

Integration of attractive and defensive phytochemicals is unlikely to constrain chemical diversification in a perennial herb

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Summary

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- Diversification of plant chemical phenotypes is typically associated with spatially and temporally variable plant–insect interactions. Floral scent is often assumed to be the target of pollinator-mediated selection, whereas foliar compounds are considered targets of antagonist-mediated selection. However, floral and vegetative phytochemicals can be biosynthetically linked and may thus evolve as integrated phenotypes.
- Utilizing a common garden of 28 populations of the perennial herb *Arabis alpina* (Brassicaceae), we investigated integration within and among floral scent compounds and foliar defense compounds (both volatile compounds and tissue-bound glucosinolates).
- Within floral scent volatiles, foliar volatile compounds, and glucosinolates, phytochemicals were often positively correlated, and correlations were stronger within these groups than between them. Thus, we found no evidence of integration between compound groups indicating that these are free to evolve independently. Relative to self-compatible populations, self-incompatible populations experienced stronger correlations between floral scent compounds, and a trend toward lower integration between floral scent and foliar volatiles.
- Our study serves as a rare test of integration of multiple, physiologically related plant traits that each are potential targets of insect-mediated selection. Our results suggest that independent evolutionary forces are likely to diversify different axes of plant chemistry without major constraints.

Introduction

Plants engage in diverse interactions with mutualistic and antagonistic insects, and selection from these insects is considered an important driver of the spectacular diversity of plant phenotypes (e.g. Ehrlich & Raven, 1964; Fenster *et al.*, 2004). At local scales, insect community composition may vary extensively in space and time, providing ample opportunity for adaptive divergence among populations of plant traits involved in these interactions (Thompson, 1999, 2005; Futuyama & Kirkpatrick, 2018). Local variation in plant traits may be the result of multiple agents of selection (Kessler & Kalske, 2018; Sletvold, 2019), and identifying what feature of the plant phenotype is under selection can therefore be difficult (Armbruster & Schwaegerle, 1996; Arnold, 2023). This is particularly complex if trait variation relevant for plant–insect interactions is constrained or facilitated by among-trait correlations (Arnold, 2023).

Plant traits are often part of integrated phenotypes, that is a group of traits that experiences stronger correlation within the group compared with between groups. Therefore, integrated traits may constrain the potential for natural selection to shape locally optimized trait combinations (Armbruster & Schwaegerle, 1996; Arnold, 2023). Strong phenotypic integration can result from genetic (e.g. Armbruster, 1991) or developmental linkage of traits through pleiotropy (Armbruster & Schwaegerle, 1996; Herrera *et al.*, 2002; Futuyama & Kirkpatrick, 2018) and from functional trade-offs. Developmentally linked traits may manifest as the production of identical phytochemicals in both floral and vegetative tissues (Dudareva *et al.*, 2013; Schiestl, 2014; Dