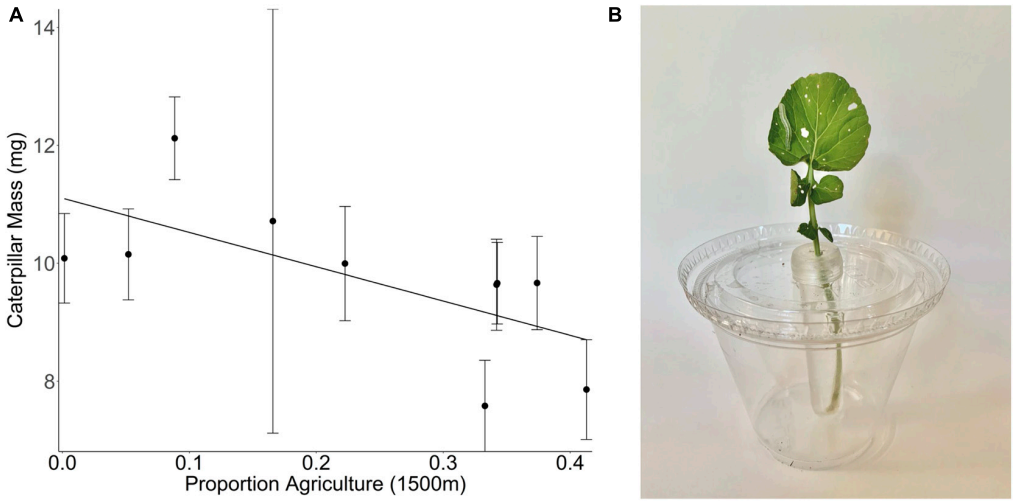

FIGURE 3 | (A) Effect of the proportion agriculture at 1500 m on the mass of *Trichoplusia ni* caterpillars that have been feeding on *Barbarea vulgaris* plants for 3 days [$F_{(1,8)} = 5.21$, $p = 0.0518$]. *B. vulgaris* plants were collected at 10 different sites that vary in their landscape composition. The proportion of agricultural cover at a 1500 m radius around the collection site is a measure of land use composition but does not provide any information regarding the intensity of the management practices within any given land use. Mean ± 1 SE values per site are calculated based on caterpillar growth on individual severed leaves from 1 to 10 different *B. vulgaris* plants per site, with two leaf samples (and therefore two caterpillars) per plant. **(B)** Experimental set up with *T. ni* caterpillar feeding on a *B. vulgaris* leaf.

TABLE 2 | Effect of land use change (from natural to agricultural or urban areas) on pollinator communities and populations of wild plant systems.

Mechanism	Outcome and Prediction	References
Management practices	⇕ Management practices such as pesticide use and tillage can have mixed effects on pollinator populations with mixed effects on wild plant pollination.	Positive effect of pesticides: Brittain et al., 2010 Neutral effect of pesticide: Chateil and Porcher, 2015 Negative effect of tillage: Shuler et al., 2005
Habitat loss	⇓ Habitat loss when pollinators do not use the matrix is expected to reduce pollinator population over the long term.	Taki et al., 2007
	⇑ When pollinators do not use the matrix and habitat loss is recent, pollinators will be transiently concentrated in remaining habitat fragments.	
Crops as alternative resources	⇓ When crops provide a higher floral resource quantity and/or quality concurrent with wild plant bloom resulting in reduced pollinator visitation to wild plants	Montero-Castaño et al., 2016
	⇑ Complementary resources use in the natural habitat and matrix results in an overall increase in resource availability and augments pollinator population	Lundin et al., 2017
Introduced pollinators	⇓ Increase in inefficient introduced pollinator visits to the wild plant	Dupont et al., 2004; Magrach et al., 2017
	⇑ Increase in efficient introduced pollinator visits to the wild plant	Aizen and Feinsinger, 1994b

Here we present the direction of the response (⇑ = positive effects on pollinators, ⇓ = negative effects on pollinators) and the potential mechanisms for that response. When examples from the literature are available that suggest those mechanisms, we cite them under references. Rows without references reflect areas of future work.

although solitary bee visitation declined. In this case, differences in responses may be due to differences in body size as large bodied bees are able to forage further (Greenleaf et al., 2007). Similarly, spillover of managed bees may substantially alter wild plant–pollinator interactions in human-dominated landscapes (Geslin et al., 2017). Both honey bees and managed bumble bees use a large proportion of non-crop pollen even when placed in crops during mass flowering (Foulis and Goulson, 2014; McArt et al., 2017) and can be more abundant than their wild counterparts in nearby natural habitats during periods of floral scarcity (González-Varo and Vilà, 2017; Trillo et al., 2019). For example, honey bee visitation to several native plants was highest in landscapes with low semi-natural cover while the opposite pattern was observed for wild bees (Steffan-Dewenter, 2002) suggesting that increased managed bee visitation may offset wild pollinator losses (Aizen and Feinsinger, 1994a).

Loss and fragmentation of natural habitat has frequently been correlated with reduced pollination and reproduction in wild plants (Aguilar et al., 2006). Because many pollinators utilize both wild plants and crops, sharing local pollinators creates

potential fitness implications through interference in visitation rates or pollen transfer (Stanley and Stout, 2014). For example, mass blooming crops can mediate the spatial distribution of pollinators in the landscape, reducing visitation and fitness in co-blooming wild plants (Holzschuh et al., 2011; Van Reeth et al., 2019). Conversely, mass crop blooms may augment pollinator populations, increasing spillover and boosting the fitness of wild plants blooming immediately after the crop (Kovács-Hostyánszki et al., 2013). Therefore, mass flowering crops can have opposing outcomes for wild plant populations via separate mechanisms suggesting that the kind of habitat conversion as well as the traits of focal wild plant populations are likely to have strong impacts on the outcome of these land use changes on wild plant populations.

In some systems, plants demonstrate resilience to land use change. This is exemplified when there is no detectable effect of land use change on plant reproductive success (Lopes and Buzato, 2007; Ekroos et al., 2015; Skogen et al., 2016; Grass et al., 2018) likely because these landscapes select for pollinator species that are tolerant to disturbance and/or have a high dispersal ability. In restored prairies, increasing proportion of agriculture in the surrounding landscape did not increase pollen limitation in a pollinator dependent forb (Ritchie et al., 2020). It is possible that the restored prairies provide habitat to support sufficient pollinators to prevent pollen limitation. Further, conflicting pressures of pollinators and herbivores could also be responsible for the failure of these studies to detect a fitness effect of landscape composition (Steffan-Dewenter et al., 2001).

In summary, land use changes have substantial impacts on the composition of pollinator communities with potential consequences on plant fitness in remaining natural habitat fragments. As a rule, loss of natural habitat is expected to reduce the abundance and diversity of wild plant pollinators and consequently cause pollen limitation in wild plants (Taki et al., 2007); yet, exceptions to this rule, such as temporal effects of mass flowering crops, will alter these dynamics. Further, loss of natural habitat is also expected to shift flower visitor communities toward disturbance-adapted and managed-pollinator species.

Land Use Change Affects Wild Plant Floral Traits

In spite of the evidence that land use change is altering pollinator communities and that these shifts can drive trait change in plants, there are few studies linking these two processes. There is some evidence that spatial variation in pollinator communities can mediate differential selection on floral traits (Ågren et al., 2013; Chapurlat et al., 2015). Irwin et al. (2018) linked pollinator mediated shifts in floral display size to land use change, with larger displays present in urban landscapes. Similarly, in scotch broom (*Cytisus scoparius*) pollinators are shown to impose selection for increased floral size in urban, but not rural landscapes (Bode and Tong, 2018). Further, in a study comparing pollinator mediated selection in insect-impoverished industrial sites and insect-rich natural habitats, plants in insect-impoverished landscapes produced smaller and fewer flowers and demonstrated higher potential for autonomous selfing than those in insect rich habitats (Brys and Jacquemyn, 2012). Overall, it seems that the effects of urbanization are inconclusive showing cases of increased, but also decreased investment in floral traits. Studies from agricultural land-use change seem to be completely missing today, leaving this field equally inconclusive. Here we present results from our own work examining the effects of agricultural land use change on flower size.

In the summer of 2017, we collected seeds of the Brassicaceae species *Thlaspi arvense* from sites across a landscape gradient from low to high agriculture. Plants from each site were grown together in a greenhouse common garden and petal length and width was measured using electronic calipers (see **Supplementary Methods**). Plants sourced from areas with greater natural area produce smaller flowers than plants sourced from areas with very low natural area (**Figure 4**). This indicates increased investment in floral display in landscapes with a greater proportion agricultural cover as a potential response to compensate for changes in pollinator community assemblage or visitation rate given that flower size has been previously demonstrated to affect visitation by both small bees and syrphid flies in another brassica species (Conner and Rush, 1996). This trait adaptation could result from genetic change or maternal effects.

LAND USE CHANGE AFFECTS INDIRECT DEFENSES, MEDIATED BY NATURAL ENEMY COMMUNITIES' RESPONSES TO LAND USE CHANGE

Natural Enemies Are Likely to Affect Plant Indirect Defenses

Plants express multiple traits that facilitate the host/prey search behavior or residence of natural enemies of herbivores, such as predators or parasitoids. These traits can indirectly increase resistance to herbivory and so potentially function as indirect defenses if the natural enemy attraction also results in relative increases in plant fitness. Experimentally demonstrating the effects of traits associated with natural enemy attraction on plant fitness has been difficult for experimental and biological reasons (Kessler and Heil, 2011). However, increased indirect resistance, the reduced performance or survival of herbivores mediated by plant traits attracting natural enemies, has been demonstrated commonly in multiple study systems (Dicke and Baldwin, 2010). Plant indirect resistance can be categorized into two groups based on the traits mediating it: resource-mediated indirect resistance and information-mediated indirect resistance (Kessler and Heil, 2011). Resource-mediated indirect resistance traits include physical structures (e.g., domatia, hollow stems and thorns) or food provisions (e.g., food bodies, extrafloral nectaries) that facilitate the residence time and abundances of natural enemies on plants (Heil et al., 2001; Llandres et al., 2019). Probably the most prominent example for such resource-mediated indirect defenses are ant plant–ant interactions, where plants provide both shelter, such as

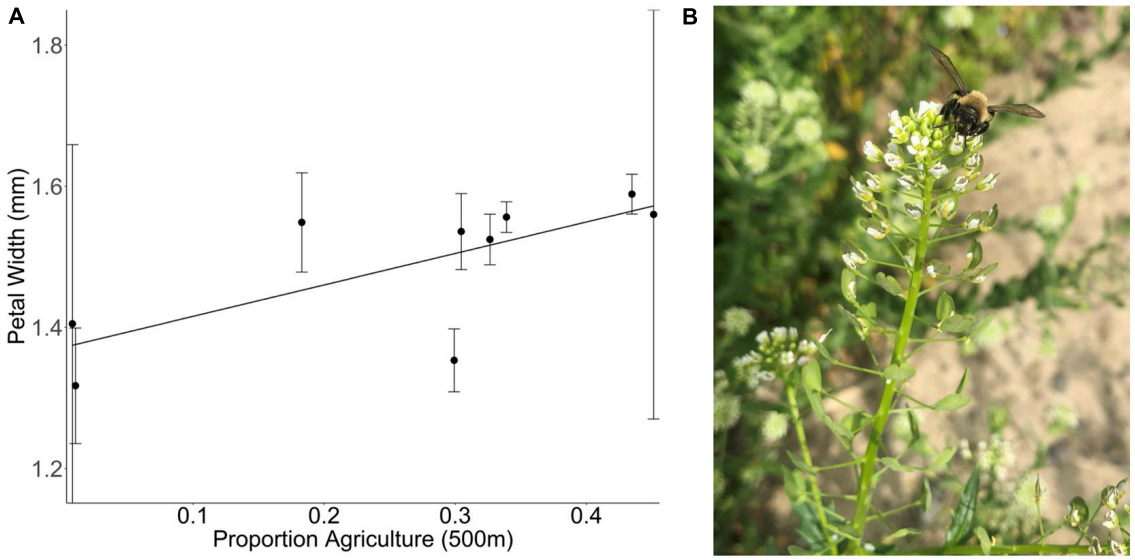

FIGURE 4 | (A) Effect of the proportion of agricultural land at 500 m on the petal width of *Thlaspi arvense* plants. *T. arvense* plants were collected at nine different sites that vary in their landscape complexity [$F_{(1,7)} = 5.5097$, $p = 0.0513$]. The proportion of agricultural cover at a 500 m radius around the collection site is a measure of land use composition but does not provide any information regarding the intensity of the land management practices within any given land use. Mean ± 1 SE values per site are calculated based on the measurement of 157 flowers of 77 different *T. arvense* plants (2–15 plants per site). **(B)** A Melandrena visiting a *Thlaspi arvense* plant in the field.

hollow stems and thorns, as well as food in the form of extrafloral nectaries and nutrient-rich food bodies. This form of indirect defense in an obligate relationship between ants and plants can be so efficient as a defense that the plants apparently lose their direct chemical defenses almost entirely (Heil et al., 1999, 2000; Dyer et al., 2001). This suggests a high functionality and efficiency of resource-mediated indirect defenses. And, indeed, such traits are taxonomically widely distributed in the plant kingdom (Beattie, 1989; Koptur, 1992). Nevertheless, demonstrating that these traits are functional as defenses requires demonstrating plant fitness differences that are associated with differential expression of those plant-derived resources via differential attraction of natural enemies and their effects on herbivory (Kessler and Heil, 2011). Other than in obligate ant plants, most of the natural enemy–plant relationships are facultative and depend on the availability of natural enemies in a given habitat. In one of the very rare cases that natural selection on one of the putative resource-mediated indirect defenses was measured, predatory ants were found as agents of selection on extrafloral nectar production of wild cotton, *Gossypium thurberi*, as nectar production correlated with reduced herbivory and increased plant fitness (Rudgers and Strauss, 2004). While questions about microevolutionary dynamics on resource-mediated indirect resistance traits remain largely open, their study along land use change gradients, is promising as such gradients provide a vast range of variation in herbivory (see above) as well as the availability of natural enemies of herbivores (see below). To our knowledge no such study on landscape-related expression of resource-mediated indirect defense traits and its ecological causes and consequences has been undertaken.

This is certainly similar for information-mediated indirect defenses. By far the best studied group of such traits is the herbivory-induced production of volatile organic compounds. The volatiles emitted from damaged plants can function as chemical information that facilitate the host/prey search behavior of natural enemies (predators or parasitoids) and so, presumably increase predation pressure on herbivores and, consequently, increase plant fitness (Dicke and Baldwin, 2010). It is probably one of the most widely distributed natural phenomena but there has yet to be a study to show evidence for natural selection on inducible volatile emission with predators as the major agents of selection (Kessler and Heil, 2011). Nevertheless, there is overwhelming evidence that differential VOC emission in response to herbivory results in differential attraction of natural enemies (Dicke and Baldwin, 2010). There is also plenty of evidence that this differential attraction has effects on herbivory (Kessler and Heil, 2011), especially when considering predators and egg parasitoids, collectively suggesting high resistance-mediating function on inducible volatile emissions. However, there are only few studies linking differential predator attraction and resulting changes in herbivory to plant fitness (Kost and Heil, 2008; Allison and Hare, 2009). In large part this is due to the fact that VOC-mediated indirect resistance is facultative, thus depending on the availability of natural enemies in the environment and subject to compromising interactions with other organisms in the environment that use the same chemical information. For example, herbivory-induced VOCs in *Brassica oleracea* do not only attract parasitoids, but also their hyperparasitoids, thus fourth-trophic level effects canceling out third-trophic level effects (Poelman et al., 2012). Similarly, the indirect effect of predation on herbivory mediated by the

plant, makes more direct agents of selection on plant inducible VOC emission, such as herbivores and neighboring plants more likely. In a recent study on tall goldenrod, *Solidago altissima*, herbivory was identified as the agent of natural selection on the inducibility of VOC emission. In this case herbivore selection increased the plants' ability to exchange chemical (VOC-mediated) information with their neighbors which allowed for a more even distribution of herbivory in the plant population and thus minimizing the damage each individual plant receives (Kalske et al., 2019). While this study supports the hypothesis that herbivory may be a more direct agent of selection on inducible VOC emission it also demonstrates that there can be strong selection on the inducibility of VOC emission. Thus, although natural enemies may not necessarily be the major agents of natural selection on inducible VOC emission, pronounced alterations in organismal interactions along environmental gradients may have significant consequences for the mean expression of information-mediated indirect defenses.

We do not currently know of any study investigating whether inducible VOC emission varies predictably along environmental gradients, such as those of land use change. The three characteristics of VOC bouquets that can vary are (I) strength of inducibility (e.g., increase in total emission), (II) induced changes in composition (e.g., relative production of certain compounds), and (III) changes in relative similarity of VOC bouquets among plants of the same population (Kessler and Shiojiri, 2016; Junker et al., 2017). The latter had been identified in a *Solidago altissima* study to increase information transfer between plants in populations of elevated herbivory and was interpreted as a convergence of chemical language (Kalske et al., 2019). A similar convergence or increased phenotypic integration was predicted for herbivore-induced VOC emission as functional attractants to predators and parasitoids. Interestingly, this increased phenotypic integration of herbivory-induced VOC emission in comparison to those of unchallenged plants has been found in a meta-analysis across a large number of study systems (Junker et al., 2017). Thus, when searching for signs of natural selection in association with altered herbivory and natural enemy availability in plant populations along environmental gradients, simple measurements of mean differences in inducibility of VOC emission may not be sufficient.

Land Use Change Affects Natural Enemies

Although the direct effect of natural enemies on plant selection remains an area to be studied, there is clear evidence that land use change impacts natural enemy communities (**Table 3**). Land use change will negatively impact natural enemies, when the natural enemies cannot use the surrounding agricultural matrix. The surrounding matrix might not be suitable for natural enemies when it does not provide the resources necessary to support natural enemy populations due to host specialization, herbivore suppression (i.e., pesticide use), or lack of alternative resources such as nectar and pollen (Tscharntke et al., 2016). Additionally, anthropogenic management practices in intensively managed agricultural systems have been shown to reduce natural enemy populations through activities such as tilling, mechanical weed control, and broad spectrum insecticide sprays (Thorbek and Bilde, 2004; Nilsson, 2010; Rusch et al., 2011). Examples of negative effects of land use change have been reported for native lady-beetles in prairies (Werling et al., 2011), for specialist parasitoids of nettle aphids (Rand and Tscharntke, 2007), and for bark beetles and their natural enemies (Ryall and Fahrig, 2005).

Interestingly land use change can differentially affect natural enemies depending on their extent of specialization and invasiveness. For example, in prairie systems in Michigan and Wisconsin, the abundance of native lady-beetles decreased, while the abundance of exotic lady-beetles did not change with increasing annual agricultural cover (Werling et al., 2011). For nettle aphids, specialist parasitoids were reduced by land use change, while generalist predators increased with an increase in the surrounding agricultural matrix (Rand and Tscharntke, 2007).

An increase in natural enemy abundance related to land use change is expected to occur when the surrounding agricultural matrix provides more resources such as hosts, overwintering sites, pollen and nectar to natural enemies than natural habitats (Tscharntke et al., 2016). For example, the irrigation of crops in dry regions or seasons can provide resources to herbivores and natural enemies at times when the natural habitats are inhospitable or have a low productivity (Costamagna et al., 2015).

The scenarios in which natural enemies prefer and thrive in agricultural matrices could lead to permanent or temporarily different outcomes for wild plants. If natural enemies prefer agricultural fields over adjacent natural areas, there could be a reduction in natural enemies and biological control for wild plants. This reduction could be permanent or limited in time if these natural enemies spill back and even increase the number of natural enemies in natural areas. Empirical work has shown that the adjacent crop habitat (Madeira et al., 2016), and management practices such as harvesting (Schneider et al., 2016), mowing and plowing (Thorbek and Bilde, 2004) can increase the movement of natural enemies from agricultural areas back into natural systems. Also, natural enemy traits and the availability of hosts have been shown to affect the intensity of the spillover effects, as was shown by Frost et al. (2015). Another mechanism through which land use change can increase natural enemies in natural systems, is through management practices, such as augmentative biological control. As with herbivores and pollinators, natural enemies introduced through agriculture can disrupt enemy–prey interactions in natural habitats (Snyder and Evans, 2006). However, that disruption can have very different consequences depending on if the natural enemies mostly control the herbivores, or if they reduce the local natural enemy population through intra-guild predation (Snyder and Evans, 2006).

Landscape-mediated increases on natural enemies have been shown to translate into reduction of herbivores (Balzan et al., 2016). For example, in grassland remnants in Nebraska, an increase in the surrounding agricultural land cover led to an increase in the abundance of lady beetles and a higher

TABLE 3 | Effect of land use change (from natural to agricultural or urban areas) on natural enemy communities and populations of wild plant systems.

Mechanism	Outcome and Prediction	References
Management practices	↓ Agricultural crop management such as tillage and insecticide use decreases natural enemy densities and results in lower biological control.	Thorbek and Bilde, 2004; Nilsson, 2010; Rusch et al., 2011
	↑ Agricultural crop management like plowing and mowing can increase predator movement to natural habitats. Also conservation biological control practices (i.e., beetle banks) could increase natural enemy populations.	Thorbek and Bilde, 2004
Habitat loss	↓ When the surrounding agricultural matrix provides no alternative resources (hosts, pollen, and nectar), natural enemy populations are decimated.	
	↑ Natural enemies do not use the landscape matrix causing the enemy population to transiently concentrate on the wild plants, increasing biological control.	
Crops as alternative resources	↓ When the surrounding agricultural matrix provides more resources than the natural habitat, natural enemies will be reduced in wild habitats.	Rand and Louda, 2006; Gladbach et al., 2011
	↑ When the surrounding agricultural matrix provides more resources than the natural habitat, the natural enemy population will increase and spill back to wild populations, increasing natural populations in the natural systems.	Rand and Louda, 2006; Madeira et al., 2016
Introduced natural enemies	↓ When introduced natural enemies, through practices such as augmentative biological control, spill over into the natural systems and lead to intraguild predation, the population of natural enemies will be reduced.	
	↑ When native natural enemies, through practices such as augmentative biological control, spill over into the natural systems and lead to an increase in the population of natural enemies.	

Here we present the direction of the response (↑ = positive effects on herbivores, ↓ = negative effects on herbivores) and the potential mechanisms for that response. When examples from the literature are available that suggest those mechanisms, we cite them under references. Rows without references reflect areas of future work.

suppression of aphids on wild plants (Rand and Louda, 2006). Similarly, in woodland fragment edges adjacent to roads and residential areas pupa parasitism of the holly leaf miner, *Phytomyza ilicis*, was higher than in woodland interiors (McGeoch and Gaston, 2000). This would suggest that natural plants at sites that have experienced land use change might benefit from the presence of natural enemies and could be investing more in indirect defenses to take advantage of the higher abundance of natural enemies in the community.

Land Use Change Affects Plant Indirect Defenses

Although we were not able to find any studies that have directly measured the effect of land use change on plant indirect defenses in natural plant populations, the little evidence we have suggests that there could be differential selection by natural enemies on indirect defense traits. In a study done on cabbage infested with herbivore *Pieris brassicae* placed in different landscapes, it was found that the induction of plant volatiles in response to herbivory can mediate the effect on parasitism (Aartsma et al., 2020). To test this, two different accessions of cabbage were used. One accession was more attractive to the parasitoid *Cotesia glomerata* while the other accession was less attractive to the same parasitoid. The attractiveness is thought to be mediated by differences in herbivore induced plant volatile blends, that have been found to be different between these two accessions (Poelman et al., 2009; Aartsma et al., 2019). As the proportion of arable land cover increased in the landscape (at a 200 m radius) the parasitism rate of the caterpillars increased. The increase in parasitism was stronger for the more attractive variety than for the variety that was less attractive. With low agricultural cover there was no difference in parasitism rate, but with higher agricultural cover, where parasitism was also higher, the attractive variety also had significantly more parasitism. This example shows that parasitoids in different landscapes are responding differently to indirect defense traits on plants, and although the plants used are cultivated plants, we could expect that the effect is similar for natural plant populations. Further work looking at the importance of indirect defense traits in plants along human-made land-use change gradients, would give us more insights into the importance of these effects.

IMPLICATIONS FOR COMPLEX PLANT-MEDIATED INTERACTIONS

Although in this review we have addressed the evolutionary consequences of each insect group on plant traits separately, in reality the landscape context influences all insect groups simultaneously yet not necessarily in the same way. Further, many plant traits mediate interactions across different insect guilds, while also changing how insects interact with each other. These complex relationships determine the outcomes of interactions and thus the effects on plant growth and reproduction. In consequence, the interactor that has the

strongest effect on plant fitness in an isolated two-species interaction may not be the major agent of selection on associated traits. For example, landscape-mediated increases in herbivory and the associated variation in the proportion of plants damaged and induced by one herbivore can make plants more susceptible or more resistant to other herbivores, which would be reflected in the relative compositional changes of the herbivore community (Kessler and Baldwin, 2004; Viswanathan et al., 2005). This can even mean that the presence of a less damaging herbivore that reduces the impact of a more damaging herbivore confers a net fitness benefit to the plant (Kessler and Baldwin, 2004), with the less damaging herbivore becoming a keystone herbivore and the strongest agent of selection on the interaction-mediating plant traits (Poelman and Kessler, 2016).

Different types of defenses can interact with each other and so dramatically affect the outcome of interactions. One of the most cited examples is the interaction of plant endogenous signaling pathways when plants respond to herbivores and pathogens, which in many cases have been shown to elicit conflicting responses (Thaler et al., 2012). When strictly considering interactions among arthropods, direct defenses can function synergistically with indirect defense traits, whereby induced direct resistance can reduce the growth and defensibility of herbivores and so expose them more to natural enemies that are simultaneously attracted by herbivory-induced volatile emissions (Kessler and Baldwin, 2004; Fatouros et al., 2014). In contrast, plant direct defenses can also reduce the survival of natural enemies as they may be directly exposed to defense metabolites on the plant surface or indirectly to plant toxins that have been sequestered by specialist herbivores (Dyer et al., 2001; Ballhorn et al., 2008). In addition, herbivore-induced VOC emissions may not only attract natural enemies of herbivores but also predators and parasitoids of the predators so erasing the indirect defense effect (Poelman et al., 2012).

One of the complex community interactions that can be particularly impactful for plant trait evolution is the plant-mediated interactions between herbivores and pollinators. As pollinators, just like herbivores, consume plant products, they are similarly exposed to plant defenses, which results in a conflict for the plant to attract mutualist pollinators while repelling antagonistic herbivores (Kessler and Chautá, 2020). Mechanisms to overcome this conflict include antagonistic pleiotropy between defense and reproductive traits altering resource allocation into and secondary metabolite concentrations in floral rewards, or shifted flowering phenology (Mothershead and Marquis, 2000; Lucas-Barbosa et al., 2016; Jacobsen and Raguso, 2018). In tobacco, *Nicotiana* spp., species with greater dependency on pollinators produce lower amounts of defense-related alkaloids in comparison to species that do not rely on animal pollination (Adler et al., 2012). In a study with a broader taxonomic scope across the Solanaceae plant family, the phylogenetic shift from self-incompatibility (e.g., strong dependency on animal pollinators) to self-compatibility was associated with decreased constitutive resistance and increased inducibility of resistance (Campbell and Kessler, 2013). Such a linkage between defense and reproductive strategy on the macro-evolutionary level seems to be reflected in microevolutionary dynamics as well. For example, in *Brassica rapa*, exposure to selection by bee pollinators increased mean floral attractiveness. However, the additional presence of herbivores in the population selected for reduced separation of stigmas and anthers (herkogamy) and the associated increase in self-compatibility and autonomous selfing. This suggests that altered selection by herbivores can strongly affect plant defense expression and so the interaction of plants with pollinators (Ramos and Schiestl, 2019). On an ecological scale plant induced responses to herbivory can result in pollinator limitation that can either be compensated for by changes in plant phenology (Schiestl et al., 2014) or mating system (Kessler and Halitschke, 2009) or result in apparent ecological fitness costs (Kessler et al., 2011). In some cases, insects may function as 'pollinating-herbivores,' shifting from larval herbivore to adult pollinator as they progress through life stages (Altermatt and Pearse, 2011). This creates further conflict as plants must defend against damage while also cultivating pollinators and maintaining pollinator attraction (Sharp et al., 2009; Lucas-Barbosa, 2016). Ultimately, as any one insect is affected by land use change, the consequences may cascade through this network of interactions altering how insects interact with plants and each other. Previous work has emphasized the effects of land use change on whole communities, yet insect functional traits are also affected by land use change. In pollinators for example, land use change can decrease body size (Persson and Smith, 2011; Renauld et al., 2016; Grab et al., 2019), which can negatively affect the pollination services provided to plants (Jauker et al., 2016). While landscape mediated functional trait evolution is not well studied, we expect these trait expression shifts are present across insect groups and important in mediating insect interactions with plants. However, the nuances of insect functional trait evolution go beyond the scope of this paper.

Though we have highlighted numerous challenges in disentangling plant-insect interactions across landscapes, we present land use gradients as a unique opportunity to use

BOX 1 | Proposed Future Work.

1. *Link land use change to the evolution of plant traits.* In a single study test all three steps highlighted in this review: (1) land use affects an insect interactor, (2) the interactor affects the plant traits, and (3) land use affects the plant traits via changes in the interactor. These studies would need to be repeated for multiple systems and sites to answer the following question: Are there broad patterns across systems and insect groups?
2. *Eco-evolutionary feedback loops in plant communities:* Determine how changes in plant traits lead to an eco-evolutionary feedback loop between the insect community and the plant community driving change beyond the plant population.
3. *Land use change and plant indirect defenses.* How does land-use change affect plant indirect defenses? Is there conflicting selection on plant indirect and direct defenses or do they function synergistically? What is the role of abiotic factors like pesticides and fertilizer in mediating the relationship between plants and natural enemies?
4. *Land use change and plant population genetics.* How does land use change affect plant populations genetics? To what extent can variation be explained by plasticity and maternal effects? Further, selection experiments along the land-use change gradients are equally important to determine if these changes in traits are adaptive.

modified landscapes as a natural experiment. The magnitude of land use change creates an experimental framework that can be utilized in systems across the globe. Experiments within this framework can reveal the selection pressure exerted by different organisms, the resulting effect on plant fitness, and how the relative importance of each interactor changes with landscape mediated changes in insect community composition. We are not aware of any one study that simultaneously examines the following three steps linking land use change to the evolution of plant traits via some insect interactor: (1) land use affects the interactor, (2) the interactor affects the plant traits, and (3) land use affects the plant traits via changes in the interactor. While descriptive studies are a crucial starting point, selection experiments are needed to establish a causal link and to test the role of maternal effects. We propose artificial selection experiments in which individuals from the extreme ends of the land use gradient are crossed and the offspring self-pollinated to segregate traits. The large variation in traits of these F2 populations can be used to evaluate which traits are selected for in given environments and which insect interactors are likely important agents of selection. Pollinator and herbivore exclusion treatments would be necessary to disentangle the role of each group. Further, abiotic factors associated with land use change such as pesticide and fertilizer runoff are also known to impact wild plant communities (Didham et al., 2015; Botías et al., 2016). These factors may act as additive or conflicting forces with insect-imposed selection and thus are also important to quantify. We present what we consider the most important questions we think should be resolved next to better understand plant-insect evolutionary dynamics (**Box 1**).

FINAL CONCLUSION

The conversion of natural habitats to human-modified landscapes disrupts the stable species interactions that evolved in these landscapes. Unlike natural disturbances where succession restores many pre-disturbance species interactions, human-modified landscapes are maintained in a modified state. Through this review and our own results, we have collected evidence suggesting that altered communities of pollinators, herbivores, and natural enemies occupy these modified landscapes and that each of these insect groups can act as a selective force. Thus, we hypothesize that wild plants are experiencing differential selection based on landscape context through interactions with the resulting community of insects. Further studies are needed to examine the outcomes of landscape-mediated selection on individual plant traits and ultimately to understand the consequences for wild plant communities on a broader scale.

Differential selection on plant traits across the landscape likely has cascading consequences for population and community dynamics driven by evo-eco feedback loops. In each landscape context, the availability of insect interactors depends on whether an insect is able to utilize the modified habitat. Plant trait evolution and changing plant community and population dynamics, in turn, will affect which members of the insect community remain or are added to interact with the plant community. Selection on traits that increase or decrease the attractiveness of a plant population to herbivores and/or pollinators may impact the entire plant community, potentially altering the insect community drawn to a patch and the relative competitive ability of plants within the patch. This may create a feedback loop where plant adaptation reinforces traits in the insect community and patterns of insect availability. There is already evidence that human-mediated land use change is linked to trait filtering in plants and insects, and selection by insects may be one additional mechanism through which this trait filtering is occurring in plants (Gámez-Virués et al., 2015; Mendes et al., 2016; Boukili and Chazdon, 2017).

Through this review we hope to motivate further studies examining the potential for wild plants to adapt with land use change, the outcomes of conflicting selection, and the ultimate consequences for plant fitness and community structure. Future work should examine plant population genetics to establish if observed phenotypic changes represent plasticity or genetic change. Additionally, while many studies suggest the mechanisms by which land use change affects natural plant communities, few actually test these claims. Of all the patterns examined here, the effects of land use change on indirect defenses remains the least studied (**Box 1**). Given that human-modified habitats dominate the global landscape, understanding how wild plants in these landscapes are adapting and how their communities are changing could help inform which species are the most vulnerable. Human-initiated land use change represents an unintended experiment in plant evolution on a global scale, creating opportunity to expand basic research in plant adaptation and evo-eco dynamics, and potentially guide conservation action.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.5061/dryad.ncjsxkst4. The data is uploaded to dryad but not publicly available while under review.

AUTHOR CONTRIBUTIONS

KP and AK conceived the idea of the manuscript. HG and KP collected the data. HS and HG analyzed the data. HS drafted the manuscript to which all authors contributed sections and revisions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.592881/full#supplementary-material

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Flower Production, Headspace Volatiles, Pollen Nutrients, and Florivory in *Tanacetum vulgare* Chemotypes

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Floral volatiles and reward traits are major drivers for the behavior of mutualistic as well as antagonistic flower visitors, i.e., pollinators and florivores. These floral traits differ tremendously between species, but intraspecific differences and their consequences on organism interactions remain largely unknown. Floral volatile compounds, such as terpenoids, function as cues to advertise rewards to pollinators, but should at the same time also repel florivores. The reward composition, e.g., protein and lipid contents in pollen, differs between individuals of distinct plant families. Whether the nutritional value of rewards within the same plant species is linked to their chemotypes, which differ in their pattern of specialized metabolites, has yet not been investigated. In the present study, we compared *Tanacetum vulgare* plants of five terpenoid chemotypes with regard to flower production, floral headspace volatiles, pollen macronutrient and terpenoid content, and floral attractiveness to florivorous beetles. Our analyses revealed remarkable differences between the chemotypes in the amount and diameter of flower heads, duration of bloom period, and pollen nutritional quality. The floral headspace composition of pollen-producing mature flowers, but not of premature flowers, was correlated to that of pollen and leaves in the same plant individual. For two chemotypes, florivorous beetles discriminated between the scent of mature and premature flower heads and preferred the latter. In semi-field experiments, the abundance of florivorous beetles and flower tissue miners differed between *T. vulgare* chemotypes. Moreover, the scent environment affected the choice and beetles were more abundant in homogenous plots composed of one single chemotype than in plots with different neighboring chemotypes. In conclusion, flower production, floral metabolic composition and pollen quality varied to a remarkable extend within the species *T. vulgare,* and the attractiveness of floral scent differed also intra-individually with floral ontogeny. We found evidence for a trade-off between pollen lipid content and pollen amount on a per-plant-level. Our study highlights that chemotypes which are more susceptible to florivory are less attacked when they grow in the neighborhood of other chemotypes and thus gain a benefit from high overall chemodiversity.

Keywords: terpenoids, gas chromatography-mass spectrometry (GC-MS), Asteraceae, protein:lipid-ratio, insect behavior, Phalacridae, chemodiversity

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INTRODUCTION

Outcrossing plants attract pollinators by various traits such as color, odor and nutritious reward traits, i.e., nectar and pollen. However, these rewards are likewise explored by florivores that consume pollen and floral tissues prior to seed maturity (McCall and Irwin, 2006), which may lead to contrasting selection pressures (Theis and Lerdau, 2003; Schiestl, 2015) and thus potentially also high variation. Indeed, the extent to which the composition and concentrations of primary metabolites such as amino acids and sugars within reward traits and specialized metabolites (i.e., compounds that are often restricted to certain plant taxa, formally known as secondary metabolites) such as headspace volatile compounds (Knudsen et al., 2006) and pigments within floral tissues (Harborne and Smith, 1972) differ across plant species is remarkable (Borghi et al., 2017). For example, more than 1700 volatile chemicals have been identified from floral headspace samples of different plant species (Knudsen et al., 2006), with terpenoids and benzenoids occurring most frequently (Knudsen et al., 2006; Farré-Armengol et al., 2015, 2020). The species-specific floral scent bouquet is in the majority of cases an attractant, but not always an honest signal for the presence of reward traits such as nectar and/or pollen (Knauer and Schiestl, 2015) and enables flower visitors to identify their host flowers. While variation in metabolic composition between plant species is well described, little is known about how this chemicals vary among individuals within species and affect the individual floral scent, reward trait composition and thus also the host finding ability of flower visitors.

Plants are bound to attract pollinators while repelling herbivores by adapting visual, olfactory cues and reward trait composition to avoid excess pollen removal by pollen-feeders (Hargreaves et al., 2009; Junker, 2016). One commonly described adaptation to avoid pollen robbery is the concurrent and fine-tuned provision of attractive combined with defensive components (Irwin et al., 2014), such as specific repellents against generalist florivores (Kessler et al., 2013). The latter study found that *Petunia* flowers emit a complex blend containing, amongst other compounds, isoeugenol and benzyl benzoate which deter florivores and methyl benzoate which attracts florivores and pollinators. In general, (mono-)terpenoids are evolutionarily regarded as antagonist-defensive compounds (Junker and Blüthgen, 2010; Junker et al., 2010; Schiestl, 2010). However, particularly terpenoids are highly diverse in function and the same compound may attract one animal but repel another, depending on context and released amount by the flowers (Raguso, 2016). Additionally, even within an individual plant, floral scent components show spatial and temporal variation. The scent bouquet may differ between flower parts (MacTavish et al., 2000; Dötterl and Jürgens, 2005; Flamini et al., 2007), may change after successful pollination (Tollsten and Bergström, 1989; Negre et al., 2003), after herbivory (Hoffmeister et al., 2015; Hoffmeister and Junker, 2017), with the time of day (Theis et al., 2007; Martel et al., 2019), or with floral ontogeny (reviewed in Piechulla and Effmert, 2010).

Apart from floral scent emission, pollen production is essential for sexual reproduction in both self-incompatible and animal-pollinated plants but also resource demanding, as flower visitors often remove huge amounts (Schlindwein et al., 2005; Müller et al., 2006). Chemically, pollen contains higher levels of proteins, lipids, vitamins, and minerals than nectar and various flower visitors are obligate pollen feeders (Nicolson, 2011). The plant's phylogenetic background and pollinator dependency shape the composition of pollen proteins and lipids, which differ greatly between plant families (Vaudo et al., 2020). Pollinators can adapt their foraging strategies to the macronutrient composition of pollen, namely the protein:lipid-ratio (Vaudo et al., 2016). In addition, pollen of various plant species also contains toxins (Rivest and Forrest, 2020), but the extent of intraspecific variation in the composition of pollen macronutrients and (toxic) specialized metabolites remains largely unknown.

The chemical ecology of Asteraceae pollen is particularly interesting. Species within Asteraceae produce comparatively high amounts of pollen per floral unit (Hicks et al., 2016) and pollinator specialization to Asteraceae pollen occurs in multiple bee lineages (Müller and Kuhlmann, 2008; Praz et al., 2008a; Cane, 2017). Paradoxically, pollen of many Asteraceae is poor in proteins (Nicolson and Human, 2013; Vaudo et al., 2020), harmful to bees (Schmidt et al., 1987; Tasei and Aupinel, 2008; Vanderplanck et al., 2020), and even Asteraceae-specialists may suffer from feeding on Asteraceae pollen from a single plant species (here: *Tanacetum vulgare;* Praz et al., 2008b).

In the present study, we investigated the intraspecific variation in flower traits of *Tanacetum vulgare* (common tansy, Asteraceae) and effects on florivores. This plant species contains an exceptional intraspecific chemodiversity (i.e., expression of various different monoterpenoid-chemotypes) in the leaves (Wolf et al., 2012a; Kleine and Müller, 2013; Clancy et al., 2018) and flowers (Gabel et al., 1992). In natural populations, mono-chemotypes, i.e., with one dominant terpenoid of more than 50% relative abundance of the overall terpenoid content, co-occur with plants of mixed chemotypes (Kleine and Müller, 2011). *Tanacetum vulgare* is self-incompatible and highly dependent on pollinators for seed production, which are rewarded with pollen (Lokki et al., 1973). Florivorous beetles of the genus Phalacridae (shining flower beetles, i.e., *Olibrus* spp.) commonly occur on *T. vulgare* inflorescences (Schmitz, 1998, own personal observation). Adult *Olibrus* beetles feed on pollen and oviposit in the flower heads, and the larvae consume unripe seeds (Müller-Schärer and Brown, 1995). Foliar herbivores such as beetles (Wolf et al., 2012b) or aphids (Völkl et al., 1999; Kleine and Müller, 2011; Benedek et al., 2015) prefer certain chemotypes and plant parts (Jakobs and Müller, 2018, 2019; Jakobs et al., 2019). However, the effects of intraspecific chemodiversity in *T. vulgare* on flower-visiting insects have yet not been elucidated.

We hypothesized that *T. vulgare* plants express chemotype-specific patterns of volatile organic compounds in the floral and pollen headspace, which differ in compound diversity, i.e., chemodiversity. Individuals with a high floral headspace chemodiversity were expected to produce lower pollen amounts, due to higher enzyme costs (Gershenzon, 1994). Furthermore, we expected the contents of pollen macronutrients to differ between chemotypes and trade-off with the blooming duration and the amount of pollen per plant individual, due to resource

limitations. Emission of attractive cues for (pollinating) insects is only relevant for plants during anthesis. In contrast, premature flowers should comprise a non-attractive or even deterrent scent bouquet to fight off florivores, which are present at all floral developmental stages. We therefore hypothesized that within-plant composition of volatile compounds changes with floral ontogeny and thus that florivorous beetles prefer volatile cues of mature, pollen-producing flower heads to those of unripe flower heads. These hypotheses were tested in laboratory experiments. In addition, we expected that *T. vulgare* chemotypes vary in susceptibility to florivores under semi-field conditions when grown in homogenous stands of the same chemotype. These differences in susceptibility should decrease in heterogenous stands comprising plants of different chemotypes, because of overlapping odor plumes, and thus lower concentrations and potential masking of single repellent or deterrent compounds and/or a higher overall chemodiversity (Riffell et al., 2014; Cai et al., 2017).

MATERIALS AND METHODS

Plant Cultivation, Chemotype Determination and Selection

For semi-field experiments, *T. vulgare* seeds were collected in November 2011 close to the experimental site in Bielefeld Ummeln (Germany, 51°58′58.52′N, 8°27′12.27′E, elevation 104 m; see section "Semi-Field Experiment: Florivore Abundance and Flower Head Miner Counts"). For laboratory and greenhouse experiments, seeds were collected in January 2019 at the same site and three additional sites nearby (51°58′51.8′N, 8°27′40.0′E; 51°58′59.3′N, 8°28′13.8′E; 51°58′42.2′N 8°28′35.5′E; elevation 98–105 m). The area of each collection site was <1 km^2 to ensure comparable local climate conditions and the distance between plants was maximized to reduce genetic, and thus chemotypic, similarity of seeds (see Lokki et al., 1973, for a chemotypic analysis of chemotype crossings). In both years, seeds of at least 10 mother plants per site were germinated 1–6 weeks after seed collection on glass beads and seedlings were transferred to pots filled with a 2:1 mixture of sterilized potting soil and river sand. Four weeks after germination, the offspring (F1) plants were in each case transferred to larger pots with the same substrate as in Jakobs et al. (2019).

Chemotypes were defined as mono-chemotypes based on the most abundant terpenoids and high similarity in satellite compounds, i.e., terpenoids present in lower abundance. Chemotype determination of all plants was conducted by the same procedure: Leaflets of the second oldest leaf of a 6–8 weeks old plant were frozen in liquid nitrogen, lyophilized and extracted by homogenization in 1 mL *n*-heptane (99% HPLC grade, Roth, D) containing 50 ng μL^{-1} 1-bromodecane (97% GC-MS grade, Sigma Aldrich, D) as internal standard with a stainless steel ball (Ø 5 mm) for 30 s at 30 Hz in a mixer mill (MM 301, Retsch, D), followed by ultrasonic bathing at 20°C for 15 min (RK 100 H, Bandelin Sonorex, D) and centrifugation at 13200 rpm, 20°C for 5 min (modified after Kleine and Müller, 2011; Wolf et al., 2012a; Jakobs and Müller, 2018). The supernatants were analyzed using gas chromatography coupled with mass spectrometry (GC-MS; see section "Measurements by GC-MS"). For the semi-field experiments in 2012, four mono-chemotypes of high abundance in the field were chosen: β-thujone, *(E)*-carvyl acetate, (Z)-chrysanthenyl acetate and camphor. For the laboratory and greenhouse experiments in 2019 and 2020, the two most common mono-chemotypes (β-thujone and artemisia ketone), and the three most common mixed-chemotypes were chosen, which contained $\geq$ 50% of all terpenoids of α- and β-thujone, artemisia ketone and artemisia acetate, and myroxide, artemisia acetate and santolina triene. In general, vegetative propagated plants of *T. vulgare* maintain a stable leaf terpenoid composition (Clancy et al., 2018, personal observations). Similarly, the monoterpenoids with highest compound abundance in leaf extracts had also the highest abundance in flower head extracts, so that the leaf chemotypes corresponded to flower chemotypes. This was verified for a random sample of 20 plants from the greenhouse, i.e., four of each chemotype. All plants were grown in the greenhouse at 21°C and 16 h:8 h, light:dark, fertilized weekly (modified solution after Arnon and Hoagland, 1940) and transferred after chemotype determination either to a greenhouse chamber with equal conditions or to the field site.

Measurements of Flower and Pollen Traits

The greenhouse-grown plants flowered over a period of 4 months with a blooming period between 2 and 4 weeks. We defined flower heads, i.e., capitula, as premature once the buds were open but pollen was not yet produced and as mature when pollen was visible but flowers were not senescent (Dupont et al., 2018). Thrice a week, premature and mature flower heads were counted for each plant ($N = 150$, 30 plants per chemotype). Additionally, the total number of flower heads, the diameter of flower heads (mm), the onset and duration of the blooming period (day number, where day 0 was defined as the day of the first observed flowering) as well as the amount of pollen per day (mg) and the total amount of pollen (mg) were determined over the whole blooming period for all flowering plants. Per chemotype, 9–16 plants were sampled from the beginning of flowering to complete withering. Pollen of remaining late-flowering plants as well as pollen of plants that showed a second blooming period were not considered for analyses but used for the florivore choice bioassay (see section "Florivorous Beetles and Four-Field Olfactometer Choice Assay"). As central and lateral flower heads within one inflorescence may differ, e.g., in the probability of setting fruit (Guitián and Navarro, 1996), pollen was pooled from all flower heads with dehisced anthers per plant. For pollen collection, the flower heads were gently shaken over a piece of weighing paper, from which the pollen was transferred to a centrifuge tube and kept on ice. The pollen was weighted within 1 h and stored at −80°C for 2–6 weeks.

Protocols after Roulston et al. (2000); Laurentin and Edwards (2003), and Vaudo et al. (2016) were modified to extract and quantify protein, carbohydrate and lipid contents (i.e., macronutrients) in microtiter plate–photometer assays. Pollen samples were dried for 24 h at 36°C and weighted again to

determine the water content. Proteins were extracted from 1 mg dried pollen in 500 μL cold 0.1 mol L^{-1} NaOH by homogenizing the pollen, followed by 15 min ultrasonic bath incubation and centrifugation for 5 min at 13400 rpm. Supernatants were 1:5 diluted in 0.1 mol/L NaOH, and 30 μL were combined with 200 μL Coomassie Brilliant Blue G-250 (AppliChem GmbH, D) in triplicates for each sample in a microtiter plate. Absorbance was measured at 595 nm in a plate reader (Thermo Scientific, United States), using a bovine serum albumin (Roth, D) concentration series as standard (Bradford, 1976). We extracted carbohydrates and lipids together from 2 mg dried pollen samples by homogenization in 200 μL 2% Na_2SO_4. After adding 1 mL of chloroform:methanol (1:1 v:v), samples were placed for 15 min in an ultrasonic bath and then centrifuged for 5 min at 13,400 rpm. Water (375 μL) was added to the supernatants (each approximately 400 μL) and samples were vortexed and centrifuged again to obtain two phases, which were separated. We doubly concentrated the upper methanol/water phase (approximately 400 μL) containing the carbohydrates under vacuum at 60°C. As carbohydrate standard, a glucose (Roth, D) concentration series in water was used. Of each carbohydrate sample and glucose standard, triplicates of 40 μL were each combined with 100 μL 2 g L^{-1} anthrone in 98% sulfuric acid in microtiter plates (Immulon 4, Dynatech Laboratories Ltd., United Kingdom), foil-sealed, shaken for 20 s in the photometer and incubated for 3 min at 92°C. The samples were left for 5 min at room temperature, incubated for 15 min at 45°C and absorbance was measured at 595 nm. As standard for lipids, a linseed oil (Naturwert Bio, D) concentration series in chloroform was used. Lipid samples (chloroform phase, approximately 165 μL) and equal volumes of linseed oil standards were dried under vacuum at 60°C and each resolved in 200 μL 98% sulfuric acid by vortexing for 10 s. Of each sample and standard, 50 μL triplicates were applied on microtiter plates, sealed, and incubated for 12 min at 92°C. After a water bath for 5 min at 18°C, plates were unsealed and 100 μL of 400 μg mL^{-1} vanillin in 34% phosphoric acid was added to each well. Then, samples were left for 10 min at room temperature and the absorbance was measured at 570 nm. For pollen terpenoid analyses, 15 ± 5 mg fresh pollen per plant was solved in 100 μL of *n*-hexane containing 50 ng μL^{-1} 1-bromodecane as internal standard and shaken at 350 rpm for 60 min at 20°C (modified after Wiese et al., 2018), then centrifuged at 13200 rpm, 20°C for 5 min and the supernatant was transferred into glass vials for analysis of compounds with GC-MS (see "Measurements by GC-MS").

Floral Headspace Volatile Collection

Floral headspace volatile organic compounds (VOC) were trapped on absorbent polydimethylsiloxane (PDMS) tubes, followed by analysis *via* thermal desorption (TD)-GC-MS (Kallenbach et al., 2014, 2015). One headspace VOC collection trial per week was performed from April to May 2020 on sunny days for 3 h between 11 am and 2 pm. Two to three plants of each chemotype with premature and mature (i.e., pollen presenting) flower heads were selected for each trial and placed 50 cm distant to one another in the greenhouse. The number of premature and mature flower heads (i.e., without and with pollen) was counted. Of each plant, 2–3 intact premature and 2–3 mature flower heads, of which no pollen had been harvested yet, were selected for VOC collection. The diameter of these flower heads was measured in each case to later normalize the measured compound abundances to the summarized flower head surface enclosed in VOC collection units (**Supplementary Figure S1**). The units were polypropylene cups of 50 mL volume (Premium Line, Tedeco-Gizeh, D), which were fixed with steel wire on wooden sticks at the exterior of the respective plant pots. The units were closed with respective lids containing holes of Ø 15 mm, through which the selected flower heads were gently threaded. The holes prevented heating and waterlogging. Two VOC collection units were fixed on each plant individual, one for premature and one for pollen-producing flower heads. In addition, two collection units per trial were fixed on plant pots containing humidified substrate and served as control to identify contaminants originating from the pots, substrate or the setup. Prior to usage, the absorbent PDMS tubes (length 5 mm, external diameter 1.8 mm, internal diameter 1 mm; Carl Roth, D) were cleaned in 4:1 (v:v) acetonitrile:methanol and then heated to 230°C for 20 min in a helium flow of 60 mL min^{-1} (Kallenbach et al., 2014, 2015). Two PDMS tubes were carefully added to each VOC collection unit and remained in the floral headspace for 3 h. Afterward, the PDMS tubes were gently removed from the units and stored in separate air-sealed glass vials (1.5 mL) with polytetrafluoroethylene (PTFE) – inlet screw caps and sealed with PTFE tape at −20°C for max. 2 weeks until GC-MS analyses (see section "Measurements by GC-MS").

Measurements by GC-MS

Samples were analyzed by GC-MS (GC 2010plus – MS QP2020, Shimadzu, JP) in electron impact ionization mode, using a VF-5 MS column (30 m × 0.2 mm ID, 10 m guard column, Varian, United States) and helium as carrier gas. The GC settings were adjusted to maximize the resolution for the three different sample types. For heptane extracts of leaf tissue for chemotype determination, the column flow was 1.5 mL min^{-1}. The GC-injection port was kept at 240°C and operated in a 10:1 split mode. The GC oven program started at 50°C for 5 min and increased to 280°C with 5°C min^{-1}, which was hold for 5 min (total run duration 25 min). For hexane extracts of pollen for determination of terpenoid composition, the column flow was set to 1.5 mL min^{-1}. The GC temperature program started at 50°C for 5 min and increased to 250°C at a rate of 10°C min^{-1} and to 300°C at a rate of 30°C min^{-1} with a hold time of 10 min (total run duration 37 min). Relative concentrations of terpenoids in leaf extracts and pollen washes were calculated by converting the obtained peak areas to sample dry weights and by normalizing the sample to the internal standard peak area (1-bromodecane). Floral headspace VOC were analyzed via TD on the same instrument (TD 30 – GC 2010plus – MS QP2020, Shimadzu, JP). Trapped VOC were desorbed from PDMS tubes at 230°C under a flow of 60 mL min^{-1} and adsorbed on a Tenax® cryo-trap with a temperature of –20°C for 8 min. From the cryo-trap, the compounds were re-desorbed at 250°C for 3 min, transferred to the GC at 250°C in a 1:1 split mode,

and migrated with a column flow of 1.6 mL min^{-1}. The GC temperature program started at 50°C for 5 min and increased to 250°C at a rate of 10°C min^{-1} and to 280°C at a rate of 30°C min^{-1} with a hold time of 3 min (total run duration 29 min). For all GC-MS samples line spectra (30 – 400 m/z) of separated compounds were acquired in quadrupole MS mode. An alkane standard mix (C8–C20, Sigma Aldrich, D) was analyzed with the same respective method like the samples in order to calculate Kovats retention indices (KI) for targeted compounds (Kováts, 1958). Compounds were identified by comparing the RI and mass spectra with those of synthetic reference compounds, where available, with library entries of the National Institute of Standards and Technology NIST 2014, Pherobase (El-Sayed, 2012), and mass spectra reported in Adams (2007). Compound quantification was based on the total ion chromatogram of peaks. For floral headspace samples, control samples (VOC collection units without flowers), and blanks (cleaned PDMS tubes) were used to identify and subtract contaminations.

Florivorous Beetles and Four-Field Olfactometer Choice Assay

Olibrus spp. (Phalacridae) beetles occur very frequently in Northern Germany on yellow flowering Asteraceae (Müller-Schärer and Brown, 1995). For bioassays, *Olibrus* spp. were collected from *T. vulgare* and *Taraxacum officinale* flower heads in August and September 2019 and April to June 2020 from two sites. The first site was close to Gütersloh (Germany, 51°52′45.2′N, 8°17′04.6′E, elevation 68 m), the second close to Bielefeld University (Germany, 52°03′07.7′N, 8°28′43.0′E, elevation 105 m). The beetles were kept in boxes with mesh lids and fed with purchased pollen (Buxtrade, D). *Olibrus* spp. determination to species-level is difficult and beetles are thereby harmed. The species was hence determined on a subset of $\geq N = 15$ beetles from each site after finishing the bioassays following the protocol described in Freude et al. (1967) and individuals could be assigned to two species, *Olibrus affinis* (Sturm, 1807) (Gütersloh approximately 67%, Bielefeld approximately 47%) and *Olibrus millefolii* (Paykull, 1800) (approximately 33 and 53%, respectively). Four premature and four mature flower heads were cut from greenhouse-grown plants (see section "Plant Cultivation, Chemotype Determination and Selection") for the florivore choice bioassays and placed upright on ice in Parafilm-sealed Petri dishes lined with moist filter paper. Four-field olfactometers with 20 cm diameter and 4 cm height were used (**Supplementary Figure S2**, Kühnle and Müller, 2009). In each trial, one single premature and one mature flower head were placed in opposing olfactometer fields, each in a 2 cm diameter PET ring, covered with moist filter paper to exclude visual cues. The remaining two fields received empty PET rings with moist filter paper and served as control. Four different beetles were tested for four different flower head combinations of the same plant, placed alternating in all four olfactometer fields to account for bias due to position preferences. In each trial, a single adult beetle was placed on a permeable mesh surface above the test fields, which was covered with a glass pane about 1 cm above the mesh. The beetle's position (i.e., olfactometer field) was recorded every 10 s for 3 min. Non-responders, i.e., beetles that did not move at all within the 180 s of a trial, were excluded. Data for the four tested beetles per plant individual were averaged prior to statistical analyses and beetles were only used once per trial.

Semi-Field Experiment: Florivore Abundance and Flower Head Miner Counts

Abundance of florivorous beetles on *T. vulgare* flower heads was observed in August 2012 and flower head miners were counted in November 2013 in the same semi-field experiment in Bielefeld Ummeln (see section "Plant Cultivation, Chemotype Determination and Selection"), which was planted in spring 2012. The field experiment site measured 6 × 55 m and was embedded within a meadow of extensively used agricultural grassland, surrounded by deciduous trees and shrubs. Spacing blocks of 6 × 11 m without intentionally planted *T. vulgare* divided the field into three blocks. Each block measured 6 × 11 m and contained four homogenous plots of each of four chemotypes and four heterogenous plots each containing all four chemotypes, i.e., eight plots of 1.5 × 1.5 m^2 per block in total with randomly allocated individuals (see section "Measurements of Flower and Pollen Traits" for details). Heterogenous plots contained two plants of each of the four chemotypes. The chemotypes were β-thujone, *(E)*-carvyl acetate, (Z)-chrysanthenyl acetate and camphor (see section "Plant Cultivation, Chemotype Determination and Selection"). The eight plants in each plot had equal distance to each other and a minimum distance of 1 m to another plot or the site border. Sampling of adult florivores on flower heads was performed between 11 am and 2 pm on eleven days in August 2012. Each plant was observed for 2 min, avoiding casting a shadow on the observed plant. Example specimen were collected for determination to the species level (Chinery, 2004; Harde and Severa, 2006; Brohmer and Schäfer, 2010). Only a subset of *Olibrus* spp. individuals was sampled for determination and most specimens were scored in the field. Flower head infestation with miners was assessed in November 2013 on 10 flower heads of four plants per homogenous plot which were randomly chosen, resulting in a sample size of 120 flower heads for each of the four chemotypes. For this purpose, the seeds of the chosen flower heads were removed by hand and infested flower heads were counted.

Statistical Analyses

The software 'R' version 4.0.3 (R Core Team, 2020) was used for statistical analyses. The number of produced flower heads per plant during the entire blooming period and the duration of the blooming period were analyzed with generalized linear mixed-effects models (GLMM) using a log-link Poisson distribution. To test for overdispersion, the dispersion of simulated residuals was compared to the observed residuals (function 'simulateResiduals' in package 'DHARMa' by Hartig, 2020). The amount of pollen per plant produced during the entire blooming period, the diameter of flower heads, pollen water content, and macronutrient contents (protein, carbohydrate, lipid, P:L-ratio) were analyzed with linear mixed-effects models (LMM) using an identity-link

Gaussian distribution. Chemotype, the amount of pollen (if not used as response variable), and their interaction was included as fixed factors. The chemodiversity of compounds in the floral headspace and from pollen surface washes was assessed by both the compound richness (number) and calculating the Shannon index (Shannon and Weaver, 1949; function 'diversity' in package 'vegan' by Oksanen et al., 2015). The Shannon index is defined as H = -sum pi log(b) pi, where pi is in our case the proportional abundance of a compound i and b is the base of the logarithm. The Shannon-diversity was analyzed with an LMM using an identity-link Gaussian distribution and the compound richness (number) was analyzed using a GLMM with log-link Poisson distribution. All models included maternal genotype and the onset of blooming as random effects. We fitted the models with a maximum likelihood approach and applied step-wise backward model selection to obtain the minimal adequate model. Fixed effect terms with $P < 0.05$ were removed based on likelihood ratio tests (LRT). All (G)LMMs were performed using the (g)lmer function from the 'lme4' package (Bates et al., 2015).

Flower and pollen traits were correlated using Spearman's rank correlation. Correlations between compound patterns of leaf extracts, pollen washes and floral volatile headspace samples were calculated by Mantel tests. For these analyses the dataset was reduced to those plant individuals of which all data were available ($N = 28$ plants) and the four datasets were transformed by Wisconsin double standardization, using the vegan package. Mantel tests were performed based on pairwise Kulczynski distances. Permutations were performed 10,000 times and Spearman rank correlations were used to compute Mantel's r and P-values. Terpenoid profiles were compared between chemotypes within different types of samples (i.e., floral headspace, pollen, leaves) by using unsupervised Random Forest (RF) models (packages 'randomForest' and 'party' by Breiman, 2001). For each RF classification tree (the number of RF trees = iterations was set to 10,000), nine randomly selected variables were accepted as candidates at each split (mtry was set to 9 = approx. square root of the number of variables, i.e., the 82 compounds found across all samples). Multi-dimensional scaling of proximity matrix (MDS) plots were used to display RF model results. In addition, the compound abundances (normalized peak areas) detected in pollen washes were visualized as a heatmap (function 'heatmap.2' in package 'gplots' by Warnes et al., 2009).

The duration of stay above fields in the four-field-olfactometers (average percentage) of *Olibrus* spp. florivorous beetles were compared between flower types (=fixed factor) by LMM with identity-link Gaussian distribution within each of the chemotypes. More precisely, the proportional duration of stay above fields in the four-field-olfactometers to the headspace of premature flower heads (without pollen), mature flower heads (post anthesis, prior senescence) and control fields (without flower heads) was measured on four different beetles for each plant individual and the four observations per plant were averaged before LMM analyses. The plant individual was included as random factor in these models. Data were normally distributed, thus no data transformation was applied. Florivore abundance in semi-field experiments was compared between chemotypes (first fixed factor) in two plot types (second fixed factor: homogenous or heterogenous) and the interaction between these factors was included in the model. Floral head miner abundance in semi-field experiments was only counted in homogenous plots; thus, the chemotype was included as only fixed factor in these models. For both field-collected datasets GLMM with Poisson distribution were used and plot nested in block were included as random factors. Also for these models, backward model selection based on LRT was applied.

RESULTS

Flower Traits, Bloom Period and Pollen Macronutrient Composition

Greenhouse-grown plants of the five *T. vulgare* chemotypes differed significantly in the number of flower heads per individual over the entire blooming period, in the diameter of individual floral heads and in the duration of the blooming period (**Tables 1**, **2** and **Figure 1**). The myroxide mix-chemotype produced most flower heads but had smaller flowers compared to the other chemotypes. In contrast, the artemisia ketone mono-chemotype had fewer but larger flowers over a relatively short period. However, the number of flower heads was not correlated with floral diameter across all chemotypes (Spearman's rank correlation, $P = 0.53$, parameter estimate $= -0.12$, $N = 29$). The bloom period was shortest in the artemisia acetate mix-chemotype and on average 40% longer for the α,β-thujone mix-chemotype. The amount of pollen per plant individual over the entire blooming period did not differ significantly between the five tested chemotypes. The macronutrient composition of pollen differed between chemotypes (**Tables 1**, **3** and **Figure 1**). There was a significant interaction of the factors chemotype and amount of pollen with the pollen protein content. On average, the pollen protein content was highest in the β-thujone mono-chemotype and lowest in the artemisia ketone mono-chemotype. Pollen of the chemotype α,β-thujone mix-chemotype had the highest protein-to-lipid (P:L) ratio, while pollen of the β-thujone mono-chemotype had the lowest P:L-ratio. Water, carbohydrate and lipid contents were not significantly different in the five tested chemotypes. The percentage of carbohydrates in pollen was negatively correlated with the duration of blooming period (Spearman's rank correlation, $P = 0.031$, parameter estimate $= -0.08$, $N = 44$, **Figure 2A**). Pollen of later blooming flower heads contained higher proportions of protein (Spearman's rank correlation, $P = 0.003$, parameter estimate $= 0.06$, $N = 44$, **Figure 2B**). Furthermore, the percentage of lipids in pollen was negatively correlated with the amount of pollen by each plant individual over the entire blooming period ($P = 0.05$, parameter estimate $= -0.02$, $N = 44$, **Figure 2C**). The number of detected compounds in pollen surface washes was not correlated with timing of flower onset ($P = 0.77$) but with pollen lipid content ($P = 0.01$, parameter estimate $= 1.54$, $N = 11$, **Figure 2D**). The Shannon diversity and compound richness in pollen washes did not differ significantly between chemotypes (**Supplementary Table S2**). However, the Shannon diversity increased as the blooming period progressed, i.e., later blooming flowers comprised a higher pollen Shannon diversity ($P = 0.01$, parameter estimate $= 0.85$, $N = 37$, **Figure 2E**). Individual plants produced flower heads for a period of up

TABLE 1 | Summary of (generalized) linear mixed-effects model analyses [(G)LMM] on traits of *Tanacetum vulgare* plants and behavioral responses of florivorous beetles toward *T. vulgare* flowers.

Response	Fixed effects	Random effects	Model	Model results	Model details
Flower heads (*n*)	CT	MG, BO	GLMM (Poisson, log link)	$\chi^2 = 82.72$, ***P* < 0.001**	**Table 2**
Ø flower heads (mm)	CT	MG, BO	LMM (Gaussian, identity link)	$\chi^2 = 12.82$, ***P* = 0.04**	**Table 2**
Bloom period (d)	CT	MG, BO	GLMM (Poisson, log link)	$\chi^2 = 63.5$, ***P* < 0.001**	**Table 2**
Pollen (mg)	CT	MG, BO	LMM (Gaussian, identity link)	$\chi^2 = 3.92$, $P = 0.81$	**Table 2**
Water (%)	CT × PA	MG, BO	LMM (Gaussian, identity link)	CT $\chi2 = 5.11$, $P = 0.32$ PA $\chi2 = 7.98$, ***P* = 0.007**	**Table 3**
Proteins (%)	CT × PA	MG, BO	LMM (Gaussian, identity link)	CTxPA $\chi^2 = 13.97$, ***P* = 0.02**	**Table 3**
Carbohydrates (%)	CT × PA	MG, BO	LMM (Gaussian, identity link)	CT $\chi^2 = 4.09$, $P = 0.39$ PA $\chi^2 = 1.75$, $P = 0.19$	**Table 3**
Lipids (%)	CT, PA	MG, BO	LMM (Gaussian, identity link)	CT $\chi^2 = 5.46$, $P = 0.24$ PA $\chi^2 = 2.12$, $P = 0.15$	**Table 3**
P:L-ratio	CT, PA	MG, BO	LMM (Gaussian, identity link)	**CT $\chi^2 = 9604$, $P < 0.001$** **PA $\chi^2 = 2765$, $P < 0.001$**	**Table 3**
Pollen Shannon diversity (H′)	CT, PA	MG, BO	LMM (Gaussian, identity link)	CT $\chi^2 = 2.17$, $P = 0.71$ PA $\chi^2 = 0.74$, $P = 0.39$	**Supplementary Table S2**
Pollen compound richness (*n*)	CT, PA	MG, BO	GLMM (Poisson, log link)	CT $\chi^2 = 1.17$, $P = 0.86$ PA $\chi^2 = 0.01$, P = 0.7	**Supplementary Table S2**
Florivorous *Olibrus* spp. stay above olfactometer chamber (*n*)	FH	Plant individual	LMM (Gaussian, identity link), separate models for chemotypes	significant: β-thu. mono: $\chi2 = 389.4$, ***P* < 0.001** art. acet. mix: $\chi2 = 219.8$, ***P* = 0.002**	**Supplementary Table S3**
Florivorous *Olibrus* spp./plant (*n*)	CT × PT	plot:block	GLMM (Poisson, log link)	CT × PT $\chi^2 = 13.1$, ***P* = 0.002**	**Supplementary Table S4**
Flower mines/ plant (*n*)	CT	plot:block	GLMM (Poisson, log link)	CT $\chi^2 = 11.35$, ***P* = 0.025**	**Supplementary Table S4**

Significant P-values (P < 0.05) are highlighted in bold. If the interaction of fixed effects factors was not significant, the interaction was removed and the statistical test result is only shown for the single factors.
MG, maternal genotype; BO, bloom onset; CT, chemotype; PA, pollen amount; FH, flower head premature/mature/none; PT, plot type; H′, Shannon diversity index.

to 79 days and individuals with a longer bloom period had more flower heads ($P < 0.001$, parameter estimate = 0.24, $N = 44$, **Figure 2F**).

Terpenoids and Other Organic Compounds in Floral Headspace and Pollen

Metabolic comparisons were conducted with greenhouse-grown *T. vulgare* plants of five different chemotypes. In total, surface washes of pollen contained 33 organic compounds (**Supplementary Figure S3**), compared to 63 compounds in leaf extracts, and 50 and 51 compounds detected in the headspace of premature and mature flower heads of the same *T. vulgare* plant individuals (**Supplementary Table S1**), respectively. In leaf extracts, the compounds with highest abundance were the compounds according to which the chemotypes were defined, i.e., particularly β-thujone in the β-thujone mono- and α, β-thujone mix-chemotypes and artemisia ketone in the artemisia ketone mono-chemotype and the artemisia ketone and artemisia acetate mix-chemotype, and the peak areas were highest for these compounds. Most abundant compounds in the headspace of mature, pollen-presenting flower heads were α-pinene and benzaldehyde, present in 94 and 91% of samples, respectively. In the headspace of premature flowers α-pinene and 3-hexen-1-ol-acetate occurred most frequently, in 97 and 87% of samples, respectively. Pollen washes contained in 87 and 89% of cases hexan-2-ol and *E*-3-hexenol, respectively (**Supplementary Figure S3**). Mantel tests revealed a correlation of the pollen compound pattern to that of the leaf extracts and the headspace of mature flower heads but not to the headspace compound pattern of premature flower heads (**Table 4**). Furthermore, the compound pattern of the leaf extracts correlated to the headspace of mature flower heads and headspace compound patterns for both ontogenetic stages of flowers also correlated. Random Forest comparisons of compound patterns did not reveal a clear separation for samples of the five chemotypes for floral headspace and pollen samples (**Supplementary Figures S4A,B**). Similarly, when comparing the compound abundances in a heat map (shown in **Supplementary Figure S3** for pollen), no clear patterns for the different chemotypes were evident.

TABLE 2 | Differences in the number of flower heads, the diameter of mature flower heads, the duration of bloom period and the pollen production between plants of different *Tanacetum vulgare* chemotypes.

Response factor	Fixed effects	Parameter estimates ± SE for chemotypes					Variance estimates ± SE of random effects	Statistical test and result
		β-thu. mono	art.ket. mono	α, β-thu. mix	art. acet. mix	myrox. Mix		
Flower heads (n)	Chemotype	46.5 ± 9.4 $N = 12$	39.4 ± 10.9▼ $N = 9$	57.7 ± 8.1 $N = 16$	44.3 ± 8.1 $N = 16$	66 ± 10.3▲ $N = 10$	MG: 0.0 ± 3.4 ($g = 12$) BO: 0.0 ± 0.0 ($g = 21$)	GLMM (Poisson, log link) $\chi^2 = 82.72$, **$P < 0.001$**
Ø flower heads (mm)	Chemotype	9.2 ± 0.4 $N = 11$	9.4 ± 0.5▲ $N = 7$	8.2 ± 0.5 $N = 7$	9.4 ± 0.4▲ $N = 12$	8 ± 0.4▼ $N = 9$	MG: 0.6 ± 0.8 ($g = 12$) BO: 0.0 ± 0.1 ($g = 18$)	LMM (Gaussian, identity link) $\chi^2 = 12.82$, **$P = 0.04$**
Bloom period (d)	Chemotype	22.8 ± 4.7 $N = 12$	15.5 ± 5.8 $N = 9$	24.0 ± 4.2▼ $N = 16$	14.4 ± 4.2▼ $N = 16$	23.6 ± 5.1 $N = 10$	MG: 0.2 ± 0.5 ($g = 12$) BO: 0.7 ± 0.8 ($g = 21$)	GLMM (Poisson, log link) $\chi^2 = 63.5$, **$P < 0.001$**
Pollen (mg)	Chemotype	18.9 ± 4.0 $N = 12$	14.8 ± 4.6 $N = 9$	20.1 ± 3.5 $N = 16$	18.3 ± 3.5 $N = 16$	27.4 ± 4.3 $N = 10$	MG: 0.0 ± 0.6 ($g = 12$) BO: 0.0 ± 0.0 ($g = 18$)	LMM (Gaussian, identity link) $\chi^2 = 3.92$, $P = 0.81$

Number of flower heads and pollen production are summed over the entire blooming period. Displayed are parameter estimates ± standard error values for the (generalized) linear mixed models [(G)LMM] and levels of significance for differences between chemotypes. Variance estimates are shown for the random effects maternal genotype (MG) and bloom onset (BO; i.e., the day on which the first mature flower was observed) and the number of groups of the respective random effect (g) is given in brackets. Significant values ($P < 0.05$) are highlighted in bold and pointing up ▲ or down ▼ triangles are added behind the highest and lowest parameter estimate, respectively.

In contrast, the compound patterns of leaf extracts clearly clustered (**Supplementary Figure S4C**).

Florivore Preferences (Four-Field-Olfactometer Assay) and Abundance of Florivorous Beetles and Mines on Flower Heads (Field)

Florivorous beetles (*Olibrus* spp.) were tested for their response to headspace volatiles of flower heads from greenhouse-grown plants. For two of the five tested chemotypes, namely β-thujone mono- and artemisia acetate mix-, the beetles spend a significantly longer time above fields containing premature flower heads compared to fields containing mature flower heads (**Figure 3**, **Table 1**, and **Supplementary Table S3**). The same trend was observed for the α,β-thujone mix-chemotype ($P = 0.065$). For the behavioral response toward flower head scent of the artemisia ketone mono-chemotype, the highest variances (standard error values) were recorded (**Supplementary Table S3**). Abundance of florivorous *Olibrus* spp. beetles on flower heads in the semi-field experiment depended significantly on the interaction of the plant chemotype with the plot type (**Figure 4** and **Supplementary Table S4**). In homogenous field plots, the abundance of beetles was higher compared to the abundance on the same chemotypes in heterogenous plots. The abundance of mines (only observed in homogenous plots) depended significantly on the chemotype. The mean abundance of both adult beetles and floral head miners in homogenous plots was highest in the *(E)*-carvyl acetate and (Z)-chrysanthenyl acetate mono-chemotypes, while plants of the camphor mono-chemotype were least frequently visited.

DISCUSSION

Patterns and Diversity in Flower and Pollen Scent Compounds

We had hypothesized that *T. vulgare* plants express chemotype-specific patterns of VOC in the floral headspace and pollen, but this hypothesis could only partially be supported by our data. Random Forest analyses revealed no clustering into chemotype-specific groups for floral headspace or pollen washes (**Supplementary Figures S4A,B**), in contrast to the clear clustering found for leaf extract compounds (**Supplementary Figure S4C**). Thus, the overlap of scent compounds in the floral headspace and pollen among the different chemotypes may distract pollinators and ensure that they visit all chemotypes and transfer pollen from one chemotype to another. In line with this hypothesis, a recent study revealed that honeybees did not discriminate between pollen scents if the pollen had a different nutritional value and/or taxonomic origin, as long as the respective plants had an overlapping bloom period (Pietrantuono et al., 2019). In *T. vulgare*, we found that surface washes of pollen contained only approximately half of the number of organic compounds detected in leaf extracts, but we cannot exclude that

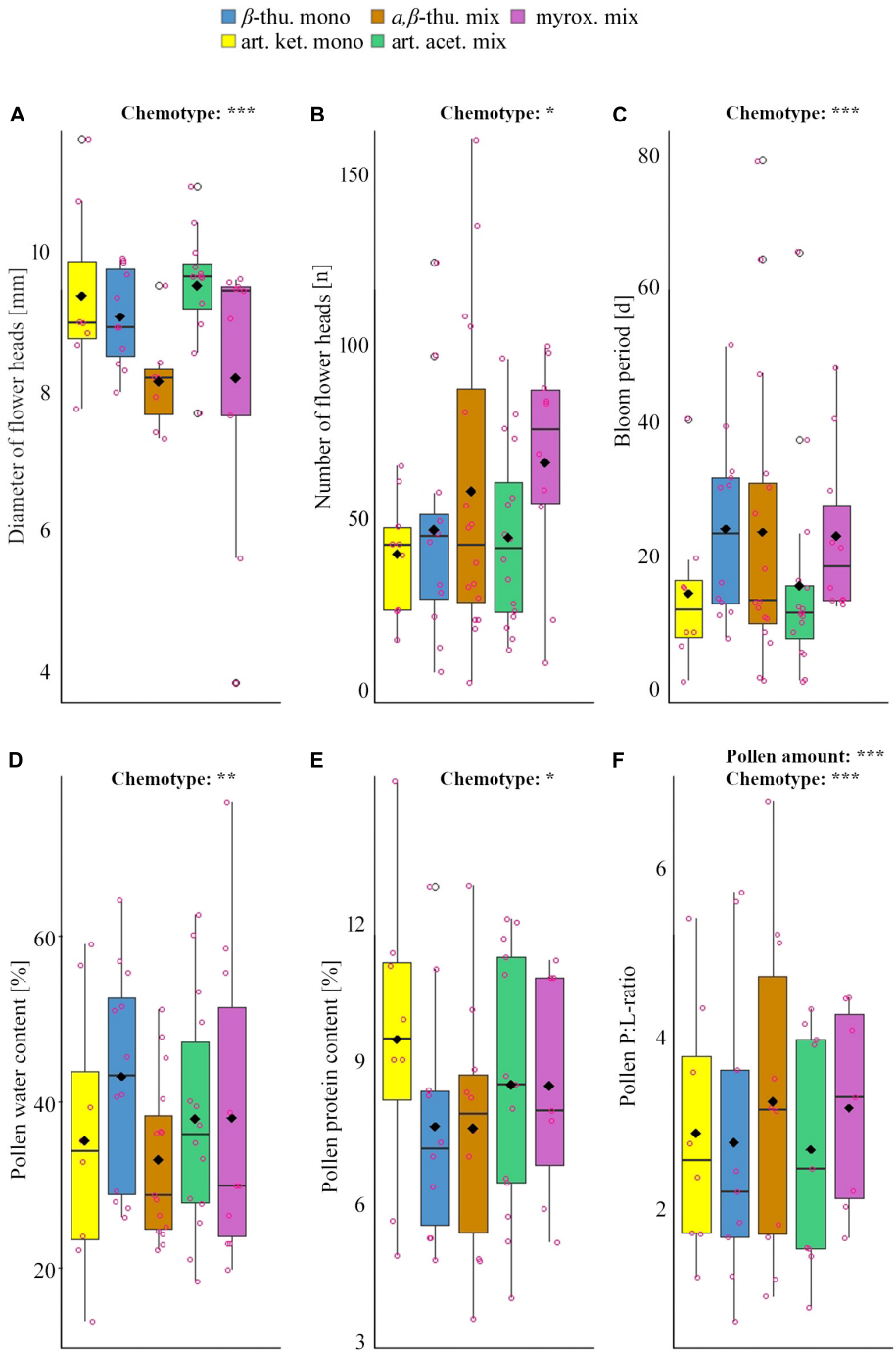

FIGURE 1 | Significant differences in flower and bloom parameters **(A–C)** and pollen constituents **(D–F)** among different chemotypes of *Tanacetum vulgare*. Colors represent chemotypes (see legend). The boxplots show the median (horizontal line), mean (filled circle), first and third quartiles (hinges), and 1.5 of inter-quartile ranges (whiskers). Jitters (pink dots) display individual observations. Significance levels: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. For detailed information on the statistical comparison of data, see **Tables 1**–**3**.

this difference is at least in part due to the different analytical methods for both sample types. Within flowers, a tissue-specific emission of volatiles is a characteristic feature of many species. In general, petals are the primary source of floral volatiles, although other tissues (stamens, pistils, sepals, and nectaries) also contribute to the floral bouquet in certain plant species (Dobson et al., 1997; Dötterl and Jürgens, 2005; Flamini et al., 2007). Moreover, pollen are often characterized by distinct scent emission profiles, as for instance found in *Citrus limon* (Rutaceae) (Flamini et al., 2007) and *Ranunculus acris* (Ranunculaceae)

TABLE 3 | Differences in pollen water and macronutrient contents between plants of different chemotypes of *Tanacetum vulgare*.

Response factor, pollen data	Fixed effects	Parameter estimates ± SE for chemotypes					Variance estimates ± SE of random effects	Statistical test and result
		β-thu. mono	art.ket mono	α, β-thu. mix	art. acet. mix	myrox. mix		
Water (%)	chemotype (CT), pollen amount (PA)	41.1 ± 4.5 *N* = 12	33.7 ± 5.4 *N* = 8	34.3 ± 4.1 *N* = 15	38.4 ± 4.28 *N* = 14	39.9 ± 4.7 *N* = 10	MG: 4.1 ± 6.4 (*g* = 12) BO: 0 ± 0 (*g* = 53)	LMM (Gaussian, ident link) CT × PA $\chi^2 = 2.8$, $P = 0.69$ CT $\chi^2 = 5.11$, $P = 0.32$ **PA $\chi^2 = 7.98$, $P = 0.007$**
Protein (%)	CT	9.1 ± 1.7▲	3.2 ± 2.5▼	5.6 ± 1.5	8.9 ± 1.3	6.3 ± 2.2	MG: 3.1 ± 5.6 (*g* = 11) BO: 4.6 ± 0.0 (*g* = 42)	LMM (Gaussian, ident link) **CT × PA $\chi^2 = 13.97$, $P = 0.02$**
	CT × PA	0.1 ± 0.1 *N* = 10	0.5 ± 0.1 *N* = 8	0.1 ± 0.1 *N* = 10	0.1 ± 0 *N* = 13	0.1 ± 0 *N* = 7		
Carbohydrates (%)	CT, PA	9 ± 1.8 *N* = 9	12.4 ± 1.8 *N* = 9	8.7 ± 1.8 *N* = 10	10.8 ± 1.9 *N* = 11	10.1 ± 2.4 *N* = 7	MG: 0 ± 0 (*g* = 11) BO: 1.3 ± 3.6 (*g* = 42)	LMM (Gaussian, ident link) CT × PA $\chi^2 = 12.97$, $P = 0.09$ CT $\chi^2 = 4.09$, $P = 0.39$ PA $\chi^2 = 1.75$, $P = 0.19$
Lipids (%)	CT, PA	4.1 ± 0.6	3.8 ± 0.5	3.3 ± 0.6	4.5 ± 0.6	3.5 ± 0.7	MG: 0.1 ± 0.3 (*g* = 11) BO: 0.2 ± 0.5 (*g* = 41)	LMM (Gaussian, ident link) CT × PA $\chi^2 = 1.94$, $P = 0.77$ CT $\chi^2 = 5.46$, $P = 0.24$ PA $\chi^2 = 2.12$, $P = 0.15$
		N = 9	*N* = 9	*N* = 10	*N* = 11	*N* = 7		
P:L-ratio	CT, PA	2.5 ± 0.4▼	3.1 ± 0.6	3.4 ± 0.5▲	2.8 ± 0.4	3.3 ± 0.6	MG: 0 ± 0 (*g* = 11) BO: 2.5 ± 1.6 (g = 40)	LMM (Gaussian, ident link) CT × PA $\chi^2 = 5.07$, $P = 0.21$ **CT $\chi^2 = 9604$, $P < 0.001$** **PA $\chi^2 = 2765$, $P < 0.001$**
		N = 9	*N* = 8	*N* = 10	*N* = 9	*N* = 7		

Displayed are parameter estimates and standard error values for the (generalized) linear mixed models [(G)LMM] and levels of significance for differences between chemotypes in pollen water, carbohydrate and lipid content (%) and the ratio of protein to lipid (P:L ratio). Random factors in all models were maternal genotype and bloom onset (i.e., the day on which the first mature flower was observed) and the number of groups for variance estimates is given in brackets. The total amount of produced pollen of each plant individual was included as fixed effect in models, to test factor interactions. The significance and parameter estimates for the factor pollen amount are shown in ***Table 2****, thus not repeated here. Parameter estimates for the interaction of the fixed factors chemotype (CT) and pollen amount (PA) are only shown if significant. Variance estimates for the random effects maternal genotype (MG) and time of bloom onset (BO) are shown for the full model including the interaction, if the interaction was significant, otherwise the variance estimates refer to the minimized model, excluding the interaction. Significant values ($P < 0.05$) are highlighted in bold and pointing up ▲ or down ▼ triangles are added behind the highest and lowest parameter estimate, respectively.*

(Bergström et al., 1995). While the compound patterns of pollen and pollen-producing flowers showed similarities to those of leaves in our investigation of *T. vulgare*, premature flowers differed in this regard. The opposite pattern regarding floral ontogeny and scent has been described for the shrub *Asimina triloba* (Annonaceae). Premature flowers of this species produce the same sesquiterpenoids as the leaves, but mature flowers emit a specific scent of fermentation volatiles (Goodrich et al., 2006). However, literature on changes in flower scent emission in the course of floral ontogeny is scarce and the example of *A. triloba* may not be representative for various other plant species.

Interestingly, terpenoid patterns also vary between above- and belowground tissues in our study plant *T. vulgare*. In the roots sesquiterpenoids dominate, whereas in the shoots mainly monoterpenoids are expressed (Kleine and Müller, 2014), indicating independent terpenoid biosynthesis in different tissues within plants of this species. The composition of the individual compounds is partly different between the tissues, and we addressed the question, whether the number or diversity of the individual compounds between the tissues in the flowers is related. In this context, for deterrent and repellent compounds in floral headspace volatiles (Raguso, 2009) and for toxins in reward compounds (Rivest and Forrest, 2020), the pleiotropy hypothesis has been proposed and controversially discussed. The hypothesis explains the evolution of the presence of floral defensive compounds by a random physiological spill-over effect of these compounds from other plant tissues in which they originally evolved as protection agents. We found no correlation between compound patterns in leaves and premature flowers but between leaves and mature flowers. However, our results support the pleiotropy hypothesis for the presence of compounds in *T. vulgare* leaves and floral tissues to some degree: Of the detected compounds approx. one third were present in all four

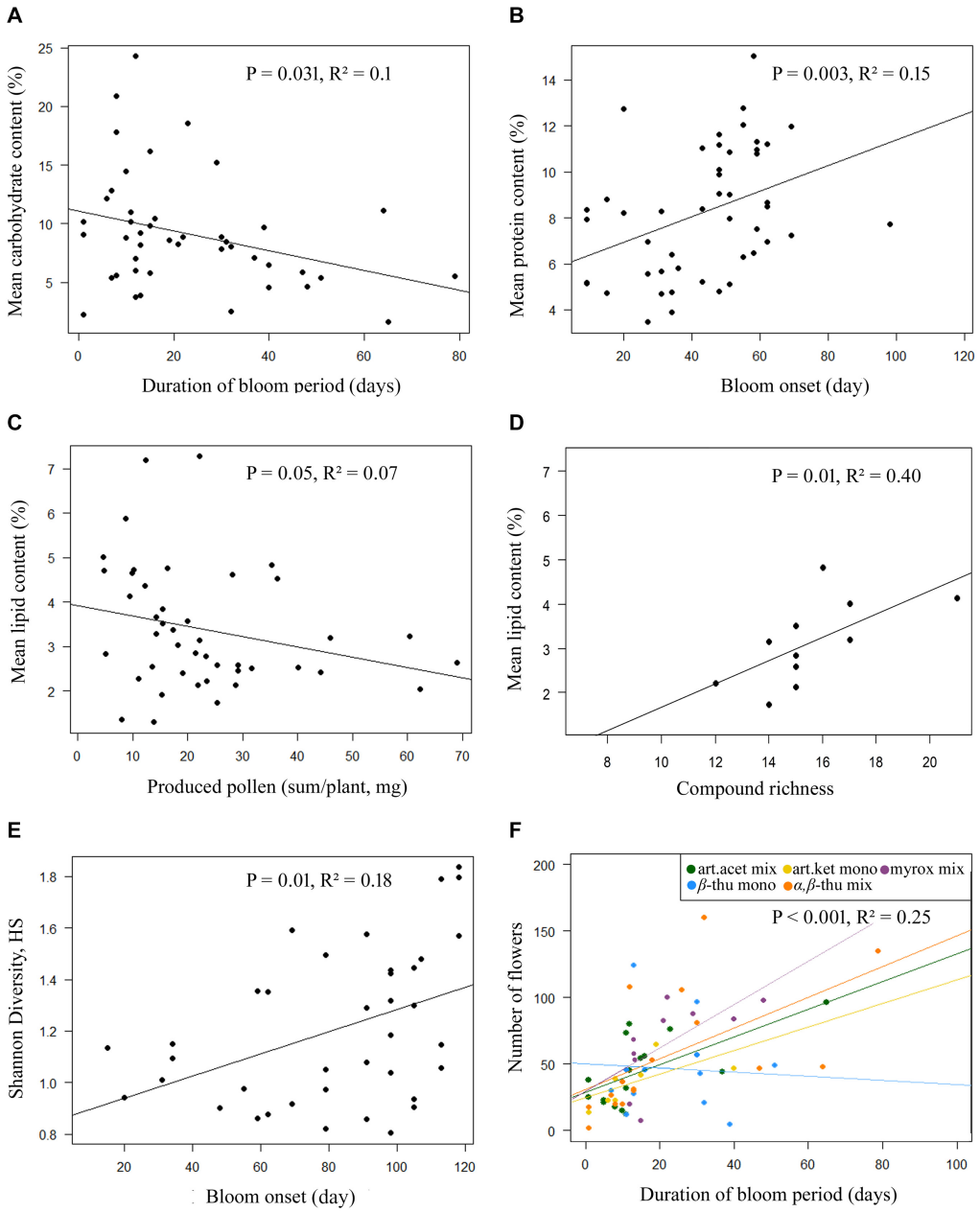

FIGURE 2 | Linear regression plots for significant correlations of pollen macronutrient contents **(A–D)**, (Shannon) diversity or richness of detected compounds in pollen hexane surface washes **(D,E)**, and the number of produced flower heads per plant of *Tanacetum vulgare* **(F)** in relation to pollen and flower phenology parameters, namely the duration of the bloom period **(A,F)**, bloom onset **(B,E)**, the amount of produced pollen per plant **(C)**, and the number of detected compounds in pollen hexane surface washes **(D)**. In the plot **(F)**, the data for bloom period duration and number of flowers are displayed with different colors for chemotypes (see legend), as these data differed particularly strong between the chemotypes.

types of samples, i.e., headspace samples of premature and mature flower heads, pollen washes and leaf extracts, indicating an overlap and potential spill-over in the metabolic composition (**Supplementary Table S1**).

Furthermore, we addressed the question, if a higher compound diversity trades off with the resource-demanding production of pollen. We found a higher Shannon diversity of pollen surface compounds and a higher pollen protein content in later blooming flowers, which contradicts our trade-off hypothesis. Thus, we propose that the reduction of pollinators at the end of the flowering season of *T. vulgare* (Dupont et al., 2018) leads to a greater need to attract the still available pollinators for late-blooming flowers, and thus pollinators are lured with higher protein levels in pollen. The higher pollen compound diversity later in the blooming season may provide a better defense of the pollen against robbery (Celedon and Bohlmann, 2019). Under

TABLE 4 | Mantel test comparisons within the same plant individuals of compounds detected in hexane pollen surface washes, leaf extracts and headspace samples for premature and mature flower heads.

	Pollen washes	Leaf extracts	HS premature flower
Leaf extract	***r* = 0.828** ***P* < 0.001**		
Headspace (HS) premature flower	$r = 0.1301$ $P = 0.152$	$r = 0.147$ $P = 0.107$	
Headspace (HS) mature flower	***r* = 0.372** ***P* < 0.001**	***r* = 0.373** ***P* < 0.001**	***r* = 0.21** ***P* = 0.032**

N = 28 plants, merged dataset for the five chemotypes used in laboratory experiments. Significant values (P < 0.05) are highlighted in bold.

natural conditions, in addition also changes in temperatures and in the occurrence of drought events later in the season may affect the composition and chemodiversity of flower volatiles (Farré-Armengol et al., 2020). We expected plant individuals with a high floral headspace chemodiversity to produce less pollen, potentially due to higher enzymatic costs. The compound diversity and pollen amount were, however, not directly linked. Estimations of costs for the production of metabolites are difficult, as most of the detected compounds in the floral headspace and in pollen washes were terpenoids, of which one single enzyme can produce multiple different ones but for some terpenoids also multiple enzymatic steps are needed (Gershenzon, 1994). Thus, a higher compound diversity is not necessarily linked to higher costs.

Flower Production and Pollen Macronutrients

Significant differences were found in the number and diameter of flower heads and the duration of blooming period between the five *T. vulgare* chemotypes investigated in laboratory and greenhouse experiments. The lowest number of flower heads with the largest diameter and a relatively short blooming period was observed for the artemisia ketone mono-chemotype (**Table 2**) and this chemotype produced only few pollen with the lowest water and protein content. Former studies showed that the number of flower heads is positively correlated with attractiveness for pollinators in the Asteraceae *Jacobaea vulgaris* (formerly: *Senecio jacobaea*) (Andersson, 1996), *Achillea ptarmica* (Andersson, 1991), and *Pertya triloba* (Kawarasaki and Hori, 1999). In a field collection of 140 plants at the same site where *T. vulgare* seeds for the present study were collected, the artemisia ketone mono-chemotype was the second most frequently represented, thus seemingly a very successful and highly competitive chemotype (Kleine and Müller, 2011). However, it remains to be elucidated if the larger flower size of the artemisia ketone mono-chemotype found in the present study attracts more pollinators or even only certain pollinator types under field conditions and can thus compensate for the low amount of flowers, pollen and pollen protein in this chemotype. Instead, this chemotype may have multiple blooming periods or invest rather in vegetative reproduction, which may explain its success in the field. But since there are hardly any extensive field studies on trophic interactions with chemotypes, this remains to be a topic for future analyses. In fact, vegetative reproduction by ramets is an important growth strategy of this perennial species (Kleine et al., 2017). The shortest bloom periods were observed for the artemisia ketone and artemisia acetate mix-chemotypes, but within this short period, plants of these chemotypes produced the highest numbers of flower heads (**Figure 2F**). These examples highlight intraspecific differences in the strategies for pollinator attraction and reproduction among the different chemotypes, which is a novel and particularly interesting finding.

We expected the proportions of pollen macronutrients to differ between the chemotypes and trade-off with the blooming duration (i.e., fewer nutrients, longer bloom period) and the amount of produced pollen per plant individual (i.e., more nutrients, lower amount of produced pollen). We did indeed find chemotype-related differences in the proportions of pollen macronutrients, i.e., water, protein and P:L-ratio. Moreover, trade-offs between pollen macronutrients and blooming parameters were detected, which suggest resource limitations in the production of pollen. In particular, plants with a shorter blooming period showed higher carbohydrate contents than plants with a longer blooming period. Pacini (1996) found differences in the pollen carbohydrate composition between dehydrated and non-dehydrated pollen and postulated that cytoplasmic carbohydrates and sucrose are protection-agents of pollen and maintain viability during exposure and dispersal. Pollen contains various sources of carbohydrates, such as starch and mono- and polysaccharides such as callose, with different function (Pacini, 1996), and further studies are required to elucidate, if only the carbohydrate contents, or also the composition differs and how differences in carbohydrate content and composition affect pollen viability and flower visitation at different time points within the bloom period. Regarding protein, previous studies of pollen macronutrient contents showed that pollen ranges from 1.5 to 61% protein by dry mass across different plant species (Roulston et al., 2000; Vaudo et al., 2020). In our intraspecific comparison, protein contents in pollen ranged from 3 to 15% between different chemotypes, with 8% on average, and similar protein contents were reported from pollen of other Asteraceae plants (Nicolson and Human, 2013; Vaudo et al., 2020). Pollen protein is supposed to be the most important macronutrient for bees; across 68 bee and six plant species, significant correlations between pollen protein content, bee abundance and visitation rate were found (Russo et al., 2019). Similarly, the P:L-ratio could be directly related to floral attractiveness for bumble bee workers, with P:L-ratios of 5:1 and 10:1 being most attractive (Vaudo et al., 2016). Thus, according to the protein content and P:L-ratios, *T. vulgare* flowers should be poorly rewarding and not very attractive to bees and other pollinators. However, *T. vulgare* flowers are visited by various generalist and specialized pollinating insects of different species and families, such as dipterans (Peach and Gries, 2016), hymenopterans (Müller and Kuhlmann, 2008; Praz et al., 2008a), and lepidopterans (Bąkowski and Boroń, 2005).

In addition to macronutrients, other constituents of reward traits may be of value for flower visitors. For instance, pollen of Asteraceae can protect bees from brood parasitism (Spear et al., 2016) and terpene-rich pollen of Lamiaceae helps

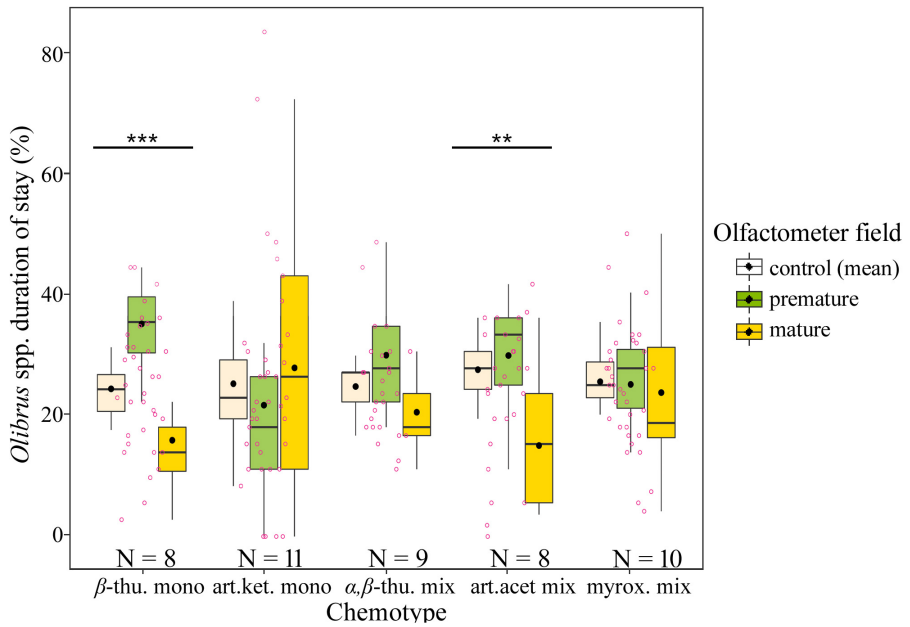

FIGURE 3 | Duration of stay of florivorous beetles (*Olibrus* spp.) above olfactometer fields without flower head (control, mean values of both control fields), with premature flower head (prior anthesis), or with mature flower head (post anthesis) of five *Tanacetum vulgare* chemotypes. Four beetle trials were conducted for each plant individual and data were averaged to the plant individual level prior display and statistical comparison to avoid pseudoreplication. The boxplots show the median (horizontal line), mean (filled circle), first and third quartiles (hinges), and 1.5 of inter-quartile ranges (whiskers). Jitters (pink dots) display individual observations. Asterisks indicate significant differences between the three chambers (control, premature and mature flower heads). Significance levels of LMM: ***$P < 0.001$; **$P < 0.01$. For detailed statistical comparison of data, see **Supplementary Table S3**.

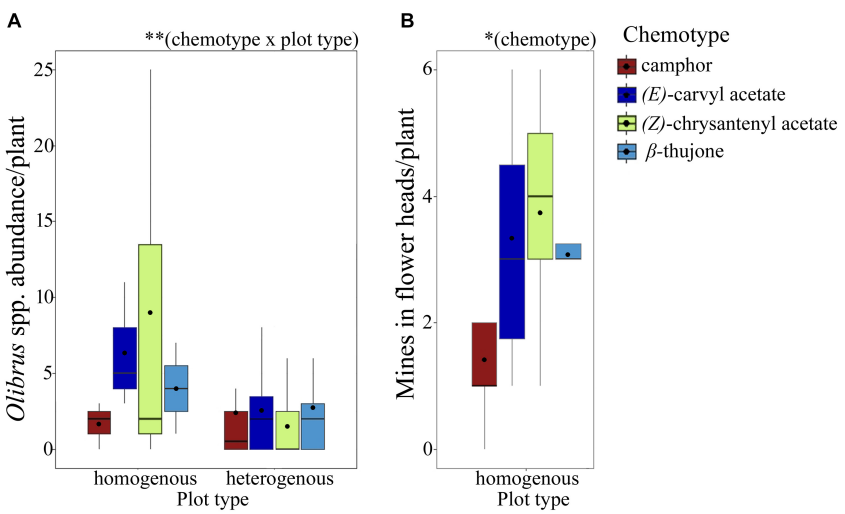

FIGURE 4 | Abundance of florivorous *Olibrus* spp. beetles on flower heads (**A**, $N = 18$ plants per chemotype) and of mining insects in flower heads (**B**, $N = 24$ plants per chemotype) of four *Tanacetum vulgare* chemotypes in the field. Florivorous beetles were counted in plots containing only individuals of the same chemotype (homogenous plots) and in plots containing all four chemotypes (heterogenous plots). The boxplots show the median (horizontal line), mean (filled circle), first and third quartiles (hinges), and 1.5 of inter-quartile ranges (whiskers). Significance levels: *$P < 0.05$; **$P < 0.01$. For detailed statistical comparison of data, see **Supplementary Table S4**.

bees to fight off pathogens (Wiese et al., 2018). Moreover, we found very high amounts of pollen of up to 70 mg fresh weight per individual in *T. vulgare* and individual plants flowered up to 80 days, which may compensate for the non-optimal nutrient composition. Pollen constituents reward and attract pollinators, but specialized metabolites in rewards also mediate interactions with microbes (Huang et al., 2012; McArt et al., 2014) and pollen proteins and lipids are

essential for pollen tube growth (Wolters-Arts et al., 1998; Ruedenauer et al., 2019). Thus, future research is required to clarify whether the differences in pollen macronutrient contents of *T. vulgare* chemotypes affect interactions with pollinating insects and microbes and whether the pollen nutrient content trades-off with the ability of the pollen to germinate and penetrate the stigma.

Florivore Attraction and Abundance

We hypothesized that florivorous beetles prefer volatile cues of mature flower heads to those of unripe flower heads, but we found the opposite effect in olfactometer assays, in which visual cues were hidden. This result indicates that florivorous beetles discriminate between the odors of flowers of different ontogenetic stages. While collecting the beetles in the field, we found them on mature and premature flowers. However, so far there are no available field studies describing at which maturation stage of the flowers the beetles settle at the beginning of the season and whether beetle preferences change over the season. Mature flowers should be particularly attractive to the beetles after birth or hibernation, because they provide pollen as food source. Premature flowers may be particularly attractive for beetles with reproductive pressure, as the females lay the eggs near immature seeds. Our results suggests that pollen is emitting repellent compounds to the beetles. However, the mature flower heads were tested in our experiments shortly after anthesis. Whether the floral scent of mature *T. vulgare* flowers becomes attractive again to the beetles with continuing maturity remains to be tested. Moreover, our field abundance data of florivorous beetles revealed preferences for certain chemotypes. These results support our hypothesis and are in line with previous studies showing similar distinct chemotype-specific preferences for foliar herbivores, such as beetles (Wolf et al., 2012b), or aphids (Völkl et al., 1999; Kleine and Müller, 2011; Benedek et al., 2015). However, we exclusively tested mono-chemotype plants. It will be interesting to compare the attractiveness of mono- and mix-chemotype plants toward flower visitors and we expect preferences for flower heads of mix-chemotype plants due to lower concentrations of individual compounds in headspace volatiles (Müller et al., 2020).

In our semi-field experiment we expected chemotype-specific differences in florivore abundance to diminish in heterogenous stands with different chemotype neighbors due to overlapping odor plumes (Riffell et al., 2014; Cai et al., 2017), increased chemical diversity of the scent bouquet per plot and a dilution of the headspace concentration and/or masking of single repellent or deterrent compounds. Our field data support this hypothesis and indicate that heterogenous plots are on average less infested by florivores. However, an additional reason besides altered odor plumes may explain the differences in beetle abundance: In our semi-field experiment, we observed differences in the flowering time of individual chemotypes, similarly to the differences that we found for our greenhouse-grown plants. Thus, in some of the homogeneous plots more flowers may have been present simultaneously, while in none of the heterogeneous plots all plants were in full bloom at the same time.

CONCLUSION

In conclusion, we showed that *T. vulgare* chemotypes differ remarkably in flower traits such as flower head diameter, bloom period and pollen P:L-ratio. Florivorous beetles were able to discriminate between flower heads in different ontogenetic stages by scent. In a semi-field experiment, the beetles showed clear chemotype-specific preferences but their choice was distracted by the presence of different chemotype neighbors. These results provide insight to the chemical ecology of a plant species with fascinating intraspecific chemodiversity and indicate that metabolic and ecologic traits are highly linked.

DATA AVAILABILITY STATEMENT

The data were now uploaded to the Knowledge Network for Biocomplexity (KNB): https://knb.ecoinformatics.org/view/urn%3Auuid%3A1cb33aae-b19e-4b48-8fae-96f00c2e354a.

AUTHOR CONTRIBUTIONS

CM, EE, and SK conceived and designed the experiments. CM and EE acquired the funding. SW, SE, and SK performed the semi-field experiments and analyzed the respective data with the help of CM. EE performed the experiments with greenhouse-grown plants, i.e., chemical analyses and olfactometer assays, analyzed respective data, conducted all statistical analyses, prepared all figures and tables, and wrote the first version of the manuscript. All the authors revised and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.611877/full#supplementary-material

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Cross-Species Metabolic Profiling of Floral Specialized Metabolism Facilitates Understanding of Evolutional Aspects of Metabolism Among Brassicaceae Species

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Plants produce a variety of floral specialized (secondary) metabolites with roles in several physiological functions, including light-protection, attraction of pollinators, and protection against herbivores. Pigments and volatiles synthesized in the petal have been focused on and characterized as major chemical factors influencing pollination. Recent advances in plant metabolomics have revealed that the major floral specialized metabolites found in land plant species are hydroxycinnamates, phenolamides, and flavonoids albeit these are present in various quantities and encompass diverse chemical structures in different species. Here, we analyzed numerous floral specialized metabolites in 20 different Brassicaceae genotypes encompassing both different species and in the case of crop species different cultivars including self-compatible (SC) and self-incompatible (SI) species by liquid chromatography-mass spectrometry (LC-MS). Of the 228 metabolites detected in flowers among 20 Brassicaceae species, 15 metabolite peaks including one phenylacyl-flavonoids and five phenolamides were detected and annotated as key metabolites to distinguish SC and SI plant species, respectively. Our results provide a family-wide metabolic framework and delineate signatures for compatible and incompatible genotypes thereby providing insight into evolutionary aspects of floral metabolism in Brassicaceae species.

Keywords: floral specialized metabolite, plant metabolomics, cross-species comparison, chemodiversity, Brassicaceae, compatible and incompatible species, flavonoids

INTRODUCTION

Self-compatibility and self-incompatibility are common reproductive systems in flowering plant species, with both modes having advantages for species reproduction and selective breeding. Self-compatible (SC) species can integrate reproductive assurance with low-cost effects (inbreeding depression; Lahiani et al., 2015) and rapidly occupy a suitable growth habitat, but lack

adaptability to sudden environmental changes. By contrast, self-incompatible (SI) species have increased individual genetic heterozygosity, which accelerates species differentiation to adapt to environmental changes (Charlesworth et al., 2005); however, as a consequence they pay higher costs, such as the need to attract pollinators. Given the benefits of outcrossing, SC species can, with the help of pollinators, disperse more seeds (Kobayashi et al., 2012). Consequently, both SC and SI species have developed techniques to attract pollinators, but SI species have a higher demand for pollinators and therefore invest more in this process. For SI plants, flowers are the most important reproductive organ and bear the considerable task of attracting pollinators. To do so they rely on signals such as attractive floral pigmentation patterns, large floral size, strong floral scent, or rich nectar-honey rewards. In *Raphanus raphanistrum* and *Antirrhinum* spp., yellow-flowered individuals attract more attention from bees, thus obtaining more visits than white-flowered individuals (Stanton et al., 1986; Jones and Reithel, 2001). In monkeyflowers (*Mimulus* spp.), the presence or absence of yellow carotenoids, which are regulated by *YUP* genes, significantly shifts the preference of bees and hummingbirds (Bradshaw and Schemske, 2003). In addition to overall flower color, colorful pigmentation patterns such as spots, stripes, and central deepening or brightening of hue can serve as a nectar guide to promote pollination success (Davies et al., 2012). In *Mimulus lewisii*, loss of "light areas," which are formed by the flux from anthocyanin pigments to colorless flavonols, results in a significantly decreased rate of bumblebee visitation (Yuan et al., 2016). In addition to the pigmentation patterns that can be recognized by human eyes, UV-absorbing areas ranging from UV-C to UV-A (~300–400 nm) on flowers serve as a UV nectar guide, which can be sensed by some insect species (Kevan, 1976). Flavonoids usually play a role in this process, promoting visitation from pollinators such as bees or flies. Some accessions of *Brassica rapa* that present a UV "bulls-eye" nectar guide pattern on their corolla gain greater preference from pollinators. The UV-absorbing compound isorhamnetin 3,7-*O*-di-glucoside underlies UV patterning in these plants (Sasaki and Takahashi, 2002; Brock et al., 2016). In *Rudbeckia hirta*, 19 flavonols from the basal UV-absorbing area and apical UV-reflecting area on petals have been characterized, most of which accumulate in the basal area. Moreover, flavonol 7-*O*-glucosides showing yellow fluorescence under UV light are found to exclusively accumulate in the basal part and may contribute to promoting the sight-sensibility of pollinators (Schlangen et al., 2009).

To adapt to demand as a reproductive organ, specialized (secondary) metabolites in flowers are also needed to ensure the success of the fertilization process, such as protecting pollen from UV-irradiation and herbivores. Pollen from flowers of SI plant species may require a long trip to drop on suitable stigma, so stronger protection against UV-B stress, which reduces viable pollen production (Demchik and Day, 1996), inhibits pollen germination and tube growth (Feng et al., 2000) and makes pollen shrivel (Koti et al., 2005), is needed. UV-absorbing flavonols or other compounds with phenolic acid moieties, as well as some phenolamides, can play an important antioxidant role in response to UV irradiation. Levels of flavonols such as quercetin and kaempferol glucoside derivatives have frequently been reported to significantly increase in *Brassica napus*, *Trifolium repens*, *Malus domestica*, and *Arabidopsis thaliana* when plants are exposed to UV radiation (Olsson et al., 1998; Hofmann et al., 2000; Solovchenko and Schmitz-Eiberger, 2003; Götz et al., 2010). Phenylacylated-flavonols (saiginols) in floral tissue of accessions of *Arabidopsis* found at low-latitude and high-altitude have been shown to confer greater UV light tolerance (Tohge et al., 2016). Moreover, levels of hydroxycinnamic acids, such as caffeic acid, ferulic acid, sinapoyl-malate, and sinapoyl-*O*-glucoside, also increase greatly in response to UV treatment in tomato, red leaf lettuce, *A. thaliana*, and *B. rapa* (Luthria et al., 2006; García-Macías et al., 2007; Meißner et al., 2008; Li et al., 2010; Brock et al., 2016). Additionally, phenolamides, a large proportion of which are phenolic acids, emerge as the major metabolites in anther and pollen grains, and also function to strengthen abiotic resistance and affect fertility (Bassard et al., 2010). To date, metabolic profiling of floral organisms has been explored to elucidate the function of flower-specific specialized metabolites. For example, spatial metabolite expression in the flower has been described for *Fragaria×ananassa*, *Crocus sativus*, and *Rumex algeriensis* (Hanhineva et al., 2008; Moraga et al., 2009; Ammar et al., 2020). However, the metabolic profiling of multiple closely related species still needs to be focused on in order to further elucidate evolutionary aspects of floral metabolism. Specific classes of specialized metabolites in flowers, such as flavonoids or phenolamides, have already been discussed. Furthermore, specialized metabolites, such as pollen-specific compound *N*',*N*''-di-(hydroxyferuloyl)-*N*'''-sinapoyl spermidine and flavonol-3-*O*-diglucosides (kaempferol/quercetin-3-*O*-(2''-*O*-glucosyl)glucosides), have also been characterized (Fellenberg et al., 2009; Yonekura-Sakakibara et al., 2014), therefore, their roles in structural and functional diversification are worthy of discussion.

Brassicaceae, which contains many agriculturally important SC and SI species, such as *A. thaliana* and many of its relatives, have SC reproductive systems (Roy et al., 2010). On the other hand, many species in the *Brassica* genus have an SI reproductive system. For instance, *B. rapa* (Br) and *Raphanus sativus* (Rs) have an SI system, yet the amphidiploid species *Brassica juncea* (Bj) and *B. napus* (Bn) have incomplete SC systems (Kobayashi et al., 2012). So far, the SI model system in Brassicaceae has been well-characterized at the molecular level. In *B. napus*, the SI reaction is initiated by allele-specific recognition of the pollen-coat protein SCR/SP11 by *S*-locus receptor kinase (SRK). The activation of SRK causes activation of E3 ligase ARC1, which degrades compatibility factors such as Exo70A1, GLO1, and phospholipase D, which results in pollen rejection (Charlesworth et al., 2005; Kitashiba and Nasrallah, 2014; Scandola and Samuel, 2019). However, to date, only a few studies have systematically compared the differences in metabolite composition between compatible and incompatible species. In this study, we employed an LC-MS-based metabolomic approach to describe the metabolic framework of specialized floral

metabolism as a whole from both compatible and incompatible species in Brassicaceae. This has provided insight into the cross-species metabolites featured among different clades, as well as the role of specific metabolites in response to the reproductive recognition system.

MATERIALS AND METHODS

Plant Materials

Plant genotypes including *A. thaliana* (accessions Col-0, C24, and *tt4* mutant), *Arabidopsis shokei* (As), *Arabidopsis lyrate* (Al), *Crucihimalaya lasiocarpa* (Cl), *Olimarabidopsis pimila*, (Op) *Lepidium sativum* (Ls), *Thellungiella salsuginea* (Ts), *Thlaspi arvense* (Ta), *Capsella rubella* (Cr), *Nasturtium officinale* (No), *Cardamine hirsute* (Ch), *R. sativus* (Rs), *Brassica oleracea* var. *alboglabra* (Boa), *Brassica oleracea* var. italic (Boi), *B. napus* (Bn), *B. rapa* (Br), *Sinapis alba* (Sa), *Diplotaxis muralis* (Dm), *Eruca sativa* (Es), and *B. juncea* (Bj) were obtained from the Arabidopsis Biological Resource Center (ABRC),[1] and the seed companies (Takii, Japan; Sakata-no-Tane, Japan and Marche, Japan). Plants were grown in the greenhouse at 22°C for long day condition (16 h light/8 h dark). Those plants were grown in a mixture of red jade soil-vermiculite-nutrient soil (1:3:7). More than two whole flowers (containing stamen, petal, pistil, and anther) were collected in individual three biological replicates from two to three individual plants at the day of full-opened, and frozen immediately in liquid nitrogen. Samples were ground by Mixer Mill TissueLyser II (Qiagen, Hilden, Germany). The frozen plant powder was stored at −80°C until use.

[1]https://abrc.osu.edu/

Phylogenetic Tree Construction

The internal transcribed spacer (ITS) sequences from Brassicaceae species were retrieved from the NCBI database. Accession numbers are displayed in **Table 1**. The sequences were aligned by the MUSCLE algorithm (Edgar, 2004). The positions comprising more than 70% unrecognized characters were discarded. A phylogenetic tree was constructed by molecular evolutionary genetics analysis version 10.0 (MEGA X; Kumar et al., 2018) by the maximum likelihood (ML) method. Model selection for ML analysis was performed by using the model selection tool as supplemented in MEGA X. The model test result showed that the general time reversible model was the most suitable for analysis. The initial tree for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories, +G, and parameter). The tree confidence was inferred by the bootstrap method containing 1,000 replicates (Felsenstein, 1985). *Cleome serrulata* (accession number DQ455804.1) was selected as an outgroup.

Metabolite Extraction

Metabolite extraction was conducted as previously described (Tohge and Fernie, 2010). About 15 mg of frozen sample was weighed. Extraction buffer i.e., 80% methanol (5 μg/ml isovitexin as an internal standard) was added at a ratio of 1 mg F.W. sample powder: 10 μl extraction buffer. The mixture was vortexed to mix thoroughly and centrifuged at 14,000 rpm at 4°C for 10 min, then supernatant was transferred into new 1.5 ml tube and centrifuged once more, the final obtained supernatant was transferred into vials for liquid chromatography-mass spectrometry

TABLE 1 | Twenty Brassicaceae genotypes analyzed in this study.

ID	Genotypes (species/cultivars)	Reproductive system	GenBank ID**	Reference
At	*thaliana*	SC*	MG886682.1	Zhang et al., 2019
As	*shokei*	SC	N/A	Indriolo et al., 2012
Al	*lyrata*	SI	DQ528878.1	Indriolo et al., 2012
Op	*O. pumila*	SC	DQ310528.1	Roy et al., 2010
Cl	*C. lasiocarpa*	SC	AF137556.1	Roy et al., 2010
Ls	*L. sativum*	SC	MN257764.1	Wadhwa et al., 2012
Ch	*C. hirsura*	SC	DQ268383.1	Hay and Tsiantis, 2006
No	*N. officinale*	SC	AY254531.1	Pink, 1993
Cr	*C. rubella*	SC	AY662286.1	Fujikura et al., 2018
Ta	*T. arvense*	SC	KM892656.1	Best and McIntyre, 1975
Ts	*T. salsuginea*	SC	DQ165371.1	Wang et al., 2015
Rs	*R. sativus*	SI	GQ268079.1	Roy et al., 2010
Bj	*juncea*	Incomplete SC	MG923970.1	Kobayashi et al., 2012
Boa	*B. oleracea* var. alboglabra	SC	GQ891870.1	Okuda et al., 1997
Boi	*B. oleracea* var. italica	SI	KX709353.1	Anstey, 1954
Bn	*B. napus*	Incomplete SC	MG923974.1	Kobayashi et al., 2012
Br	*B. rapa*	SI	MG923989.1	Roy et al., 2010
Sa	*S. alba*	SC	AF128106.1	Fan et al., 2007
Dm	*D. muralis*	SC	DQ983972.1	Kokichi and Noboru, 1976
Es	*E. sativa*	SI	AY254536.1	Wang et al., 2007

*SC indicates self-compatible; SI indicates self-incompatible; Incomplete SC indicates incomplete self-compatible.

**N/A indicates not available in NCBI database.

(LC-MS) and high performance liquid chromatography-photodiode array detector (HPLC-PDA).

LC-MS Analysis

The flower metabolite extracts of 20 genotypes *A. thaliana* C24, *A. shokei*, *Arabidopsis lyrata*, *C. lasiocarpa*, *O. pimila*, *L. sativum*, *T. salsuginea*, *T. arvense*, *C. rubella*, *N. officinale*, *C. hirsute*, *R. sativus*, *B. oleracea* var. alboglabra, *B. oleracea* var. italic, *B. napus*, *B. rapa*, *S. alba*, *D. muralis*, *E. sativa*, and *B. juncea* were used for LC-MS analysis. LC-MS was carried out as described previously (Calumpang et al., 2020). Chromatographic separations were conducted on Nanoflow-HPLC "Paradigm MS4 system" (Michrom BioResources, Inc., Auburn, CA, United States), equipped with a Luna C18 column (150 by 2.00 mm i.d. 3 micron particle size, Phenomenex, Torrance, CA, United States). The mobile phase consisted of Solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). For each injection, 10 µl sample was loaded, and following gradient was applied at a flow rate of 200 µl min^{-1}: 0–1 min, from 100% A to 93%; 1–8 min, to 80% A; 8–17 min, to 60% A; 17–21 min, to 15% A; 21–25 min, to 0% A; 25–28 min, column wash; 28–31 min, to 100% A for equilibration of the column. Compounds were detected from *m/z* 200–1,500 by MS TSQ Vantage (Thermo Fisher Scientific, San Jose, CA, United States) using full scan mode covering both positive and negative ion detection. The transfer capillary temperature was set to 350°C and the spray voltage was fixed at 3.00 kV. The chromatograms were analyzed by Xcalibur software version 4.1.31.9 (Thermo Fisher Scientific, San Jose, CA, United States), the *m/z* value, retention time, and detection mode information of 228 characteristic peaks from all 20 genotypes were extracted and listed for peak picking process. Peak picking was conducted by using the process program in Xcalibur software to deal with the raw files of 20 genotypes with the parameter of retention time tolerance window (20 s), base window 100, area noise factor 5.0, peak noise factor 10, mass tolerance 0.5 *m/z*, and "nearest RT." A data matrix of areas of extracted ion chromatogram (EIC) was exported into Microsoft Excel and used for statistical analysis. Peak annotation of major flavonols, phenolic acid derivatives, phenolamides, and glucosinolates (GSLs) were conducted *via* by combined approach of literature survey, databases, co-elution of reference plant-extracts of *Arabidopsis* (Col-0, C24, and *tt4* mutant) and profiling of specific in-source fragments detected in positive ion detection as well as retention time referring to the metabolite information from the literature (Hanhineva et al., 2008; Fellenberg et al., 2009; Tohge et al., 2016) and databases including m/zCloud,[2] KNApSAcK (Afendi et al., 2012),[3] and PubChem.[4] Consequently, 82 peaks and 16 peaks were annotated or classified to compound class out of 228 characteristic peaks (**Supplementary Table S1**).

HPLC-PDA Analysis

The flower metabolite extraction of *R. sativus* and *A. thaliana* accession C24, Col-0 as well as *tt4* mutant were used for HPLC analysis. The HPLC analysis was conducted on Waters alliance HPLC system (Waters, Milford, MA, United States) controlled by Empower™ 25 (Waters, United States) software. Chromatographic separation was achieved on Waters 2695 separations module equipped with a 00F-451-B0 column (150 by 2.0 mm i.d. 3 micron particle size, Phenomenex, Torrance, CA, United States). The Waters 2996 photodiode array detector was employed for UV/VIS-detecting at 200~550 wavelength range. The mobile phase consisted of Solvent A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The sample injection volume was 10 µl each time. The flow-rate was set to 200 µl min^{-1}. Following elution program was performed for separation: 0–2 min, from 0% B to 10%; 2–15 min, to 25% B; 15–27 min, to 55% B; 27–30 min, to 100% B; 35–35.01 min, to 0% B; 35. 01–40 min, column wash.

Statistical Analysis

The peak area value of 228 characteristic peaks in each genotype was normalized by multiplication with the ratio of the average peak area of the internal standard to the peak area of the internal standard. The average of three replicates of normalized peak area of 82 annotated metabolites was then calculated and used for creating percentage stacked column shown in **Figure 2** by using Microsoft excel 2016 to represent the proportion of each annotated compound in different groups. The average of three replicates of normalized peak area of 228 characteristic peaks was scaled by $\log_2$ (mean/average_mean) and used for heatmap analysis. Heatmap visualization of metabolite data was performed by MeV software version 4.9.0 (Dana Farber Cancer Institute, Boston, MA, United States).[5] Metabolites and genotypes in heatmap were clustered using hierarchical clustering method (HCL). The normalized peak area value with three replicates of 228 characteristic peaks was used for the K-means test, principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), which were conducted by MetaboAnalyst 4.0 (Xia and Wishart, 2011).[6] The missing value was replaced by 1/5 of the min positive value for each variable. The peak area value of metabolites with high variations (RSDs > 20% in QC samples) were excluded and data was normalized by following parameters: none row-wise normalization, logarithmic 10 transformation, and none-data scaling. The significant component of PLS-DA was calculated using 10-fold cross-validation, and the predictive ability was evaluated by Q^2 value. The metabolites with variable importance in projection (VIP) scores greater than 1.7 were considered as significant feature metabolites. The absorption wavelength value (250~520 nm) of HPLC data was extracted by Empower™ 25 (Waters, United States) software, and scaled by setting the lowest value and highest value as 0 and 1. The cluster analysis of HPLC spectrum was conducted by K-means in MeV software.[7] The number of clusters was decided by formula $\sqrt{\frac{n}{2}}$ (n represent the number of input data; Joseph et al., 2010).

[2]https://www.mzcloud.org/
[3]http://www.knapsackfamily.com/KNApSAcK_Family/
[4]https://pubchem.ncbi.nlm.nih.gov/
[5]http://www.mev.tm4.org/
[6]http://www.metaboanalyst.ca
[7]http://www.mev.tm4.org/

RESULTS

Phylogenetic Relationship and Floral Phenotype of 20 Brassicaceae Genotypes

In this study, 20 genotypes encompassing species and cultivars of the different subfamilies of the Brassicaceae were selected, including 12 SC species (two cultivars for *B. oleraceae*), two incomplete SC species, and five SI species (**Table 1**). To construct phylogenic relationship among selected Brassicaceae plant species, the phylogenetic tree was constructed by the sequence of the ITS gene of each species (**Figure 1**). Furthermore, visible color of flower petal was checked for consideration of indication to plant speciation, floral pigmentation, and pollinators' recognition. Fourteen of twenty species had white flowers including *A. thaliana* (At), *A. shokei* (As), *A. lyrata* (Al), *C. lasiocarpa* (Cl), *L. sativum* (Ls), *C. hirsuta* (Ch), *N. officinale* (No), *C. rubella* (Cr), *T. arvense* (Ta), *T. salsuginea* (Ts), *R. sativus* (Rs), *B. oleracea* var. alboglabra (Boa), *B. napus* (Bn), and *E. sativa* (Es). Among these species, *E. sativa* had special purple stripes. Six species had bright yellow flowers including *Ophiorrhiza pumila* (Op), *B. juncea* (Bj), *B. oleracea* var. italica (Boi), *B. rapa* (Br), *S. alba* (Sa), and *D. muralis* (Dm) (**Figure 1**). Within six SI plant species, four Brassicaceae plants except *A. lyrata* (Al) and *R. sativus* (Rs) were species producing pigmented petals.

Flower Metabolite Profiling of the 20 Brassicaceae Genotypes

To evaluate the metabolic variance across these genotypes a non-targeted metabolite profiling of entire flowers was conducted based on LC-MS. In total, 228 peaks were detected, including 82 peaks annotated as 46 flavonoids, three hydroxycinnamate

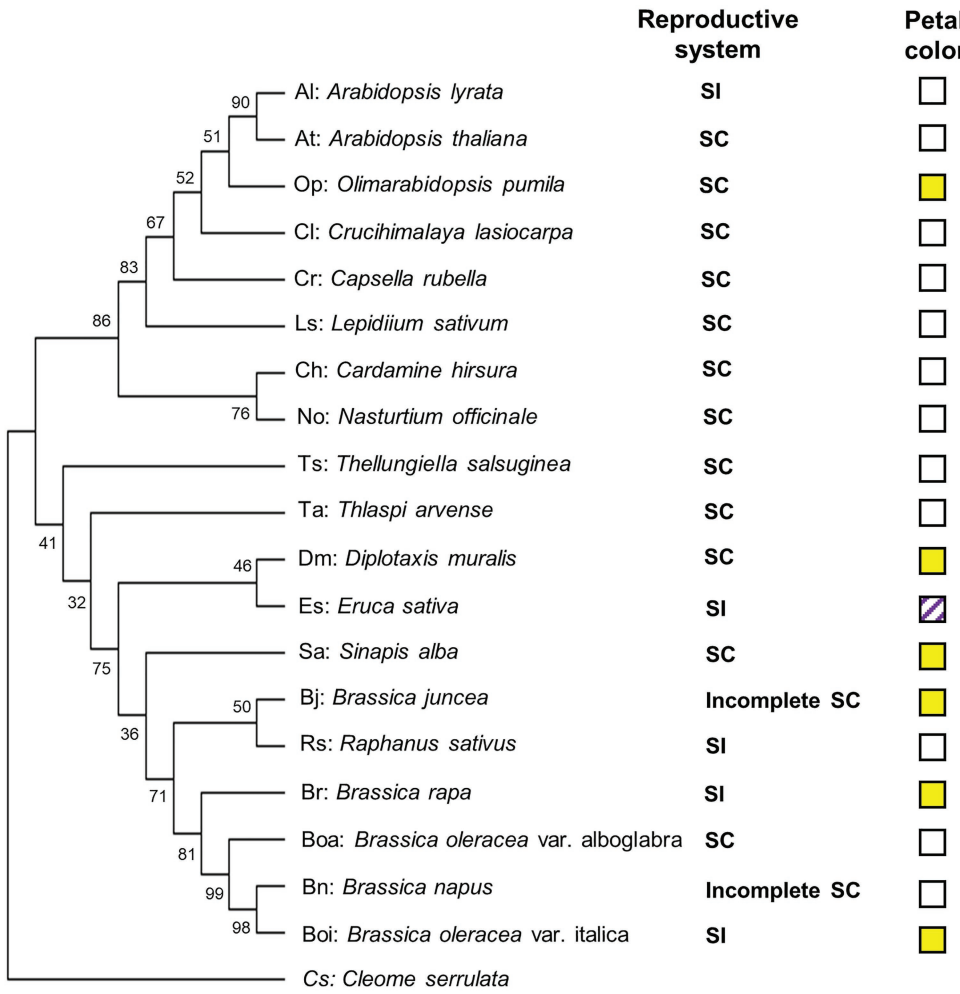

FIGURE 1 | Phylogenetic diagram of the relationships among selected Brassicaceae genotypes. The phylogenetic tree was constructed by MEGA X (Kumar et al., 2018) using the sequence of the internal transcribed spacer (ITS) gene of each genotype. *Cleome serrulata* was set as outgroup. The maximum likelihood (ML) method was used with the following parameters: General time reversible model, complete deletion and bootstrap (1,000 replicates). Values on the branches indicate bootstrap support in percentages. SC indicates self-compatible. SI indicates self-incompatible, incomplete SC indicates incomplete self-compatible. White square indicates white flower color, yellow square indicates yellow flower color, and white squares with purple stripes indicate white flower with purple stripes.

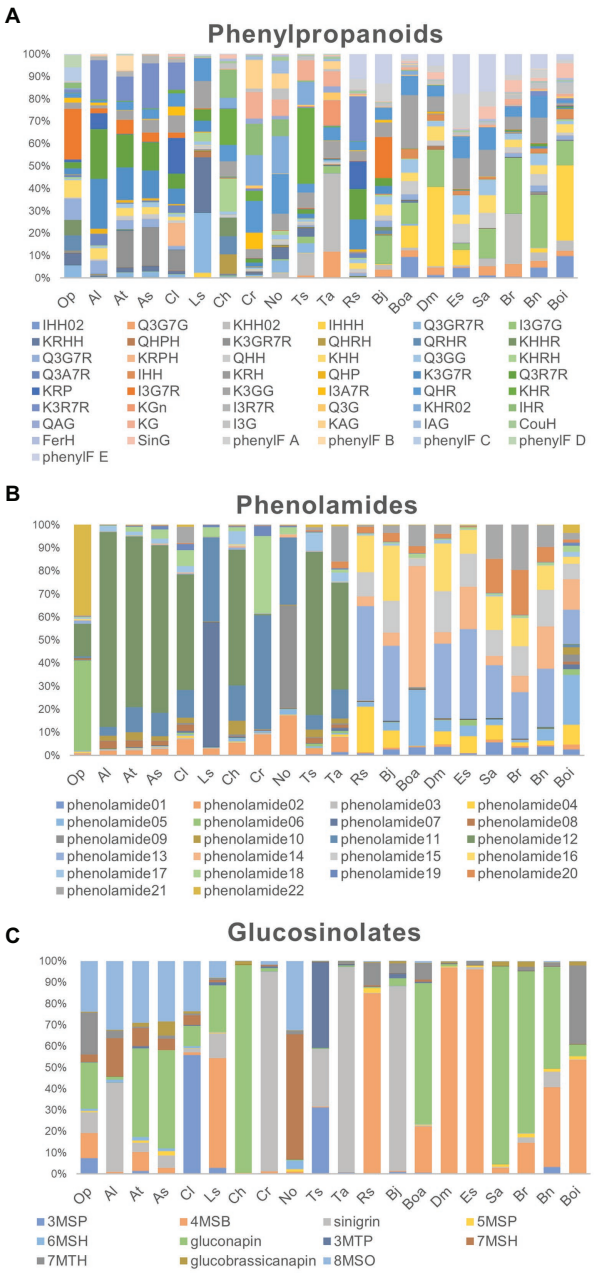

FIGURE 2 | Relative proportion of metabolites in each genotype. **(A)** The proportion of 49 phenylpropanoid compounds in each genotype; **(B)** The proportion of 22 phenolamides in each genotype; **(C)** The proportion of 11 glucosinolate compounds in each genotype.

derivatives, 22 phenolamides, and 11 GSLs by co-elution of *Arabidopsis* Col-0 flower extracts and combination approach with literature-based peak annotation (**Figure 2**; Tohge et al., 2016; de Souza et al., 2017). The relative abundance level of each peak was evaluated and presented in **Supplementary Figure S1** and **Supplementary Table S1**. **Figure 3** shows a heatmap presenting relative abundance of 82 annotated specialized metabolites. According to the clustering result of annotated peaks using hieratical clustering analysis (HCA), plant species were clustered into three clades (**Table 2**), which were mostly similar to their phylogenetic relationship. In the result of classification, plant species of clades A and B, with the exception of *A. lyrata*, were all self-compatible species. In clade C with the exception of *B. oleracea* var. alboglabra, *S. alba* and *D. muralis*, which were self-compatible and *B. juncea*, *B. napus* were incompletely self-compatible species, all other genotypes were self-incompatible.

Diversity in Floral UV-Absorbing Phenylpropanoids Across Genotypes

Differences in the abundance of phenolic acid derivatives and flavonols were analyzed among the 20 genotypes in order to evaluate evolutionary changes in the productivity of UV-absorbing compounds. In this analysis, three hydroxycinnamate-glucosides were annotated. Sinapoyl-*O*-glucoside, a major compound in UV-nectar area in *B. rapa*, largely accumulated in the compatible species *A. thaliana*, *A. shokei*, *C. lasiocarpa*, *C. hirsuta*, *S. alba*, and *D. muralis* as well as the incompatible species *B. rapa*. The other two common compounds coumaroyl-hexose and feruloyl-hexose, were ubiquitously present across the genotypes studied, however, they were present at considerably lower abundance in clade A species. Asides from phenolic acid compounds, 46 flavonols have been detected, mostly being the mono-, di-, and tri- hexose or pentose substituents of quercetin, kaempferol, and isorhamnetin aglycones (**Figure 3**). The ubiquitous "UV-nectar" compound namely isorhamnetin 3,7-*O*-glucoside was found to mainly in clade C species (with the exception of *R. sativus*). The *Arabidopsis* (C24) floral phenylacyl-flavonol glycoside kaempferol-3-*O*-(2"-*O*-rhamnosyl-6"-*O*-sinapoyl)glucoside-7-*O*-rhamnoside, which was supposed to have stronger UV-defense ability because of its additional phenyl moiety specifically accumulate in *A. thaliana* (C24) and *A. lyrata*. Flavonols quercetin-3-*O*-(6"-*O*-glucosyl) glucoside and kaempferol-3-*O*-(6"-*O*-glucosyl) glucoside, which have been reported being pollen specific compound in *A. thaliana* (C24), were found to accumulate in all species except *B. oleracea* var. italica, while kaempferol-3-*O*-(rhamnosyl)glucoside-7-*O*-rhamnoside, which was detected in *A. thaliana*, only accumulated to high levels in *A. thaliana*, *A. shokei*, *C. lasiocarpa*, *L. sativum*, and *B. oleracea* var. alboglabra.

According to the flavonol composition ratio in floral organs in each species, the flavonols which contributed the greatest proportion to the total flavonol level and the changes in these rates along with evolutionary relationships were investigated. In clade A genotypes, kaempferol derivatives such as kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside, and kaempferol-3-*O*-(2"-*O*-rhamnosyl)-glucoside-7-*O*-rhamnoside constituted a large proportion of flavonols. For clade B, the major flavonols were still kaempferol however with different sugar moiety substitutions such as kaempferol-hexose-rhamnoside and kaempferol-hexose. Although the proportion of the isorhamnetin-hexose, isorhamnetin-hexose-rhamnoside, and isorhamnetin-3,7-*O*-di-glucoside, is low in clade B genotypes, the compounds are present whereas they are not in clade A genotypes, and expanded to represent a large proportion

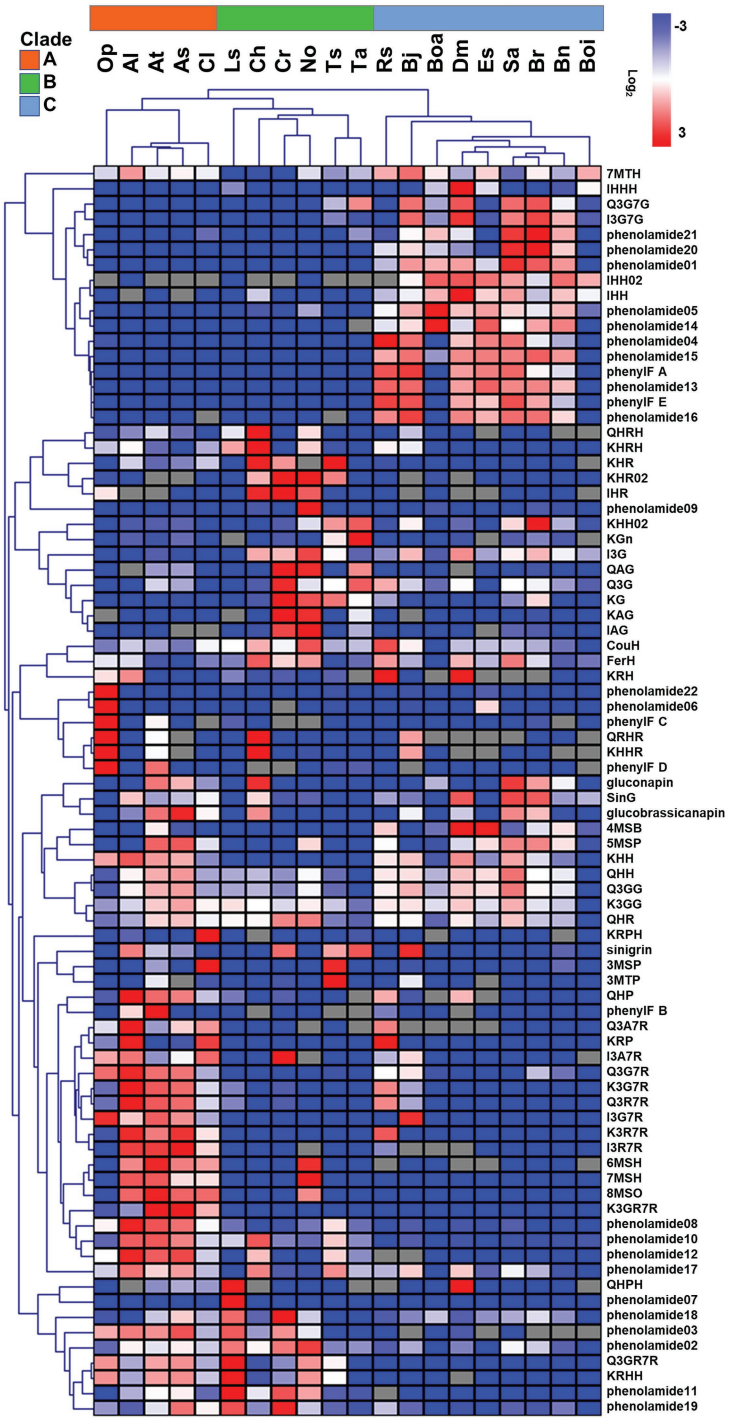

FIGURE 3 | Relative abundance of 82 annotated metabolites in floral organ of 20 genotypes. Color indicates the level of $\log_2$ (mean/mean_average). Gray grids indicate no detection. Metabolites and genotypes in heatmap were clustered using hierarchical clustering method (HCL).

clade C along genotypes. The latter expansion was coupled to an obvious decrease in the proportion of quercetin and kaempferol-derivatives. In addition, two phenylacylated-flavonols were specifically detected in clade C genotypes where they constitute a large proportion of the total flavonols.

Genotype-Specific Changes in the Type and Abundance of Floral Phenolamides

Comparison of phenolamide content was next conducted in order to investigate the evolutionary changes adapt to different environments and the divergence of reproductive system type

TABLE 2 | Clustering result using K-means.

Cluster	Species
A	At, As, Al, Cl, Op
B	Ls, Ch, No, Cr, Ta, Ts
C	Rs, Bj, Boa, Boi, Bn, Br, Sa, Dm, Es

because of their important roles in abiotic stress defense and fertility, respectively. In this study, 22 phenolamide compounds were annotated and their variance was studied in the same manner as for the flavonols described above. All genotypes in clade A and *Cardamine hirsta*, *T. salsuginea* in clade B displayed a high proportion of the well-known pollen coat constitution compound *N*',*N*''-bis-(5-hydroxyferuloyl)-*N*'''-sinapoyl spermidine that was previously found from *A. thaliana* (Fellenberg et al., 2008). By contrast, the proportion of *N*',*N*''-bis-feruloyl-*N*'''-(5-hydroxyferuloyl) spermidine was higher in clade B compared to clade A, being absent in clade C and *O. pumila* whose major constituent was *N*', *N*''-di-coumaroyl spermidine. In clade C species, the proportion of *N*',*N*''-bis-coumaroyl spermidine was greatly enhanced. In addition, the (5-hydroxyferuloyl) polyamine derivatives and caffeoyl phenolamide derivatives were specifically observed in clade C genotypes where they accounted for a large proportion of total phenolamides.

Aliphatic Glucosinolates Are the Major Type of Glucosinolate in Floral Organs

As a unique group of specialized metabolites in Brassicaceae, GSLs were focused because of their unique and diverse roles in biotic stress defense and interaction with insects. In this study, 11 GSLs were annotated among the 20 genotypes indicating the major type to be chain-elongated aliphatic GSLs derived from methionine. In general, four glucosinolates sinigrin (2-propenylglucosinolate), gluconapin (3-butylglucosinolate), 8-methylsulfinyloctylglucosinolate (8-MSO), and 4-methylsulfinylbutylglucosinolate (4-MSB) constitute the majority of glucosinolates, however, some genotype specific differences were observed. Sinigrin and gluconapin showed relatively universal existence cross clade, but sinigrin was present at considerably higher proportions in *B. juncea*, *T. arvense*, and *C. rubella*, while gluconapin was the major glucosinolate in *A. shokei*, *C. hirsuta*, *B. rapa*, and *S. alba*. By contrast, 4-MSB was considerable in clade C genotypes especially in *E. sativa*, *D. muralis*, *R. sativus*, *B. oleracea* var. italica and part of clade A or B genotypes like *A. thaliana* and *L. sativum*. While 8-MSO occupied certain proportion mostly in *A. thaliana* relatives.

PCA and PLS-DA Analysis to Identify Metabolite Features Cross-Clades

To assess specific floral metabolites which contribute to the discrimination of clades, the peak area value of the 228 characteristic peaks were subjected to PCA (**Figure 4A**). The R^2 value (which is defined as the proportions of variances explained by model was used to describe the goodness of fit) of 0.401 [0.28 value of variance was captured by principal component 1 (PC1)] was generated from the PCA model using two components, unraveling the existence of differences among these three clades. To investigate the contributors to the principal components, loading plots were visualized to reveal the role of metabolites in separating clades (**Figure 4B**). The metabolic loadings with highest values were caffeoyl-phenolamide derivative in PC1, indicating its strong impact on separation.

To optimize the separation among three clades, PLS-DA was applied. The predictive ability of PLS-DA model was estimated by Q^2 value, which was calculated *via* cross-validation (CV). As a result of 10-fold CV, the model was built with a Q^2 value of 0.937, indicating this model having well predictive ability since $Q^2 > 0.9$. As observed in the score plots of PLS-DA, species were clearly separated into three groups with 31.9% of variance was captured by PC1 (**Figure 4**). The VIP, a weighted sum of squares of the PLS loadings, was calculated in PLS-DA model, and metabolites with VIP > 1.7 were considered to be significantly distinguished. As a result, fifteen out of 228 characteristic peaks were detected as significant features (**Figure 4**). The relative levels of these significant metabolites were investigated in each clade and found to show specific pattern of presence/absence across clades. The phenylacylated-flavonoid, caffeoyl phenolamide derivatives, and (5-hydroxyferuloyl) polyamine derivative displayed high accumulation in the clade C group. By contrast, the *N*',*N*''-bis-(5-hydroxyferuloyl)-*N*'''-sinapoyl spermidine, 6-methylsufinylhexyl GSL, 7-methylsufinylheptyl GSL, and 8-methylsulfinyloctyl GSL displayed high accumulation in the clade A group, while species in clade B group almost contained medium levels of these compounds.

HPLC Verification of Phenylacylated-Flavonoid Derivatives in *Raphanus sativus* From Clade C

From PLS-DA analysis, several putative phenylacylated-flavonoid derivatives were supposed to be the significant feature to distinguish three clades. To provide further support that they are phenylacyl-moiety decorated flavonoids, *R. sativus* which accumulated the highest abundance of these compounds was selected for performing validatory HPLC-PDA analysis. The spectrums of saiginol A (Tohge et al., 2016), a group of phenylacylated-flavonoid compounds from the C24 accession of *A. thaliana* were used as positive control. Additionally, the spectrums of major flavonol-glycosides, kaempferol-3-*O*-(rhamnosyl)glucoside-7-*O*-glucoside, kaempferol-3-*O*-glucoside-7-*O*-rhamnoside and kaempferol-3-*O*-rhamnoside-7-*O*-rhamnoside, and hydroxycinnamate, sinapoyl-malate of *Arabidopsis*, were used as a negative control. Clustering by HCA of the resultant HPLC spectrum showed that the absorbance peaks at 17.57, 18.21, and 18.68 min on the chromatogram of *R. sativus* compared with saiginol A, suggesting them to be phenylacylated compounds (**Supplementary Table S2**). Further comparison of the absorption spectrum supported that peaks at 17.57 and 18.68 min were phenylacylated-flavonoids similar to saiginol A, while peak at 20.26 min corresponding to sinapoyl-malate shows different absorption pattern (**Supplementary Figure S2**).

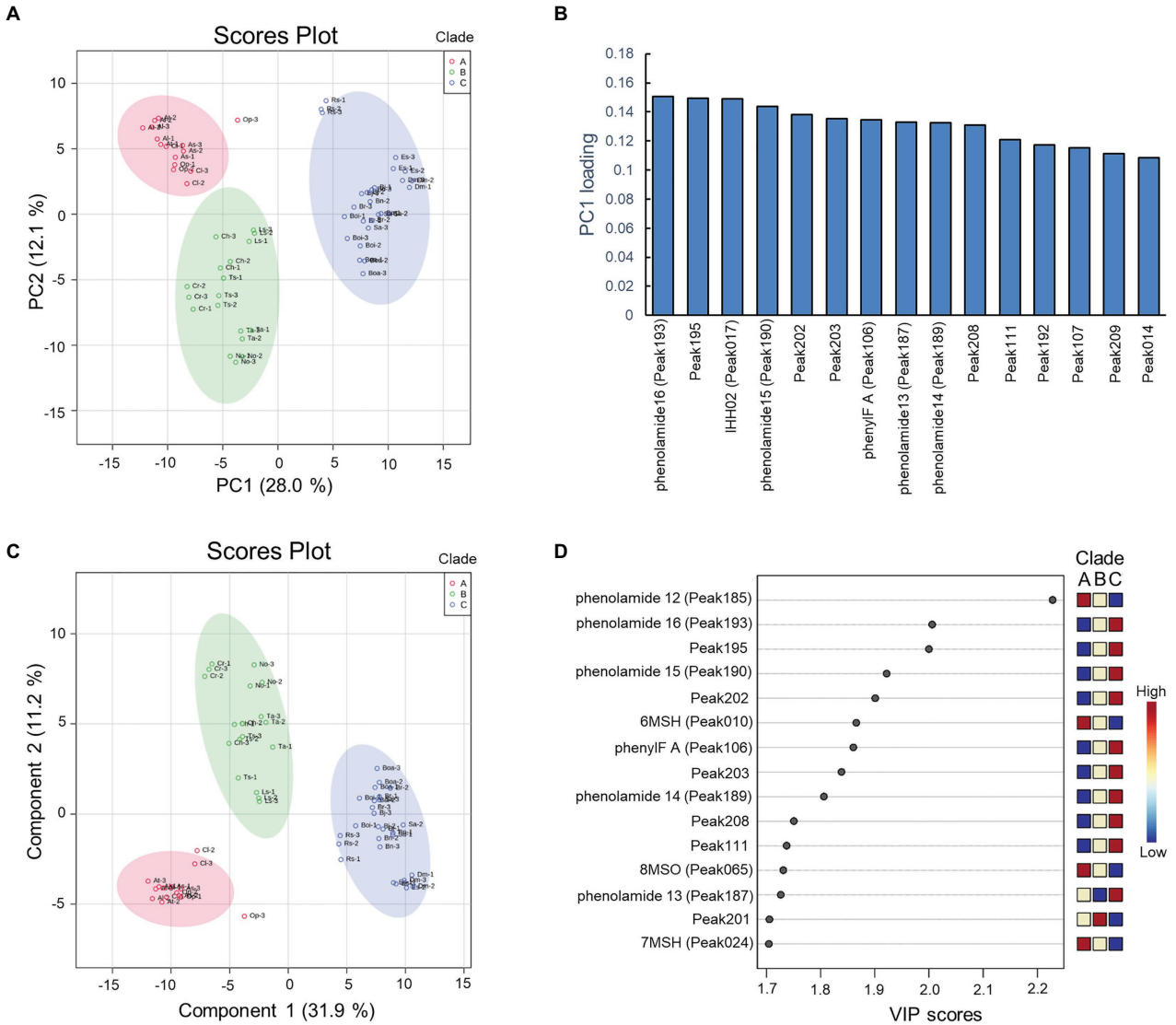

FIGURE 4 | Results of principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) of the metabolite data. **(A)** PCA scores plot between the principal component 1 (PC1) and PC2. The explained variances are shown in brackets, colors of circle indicate three replications of metabolite data of species; **(B)** PCA loading plots between the PC1 and PC2; **(C)** PLS-DA scores plot between the PC1 and PC2. The explained variances are shown in brackets; **(D)** Important features identified by variable importance in projection (VIP) scores. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each clade.

DISCUSSION

With few exceptions flowers in angiosperms are responsible for producing the next generation, therefore specialized metabolites in flowers serve the purpose of attracting pollinators, playing protective roles to against biotic or abiotic stress such as UV-irradiation or herbivory, and being an important constitution allowing the maintenance of normal fertility (Tohge et al., 2018; Borghi et al., 2019; Borghi and Fernie, 2020). Successful fertilization involves certain metabolite groups, for example, phenolic acids derivatives and flavonols function as important UV-absorbing compounds, some of which are essential to maintain pollen activity (Xue et al., 2020), phenolamides play unique role for fertility (Bassard et al., 2010), and glucosinolates are involve in plant-insect interaction (Liu et al., 2020). In this study, specialized metabolites from flowers of 20 Brassicaceae genotypes were investigated in order to study the metabolite evolutionary changes concomitant to the divergence of the different clades and the underlying different reproductive recognition systems.

To attract pollinators, bright color helps the flower to be more visible. With the exception of *O. pumila* in clade A, all clade A and clade B genotypes which includes the majority of SC genotypes had white flowers, while species in clade C mostly

had yellow flowers, or specific pigmentation pattern such as that exhibited by *E. sativum*, indicating that they may be more attractive to pollinators. Hence this clade C harbors predominantly SI or incomplete SC genotypes. Besides the human-visible bright colors of flowers, UV-nectar also greatly promotes pollinator visits. Indeed, sinapoyl-*O*-glucoside and isorhamnetin 3,7-*O*-di-glucoside were characterized to highly accumulate in the UV-nectar area in incompatible species *B. rapa* (Brock et al., 2016). While in our study, with the exception of *B. rapa*, sinapoyl-*O*-glucoside accumulated in self-compatible genotypes, but isorhamnetin-3,7-*O*-di-glucoside accumulated in clade C species, most of which being self-incompatible genotypes. In general, genotypes in clade C had brighter flower color and specific UV-nectar compound, in accordance with the demand of being more attractive to pollinators for most of species in clade C were self-incompatible genotypes.

As a crucial step of the fertilization process, pollen production and its activity are easily affected by UV-irradiation since enhanced UV-B levels reduce the pollen viability (Koti et al., 2005). Flavonols not only play a role in presenting UV nectar patterns on petals attracting pollinators, but also function as UV-defense compounds that protect reproductive tissues such as pollen, and be essential for fertility and pollen tube germination. Flavonols such as phenylacylated-flavonols, kaempferol, and quercetin are frequently reported to response to UV-irradiation and promote the UV tolerance (Hofmann et al., 2000; Tohge et al., 2016). As a result of the PCA and PLS-DA analysis in this study, phenylacylated-flavonoids was found as metabolite features, which discriminated the three clades, being specifically highly accumulated in clade C genotypes, indicating genotypes in clade C may have a higher demand, and therefore have evolved a higher ability, for UV-resistance.

Phenolamides accumulate in different floral organ, with some members of this compound class considered to be markers of fertility and play an important role in pollen development (Bassard et al., 2010). Moreover, phenolamides help to adapt to abiotic stress due to their antioxidant and radical scavenging ability. Additionally, spermidine derivatives are stamen-specific compounds, and their content may decrease due to the sharply decreased ratio of pollen grains to ovules, which increases the tendency of self-fertilization. *N*', *N*''-bis-(5-hydroxyferuloyl)-*N*'''-sinapoyl spermidine is a well-known major pollen coat constituent in *A. thaliana*, and this pathway was evolved by the generation of an enzymatic cascade of six successive hydroxylations by two partially redundant cytochromes P450 genes *CYP98A8* and *CYP98A9*, which are duplications of an ancestor *CYP98A3*, showing new functionalization under Darwinian selection (Matsuno et al., 2009). In the current study, *N*',*N*''-bis-(5-hydroxyferuloyl)-*N*'''-sinapoyl spermidine has low abundance in most genotypes of clade B and clade C, indicating possible functional differentiation of the CYP98A family in those genotypes. Di-coumaroyl and tri-coumaroyl spermidine, which are considered as markers of male fertility are abundant in anther (Werner et al., 1995), and rape bee pollen nectar (Zhang et al., 2020). In this study, the proportion of di-coumaroyl spermidine in clade C genotypes was much higher than that in clade A and B, indicating the species in clade C may attract higher bee visitation. Di-feruloyl polyamine derivatives are usually transiently detected following fertilization (Bassard et al., 2010), indicating a possible earlier fertilization of clade A and clade B genotypes than clade C genotypes.

Glucosinolates are sulfur- and nitrogen-containing specific specialized metabolites of Brassicaceae species, which have a defense activity against herbivorous insects and serve as sulfur sources under sulfur-deficiency (Aarabi et al., 2016). Glucosinolates are derived from a variety of amino acids, therefore, comprising a diverse group whose chemical diversity is defined by the side-chain modification and amino acid elongation (Kliebenstein et al., 2001). They are divided into aliphatic GSLs, indole GSLs, and aromatic GSLs according to their chemical structure. Physiological experiments in *A. thaliana* have revealed that, aliphatic GSLs function as the sulfur source during sulfur-deficiency, indole GSLs are essential for the defense and symbiosis balance toward soil fungi and nematode (Hiruma, 2019), and aromatic GSLs have specifically high accumulation in seeds involved in the inhibition of seed germination (Brown et al., 2003). Here, we demonstrate that aliphatic GSLs were the majority in floral organ. Usually, the glucosinolates are toxic to herbivores, and more recent reports have uncovered the diversity in their toxicity. For example, low-sinigrin content in plants tends to increase caterpillar attack, while glucoiberin (3-methylsulfinylpropylglucosinolate) has the opposite effect (Olsson and Jonasson, 1994). In *B. juncea, T. arvense*, *C. rubella*, and *A. lyrata*, sinigrin was the major GSL in flowers, indicating a possible lower attack rate of these species from caterpillar. A recent report shows that their corresponding diverse breakdown products volatile isothiocyanates (ITCs) can drive the host preference of crucifer-specialist moth (Liu et al., 2020). In this study, the 3-methylthiopropylglucosinolate and 4-pentenylglucosinolate, corresponding breakdown products of which are the crucifer-specialized moth-attracting volatiles ITCs iberverin and 4-pentenyl ITC, actually constituted d a very small proportion of glucosinolates in all genotypes. We suggest that this result may be the consequence of the balance between the pressure from crucifer-specialized moths and the need for toxic defenses towards other insects.

CONCLUSION

In this study, we performed non-targeted metabolite profiling of 20 genotypes from different genus in Brassicaceae to explore the evolutionary aspects of floral metabolites corresponding to different reproductive recognition system. More than 228 characteristic peaks with 82 annotated specialized metabolites were detected including those from the phenolics, phenolamides, and glucosinolates class. We demonstrated that although diversity of specialized metabolites presented among species, these metabolites exhibited certain metabolic structure characteristics among different clades, which we speculate is linked to their different roles in the different fertilization processes alongside divergent evolution under biotic/abiotic stress. Through PCA

and PLS-DA analysis, 15 metabolites including one phenylacylated-flavonoid and five phenolamides were indicated as significant contributors to this discrimination. These polyphenolic compounds are suggested as metabolites, which possibly have evolved during metabolic evolution conferring the physiological function as pollinator attraction and/or light protection. As such our study provides new insights into the evolutionary aspects of floral specialized metabolism and relationship to the environment.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

YL, MW, and TT conceived, designed, and conceptualized the outline of the review manuscript. MW, SY, and YK performed plant cultivation and metabolite profiling. YL and TT performed data analysis and peak annotation. YL, AF, and TT wrote the manuscript. YL, MW, CA, AF, and TT supervised and edited throughout the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.640141/full#supplementary-material

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Cycad-Weevil Pollination Symbiosis Is Characterized by Rapidly Evolving and Highly Specific Plant-Insect Chemical Communication

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Coevolution between plants and insects is thought to be responsible for generating biodiversity. Extensive research has focused largely on antagonistic herbivorous relationships, but mutualistic pollination systems also likely contribute to diversification. Here we describe an example of chemically-mediated mutualistic species interactions affecting trait evolution and lineage diversification. We show that volatile compounds produced by closely related species of *Zamia* cycads are more strikingly different from each other than are other phenotypic characters, and that two distantly related pollinating weevil species have specialized responses only to volatiles from their specific host *Zamia* species. Plant transcriptomes show that approximately a fifth of genes related to volatile production are evolving under positive selection, but we find no differences in the relative proportion of genes under positive selection in different categories. The importance of phenotypic divergence coupled with chemical communication for the maintenance of this obligate mutualism highlights chemical signaling as a key mechanism of coevolution between cycads and their weevil pollinators.

Keywords: coevolution, brood-site mutualism, chemical signaling, Coleoptera, Caribbean

INTRODUCTION

Obligate mutualistic associations involving a high degree of specialization by both partners such as those between figs and fig wasps and yuccas and yucca moths are less common in nature, and yet the analysis of such systems is also vital to our understanding of mechanisms underlying the evolution of plant/insect interactions (Pellmyr, 2003; Herre et al., 2008). Studies of these and other pollination systems (Holland and Fleming, 1999; Kawakita, 2010) have highlighted morphological and behavioral traits associated with mutualism, and begun to identify mechanisms of diversification and speciation associated with these lineages (Althoff et al., 2012; Hembry et al., 2013; Suinyuy et al., 2015). Plant volatile production for attracting pollinators has been hypothesized as a likely mediator of specialized brood-site mutualisms (Pellmyr, 2003; Okamoto et al., 2007), yet has been largely overlooked in its contribution to diversification (Suinyuy et al., 2015). In order to fully understand the process of diversification in mutualistic, coevolving lineages,

we need to learn more about the causes and consequences of species interactions. Specifically, we are interested in identifying: (1) traits driving these relationships, (2) whether these traits are diverging across closely related taxa, and (3) the role of selection in trait divergence. *Zamia* cycads and their *Rhopalotria* pollination mutualists are an ideal study system as they represent an understudied obligate brood-site pollination mutualism where the importance of volatile signaling is clear.

The ancient plant order Cycadales is an early diverging lineage of gymnosperms (Ran et al., 2018) and, unlike most other gymnosperms requires insect pollination instead of wind pollination (Terry et al., 2012). The pollination mechanism of most cycads appears to be an obligate brood site mutualism whereby highly specialized pollinators live out their lifecycle within pollen cone tissue (Mound and Terry, 2001; Terry, 2001; Terry et al., 2005, 2007). This mutualism is hypothesized to be driven by host plant volatile organic compounds (VOCs) through a push-pull pollination mechanism (Terry et al., 2007; Salzman et al., 2020). Pollinators are attracted to mid-level amounts of the host plant compound but are repelled by high levels of VOCs. Pollen and ovulate cones have a daily cycle of VOC production causing pollinators to be repulsed by the male cones and attracted to the female cones, and then attracted to the male cones once again. The pattern of plant volatile release is conserved across the Cycadales, and the behavioral response of pollinators to the daily change in expression of host plant VOC has converged between *Cycadothrips chadwicki* (Thysanoptera) and *Rhopalotria furfuracea* (Coleoptera) suggesting that this push-pull pollination mechanism is the likely pollination syndrome across the Cycadales (Salzman et al., 2020). Much of the Neotropical cycad genus *Zamia* are pollinated by *Rhopalotria* weevils and closely related genera within the Allocorynina (Tang et al., 2018). These weevils feed and reproduce on the pollen cone of their host *Zamia* (Norstog and Fawcett, 1989; Norstog et al., 1992). Conversely, the host *Zamia* species are completely dependent upon pollination services by the weevils (Norstog et al., 1986; Tang, 1987). The *Zamia—Rhopalotria* symbiosis appears to be fully mutualistic for both lineages, and the relationship has been hypothesized to exhibit phylogenetic congruence between specialized partners (Norstog et al., 1992; Stevenson et al., 1998; Tang et al., 2018). However, studies remain to be done that provide information about potential coevolutionary mechanisms involved, including those documenting reciprocal evolutionary change in each lineage (e.g., Janzen, 1980).

Chemical communication mediating the relationship between plant and pollinator has been described for one species pair, Mexican *Z. furfuracea/R. furfuracea*, and is hypothesized to be driving relationships across the two lineages (Salzman et al., 2020), but we do not know whether the plant/pollinator behavior is shared across other *Zamia/Rhopalotria* pairs, whether volatile production and perception traits are diverging in the lineages, or whether any of these traits are driven by positive selection. Here we describe the chemical communication that underlies a distantly related species pair, the Caribbean *Z. integrifolia—R. slossoni*. We then ask if there has been evolution of specificity in volatile perception in these two *Rhopalotria* species. Finally, we investigate phenotypic and genotypic evolution across the Caribbean *Zamia* clade to look for evidence of rapid evolution and positive selection associated with volatile production. By analyzing reciprocal evolutionary change in volatile production and insect perception, we provide investigation of coevolution in what is arguably the earliest example of an insect mediated "pollination syndrome" (Cai et al., 2018; Salzman et al., 2020).

MATERIALS AND METHODS

Study System and Design

Rhopalotria and *Zamia* form a tight obligate mutualism. *Rhopalotria* and their close relatives are found across much of *Zamia* diversity where they provide necessary pollination services to their respective host *Zamia* species, which in turn provide food, brood sites, and shelter. Feeding damage does not affect reproduction or potential fitness of *Zamia* as *Rhopalotria* feed only on the disposable pollen cone parenchyma tissue. This mutualism is mediated by plant volatile production (Salzman et al., 2020), a cue that is important for lifecycle completion in both lineages. The strength of the mutualism is such that pollinators go into diapause during the 10 months of the year when reproductive services are not required by the host plant (Norstog and Fawcett, 1989; Norstog et al., 1992).

Caribbean *Zamia* species are distributed across Florida, Cuba, Jamaica, the Bahamas, Cayman Islands, Dominican Republic (but not Haiti) and Puerto Rico (Calonje et al., 2020). *Rhopalotria* species have been found associated with all populations of Caribbean *Zamia* except those on Puerto Rico (Tang et al., 2018). *Zamia integrifolia* as currently circumscribed is distributed across the southeastern United States (Florida and Georgia), the Bahamas, Cayman Islands, and Cuba (Calonje et al., 2020) although these populations are not monophyletic (Calonje et al., 2019). *Rhopalotria slossoni* is distributed across southern Florida (O'Brien and Tang, 2015) where it provides pollination services to populations of *Z. integrifolia* (Tang, 1987; Norstog et al., 1992).

Plant Volatile Analysis

To quantify the diversity of VOC profiles in the *Zamia* clade, we collected plant volatiles from dehiscing pollen cones of 10 Caribbean taxa using headspace collection methods (see Salzman et al., 2020). We quantified volatiles from 3 to 7 plants per taxa. These plants were wild collected as seeds from native populations across the Caribbean (**Supplementary Figure 1**) and cultivated in a common garden at Montgomery Botanical Center (MBC) in Coral Gables, Florida. All volatiles were collected between the hours of 13:30 and 16:00 (**Supplementary Table 1**) across 7 days in 2019. *Zamia* phenology across the phylogeny is highly varied at MBC, but most Caribbean *Zamia* are reproductive in January/February (Griffith et al., 2012). We also captured volatiles from one pollen and two ovulate cones of *Z. integrifolia* collected from Levy County, Florida at hour and a half time points from 8:00 until 20:45. MBC accession numbers for these plants are 20050825*A, 20050831*A, and 20050820*C. Samples were eluted as described in Salzman et al. (2020) and were run on a combined Agilent technologies 6890 network gas chromotograph and 5973 mass-selective detector using a DB-5 column (J&W Scientific

Inc., Folsom CA; 30 m × 0.25 mm I.D.; film thickness, 0.25 um; splitless mode) (GC-MS). Helium was the carrier gas at a constant flow rate of 0.7 ml/min. the injector temperature was 250°C, oven temperature was held at 50°C for 1 min, and then increased 10°C /min to 170°C where it was held for 5 min.

Volatile peaks were standardized and calibrated manually to include all peaks to be comparable across samples. Bag, filter, and DCM controls showed a few contaminant peaks after 15 min retention time that were removed during integration. The ChemStation Integrator was used with the following parameters: Initial area reject = 0, initial peak width = 0.300, initial threshold = 16.0, shoulder retention = off. Integration began at 3,550 min after the solvent peak and ended at 15,000 min (ChemStation software, Agilent technologies). Peak areas were calculated to percent composition (size of individual volatile compound peak/total peak area of the sample) and were treated as phenotypic traits in subsequent trait analysis.

Percent compositions of volatile peaks were analyzed for dissimilarities within and between species (Bray-Curtis dissimilarity index with 999 permutations) using the non-parametric test ANOSIM (analysis of similarities), vegan v2.3-5 package (Oksanen et al., 2019) in R version 3.6.3 (R Core Team, 2020).

Weevil Electroantenograph Detection (EAD)

Gas Chromotrography-Electroantenograph Detection (GC-EAD) can be used to determine the physiological capability of an insect to perceive a volatile compound. We used this technique to identify volatile compounds produced by *Zamia integrifolia* that trigger responses in the antennae of *Rhopalotria slossoni*. We also tested the specificity of pollinator response using two species of *Rhopalotria* and their respective host plants.

We used headspace collection methods (see Salzman et al., 2020) to capture volatiles from receptive ovulate (Montgomery Botanical Center (MBC) accession number 20050831*A) and dehiscent pollen (MBC accession numbers 20050815*1 and 20050815*2) cones of *Z. integrifolia*. We then used GC-EAD (see Crook et al., 2008; Salzman et al., 2020) to determine which host plant volatiles elicited responses from the antennae of *R. slossoni*. We injected 2 μl plant volatile eluate and used a GC method where the oven temperature was held at 50°C for 1 min, and then increased 10°C/min to 300°C where it was held for 5 min. The injector temperature was 280°C and the GC outlets for the EAD and FID were 300°C. For mass spec identification of individual plant volatile compounds, samples were run separately as described above for plant volatile analysis.

Eleven *R. slossoni* weevils were tested using GC-EAD against *Z. integrifolia* cone aerations. The antennally active peak was identified on the basis of its mass spectra using the universal NIST chemical library (NIST version 2.0, 2002), Kovats index (van Den Dool and Kratz, 1963; Kovats, 1965), and by comparing its retention time and mass spec with an authentic standard obtained from Sigma-Aldrich (St. Louis, MO, catalog no. M2047). The positive EAD response was tested using GC-EAD of nine weevils against a standard of methyl salicylate (Sigma-Aldrich, St. Louis, MO, catalog no. M2047). For these standard GC-EAD runs, the oven temperature was held at 50°C for 1 min, and then increased 20°C /min to 320°C, where it was held for 5 min. All other settings remained the same.

To determine the specificity of weevil perception of potential host plants, the GC-EAD physiological responses of two species of *Rhopalotria* were compared using the volatile compounds of their host and non-host *Zamia* species. The active compound identified using antennae of *R. slossoni*, methyl salicylate, differs from the active compound identified in another *Rhopalotria-Zamia* species pair involving *Zamia furfuracea*. In the latter, the plant volatile compound 1,3-octadiene was identified as the physiologically active component eliciting a response from the mutualistic weevil partner, *R. furfuracea* (Salzman et al., 2020). Thus *R. slossoni* (n = 2) and *R. furfuracea* (n = 3) were tested against a mixture of 1,3-octadiene (ChemSampco Inc. Dallas, TX, catalog no. 7015.90) and methyl salicylate (Sigma-Aldrich, St. Louis, MO, catalog no. M2047) using the same GC-EAD set up and oven settings used for the cone aeration GC-EADs.

Weevil Behavior

Using pitfall tests, we confirmed that the behavioral response of *R. slossoni* to the volatile compound methyl salicylate follows push-pull pollination in the same manner observed in *R. furfuracea* (see Salzman et al., 2020). To determine whether methyl salicylate acts as an attractant, and whether weevil attraction changes with differing amounts of the volatile compound, behavioral assays were carried out consisting of a hexane control and five dilutions of methyl salicylate in HPLC grade hexane (concentrations: 1 ng/μl, 10 ng/μl, 100 ng/μl, 1 μg/μl, 10 μg/μl) (see Salzman et al., 2020 for detailed methods). 10 μl of each dilution was used to cover the natural emission of methyl salicylate production found in *Z. integrifolia* (**Figure 1C**). Methyl salicylate values at peak plant expression ranging from 1 to 50 μg (**Figure 1C**) are expected to be repellent to *R. slossoni*. All concentrations plus the control were run simultaneously for each trial (n = 10). Prior to the trial, weevils were allowed to feed freely on *Z. integrifolia* cones so they would not be stressed before the trial started. Each trial was carried out by placing 4 *R. slossoni* weevils into the arena away from the pit. Arenas were closed and the entire trial was placed in the dark at room temperature (21°C) for 30 min, after which time the number of weevils in the pits were counted. Dead or copulating weevils were not counted.

As with *R. furfuracea* (Salzman et al., 2020), *R. slossoni* behavioral response to host volatile did not follow a normal distribution and therefore the raw data plus 0.5 (to account for zero values) was log transformed for statistical tests of differences in attraction to the different concentrations of methyl salicylate. Weevil attraction to different amounts of methyl salicylate was determined through an ordinary least squares model when no significant difference was found between ordinary least squares and weighted least squares models (p = 0.54) using the nlme package (Pinheiro et al., 2020) in R version 3.6.3 (R Core Team, 2020). P-values were then corrected for family-wise error using sequential Bonferroni correction.

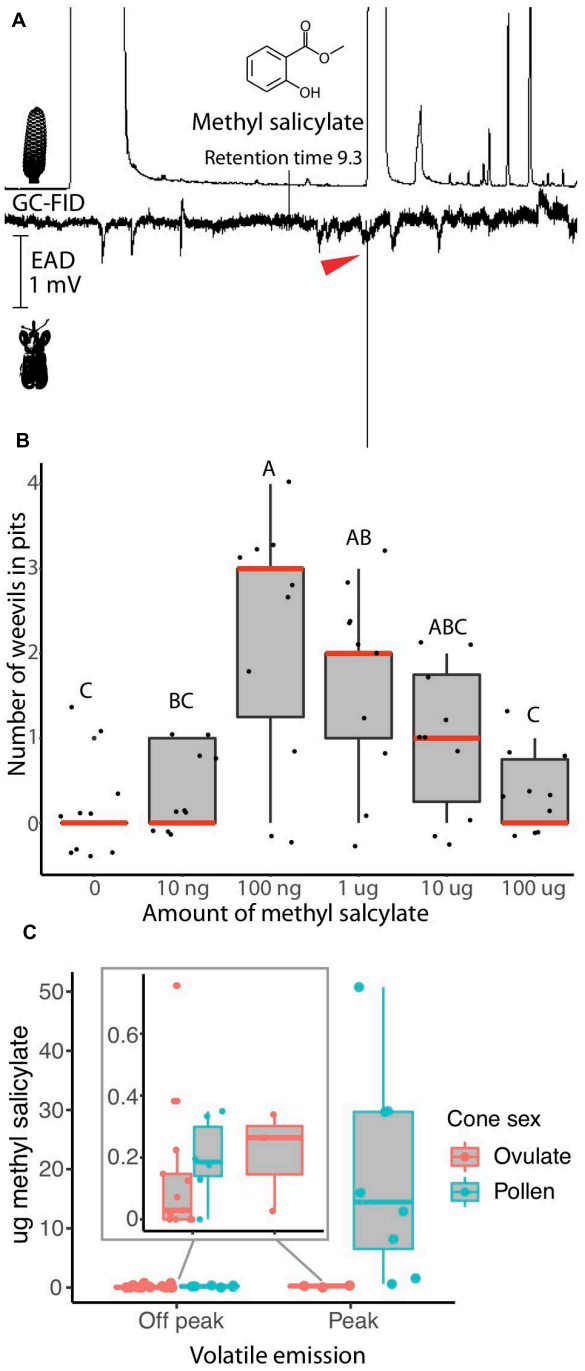

FIGURE 1 | *Rhopalotria slossoni* perception and behavioral response to *Zamia integrifolia* production of methyl salicylate follows push-pull pollination (Salzman et al., 2020). **(A)** Electroantenograph detection (EAD) shows methyl salicylate elicits responses from antennae of *R. slossoni*. Plant gas chromatography flame ion detection (GC-FID) on the top, weevil electroantenograph detection (EAD) on the bottom with red arrow denoting a positive response (n = 11 and confirmed with a standard n = 9). **(B)** Pitfall test of behavior shows that methyl salicylate acts as an attractant with a non-linear response. Median values are shown in red and p-values are in **Supplementary Table 5**. **(C)** Methyl salicylate emissions from *Zamia integrifolia* pollen and ovulate cones change over the course of the day.

(Continued)

FIGURE 1 | Continued
Volatiles were sampled from 8:00 to 20:45. Peak emissions are between 13:30 and 16:00 h. Methyl salicylate production in pollen cones during off peak hours (inset) coincides with the amount most attractive to weevils in pitfall tests. Production of methyl salicylate during peak hours coincides with amounts found to be less attractive in pitfall tests. Ovulate cones also increase their emission of methyl salicylate, reaching peak attractive amounts at the same time that pollen cones become less attractive.

Morphological Trait Analysis

To quantify morphological variation across the *Zamia* clade, we photographed Caribbean *Zamia* herbarium sheets from 12 Herbaria and processed them for morphological leaflet measurements in ImageJ (version 2.0.0-rc-69/1.52n) (Rueden et al., 2017) through Fiji (Schindelin et al., 2012). Leaflet length/width were chosen as comparative morphological traits because they have been shown to be fairly consistent within and divergent between Caribbean populations (Eckenwalder, 1980). Herbarium sheets were used from A, ANS-PHILA, BNH, FTG, GH, MAPR, MICH, MO, NYBG, UPRRP, US, and USNH herbaria. We also collected leaflets from natural populations on Eleuthera, Andros Island, Grand Bahamas, New Providence and Long Island. Leaflets from the middle of the leaf rachis were selected from each herbarium sheet or individual field collection and measured for length and width. Cycad leaflets unfurl and all leaflets expand at the same time resulting in a leaflet shape that is mostly uniform across the leaf while there may be variation in size especially at the top and bottom of the leaf rachis. Size increases as the leaflet grows and fully expands. Due to the limitations of the available material and not knowing the age or developmental stage of the leaflets in herbarium material, we calculated ratios of leaflet length by leaflet width to remove any influence of size. The number of measured leaflets per population are: *Zamia* sp. (Andros Island): 34, *Z. angustifolia* (Eleuthera): 10, *Z.* sp. (Eleuthera) 48, *Z.* sp. (Grand Bahamas): 51, *Z.* sp. (New Providence): 37, *Z. lucayana* (Long Island): 25, *Z. integrifolia* (Florida): 19, *Z. pumila* (Puerto Rico): 12, *Z. erosa* (Puerto Rico): 6. GPS point localities were either measured in the field, copied from herbarium sheets, or manually generated using herbarium data sheet locality information and Google Maps to determine their inclusion in the studied populations. Herbarium sheets were not available for the Jamaican populations of *Z. aff. amblyphyllidia* or *Z. aff. portoricensis* and so these species were not included in the leaflet morphology analysis.

We analyzed the leaflet measurements using R version 3.6.3 (R Core Team, 2020) to determine dissimilarities (Bray-Curtis dissimilarity index with 999 permutations) using within and between populations using the non-parametric test ANOSIM (analysis of similarities) as implemented in the vegan v2.3-5 package (Oksanen et al., 2019).

Selection in the Zamia Genome

Transcriptomes

We collected Plant RNA from dehiscing pollen cones of wild collected Caribbean *Zamia* seeds grown at MBC for three purposes: (1) to detect signatures of positive selection

across the Caribbean clade, (2) to identify genes whose expression correlates with volatile production, and (3) to identify genes whose expression correlates with cone development. To detect signatures of selection across the Caribbean clade, microsporophyll (cone scales) were collected from dehiscing pollen cones of 10 Caribbean *Zamia* (**Supplementary Table 2**). To identify genes whose expression correlates with volatile production, microsporophyll cone scales were collected from *Z. integrifolia* at six time points across a day in concert with volatile headspace sampling. This sampling was done at the same time as the daily volatile collection described above using MBC accession 20050825*A. Volatiles were collected for 45 min, at which point one microsporophyll cone scale was removed from the center of the cone for RNA extraction. To identify genes whose expression correlates with cone development, entire cones of *Z. furfuracea* were collected at three developmental stages: just emerging cones, young half sized cones, and immature almost full-sized cones. Two separate plants were sampled, with one cone of each stage collected on each plant.

All RNA samples were flash frozen in liquid nitrogen in the field and then stored at −80 until RNA extraction. Accession information and initial collection locality for all plants are listed in **Supplementary Table 2**. Cone tissue was ground to a powder in liquid nitrogen and RNA extraction was performed using the Qiagen RNeasy Plant MiniKit (Qiagen, Hilden, Germany) with the addition of 100 μl polyethylene glycol 4000 and 10 μg polyvinlypyrrolidone 40. RNA purity and integrity were evaluated using a 2100 bioanalyzer (Agilent Technologies, Santa Clara CA) using the plant RNA Nano Assay (version 1.3). Libraries were prepared from samples with a RNA Integrity Number greater than 7 using the NEBNext Ultra RNA library prep kit for Illumina (# E75305, New England Biolabs, Ipswich MA), NEBNext Poly(A) mRNA magnetic isolation module (#E7490, New England Biolabs, Ipswich MA), NEBNext multiplex oligos for Illumina (#E7335, #E7500, New England Biolabs, Ipswich MA), and Agencourt AMPure XP beads (#A63881, Beckman Coulter, Pasadena CA). Library purity and Integrity was assessed using a 2100 bioanalyzer (Agilent Technologies, Santa Clara CA) using the high sensitivity DNA Assay (version 1.03). Libraries averaged 350–500 base pairs. All 23 samples were combined in equal concentration and run across three lanes of 125 paired end reads on Illumina HiSeq2500 (San Diego CA) at Cold Spring Harbor Labs (Cold Spring Harbor NY).

Transcriptomes were filtered to remove low-quality paired-end sequence reads with Timmomatic (Bolger et al., 2014) and individually *de novo* assembled using Trinity v2.3.2 (Grabherr et al., 2011; Haas et al., 2013; **Supplementary Table 3**). Assembly quality was assessed using the embroyphyta lineage gene set in BUSCO v3.0.2. (Seppey et al., 2019). Through the Galaxy platform (Afgan et al., 2018; **Supplementary Figure 2**). Coding sequences were identified with Transdecoder v3.0.0 (Haas et al., 2013) and sequence redundancy reduced with CD-HIT-EST v4.6.4 (Li and Godzik, 2006). Orthologs were identified using the tree based ortholog identification pipeline described in Yang and Smith (2014) and briefly described here. This involved using all-by-all BlastN (Altschul et al., 1997) and Markov cluster algorithm (MCL) (Enright et al., 2002; Van Dongen and Abreu-Goodger, 2012) on all non-redundant coding sequences to obtain clusters of similar sequences. These clusters are aligned using MAFFT v7.309 (Katoh and Standley, 2013) and trimmed with Phyutility (Smith and Dunn, 2008). Cluster trees were estimated using FASTTREE v2.1.9 (Price et al., 2010). In order to account for long branches resulting from misassembly, paralogy, or recombination branches longer than 10 times the average distance to tips in its sister clade and longer than 0.4 substitutions per site were trimmed from these trees. The resulting sequence files were then realigned using MAFFT (Katoh and Standley, 2013) and used to infer gene trees with Randomized Axelerated Maximum Likelihood (RAxML) v8.2.10 (Stamatakis, 2014). These trees were subjected to an additional long branch trimming and subsetted to a taxon occupancy of 12 or more for phylogenetic analysis and 10 or more for tests of positive selection that excluded the out-group, *Zama furfuracea*, and the Caribbean *Z. lucayana* where many genes were missing due to poor transcriptome assembly. These homologous gene trees were then further pruned to a single orthologous sequence per sample using the maximum inclusion method (MI) which isolates the subtree with the highest number of taxa without taxon duplication (Dunn et al., 2008, 2013; Smith et al., 2011; Yang and Smith, 2014).

Plant Genomic Positive Selection

Transcriptomes of dehiscing pollen cones of ten Caribbean *Zamia* species were used to determine whether genes related to volatile production were experiencing higher rates of positive selection than other genes in the genome. The Mexican *Zamia furfuracea* was included as an outgroup. This was done in three steps briefly described here and further described below. First, genes that were found in all in-group species transcriptomes were tested for evidence positive selection. Second and separately, differential expression analysis was run on *Z. furfuracea* developmental cones to identify differentially expressed (DE) genes related to cone development and on *Z. integrifolia* cone scales collected in correlation with volatile production to identify DE genes related to volatile production. These two groups of DE genes were then used to define three gene "bins": DE genes related to *Z. furfuracea* cone development = "reproductive development genes," DE genes related to *Z. integrifolia* volatile production = "volatile associated genes," and all remaining genes = "other genes." Finally, these two data sets were brought together and the genes previously found to be experiencing positive selection were divided amongst gene "bins" to look for increased positive selection on volatile associated genes.

First, we estimated a species tree for all Caribbean *Zamia* included in this study using 829 orthologous genes found in all 11 in-group samples and the *Zamia furfuracea* out-group. Orthologs were concatenated for a total of 797,745 aligned columns with an overall matrix occupancy of 0.96 (Statistics per sample are found in **Supplementary Table 4**). Models of evolution for each gene were determined using ModelFinder plus in IQ-TREE v 1.6.1 (Nguyen et al., 2015; Kalyaanamoorthy et al., 2017). All partitions were set to share the same branch lengths, with each partition allowed to have its own evolution rate. A maximum likelihood (ML) tree and bootstrap tree were then estimated together using

200 runs and random bootstrap and parsimony seeds in RAxML v8.2.10 (Stamatakis, 2014; **Figure 2A**).

We then investigated evidence of positive selection on the 2,487 genes that were found in 10 in-group Caribbean species using the site models (Nielsen and Yang, 1998; Yang et al., 2000) implemented in the program Phylogenetic Analysis by Maximum Likelihood v4.8 (PAML) (Yang, 2007). The species tree was pruned to remove the two taxa not used in PAML selection analysis, the out-group *Z. furfuracea* and in-group *Z. lucayana* that had a poor quality assembly as determined with BUSCO scores. Nucleotide sequences for these genes were aligned using the codon model in prank v.170427 (Löytynoja and Goldman, 2008). Individual PAML analyses for each of the 2,487 genes were run using scripts from Schultz and Sackton (2019). In tests of positive selection, ω (the ratio of non-synonymous/synonymous mutations) is determined for sites along an alignment. We tested four site models that allowed for the ω ratio to vary among codons or amino acids in the protein. These models were M1a (neutral), M2a (selection), M7 (beta), and M8 (beta and ω). We then computed likelihood ratio tests comparing likelihood scores between M1a vs. M2a and M7 vs. M8 (Nielsen and Yang, 1998; Yang et al., 2000; Wong et al., 2004) and computed *p*-values according to a χ^2 distribution with two degrees of freedom. A false discovery rate (FDR) was applied to correct *p*-values for multiple testing using the fdrtool R package (Strimmer, 2008). 466 genes were statistically significant at 95% confidence for the preferred M2a model of selection verses the M1a neutral model and 463 genes were statistically significant for the preferred M8 model of selection verses the M7 neutral model. The 463 genes where both comparisons favored the selection model were considered to be those genes expressed in the dehiscing pollen cone and shared across Caribbean *Zamia* that are experiencing positive selection.

Separately, we used gene expression patterns to create three gene groupings: "volatile associated genes," "reproductive development associated genes," and "other genes." For the "volatile associated genes," we determined gene expression correlated with volatile production using *Z. integrifolia* samples that were collected in concert with volatile collection. For the "reproductive development associated genes" we determined gene expression correlated with pollen cone development using *Z. furfuracea* samples. For both *Z. integrifolia* volatile and

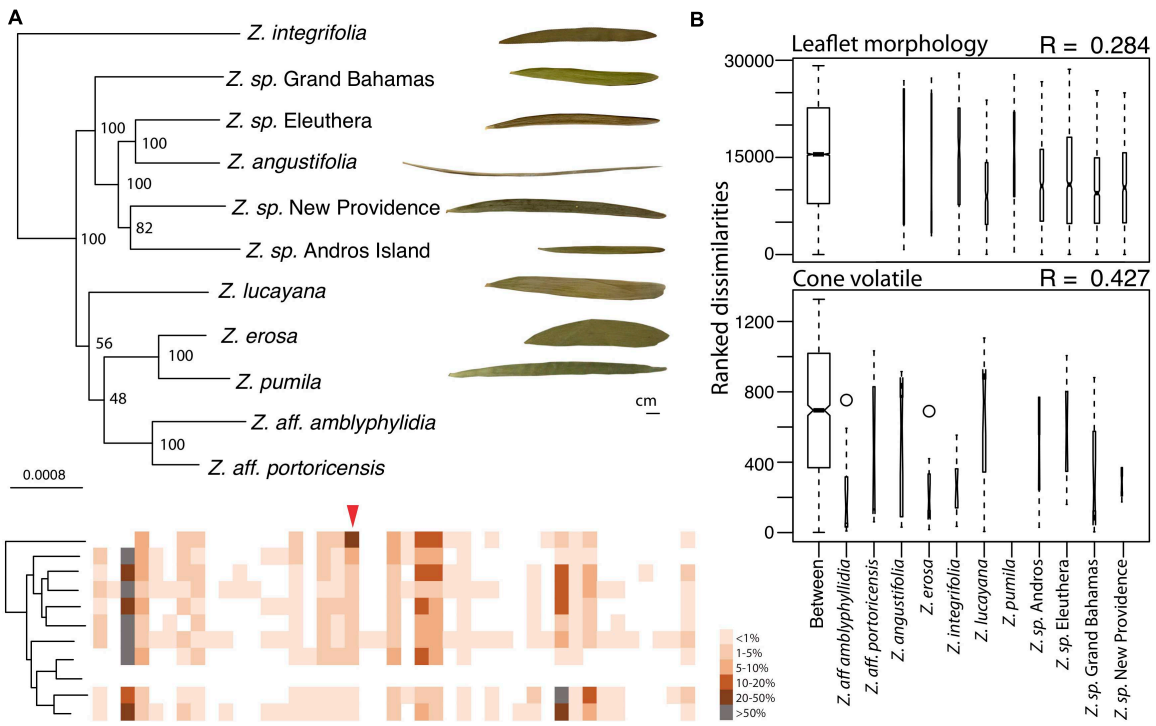

FIGURE 2 | Cone volatile diversity is more different between Caribbean *Zamia* species than leaflet morphology. **(A)** Maximum likelihood phylogeny using 829 orthologous genes of Caribbean *Zamia* showing leaflet and volatile phenotypic diversity. Bootstrap values are placed at the nodes. The out-group *Z. furfuracea* has been removed from the image for clarity. Representative leaflets are included to scale for each population where living collection or herbarium records were available and a schematic of cone volatiles is provided. Individual volatile compounds are represented by boxes along the schematic and are aligned between species. Volatiles are presented as present or absent (colored or white box) and percent composition is averaged across all individuals for the species (intensity of color). Methyl salicylate is denoted with a red arrow. A close-up of these aligned volatile representations with corresponding GC-MS retention times is presented in **Supplementary Figure 3**, along with a dendrogram heatmap of each sample in **Supplementary Figure 4**. **(B)** Analysis of similarity (ANOSIM) results using leaflet shape (length/width) measurements taken from herbarium and living collections (top) and percent compositions of all volatile compounds (bottom). Boxplots of ranked dissimilarities are shown for each taxon as well as between taxa and widths are proportional to sample size. The higher the *R*-value, the more dissimilar samples are between species than within. Species without data are not included in the analysis.

Z. furfuracea development, expression for each individual sample was calculated using RSEM v1.2.29 (Li and Dewey, 2011) and the resulting count matrices were analyzed using the EBSeq package v1.28.0 (Leng et al., 2013; Leng and Kendziorski, 2020) in R v4.0.3 for conditional analysis. To determine gene expression pattern conditions for the "volatile associated genes," we used the total ion abundance of methyl salicylate present in volatile samples collected in concert with *Z. integrifolia* assembled transcriptomes. Six time points from one individual were analyzed and found varying expression of methyl salicylate (**Supplementary Figure 5A**). However, time points one and two had similar total ion abundances (79,531 and 83,357, respectively) as did time points three and five (46,720 and 41,758, respectively). Therefore, gene expression patterns that were considered correlated with volatile production were the three in which these time point pairs, one and two and three and five, showed similar expression (**Supplementary Figure 5A**). Additionally, two gene expression patterns considered non-volatile associated were included, those where expression patterns were all the same or all different across all time points.

To determine gene expression pattern conditions for the "reproductive development associated gene," we used three developmental stages of *Z. furfuracea* pollen cones: just emerged, young half sized cones and immature almost full-size cones. Gene expression patterns that were considered correlated with pollen cone growth were those that differed by developmental stage as either all stages different or the first or last stage different (**Supplementary Figure 5B**). The expression pattern with no difference between reproductive stages was included and considered not associated with reproductive structure growth. Gene expression conditions were determined using EBSeq as libraries were set to vary between samples and 25 iterations were run on both datasets separately until convergence was reached as determined by a difference of less than 0.001 in the hyper-parameters α and β and the mixture parameter ρ for each run. Genes were considered volatile associated if they had a greater than 0.95 posterior probability of being in one of the three volatile associated gene expression patterns (**Supplementary Figure 5A**). Genes were considered reproductive development associated genes if they had a posterior probability greater than 0.95 of being in one of the three reproductive stages gene expression patterns (**Supplementary Figure 5B**).

The volatile and reproductive associated genes were then filtered to include only those genes that were present in the 2,487 genes used in the positive selection PAML analysis. Additionally, any genes that were found in both the volatile and the reproductive associated gene bins were removed from both, generating the final "volatile associated" bin with 146 genes and "reproductive development associated" bin with 674 genes. The "other" gene bin includes all remaining 1,667 genes not found in either the "volatile associated" and "reproductive development associated" gene bins as well as those genes that were found to overlap. Finally, we determined the distribution of positive selection in Caribbean *Zamia* pollen cone transcriptomes across gene bins by blasting the 463 genes found to be experiencing positive selection against the three gene bins (**Supplementary Figure 5C**). The 30 positively selected genes in the "volatile" bin were annotated using Trinnotate v3.2.1 (Bryant et al., 2017). The 21 identified gene ontology molecular functions (Ashburner et al., 2000; Gene Ontology Consortium, 2021) were then used to categorize genes into 7 functional categories (**Supplementary Figure 6**).

RESULTS

We investigate the coevolution of the *Rhopalotria* and *Zamia* lineages by determining that the mechanism of interaction is consistent across two species pairs and provide evidence of reciprocal evolution in traits related to this mechanism in both lineages. Using insect physiology and behavior, we demonstrate that plant-insect chemical communication and push-pull pollination is indeed consistent across these two species pairs and is driving the relationship of the *Rhopalotria/Zamia* mutualism. We then show that these traits are diverging in both lineages and that there appear to be higher rates of positive selection associated with volatile production.

Chemical Communication Is a Mechanism of Mutualism Across Rhopalotria and Zamia Species Pairs

We identify the chemical communication underlying the *R. slossoni/Z. integrifolia* mutualism and find the push-pull mechanism to match that demonstrated in *R. furfuracea/Z. furfuracea* (Salzman et al., 2020) in which weevils have specialized to perceive only the signal emitted by their respective host plant. To do this, we first identified plant volatile compounds that are physiologically perceived by the pollinator in the *R. slossoni/Z. integrifolia* species pair. Using electroantenograph detection (EAD) we find that *R. slossoni* is physiologically capable of perceiving only one compound emitted by its host plant, and we identified this as methyl salicylate (**Figure 1A**), a compound previously reported from *Z. integrifolia* (Pellmyr et al., 1991).

In order to begin to determine whether chemical communication and push-pull pollination is indeed the mechanism of interaction across *Rhopalotria/Zamia* lineages, we compared the volatile behavioral response and plant volatile production in *R. slossoni/Z. integrifolia* with those identified in an earlier study of *Rhopalotria/Zamia* mutualism. Using the same pitfall tests we used in the earlier work, we found that *R. slossoni* are attracted to methyl salicylate but that the response is non-linear (**Figure 1B**). Weevils are significantly more attracted to mid-level amounts of methyl salicylate than to low or high amounts (*p*-values in **Supplementary Table 5**). To determine if methyl salicylate could be performing the push-pull pollination function in *Z. integrifolia*, we analyzed the production of methyl salicylate across the day. We found that methyl salicylate production does undergo a large increase in the pollen cone (**Figure 1C**).

To test whether insect perception of host plant compounds may be diverging across these lineages, we performed a reciprocal test of volatile perception between *R. slossoni* and *R. furfuracea*, an obligate pollinator of *Z. furfuracea* previously found to

perceive and respond to the *Z. furfuracea* compound 1,3-octadiene (Salzman et al., 2020). Using mixtures of methyl salicylate and 1,3-octadiene, we find that weevils show a positive electroantenographic response to the compound produced by their host plant, but that neither weevil responds to the electroantennally active compound of the other.

Zamia Volatile Phenotypes Are More Diverged Than Morphological Phenotypes

We compared volatile and morphological divergence across the Caribbean *Zamia* clade to look for evidence of trait evolution related to the mutualism in the plant lineage and we found a greater level of dissimilarity between Caribbean *Zamia* cone volatiles as compared to leaflet morphology (**Figure 2**). Our analysis uncovered 48 volatile compounds across the entire Caribbean clade with an average of 15 compounds per individual, ranging from 9 to 26. *Zamia integrifolia* had the lowest average number of VOCs per species at 12.67 and *Z. lucayana* had the highest average at 18.14. For leaflet morphology, ratios of leaflet length by width (L/W) range from 4.232 at the widest to 68.508 at the thinnest, with an average of 15.51. *Zamia angustifolia* had the thinnest leaflets with an average L/W ratio of 49.5 and *Z. erosa* had the widest leaflets with an average ratio of 6.70, with all others falling within 9.5 (*Z. lucayana*) to 19.0 (*Z. integrifolia*).

We used an analysis of similarity to determine if volatile phenotypes are more dissimilar between taxa than leaflet morphology. The ANOSIM *R*-value compares the mean of ranked dissimilarities between groups to the mean within groups. Values below 0 indicated dissimilarities are greater within groups than between groups, whereas those close to 1.0 suggest dissimilarity between groups and those close to 0 indicate that the distribution of values is even between and within groups. Our analysis showed a low level of dissimilarity between Caribbean *Zamia* leaflet morphology using Bray-Curtis distance ($R = 0.284$) with a significance of 0.001. Conversely, we found an *R*-value closer to 1, and therefore, more dissimilar between groups for plant volatile profiles ($R = 0.4268$, Bray-Curtis distance) with a significance of 0.001.

Searching for Selection on Zamia Volatile Associated Genes

To investigate the role of selection on divergence in plant volatile traits, we sequenced *de novo* transcriptomes of pollen dehiscing cones of Caribbean *Zamia* and looked for evidence of positive selection in three gene "bins" determined by the differential expression pattern of genes compared to methyl salicylate production or cone developmental stage. Transcriptome assembly overall was of high quality with BUSCO scores for 11 of the 12 species all showing 70.3–84.6% complete BUSCO genes (**Supplementary Figure 2**). The single poor quality assembly was *Zamia lucayana* with only 8.6% complete BUSCO genes, so we included this species in the phylogeny but not in subsequent selection analysis. The maximum likelihood score for the phylogeny was −1,237,542.0107. The remaining 10 in-group Caribbean species transcriptomes had 2,487 orthologous genes, of which we measured 463 (18.6%) experiencing positive selection. Our differential expression analysis and filtering identified 146 "volatile associated" genes, 674 "reproductive development associated" genes, and 1,667 "other" genes among the 2,487 orthologs. The ratio of positively selected genes among the "volatile associated" genes was 30 out of 146 (20.6%), among the "reproductive development associated" genes was 144 out of 674 (21.4%), and among "other" genes was 289 out of 1,667 (17.3%) (**Supplementary Figure 5**). Although it is tempting to interpret this as showing a trend for more of the volatile or reproductive development associated genes to be under positive selection than other genes, the difference is not significant with a proportion test ($p = 0.1373$). and our initial hypothesis was not supported by these data. Gene annotation of the 30 positively selected genes in the "volatile" bin described 21 molecular functions that fell into 7 categories. The highest percentage was uncategorized genes (41.46%, $n = 17$), followed by enzymatic activity (24.39%, $n = 10$) and nucleotide binding (12.19%, $n = 5$) genes. The remaining categories are metal ion binding (9.76%, $n = 4$) transcription (4.88%, $n = 2$), ATP associated (4.88%, $n = 2$), and transport (2.44%, $n = 1$) genes (**Supplementary Figure 6**).

DISCUSSION

Our results provide evidence in support of co-evolutionary reciprocal evolution in the *Rhopalotria-Zamia* pollination mutualism. The relationship between two distantly related species pairs of *Rhopalotria* pollinators and their *Zamia* cycad hosts is characterized by a highly specific chemical communication that is potentially evolving under positive selection in the host plants, with phenotypes related to volatile production in the plants and perception in the insects displaying significant differences. We first identified a key trait underlying the relationship and confirmed that chemical communication mediated the interaction by showing that the physiological and behavioral responses of *R. slossoni* to a primary compound in the volatiles expressed by their host plant (**Figure 1**) mirror the push-pull pollination mechanism previously described (Terry et al., 2007; Salzman et al., 2020). The behavioral response to methyl salicylate matches the behavioral response of *R. furfuracea* to its host plant compound 1,3-octadiene (Salzman et al., 2020). In the *R. furfuracea/Z. furfuracea* pollination system, *Z. furfuracea* initiates pollination by instigating the movement of *R. furfuracea* out of the host pollen cone through cyclical increases in the amount of 1,3-octadiene (Salzman et al., 2020). Here we found that methyl salicylate production undergoes a large increase in the pollen cone (**Figure 1C**) similarly to the pattern observed with 1,3-octadiene in *Z. furfuracea* (Salzman et al., 2020). This burst in production of methyl salicylate occurs in the mid-afternoon and coincides with the time that *R. slossoni* are most active (Tang, 1987). We infer from these results that that chemical communication and push-pull pollination is the usual mechanism underlying *Rhopalotria* and *Zamia* associations and is likely to be found throughout the Cycadales (Salzman et al., 2020). We then determined that these traits are diverging between closely related taxa in both lineages. We

find that *Rhopalotria* pollinators respond to different chemicals in the volatiles released by their respective host plants, and do not respond to an inappropriately matched chemical; they appear to be physiologically uncapable of perceiving their non-host plant. Plant volatiles differ more between species than their leaf morphology does (**Figure 2**), suggesting an increase in diversification in volatile phenotype compared to other morphological phenotypes, although without additional data about trait evolution within and between these taxa of *Zamia*, it is difficult to conclude much about these relative differences.

Disentangling the contribution of mutualistic species interactions from the myriad of influences on diversification remains elusive (Maron et al., 2019), yet fine scale investigations of obligate mutualisms requiring a high degree of specialization between both partners offer some of the best potential insights into the role of coevolution in diversification. The yucca/yucca moth system is the only brood site pollination mutualism where these questions have been explicitly addressed so far, and research has shown that pollinating sister species of *Tegeticula* moths are driving divergence in floral traits related to pollination (Godsoe et al., 2008) and contributing to reproductive isolation (Smith et al., 2009) in two varieties of *Yucca brevifolia*. Our data suggest that chemical communication is likely to play a central role in lineage diversification for both parties involved here as well. Selection can drastically alter plant volatile profiles over the course of just a few generations (Zu et al., 2016; Gervasi and Schiestl, 2017; Ramos and Schiestl, 2020) and the striking differences in volatile production and perception shown here suggest that these traits are essential in securing species specificity.

Volatile variation across populations has been described in two cycad species, *Encephalartos ghellinckii* (Suinyuy and Johnson, 2018) and *E. villosus* (Suinyuy et al., 2013, 2015). The former species has diverged in pollinator assemblage between populations while the later has not, but pollinators do show preferences for volatiles from their host populations both physiologically and behaviorally. Here, we show that two species of *Rhopalotria* weevils are physiologically specialized to perceive the active component of only their own host plant VOCs, thereby exhibiting adaptation to their respective host plants. Scanning electron micrograph images of the antennae of different species of *Rhopalotria* show high variation in the distribution and density of sensory pockets (O'Brien and Tang, 2015) that are likely to correspond with the physiological and behavioral differences described here. Together, the evidence is consistent with population divergence in volatile production or perception likely acting as an inhibitor to gene flow and thereby facilitating diversification. We therefore looked for evidence of selection operating on genes related to volatile emission. Although a fifth of these genes were under positive selection, we found no significant differences between the proportion of genes under selection in different categories (**Supplementary Figure 5**). It is possible that this was due to the limitation of sample size of gene bins or because our process of binning volatile vs. reproductively related gene expression was not sufficient to capture relevant differences. Overall, while we were unable to confirm positive selection on volatile associated genes, our data nevertheless identify significant differences in volatile production and perception in these interactions, and point toward additional research that could be done to investigate how these interacting lineages may be affecting each other's evolutionary trajectories.

Controversy still exists regarding the potential evolutionary trajectory of obligately coevolved mutualisms such as the push-pull pollinations of cycads (e.g., Vamosi et al., 2014; Day et al., 2016) and with further research, this system is likely to provide important insights. Divergence in chemical communication traits in both lineages has clear potential for contributing to lineage diversification, and while the fossil record supports a scenario of plant coevolution with insect pollinators occurring before the rise of angiosperms (Crepet, 1979) and even specifically involving cycads (Cai et al., 2018), the *Rhopalotria/Zamia* mutualism is likely to be much younger than these associations. *Zamia* is thought to have arisen ∼ 85 Ma (Condamine et al., 2015; Calonje et al., 2019), about the same time as the earliest potential divergence time for *Rhopalotria* (McKenna et al., 2009). However, crown diversification of *Zamia* occurred much more recently at 22–9 Ma, with a rapid radiation occurring within the last ∼5 Ma (Calonje et al., 2019).

To date, phylogenetic relationships between species of *Rhopalotria* have been based on a cladistic analysis of 89 morphological, behavioral, and host characters, together with 300 base pairs of 16s (Tang et al., 2018). This analysis described one species in Florida, *Rhopalotria slossoni*, one species, *R. dimidiata*, in the Bahamas and Cayman Islands, and one in Jamaica, *R. meerowi*, with relationships between them unresolved. However, our initial analysis of 650 base pairs of CO1 (unpublished) suggests greater diversity within these populations than has been previously appreciated. Larger scale phylogenetic analysis is needed to determine the diversity of *Rhopalotria* and related cycad pollinating members of the tribe Allocorynina in order to assess the potential co-diversification history of the two lineages. The interaction with specialized cycad herbivores on the evolutionary trajectory of plant volatiles may also be important. The interactive effects of pollinator attraction and herbivory can have major and rapid effects on the evolution of plant traits (Ramos and Schiestl, 2019, 2020). Methyl salicylate is involved in reproduction for *Zamia integrifolia* yet likely also has other ecological functions as it is known to play a great many roles in insect behavior from attraction (James, 2003) to deterrence (Ninkovic et al., 2003) and even as an anti-aphrodisiac transferred in nuptial gifts (Andersson et al., 2000). Larvae of the lycaenid butterfly, *Eumaeus atala* (Lepidoptera) larvae in Florida and the Caribbean are major pests of Zamia (Whitaker and Salzman, 2020) and while the chemical ecology of host plant localization or response to methyl salicylate remains unknown in *E. atala*, it likely further impacts the evolution and diversification of both *Zamia* and *Rhoplaotria* lineages.

DATA AVAILABILITY STATEMENT

The sequencing data has been deposited into a publicly accessible repository (link: https://dataverse.harvard.edu/dataverse/CaribbeanZamia).

AUTHOR CONTRIBUTIONS

SS, MC, DS, NP, and RH conceived of the project. SS performed all field, laboratory and behavioral experiments, GCMS, and statistical analysis. DC performed EAD analysis. MC collected specimens in the field for morphological analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.639368/full#supplementary-material

Supplementary Figure 1 | Map of Caribbean Zamia cultivated at Montgomery Botanical Center.

Supplementary Figure 2 | BUSCO genes recovered in assembled transcriptomes.

Supplementary Figure 3 | Volatile diversity across the Caribbean *Zamia* clade.

Supplementary Figure 4 | Heatmap and dendrogram of Caribbean *Zamia* volatiles.

Supplementary Figure 5 | Caribbean *Zamia* gene expression and positive selection.

Supplementary Figure 6 | Functional analysis of volatile associated genes experiencing positive selection.

Supplementary Table 1 | Accession information on Caribbean *Zamia* used in volatile analysis.

Supplementary Table 2 | Accession information on Caribbean *Zamia* used for RNA.

Supplementary Table 3 | Summary of transcriptome assemblies.

Supplementary Table 4 | Matrix occupancy stats for phylogenetic reconstruction.

Supplementary Table 5 | Sequential Bonferroni corrected *p*-values for pitfall test.

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Humans Share More Preferences for Floral Phenotypes With Pollinators Than With Pests

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Studies on the selection of floral traits usually consider pollinators and sometimes herbivores. However, humans also exert selection on floral traits of ornamental plants. We compared the preferences of bumblebees (*Bombus terrestris*), thrips (*Frankliniella occidentalis*), and humans for flowers of snapdragon. From a cross of two species, *Antirrhinum majus* and *Antirrhinum linkianum*, we selected four Recombinant Inbred Lines (RILs). We characterised scent emission from whole flowers and stamens, pollen content and viability, trichome density, floral shape, size and colour of floral parts. We tested the preferences of bumblebees, thrips, and humans for whole flowers, floral scent bouquets, stamen scent, and individual scent compounds. Humans and bumblebees showed preferences for parental species, whereas thrips preferred RILs. Colour and floral scent, in combination with other floral traits, seem relevant phenotypes for all organisms. Remarkably, visual traits override scent cues for bumblebees, although, scent is an important trait when bumblebees cannot see the flowers, and methyl benzoate was identified as a key attractant for them. The evolutionary trajectory of flowers is the result of multiple floral traits interacting with different organisms with different habits and modes of interaction.

Keywords: o-acetanisole, agriculture, floral selection, humans, pest, pollinator, morphology, β-myrcene

INTRODUCTION

The floral phenotype is shaped by diverse selective forces including pollinators and pests, and also – at least in the case of ornamental flowers – humans (Gessert, 1993; Ågren, 2019; Ramos and Schiestl, 2019). To maximise fitness, plants need to find a balance between attracting pollinators and repelling antagonists (Junker and Blüthgen, 2010). Thus, both pollinators and herbivores exert pressures on the selection of floral traits, driving the evolution of angiosperms in natural ecosystems. However, the trade-off between attraction and defence is also important in agricultural systems and thus is of economic relevance.

Humans have long selected flowering plants (8,000–10,000 years) not only as a food resource, but also for their ornamental attributes (Milla et al., 2015). There is evidence of the study of ornamental plants dating from C.E. 1,090 (Stoskopf et al., 1994) and wherever humans live, the use of plants as ornaments is ubiquitous in their urban and periurban areas (Kowarik, 2005). The domestication of wild plants through selective breeding is the basis of agriculture, and it allows wild plants to be transformed into economically desirable crops (Stoskopf et al., 1994). Nonetheless, artificial selection, and thus domestication, is constrained by the interaction of plant genotypes with biotic and abiotic factors in the environment, as well as by biophysical, physiological, developmental, and genetic factors (Milla et al., 2015). For instance, in the case of ornamental flowering plants, which are presumably selected by humans, biotic selection of the floral phenotype is affected by the perception and preferences of the tripartite forces of humans, florivores, and pollinators.

Humans respond to and interact with their environment based on a multimodal comprehension of it (Storms and Zyda, 2000; Lindemann-Matthies et al., 2010). Although, ornamental plants have been studied from several perspectives (Reichard and White, 2001; Kingsley et al., 2009; Kendal et al., 2012; Erickson et al., 2019), little is known about the specific floral traits that might be more relevant for humans when they choose which flowers to grow (Rahnema et al., 2019). Studies on the preferences of plant breeders can provide information about the most important floral attributes guiding their decision-making based on aesthetic values. On the other hand, bumblebees (*Bombus* spp.) are model organisms for studying pollination in an ecological and evolutionary context (Wilmsen et al., 2017). Numerous studies have revealed that multiple floral phenotypes are perceived by bumblebees and affect their behaviour when selecting which flowers to visit (Katzenberger et al., 2013; Whitney et al., 2013; Bailes et al., 2018). Furthermore, they are also of major importance in agriculture, pollinating crops and increasing yield quantity and quality (Bailes et al., 2018). In contrast, although, thrips are considered secondary pollinators (Terry, 2002), they are more widely known for causing damage to plants. Their perception and selection of flowers is known to be influenced by multiple plant stimuli (Cao et al., 2018). In the context of agriculture, the western flower thrip, *Frankliniella occidentalis*, is a globally dispersed pest with a wide plant host range (Reitz, 2009; He et al., 2020). The interaction of these three organisms with plants is of great economic relevance, since they may synchronously exert selective pressures on the floral phenotype.

It has been demonstrated that both mutualistic bumblebees and antagonistic visitors exert selective pressures on floral phenotypes (Ramos and Schiestl, 2019). However, whether humans reinforce the selection by mutualists or antagonists, or exert opposing selective pressures on the floral phenotype, has been overlooked. Studies integrating behavioural responses of insects and humans to plants can reveal the traits under selection by each of these organisms. Since responses of humans, bumblebees, and thrips to flowers are the result of multimodal decisions (Storms and Zyda, 2000; Katzenberger et al., 2013; Terry et al., 2014; Telles et al., 2017; Wilmsen et al., 2017), holistic approaches are required to pinpoint, which are the most important floral traits underlying the floral selection of these organisms. In addition, behavioural experiments are needed to assess single traits that may be under selection (Junker and Parachnowitsch, 2015).

An ideal model for studying floral traits under pressure for selection is *Antirrhinum*. The genus was described by Plinius as a classical Roman ornamental (Stubbe, 1966). *Antirrhinum* has been long studied for its interest from ecological and evolutionary standpoints (Glover and Martin, 1998; Schwarz-Sommer et al., 2003), but also for its use in agriculture in the emerging market of edible-flowers (Rop et al., 2012; González-Barrio et al., 2018; Stefaniak and Grzeszczuk, 2019) and its historical and economic value as an ornamental (Kowarik, 2005). In this study, we assessed the preferences of *Bombus terrestris*, human plant experts and *F. occidentalis* for different flowers of *Antirrhinum* sp., including four Recombinant Inbred Lines (RILs) displaying a range of floral phenotypes. We phenotyped flowers with respect to colour, morphology, sizes of different floral parts, composition and quantity of scent emission in stamens and flowers, pollen content and viability, and trichome density. Our aim was to compare the preferences of pollinating bumblebees, antagonistic thrips, and humans for *Antirrhinum* flowers and to identify the flower traits potentially under selection by these organisms. These findings may be helpful for the understanding of evolutionary trajectories and also may inform agricultural practices.

MATERIALS AND METHODS

Flowers

We performed a cross between *Antirrhinum majus* line 165E, a line deriving from the John Innes JI75 line (Schwarz-Sommer et al., 2003), and *Antirrhinum linkianum* (Botanic Garden, University of Coimbra, Portugal; Ruiz-Hernández et al., 2017). A single F1 plant was used to obtain an F2. The recombinant inbred line was further developed in a standard way i.e., plants were selfed and maintained by single-seed descent. We selected four RILs, with contrasting scent profiles, on F5–F7 populations: these were identified as lines 9, 80, 112, and 113. We used

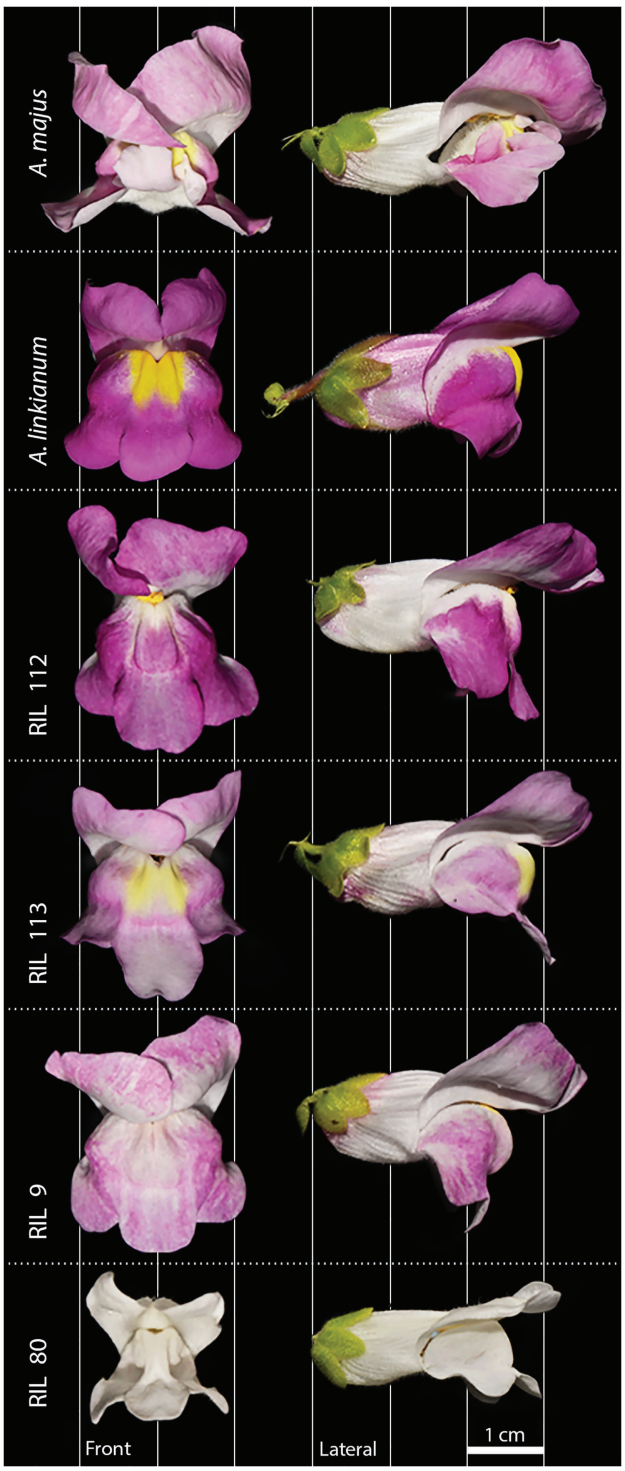

FIGURE 1 | Flowers from lines used for experiments. Parental species: *Antirrhinum majus* and *Antirrhinum linkianum*. Recombinant Inbred Lines (RILs): 112, 113, 9, and 80. Front and lateral view.

fully developed flowers, at their maximum stage of scent emission (3–4 days after anthesis; Weiss et al., 2016) for all analysis and experiments (**Figure 1**).

Plant Phenotyping

Colour of Floral Parts

We dissected the flowers, flattened them and glued each petal section to black, non-reflective cardboard to prevent light scattering from curved petal surfaces. Each mounted petal was analysed, whilst placed in a box lined with black cardboard and illuminated with a Deuterium-Halogen light source (Ocean Optics DH 2000). The background material was corrected for and the reflectance spectrum of each petal section was measured, relative to a white standard, with an Ocean Optics USB2000+ Spectrometer at an integration time of 10 ms. The petals were flattened to reduce any artefact caused by light scattering-off of curved petal surfaces. Reflectance spectra were analysed with SpectraSuite Version 1.0 (Ocean Optics). We measured five floral parts: upper and lower lateral petal, lower middle petal, palate, and outer corolla tube (**Figure 2A**). Flower colour was measured in 21 different spots, with 15–19 replicates per line. Colour spectral data was processed using the R package *pavo* (Maia et al., 2019), and measurements were restricted to the range of wavelengths visible to insects and humans (300–700 nm). Flowers of the genus *Antirrhinum* are usually multicoloured and the multiple measurements on each flower resulted in $n = 21$ different reflectance spectra. In order to best represent colour differences between flowers, we calculated the Euclidean distances between the reflectance spectra per spot, which means the colour of the same petal position was compared between two flowers. This procedure resulted in $n = 21$ Euclidean distances per pair of flowers, the mean value of these distances was defined as the average colour distance between two flowers.

Morphology

We dissected the flowers by cutting along the tube with a razor blade, in correspondence with the hinge of the dorsal and lateral petal lobes (**Figure 2B**). As a proxy for floral morphology, we estimated the shape of the ventral petal (**Figure 2C**). Following (Cui et al., 2010), we flattened the dissected three-lobed lips by gluing them onto black paper and photographed them in a standardised manner. The software tpsDig2 2.31 and tpsUtil 1.76 (Rohlf, 2015) was used to digitize 139 landmark and semilandmark points (**Figure 2C**). Using the R-package GEOMORPH 3.1.0 (Adams and Otárola-Castillo, 2013), landmark coordinates were subjected to a Generalised Procrustes Superimposition with semilandmarks slided based on minimising bending energy. The effect of asymmetry was removed by extracting the symmetric component of the shape (Klingenberg et al., 2002), using *bilat.symmetry* from GEOMORPH.

Size of Floral Parts

We used the image processing package Fiji (Schindelin et al., 2012) to measure the sizes of complete and dissected flowers. We took scaled pictures of 9–17 replicates per line. We measured the dimensions of 10 floral parts: flower front (M1–5), flower lateral (M6), lower petal lobe (M7), short and long stamen (M10 and M11), and gynoecium (M12); and the area of the upper lateral petal lobes (M8) and palate of each flower (M9; **Figure 2D**).

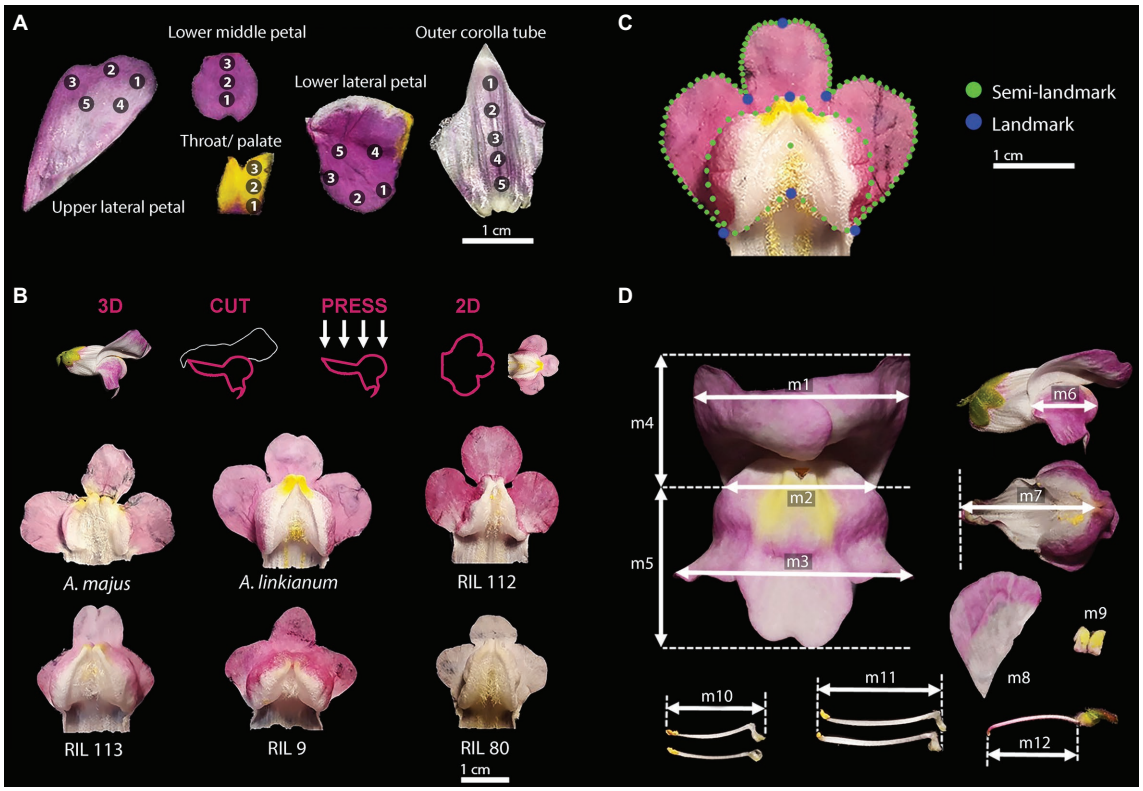

FIGURE 2 | Floral parts phenotyped. **(A)** Floral parts used to measure the colour of flowers. Numbers indicate the location of measurements on each floral part. **(B)** Dorsal and lateral petal lobe dissection used to analyse floral morphology. **(C)** Landmarks and semilandmarks used to analyse floral morphology. **(D)** Location of floral size measurements for each flower. Length of front (M1–5), lateral (M6), lower lobe (M7), short and long stamen (M10 and M11), and gynoecium (M12). Area of upper lateral petal (M8), throat/palate (M9).

Volatile Organic Compound Analysis

Scent emission was analysed using GC-MS and Twisters™ (Gerstel, Mülheim an der Ruhr, Germany) as detailed in Ruiz-Hernández et al. (2018). Cut flowers were placed inside glass beakers and cut stamens were placed inside 10 ml headspace vials (**Supplementary Figure S1**). Beakers and vials were positioned in 2 L desiccators for 24 h inside a growth chamber under a regime of 16:8 h light-dark and 23–18°C conditions. A minimum of 10 replicates (in the case of RILs: five from F5 and five from F7) from different plants within each line were analysed by GC-MS.

Scent profiles were determined using the R package gcProfileMakeR (Pérez-Sanz et al., 2020). Compounds present in at least 90% of the replicates, with a minimum average quality of 90%, were selected as representatives of the scent profile of each line. Linear retention indexes (LRI) were calculated for tentatively identifying compounds by comparing available LRIs in the literature. For that purpose, we used the retention times (RT) of C8–C20 alkanes (Sigma Aldrich, 04070), analysed under the same chromatographic conditions as flower samples (Zellner et al., 2008; Ruiz-Hernández et al., 2018; **Supplementary Table S1**). We used acetophenone, methyl benzoate, methyl cinnamate, methyl salycilate, and ocimene, (Sigma-Aldrich, 42,163, 18,344, 96,410, 240,826 and W353901, respectively) as standards for chromatographic identification. Average total scent emission (mg) was calculated by using an external calibration curve obtained by adding standards to the sampling system ($y = 5.247*10^{8}*x$; Ruiz-Hernández et al., 2018; **Table 1**). Multivariate analysis and Random Forest analysis were performed using the total peak area divided by the fresh weight of samples (Ruiz-Hernández et al., 2018). Due to the difficulties found when exactly quantifying the emission of single volatile organic compounds (VOCs) in complex matrices (Ruiz-Hernández et al., 2018), we decided to use a general dilution of 1:1,000 of VOCs in bioassays with thrips and bumblebees. Differing dosages were chosen to test hypotheses generated from preference tests and Random Forest analyses. Synthetic standards used in bioassays with thrips and bumblebees were: α-farnesene (W383902, Sigma-Aldrich, mix of isomers), methyl benzoate (M29908 Sigma-Aldrich, 99%), α-acetanisole, (M9203 Sigma Aldrich, 99%), cinnamyl alcohol (standard provided by Prof. em. Manfred Kaib, University Bayreuth, Germany), methyl cinnamate (standard provided by Günther Gerlach, SNBS Munich, Germany), and β-myrcene (EGA-Chemie M10,000-5, technical quality).

Pollen Viability and Content

To determine the percentage of viable pollen grains in each flower, the fluorescein diacetate staining method was used (Heslop-Harrison and Heslop-Harrison, 1970; Li, 2011). Flowers were inspected

TABLE 1 | Mean percentage of VOCs in scent profiles of *A. majus*, *A. linkianum*, and RILs 112, 113, 80, and 9.

VOC	*Antirrhinum majus* $n=10$	*Antirrhinum linkianum* $n=10$	RIL 112 $n=13$	RIL 113 $n=10$	RIL 9 $n=10$	RIL 80 $n=10$
o-acetanisole	0.4					
Acetophenone	17.8	3.1	8.5	44.2	4.5	41.1
o-acetylphenol	0.6		1.3	5.1	0.8	
Benzenepropanol			3.7		0.3	
(E)-cinnamaldehyde					0.9	
Cinnamyl alcohol			7.1	2.1	11.4	8.6
Decanal	0.2			0.3		0.3
3,5-dimethoxytoluene						2.3
Ethyl benzoate	0.7		0.9	0.3		
Eremophilene		3.5			8.4	
α-farnesene	1.3	20.4	11.7	14.1	11.2	15.7
Hexahydrofarnesyl acetone			0.5		0.2	0.3
Linalool					1.6	
Methyl benzoate	33		22.4	11.0	7.6	11.0
Methyl cinnamate		6.8	8.5	3.0	2.5	2.5
Methyl hydrocinnamate					3.8	0.3
Methyl 2-methyl butyrate				0.2		
Methyl salicylate				4.9	4.1	4.2
β-myrcene	7.4	6.4	5.0	0.8	5.7	3.2
Nonanal				0.4		0.3
(E)-ocimene	38.2	57.7	29.5	12.2	36.7	8.1
Sabinene		1.0		0.2		
St. Acetophenone				1.0		0.8
St. Hexahydrofarnesyl acetone		0.9	0.7	0.3	0.2	
St. α-farnesene						0.4
St. Methyl benzoate	0.3				0.1	0.6
St. Nonanal						0.1
St. β-pinene		0.3				
Average total scent emission (mg)	3.074 ± 1.125 ab	1.847 ± 0.879 bc	1.394 ± 0.709 c	2.669 ± 1.097 abc	3.804 ± 1.811 a	3.155 ± 0.680 ab

Volatile organic compounds obtained from stamens are indicated with "St.". VOCs in bold were identified with authentic standards and linear retention indexes (LRIs), the rest were tentatively identified with LRIs. Last row shows the average total emission of each line (mg) and its SD. Different letters indicate statistical differences between lines, according to Tukey test.

daily and all the stamens were removed from a flower on the day of dehiscence. Stamens were placed in 1.5 ml tubes with 250 µl of fluorecein diacetate in BK-Buffer. Tubes were vortexed for 60s and stamens were removed from tubes and visually inspected to ensure that all pollen had been released from the anthers. Each tube was briefly vortexed again to ensure even distribution of pollen grains and a 20 µl subsample of the pollen suspension was immediately pipetted into each of the two 9 mm^2 grids with a depth of 0.2 mm of a Modified Fuchs Rosenthal Chamber (MFS; Rohem, India). Pollen samples were illuminated using a CoolLED pE300 White Fluorescence illumination system and imaged using a Nikon Eclipse 50i microscope with a 40X objective and a mounted GT Vision GXCAM HiChrome-S tablet. Viable, fluorescent pollen and non-viable, non-fluorescent pollen (**Supplementary Figure S2**) was counted separately to determine the percentage of viable pollen using CountThings from Photos (Dynamic Ventures, Inc. d/b/a CountThings). The total amount of pollen was calculated adding viable and non-viable pollen. The number of flowers phenotyped per line was 8–14. At least three different 1 mm^2 chambers were counted for each flower.

Total pollen content of each flower was calculated by using the average pollen count for the sample (n) divided by the volume of the grid of the Modified Fuchs Rosenthal Chamber on which the pollen grains were counted (1.8 µl) and multiplied by the volume of the fluorecein diacetate solution in which the pollen was suspended (250 µl):

$$Total\ number\ of\ pollen\ grains\ per\ flower = 250\frac{n}{1.8}$$

Density of Trichomes

Using a scanning electron microscope (HITACHI S-3500N), we counted the number of trichomes on the palate of flowers. We had three replicates per line and we dissected 5 mm^2 of each flower. We took three images in different areas of each replicate (scale: 200, magnification: x180, size: 910 × 683 µm). We followed a protocol for critical point drying with glutaraldehyde, ethanol, and acetone (Manchado-Rojo et al., 2014). Due to the length of trichomes, we cut them using tape and tweezers (**Supplementary Figures S3A–D**). We counted the number of trichome bases as a proxy for trichome number in the area examined, giving trichome density.

Experimental Design With Flowers

We tested preferences of bumblebees, humans, and thrips for flowers of the different *Antirrhinum* lines studied.

Preferences were assessed pairwise, contrasting parental line *A. majus* against *A. linkianum*, RIL 112, RIL 113, RIL 9, and RIL 80. In the case of bumblebees and humans, two types of experiments were performed: (1) Using whole flowers and (2) Using floral scent in isolation. In contrast, assays with thrips were performed after first separating flowers from stamens and testing flowers without stamens and stamens, independently.

Whilst bumblebees are well known for pollinating *Antirrhinum* plants (Jaworski et al., 2015) and humans have used this genus long as ornamental (Kowarik, 2005), the antagonistic effect of thrips on these plants may be either caused by a strong pollen reduction or by the transmission of pathogens (Ullman et al., 2002).

Bumblebee Experiments With Flowers

For experiments with flowers, *B. terrestris audax* colonies were obtained from Biobest Group NV (Westerlo, Belgium), supplied by Agralan (United Kingdom), and connected by a transparent tube to the flight arena, a $0.3 \times 0.75 \times 1.12$ m plywood box with a clear UV- transparent Plexiglass lid (Bailes et al., 2018). Colonies were fed *ad libitum* with ~30% w/v sucrose solution, which was also used as a reward in experiments. At least 10 bumblebees performed each pairwise-experiment, but each bee was tested independently in the flight arena. Bumblebees were pre-trained to feed from 13 cm tall feeding towers composed of black card wrapped with black tape sitting within "Aracon" bases (Lehle, Roundrock, TX). Towers were covered with plastic mesh supporting a microcentrifuge tube lid (**Supplementary Figure S4A**) containing sucrose solution. Tower-feeding foraging worker bumblebees were marked on the thorax with water-soluble paints and used for further experiments. Some bumblebees were used more than once and, in those cases, at least 7 days were left between assays to allow short term learning-associations to disappear from their memories (Jaworski et al., 2015). Consequently, we consider our results in the context of flower naive responses of bumblebees, here testing their innate preferences to the different floral traits. Scent experiments were carried out by hiding flowers inside cardboard towers (1:1). **Supplementary Figures S4A,B** illustrate how flowers were displayed for experiments with floral scent alone and with whole flowers, respectively. Flowers were kept in contact with cotton dampened in a 5% w/v sucrose solution to keep the turgor pressure. Each time a bee fed from a tower or a flower, sugar solution was refilled and distribution of towers/flowers was changed.

Bumblebee Preference Assessments

To test the innate preferences of bumblebees for whole flowers, we displayed five flowers of each pair pseudo-randomly in the arena and let them choose to feed on them 10 times. Inside each flower, we placed a cut yellow micropipette tip supplied with 20 µl of sucrose solution to ensure equal availability of reward (**Supplementary Figures S4C,D**). We counted each time bumblebees fed from a flower-tip as a positive choice, refilled the tip and changed the layout of flowers in the arena. Some flowers were used for testing bumblebees more than once. In these cases, flowers were not used for at least 2 h between experiments. Bumblebees deposit cuticular hydrocarbons, whilst visiting flowers, and can subsequently use these "scent marks" as a cue, influencing their floral choices (Goulson, 2010). Although, the cues themselves may last for over 24 h (Witjes and Eltz, 2009), *B. terrestris* appear to stop using the cues by the time 1 h has passed since the flower was previously visited (Stout et al., 1998). Bumblebees may be able to learn to use scent marks that are older than 1 h (Goulson, 2010); however, here, we were examining innate rather than learned preferences, and so leaving the flowers for 2 h should avoid any potential influence of scent markings.

For floral scent experiments, 10 towers, five with a flower of each line from the pair being tested, were distributed pseudo-randomly in the flight arena. Microcentrifuge tube lids were supplied with 20 µl of sucrose solution. Each bee was allowed to feed from towers 10 times and choices were recorded. Between bumblebees, pairwise comparisons and changes of flowers contained in the towers, towers were cleaned with a 40% ethanol solution and left to dry to remove scent marks.

To test whether presence/absence of floral scent affects bumblebee selection of feeding places, we assessed the innate preference of bumblebees for towers with and without flowers ($5 + 5 = 10$ towers). For that purpose, different lines of flowers were used in each replicate ($n = 10$). The towers without flowers were previously unused and had dampened cotton added.

In addition, we tested the innate preferences of bumblebees (*B. terrestris*, Biohelp, Austria) for some VOCs found in the *Antirrhinum* flowers: methyl benzoate, o-acetanisole, ethyl benzoate, o-acetylphenol, sabinene, decanal, and methyl cinnamate. We introduced two filter paper artificial flowers attached to 1.5 ml tubes, in a 5 L clean container, which was orientated upside down (**Supplementary Figure S5**). One artificial flower was supplemented with a VOC diluted in acetone and the other was used as control with just acetone. The same quantities of acetone for the control and the diluted VOC were used. Around 1.5 ml tubes were supplied with 10 µl of sucrose solution. Bees were tested individually and each bee was only tested once. Bumblebees did not repeat experiments and when bumblebees fed from sugar-supplied tubes, choices were recorded ($n \geq 10$).

Experiments With Thrips

Field populations of thrips (*F. occidentalis*) collected in Murcia (Spain) were reared for two generations in the lab (Espinosa et al., 2002). Thrips used were females and flower-naïve. For experiments with flowers, we used new 0.5 L plastic boxes with one flower (without stamens) from each line (**Supplementary Figure S6A**). We added 30 thrips in each pairwise analysis (*n*: 4–9). In the case of stamens, they were introduced in Petri dishes (Ø = 14 cm; **Supplementary Figure S6**) and 20 thrips were included in each dish (*n*: 3–5). We controlled for the effects of ambient light/Petri dish orientation. Thrips were kept inside the plastic boxes/Petri dishes for 24 h, at 25°C and in 16:8 h of light/dark. Then, thrips were placed in a freezer (5 min) to stun them before counting the number on each flower/stamen and its immediate surroundings: 2 cm distance from the flower/stamens.

To test the effect of some VOCs on thrips, we introduced two filter paper artificial flowers into 5 L sealed containers. One flower was supplemented with a VOC (diluted in acetone) and the other with acetone (**Supplementary Figure S7**). VOCs tested were: α-farnesene, methyl benzoate, o-acetanisole, cinnamyl alcohol, methyl cinnamate, and β-myrcene. Concentrations used are indicated in **Table 2**. About 10 thrips were introduced and left inside for at least 60 min. Then, we counted the number of thrips in each artificial flower.

Experiments With Humans

Plant experts (14 women and 16 men) were recruited amongst horticulturists from the Cambridge University Botanic Garden, and plant scientists from the University of Cambridge – Department of Plant Sciences. The experts were asked to interact with each studied pairing of *Antirrhinum* flowers. Three flowers of each pairing were placed at an individual station in an indoor area at the Botanic Garden. Each pairing and each station were identified by a code. There were 11 stations in total and experts were asked to complete a survey with one question addressing each station. Experts answered the questions in the survey by moving between stations haphazardly until all were completed. The survey was divided into two parts, a preference assessment of whole flowers presenting multimodal displays and a preference assessment of the isolated scent of flowers. In stations testing preferences for whole flowers, the experts were instructed to look at and smell all replicates of each pair (**Supplementary Figure S8A**). They then answered the question "If you had to choose flowers from one group only, which flowers would you choose to grow?", by writing down the code of flowers. For the scent-only part, experts were instructed to smell pots, in which three flowers of each pairing were hidden. Pots were covered by a fabric mesh allowing scent to escape but impeding sight (**Supplementary Figure S8B**). Experts then answered the question "If you had to choose one type of flower only, which flower would you choose to grow?". An additional scent preference test was performed comparing containers with flowers and without flowers, to test if the experts prefer places with floral scent or not. All experts completed the surveys alone, and not at the same time.

Statistical Analyses

Statistical analyses were performed in R (R Core Team, 2018) using version 3.3.3. For experiments, in which an individual represented one choice, preferences were assessed by χ^2 Goodness of Fit test (Pérez-Hedo et al., 2015): thrips (flowers and artificial flowers), humans, and bumblebees with artificial flowers. In the case of bumblebee experiments with flowers, we calculated the proportion of choices made for each line by each bee, and analysed preferences for each pairwise comparison using a Wilcoxon test (Strauch et al., 2014). We did not correct for multiple comparisons at this stage and these individual tests should be considered in this light; however, our main conclusions and subsequent consideration of the results are drawn from the combined analyses described below.

In order to be able to compare preferences of organisms for each of the lines, we standardised the preference for the lines relative to the preference for *A. majus*, since all preferences were tested in pairwise with *A. majus*. Therefore, we calculated

TABLE 2 | Effect of isolated VOCs tested with each organism, at indicated concentrations.

Organism	VOC	Concentration (ppm)	Volume (µl)	No. VOC	No. control	Replicates	Chisquare (χ^2)	Chisquare (value of *p*)
Bumblebees	Methyl benzoate	1,000	10	12	4	16	4.000	**0.046**
	o-acetanisole	1,000	10	3	11	14	4.571	**0.033**
	o-acetanisole	100	10	5	6	11	0.091	0.763
	o-acetanisole	10	10	5	6	11	0.091	0.763
	Ethyl benzoate	1,000	10	5	9	14	1.142	0.285
	o-acetylphenol	1,000	10	9	5	14	1.142	0.285
	Sabinene	1,000	10	8	6	14	0.286	0.593
	α-farnesene	1,000	10	9	5	14	1.143	0.285
	Decanal	1,000	10	8	6	14	0.286	0.593
	Methyl cinnamate	1,000	10	8	6	14	0.286	0.593
Thrips	α-farnesene	1,000	5	49	52	101	0.089	0.765
	Methyl benzoate	1,000	5	29	31	60	0.067	0.796
	o-acetanisole	1,000	5	27	26	53	0.019	0.891
	Cinnamyl alcohol	1,000	5	19	19	38	0	1.000
	Methyl cinnamate	1,000	5	15	17	32	0.125	0.724
	β-myrcene	1,000	2	21	20	41	0.0024	0.876
	β-myrcene	1,000	5	46	38	84	0.762	0.383
	β-myrcene	1,000	10	39	29	68	0.225	0.225
	β-myrcene	no dilution	10	8	17	25	0.072	0.072
	*β-myrcene	no dilution	100	9	2	11	4.456	**0.035**
	**β-myrcene			4	13	17	2	**0.029**

Number (No.) VOC and No. control show the number of individuals of each organism, which chose the VOC being tested or the control, respectively. In the experiments with thrips, individuals, which did not choose either the VOC or control were discounted. Number of replicates are indicated along with Chi-square p-values. In β-myrcene experiments: single asterisk (*) indicates results for dead thrips found and double asterisks (**) indicate results for thrips alive on artificial flowers. Significant results ($p < 0.05$) are in bold.

the effect size of the responses to each line relative to *A. majus* as log response ratio: $[L = \ln(\bar{X}_E/\bar{X}_C)]$, with $\bar{X}_E$ as the mean response of the organism to *A. linkianum*, RIL 112, RIL 113, RIL 9, or RIL 80 and $\bar{X}_C$ as the mean response to *A. majus* (**Supplementary Table S2**; Hedges et al., 1999; Junker and Blüthgen, 2010). Effect sizes range from 1 to −1, with positive values indicating a higher preference of the organism for the line under consideration compared with *A. majus*.

Euclidean distances were assessed by using the R package *vegan* (Oksanen et al., 2019) for colour, floral sizes, and scent (*vegdist*), and GEOMORPH for the morphology (*gpagen*) based on the Procrustes coordinates (Dryden and Mardia, 1993; Lockwood et al., 2004). Non-metric multidimensional scaling (NMDS, *vegan: metaMDS*) was used to analyse multivariate traits: colour, floral sizes, scent, and morphology. Due to low dimensionality, we used classical MDS (*vegan: cmdscale*) to ordinate pollen data. Ordinations (NMDS and MDS) represent how similar the analysed flowers are for a given trait. Thus, the closer two points are, the more similar is their multidimensional phenotype. We obtained environmental vectors (*vegan: envfit*), referred to as vectors in the manuscript, and fitted them into ordinations. These vectors represent the correlations between the effect sizes of each organism and the multidimensional trait under consideration. Thus, ordinations represent how similar/dissimilar flowers are for a given trait, and if the preferences of animals were significantly correlated with that trait. We used Pearson's correlations with trichome density and vectors representing the preferences for flowers of the different animals.

A machine-learning algorithm (*randomForest* package, RF) was used to pinpoint VOCs that best explain the preferences of studied animals for floral scents (Breiman, 2001; Helletsgruber et al., 2017). We performed an RF for regression using the effect sizes of each line as the dependent variable, and the VOC emission of flowers from each line as the explanatory variable. We used the square root of the total number of variables as m_{try}, and grew a total of $n_{tree} = 20{,}000$ trees. In regression tasks, function *importance* provides the mean square error (%IncMSE), which informs about the variables that explain the preferences of animals. We used %IncMSE for the preferences of bumblebees and humans for the scent of flowers with stamens and the preferences of thrips for the scent of flowers without stamens and stamens separately.

Bipartite network analysis was performed with *bipartite* package (Dormann, 2011). Coefficients of determination (r^2) from vectors, in each ordination, and Pearson's correlations were used to create a bipartite network between organisms and groups of floral traits. We used the r^2 of significant ($p < 0.05$) results for each trait vs. each organism. Displayed width of edges in the bipartite network is proportional to coefficients of determination r^2 of significant traits for each organism.

Function *rcorr* with default values (R package *Hmisc*; Harrell et al., 2019) was used to obtain a correlation matrix between all phenotypic traits (except colour and morphology). We represented significant ($p < 0.05$) correlations using corrplot R package (Wei and Simko, 2021) and function. Phenotypic data used for the correlation matrix is available in **Supplementary Table S3**.

RESULTS

We tested the preferences of bumblebees, human plant experts, and thrips for different *Antirrhinum* flowers. Parental *A. majus* flowers are larger and, to human vision, lighter in colour than those of parental *A. linkianum*. Out of the four RILs studied, RIL 112 is more similar in colour and shape to *A. linkianum* than the other RILs. RILs 113 and 9 look similar, whereas RIL 80 is notably different to all other lines studied, since it is smaller and completely white (**Figure 1**).

Preference Assessments

The assessment of the preferences of bumblebees for whole flowers showed that they made more choices for *A. linkianum* (Wilcoxon $v = 21$, $p = 0.035$) and RIL 112 flowers (Wilcoxon $v = 45$, $p = 0.009$), than *A. majus* blossoms. *Antirrhinum linkianum* ($\chi^2 = 8.533$, $p = 0.003$) flowers were also significantly more appealing to humans, along with RIL 9 ($\chi^2 = 7.759$, $p = 0.005$). In contrast, thrips showed preferences for visiting more flowers of RILs 112 ($\chi^2 = 7.251$, $p = 0.007$) and 113 ($\chi^2 = 9.717$, $p = 0.002$; **Figure 3A**).

Contrastingly, experiments testing the preferences for floral scent in isolation indicate that bumblebees made more choices for feeders (towers) with the scent of *A. majus* vs. *A. linkianum* (Wilcoxon $v = 28$, $p = 0.021$). Humans preferred to grow flowers with the scent of *A. majus* compared to flowers of RILs 112 ($\chi^2 = 16.133$, $p < 0.001$) and 80 ($\chi^2 = 7$, $p = 0.008$). Furthermore, both bumblebees and humans showed preferences for floral scented rather than non-scented towers (Wilcoxon $v = 42$, $p = 0.021$ and $\chi^2 = 19.2$, $p < 0.001$, respectively; **Figure 3B**).

Finally, thrips preferred to visit stamens from RILs 9 ($\chi^2 = 8.895$, $p < 0.001$) and 80 ($\chi^2 = 14.735$, $p < 0.001$; **Figure 3C**). However, when they interacted with flowers from RILs 9 and 80, which did not contain stamens, they did not show preferences for them (RIL 9: $\chi^2 = 0.485$, $p = 0.486$; RIL 80: $\chi^2 = 1.884$, $p = 0.170$; **Figure 3A**).

Results for bumblebees suggest that visual cues override scent emission when guiding their preferences for floral visitation (**Figures 3A,B**). Results for thrips indicate that their attraction differs between the different floral parts, since they showed preferences for either the stamens of flowers, or the flowers independently of the stamens (**Figure 3**). Altogether, bumblebees showed preferences for parental species and RIL 112, whereas humans generally preferred parental species and RIL 9. In contrast, thrips preferred to visit all RILs over the parental lines.

Deconstructing the Floral Phenotype in Relation to Attraction

Colour, Morphology, and Floral Sizes

To the human eye, lines used in this study range from dark pink to white, with parental *A. majus*, RIL 113 and, to a lesser extent, RIL 9, presenting veined patterning in the floral lobes. In addition, *A. majus*, *A. linkianum*, and RIL 113 all present yellow palates (**Figure 1**). NMDS ordination of colour data clearly separates *A. linkianum* and RIL 80 from each other as well as from the other lines. In contrast *A. majus*,

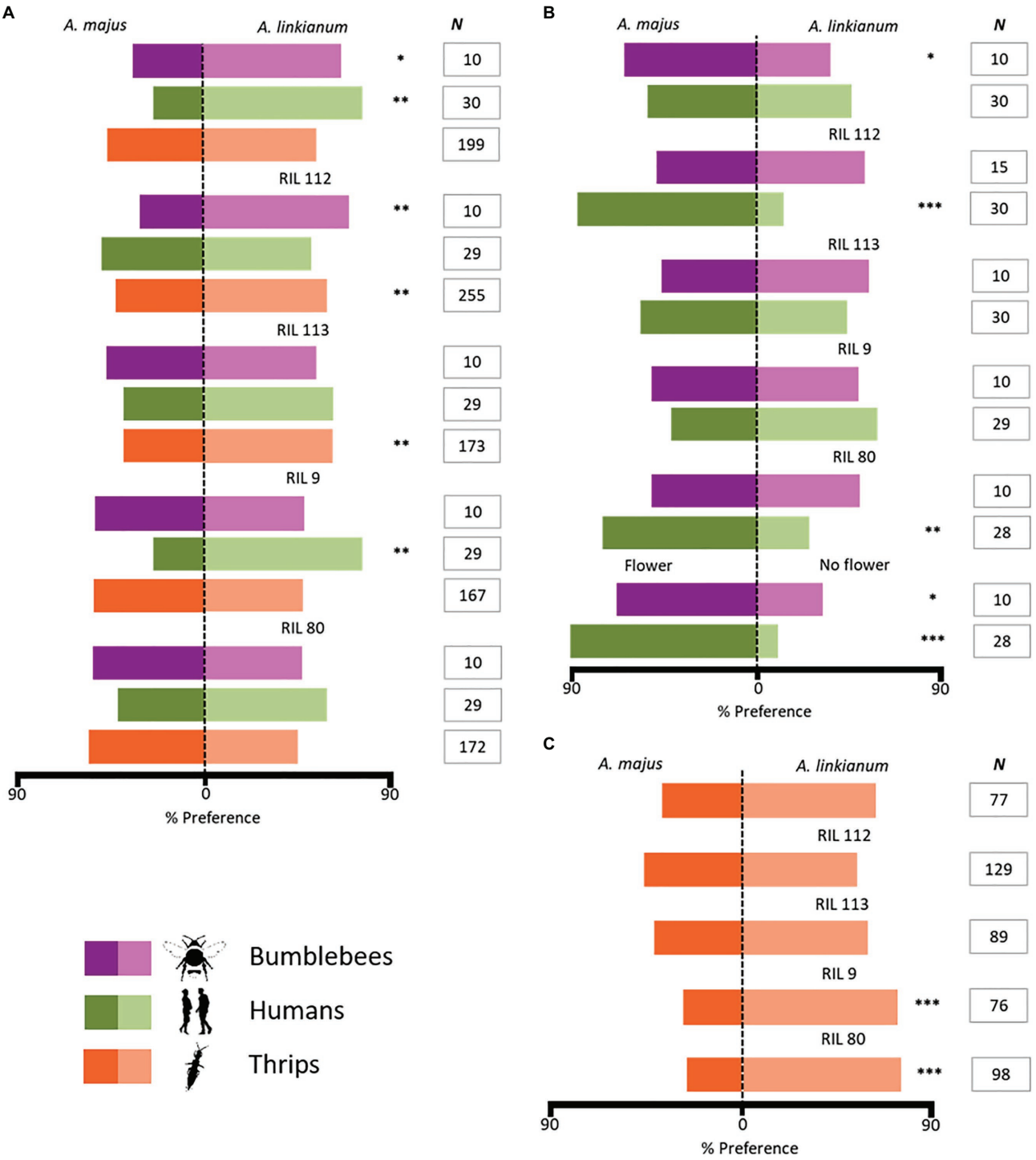

FIGURE 3 | Preference results of animals for flowers of *Antirrhinum*. **(A)** Preferences of bumblebees, humans, and thrips for whole flowers. **(B)** Preferences of bumblebees and humans for just the scent of flowers. **(C)** Preferences of thrips for stamens of flowers. All lines were compared with *A. majus* (except for the isolated scent from flower vs. no flower comparison in **B**). Bumblebees (magenta), humans (green), and thrips (orange). Asterisks indicate the level of significance in statistical results: (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$. Non-significant results are not indicated. Number of replicates (*N*) per experiment is indicated.

RIL 9, RIL 112, and RIL 113, are not clearly separated by colour in the ordination (**Supplementary Figure S9A**). Vectors fitted in ordinations indicate that this floral attribute is statistically significant for bumblebees ($r^2 = 0.356$, $p = 0.001$), humans ($r^2 = 0.269$, $p = 0.001$), and thrips ($r^2 = 0.168$, $p = 0.001$). Bumblebees and humans preferred the dark-pink lines *A. linkianum* and

RIL 112 as the vectors pointed towards these lines in the ordination. In contrast, thrips seem to prefer lines with lighter colours such as *A. majus*, RIL 113, and RIL 9 (**Supplementary Figure S9A**).

Ordination of Euclidean distances of morphological data distinctively separates each line (**Supplementary Figure S9B**). Vectors representing significant preferences associated with morphology indicate that this trait affects bumblebee foraging decisions, with the vector pointing towards flowers shaped like *A. linkianum* and RIL 112 ($r^2 = 0.354$, $p = 0.001$). In addition, floral morphology is also behaviourally significant for humans when they are asked to choose which type of flowers they would prefer to grow ($r^2 = 0.346$, $p = 0.003$), whilst it is not for thrips when they choose which flowers to visit ($r^2 = 0.010$, $p = 0.872$; **Supplementary Figure S9B**).

Finally, the ordination of floral size data clearly differentiates lines *A. majus*, *A. linkianum*, and RIL 80. In addition, vectors representing significant associations of animal preferences for flowers indicate that the size of different floral parts affect the choices of flowers of both bumblebees ($r^2 = 0.087$, $p = 0.012$) and humans ($r^2 = 0.230$, $p = 0.001$; **Supplementary Figure S9C**).

Whilst colour is a relevant trait for bumblebees, humans, and thrips, the morphology of flowers and the size of different floral parts affect the choices of bumblebees and humans. This indicates that very small animals such as thrips would not be so influenced by the size and shape of flowers.

Scent

A total of 29 different compounds were found in the scent profiles of studied flowers (**Table 1**). The most scented line (average total emission) was RIL 9, whilst the lowest emitter was RIL 112 (Levene test: $F = 1.608$, $p = 0.173$; ANOVA: $F = 6.633$, $p < 0.001$). Some VOCs, like (E)- β-ocimene and β-myrcene, were emitted by all lines. However, some VOCs that were not emitted constitutively in parental lines, were found in RILs, such as methyl salicylate (RILs 113, 80, and 9), benzenepropanol (RILs 112 and 9), 3,5-dimethoxytoluene (RIL 80), or linalool (RIL 9).

We used the scent composition of flowers with stamens (whole flowers) to analyse the preferences of bumblebees and humans. NMDS ordination of the scent profiles of whole flowers clearly separates the scent emission of *A. linkianum* from RIL 113 and 80, which are located at the bottom and the top of the ordination, respectively (**Supplementary Figures S10A,B**). In the centre of the ordination is the parental species *A. majus*, which is more similar in its scent bouquet to RILs 112 and 9 (**Supplementary Figures S10A,B**). We tested the preferences of bumblebees and humans for both whole flower multimodal displays (animals interacting with flowers) and just the scent of whole flowers. Thus, we fitted vectors representing preference results into two different ordinations (**Supplementary Figures S10A,B**).

Vectors representing the preferences of bumblebees and humans for the scent of whole flowers (**Supplementary Figure S10A**) indicate that this trait is important for both types of animals when they can see and smell the flowers: bumblebees ($r^2 = 0.481$, $p = 0.001$), humans ($r^2 = 0.375$, $p = 0.001$). In this case, bumblebees and humans have similar preferences for the scent of whole flowers (**Supplementary Figure S10A**), with vectors pointing towards *A. linkianum*.

When bumblebees and humans are unable to see the flowers, vectors representing their preferences for the scent of flowers (**Supplementary Figure S10B**) indicate that this trait is still relevant for them ($r^2 = 0.585$, $p = 0.001$ and $r^2 = 0.191$, $p = 0.002$, respectively). Interestingly, results for bumblebees with regard to the isolated scent of flowers show a completely diametrical configuration in the NMDS ordination, compared with the scent of flowers when they can interact with the whole flowers (**Supplementary Figures S10A,B**). Results indicate that visual cues prevail over scent preferences of bumblebees. In the case of humans, the direction of the vector does not change in the NMDS ordination for scent in isolation compared to the scent of whole flowers (**Supplementary Figures S10A,B**), revealing that scent is a trait strongly affecting the preferences of plant experts for flowers.

We analysed the scent emission of flowers without stamens. NMDS analysis of the scent profile of flowers without stamens shows the same configuration as previously described for the different lines, with *A. majus* in the centre of the ordination and *A. linkianum* at the bottom (**Supplementary Figures S10A–C**). Hence, the scent bouquet of flowers is barely affected by the removal of stamens (**Supplementary Figures S10A,C**). Fitting the vectors representing the preferences of thrips into the ordination (**Supplementary Figure S10C**; $r^2 = 0.267$, $p = 0.001$), indicates that thrips have opposing preferences for flowers than bumblebees and humans when they can see and smell whole flowers (**Supplementary Figure S10A**). We also analysed the scent emission of stamens removed from flowers and tested the preferences of thrips for detached stamens. Ordination of the scent emission from stamens of flowers studied generally separates each line clearly, with *A. linkianum* and RIL 112 overlapping in some points. The vector representing the preferences of thrips for stamens detached from flowers indicates that this trait is statistically significant ($r^2 = 0.220$, $p = 0.016$; **Supplementary Figure S10D**).

We used RF for the selection of the most important VOCs correlating with the effect sizes representing the preferences of studied animals. We used this approach with the results of experiments based on preferences for the isolated floral scents of bumblebees and humans (**Figures 4A,B**). We also used this approach for testing the preferences of thrips for the scent of flowers without stamens and detached stamens (**Figures 4C,D**). Results indicate that the behaviour of bumblebees might be affected by some VOCs including methyl benzoate, sabinene, eremophilene, or cinnamyl alcohol (**Figure 4A**). In contrast, hexahydrofarnesyl acetone, eremophilene, benzenepropanol, sabinene, β-myrcene, or β-ocimene may affect the preferences for the scent of flowers of human plant-specialists (**Figure 4B**). Finally, methyl hydrocinnamate, o-aceylphenol, cinnamyl alcohol, or β-myrcene may affect the behaviour of thrips towards flowers (**Figure 4C**), whilst methyl benzoate or nonanal may influence the decisions of thrips when choosing just stamens (**Figure 4D**).

We were not able to test the effect of all VOCs identified by RF on bumblebees and thrips but we were able to test the effect of some VOCs with high %IncMSE (**Figure 4**), for which,

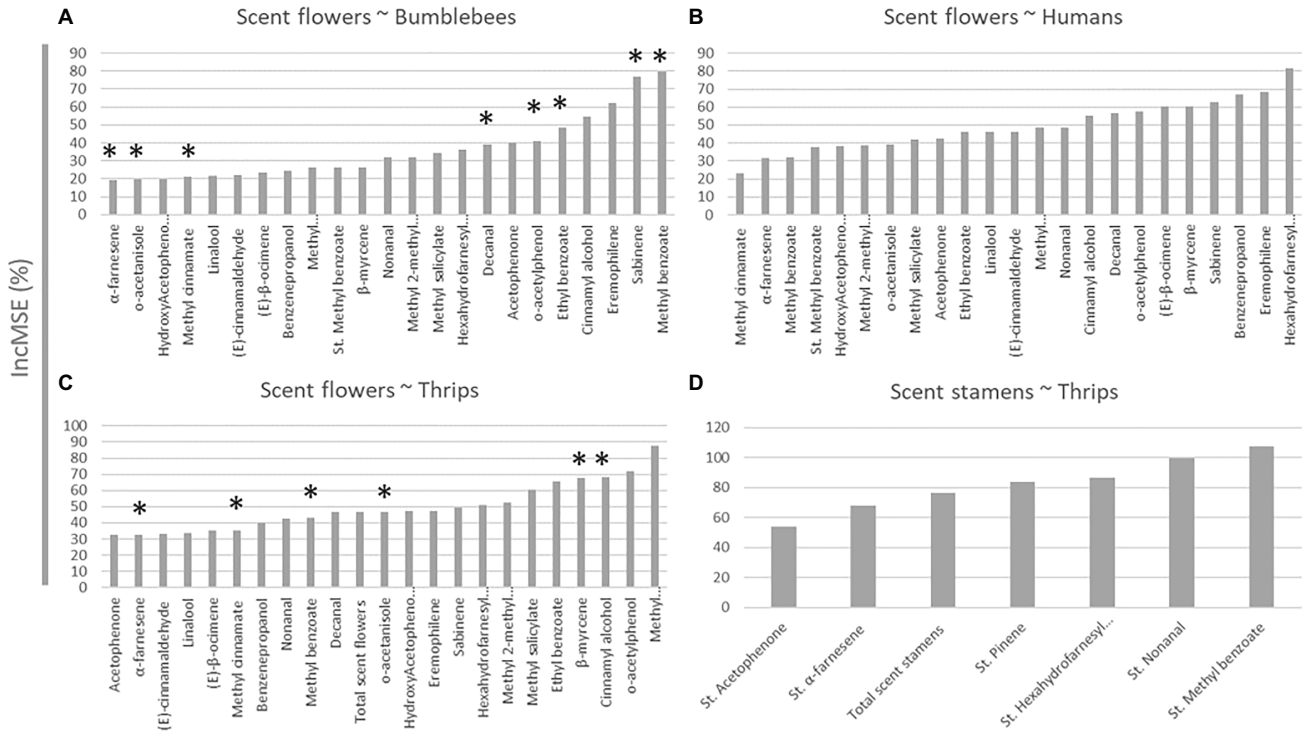

FIGURE 4 | Percentage of the mean square error (%IncMSE) resulting from random forest regression analysis of the preferences of studied animals as the dependant variable and the emission of volatile organic compound (VOCs) as the explanatory variable. **(A)** Bumblebee preferences against the scent of flowers with stamens. **(B)** Human preferences against the scent of flowers with stamens. **(C)** Preferences of thrips for the scent of flowers without stamens. **(D)** Preferences of thrips for the scent of stamens. Asterisks indicate isolated volatiles that have been used for behavioural assays with bumblebees and thrips.

there are commercially available authentic standards, on the behaviour of bumblebees and thrips (**Table 2**). Results indicate that, in the case of bumblebees, methyl benzoate seems to be an attractant. However, o-acetanisole in high concentrations is a repellent. Testing concentrations within those between the levels of methyl benzoate (33%) and o-acetanisole (0.4%) found in the flowers (**Table 1**) indicates that below 100 ppm o-acetanisole does not have an effect on the behaviour of bumblebees. Additionally, ethyl benzoate, o-acetylphenol, sabinene, α-farnesene, decanal, and methyl cinnamate did not show any effect under our experimental conditions (**Table 2**). Similarly, we tested the effect of some VOCs on the behaviour of thrips. Results obtained show that α-farnesene, methyl benzoate, o-acetanisole, cinnamyl alcohol, methyl cinnamate, and β-myrcene do not have any effect on thrips behaviour at concentrations of 1,000 ppm. However, the use of pure β-myrcene may kill these insects (**Table 2**).

Scent is a relevant floral trait for all organisms studied. In the case of bumblebees, visual cues are more relevant than the isolated scent of flowers, methyl benzoate can be an attractant and o-acetylphenol a repellent. Human preferences for the scent of flowers do not change when they can see the flowers. On the other hand, thrips are attracted differentially by the scent emitted by the flowers and by the stamens, and their attraction towards flowers by floral scent contrasts with that of bumblebees and humans. Finally, β-myrcene can kill thrips at high doses.

Pollen and Trichomes

Our experiments with thrips and stamens allowed the insects to interact with the pollen and thus, potentially, develop preferences for it, which is supported by the significant results ($r^2 = 0.156$, $p = 0.003$; **Supplementary Figure S10**). Surprisingly, our results appear to indicate that human plant experts' preferences for *Antirrhinum* flowers also were influenced by pollen traits ($r^2 = 0.131$, $p = 0.022$, **Supplementary Figure S10**) and trichome density ($r = 0.299$, $r^2 = 0.089$, $p = 0.019$; **Supplementary Figure S9B**). Since humans did not touch flowers to open them and see the trichomes or the pollen closely, this result might be caused by the correlation of these traits with other floral attributes, such as the scent of flowers (**Figure 5**).

Although, our results appear to indicate that pollen is a relevant trait for thrips and humans, whereas trichomes are important for plant experts, careful interpretation of these results in the context of the experimental design suggests that they represent coincidental correlations.

Relative Contribution of Phenotypes to the Choices of Animals

Bipartite network analysis of significant results (*envfit*, *cmdscale*, and correlations of univariate traits) reflects the relative importance of analysed floral traits for the three groups of organisms studied (**Figure 6**). Preferences seem to be multimodal

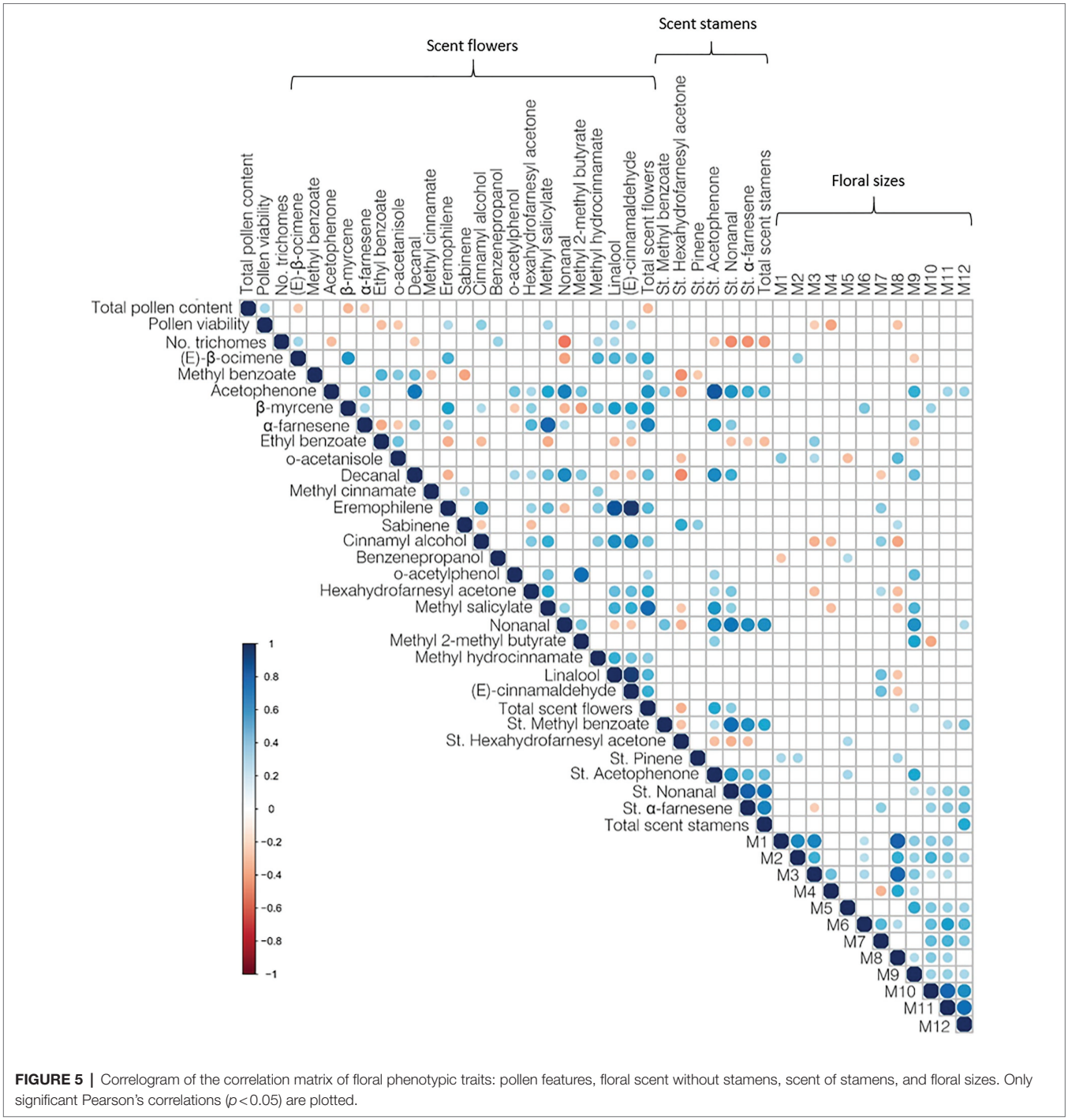

FIGURE 5 | Correlogram of the correlation matrix of floral phenotypic traits: pollen features, floral scent without stamens, scent of stamens, and floral sizes. Only significant Pearson's correlations ($p < 0.05$) are plotted.

responses with increasing complexity regarding the number of traits involved, from thrips with the lowest number of traits, to humans with the most. From the total trait spectrum studied, the most important traits affecting all organisms seem to be floral scent and colour. Choices of humans and bumblebees show responses towards the size of floral parts, the morphology of flowers, and the isolated scent of flowers, whilst the choices of humans and thrips seem to be affected by (or correlated with) pollen features. Our data suggest that the responses of thrips towards flowers are more affected by their scent and the scent of stamens. Whereas the most important traits for humans seem to be the scent of flowers and their morphology, bumblebees seem to be more influenced by the scent of flowers, either in combination with other traits or in isolation. Correlations found amongst floral phenotypic traits might underlay some of these associations (**Figure 5**), such as correlations between pollen viability and several scent compounds. Altogether, bumblebees and humans seem to be attracted by more similar

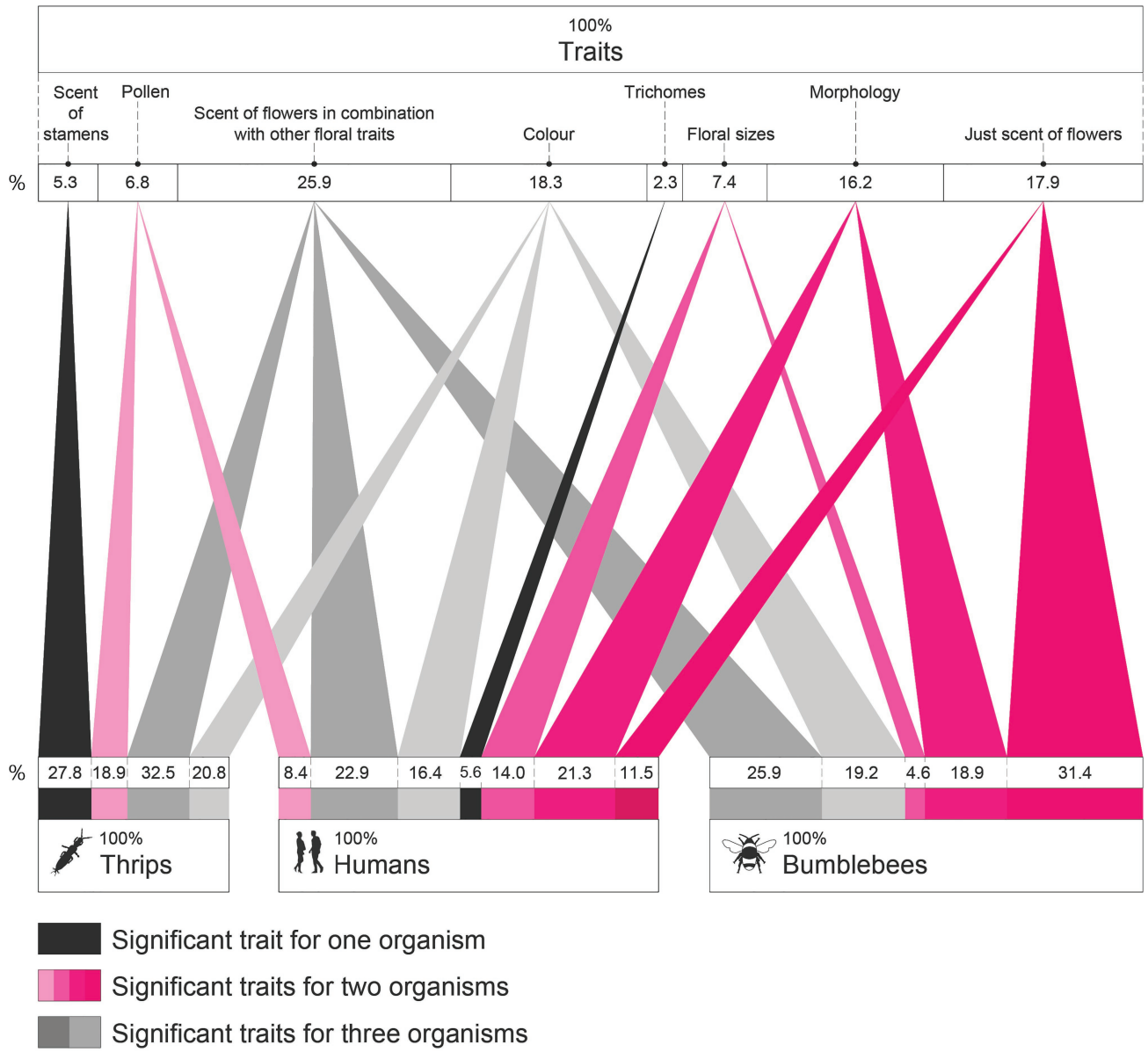

FIGURE 6 | Bipartite network of statistically significant relations of the preferences of bumblebees, humans, and thrips for groups of floral traits. Colour of edges represents: traits relevant for all organisms (grey), for two organisms (pink), or for just one organism (black). Only significant traits ($p < 0.05$) are represented for each organism. Width of edges is proportional to r^2 for each organism and trait. Percentages indicated are calculated based on the width of each trait. In the upper part of the graph, percentages represent the relative importance of each trait analysed for the three kind of organisms together. In the lower part of the graph, percentages represent the relative importance of traits for each kind of organism separately (thrips, humans, and bumblebees).

floral attributes than the floral traits that are relevant for thrips with regard to *Antirrhinum* flowers.

DISCUSSION

The ways in which humans interact and interfere with nature and plants are shaped by discernment through multiple sensory modalities (Storms and Zyda, 2000; Lindemann-Matthies et al., 2010). As our results indicate, human selection of ornamental flowers is also the consequence of decisions based on several different floral attributes. Similarly, mutualistic and antagonistic flower visitors might favour or disfavour multimodal floral traits (Katzenberger et al., 2013; Terry et al., 2014), which affects floral selection and evolution (Ramos and Schiestl, 2019). However, the traits affecting the behaviour of animals may vary between different organisms such as humans, bumblebees, and thrips. Here, we aimed to gain a more complete understanding of the mechanisms by which specific floral traits could impact plant fitness. We found that in general, humans and bumblebees

share preferences towards floral traits, and that these preferences contrast with those of thrips.

Floral Traits Affecting the Choices of Humans, Pollinators, and Pests

Findings reported here are similar to previous studies informing about the relevance of colour (Odell et al., 1999; Sampson and Kirk, 2013; Moyroud et al., 2017), floral sizes, morphology (Mainali and Lim, 2011; Moyroud and Glover, 2017), pollen (Kirk, 2009; Wilmsen et al., 2017), and scent (Suchet et al., 2010; Abdullah et al., 2014; Larue et al., 2016) for the attraction of bumblebees and thrips. We additionally examined the preferences of humans for the same floral phenotypes, testing whether human preferences match the preferences of the insects. Our study indicates that floral colour, morphology, and scent might be relevant traits guiding the selection of flowers by humans. These attributes have previously been observed as being important for humans and, for example, used in marketing (Bruce et al., 2003; Morrin, 2011).

We were able to directly assess the effect of some single floral traits, such as scent, isolated VOCs or detached stamens from flowers. However, the relative importance of distinct floral traits should be tested with comparative choice experiments to gauge the relative effects of all traits. Something that impedes discriminating relative effects in a multimodal scenario is the correlation between variables. For instance, our results indicate that the size of floral parts, pollen features, or the density of trichomes might influence the selection of flowers by humans. The size of floral parts can be related to their morphology (Moyroud and Glover, 2017), and that might partially explain why these traits seem to be important for both bumblebees and humans. Similarly, correlation of trichome abundance and pollen features with other floral traits, measured and not measured, could underlie the counterintuitive human preferences for these traits.

Herbivores are known to change floral traits, which affects pollinator behaviour, and thus, the community dynamics (Rusman et al., 2019). Consequently, studies working with flowers attached to plants, as well as the synchronised visitation of thrips and bumblebees, may yield differing results.

Traits Under Pressure for Selection

Over the past 200 million years, the evolution of flowering plants (Li et al., 2019) has been guided by their interactions with the floral visitors commonly investigated by the scientific community, such as pollinators, herbivores, microbes, or natural enemies of herbivores (Armbruster, 1997; Herrera et al., 2002; Junker and Tholl, 2013; Borghi et al., 2017; Knauer and Schiestl, 2017). Much more recent is the selection of plants exerted by humans, present for just a few millenia (8.000–10.000 years; Milla et al., 2015). In the case of ornamental plants and humans, this interaction has been proposed to be mutualistic (Wilson et al., 2016). At least in the context of these experiments, humans exert positive selection since they choose the flowers that they would prefer to grow. Our study suggests that humans and bumblebees have more similar preferences towards floral traits compared to those of thrips. This finding suggests that human *Antirrhinum* floral selection, for aesthetic reasons, could enhance the selection of phenotypes more attractive to bumblebees and less appealing to thrips. Correspondingly, selective forces exerted by bumblebees may boost the selection of flowers by plant breeders, whilst reducing visitation of thrips. When multiple selective forces are present, evolution of floral traits is not straightforward. Indeed, plant size, several volatiles such as methyl benzoate, p-anisaldehyde, and benzyl nitrile have been found to evolve rapidly in response to two pollinators, bumblebee, and hover flies in *Brassica* (Gervasi and Schiestl, 2017). However, the actual composition of volatiles differs significantly indicating a specificity of scent profile changes in response to differing pollinators. Furthermore, using the same system it has been shown that floral attractiveness i.e., they were more fragrant and displayed larger flowers when evolving in the presence of bumblebees as pollinators. In contrast, when plants grow in the presence of bumblebees and the hervibore *Pieris brassicae* (Ramos and Schiestl, 2019, 2020), volatile evolution, production of glucosinolates and autogamy evolve differently. The current emerging hypothesis is a possible coevolution of floral and defense traits. In our case, the differing choices of bumblebees and thrips indeed indicate a basic level of complexity whereupon evolutionary forces may act.

Finally, plants displaying floral phenotypes appealing to thrips might be at a competitive disadvantage due to both being less attractive to beneficial selectors and relatively more visited by herbivores. Remarkably, parental species *A. majus* and *A. linkianum* possess traits relevant for the fitness of the species, being more attractive to beneficial selectors than to antagonists.

Implications of the Study: Agricultural Perspectives

Domesticated plants are the result of the directed artificial selection of plants by humans and the natural selection under cultivation exerted by beneficial visitors and antagonists (Milla et al., 2015). The study of plant traits that can enhance the survival and quality of crops, by attracting pollinators and repelling pests, is very important from an agricultural perspective. For instance, some studies have reported the avoidance of tomato flowers in greenhouses by bumblebees due to a deterrent effect of flowers (Whittington et al., 2004; Morse et al., 2012). Our results regarding the attractive effect of methyl benzoate and the repellent effect of o-acetanisole could interest breeders seeking to improve pollination of greenhouse crops (Morse et al., 2012; Ruiz-Hernández et al., 2017). On the other hand, drawbacks derived from pesticide use (Gierer et al., 2019; Varah et al., 2020) could potentially be replaced by growing naturally pest-repellent plants or the use of auxiliary plants to control thrips populations in greenhouses. Moreover, the use of high doses of β-myrcene could be a resource to control thrips on crops (Terry et al., 2014). Finally, in the case of ornamental plants, human preferences are the main driving factor selecting flowers. However, a number of factors affect the human perception of flowers such as gender, cultural background, or education (Kendal et al., 2012). Therefore, for studying human preferences

under a market niche perspective (or evolutionary context) such factors should be considered.

CONCLUSION

Our work suggests that instead of a single trait playing a key role in the selection of flowers, there are several floral traits interacting with different floral visitors. The relative importance of floral traits for floral visitors may change with different plant and/or visitor taxa. However, our work shows that the interactions of insects and humans with floral phenotypes could ultimately drive the evolution of flowering plants in natural and human-influenced environments. Our comparisons of the floral preferences of humans and insect flower visitors represent a novel approach that yields intriguing insights into how plant breeders may inadvertently influence insect-flower interactions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee from the Universidad Politécnica de Cartagena (CEI19_013). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

VR-H, PG, JW, PB, BG, RJ, and ME-C planned and designed the research. VR-H, LJ, AR-G, SA, JP, AL-Z, JB, SE, SB, and CT-R performed the experiments with plants and animals. VR-H, LJ, SA, JP, LM-R, and RJ analysed the data. VR-H and RJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.647347/full#supplementary-material

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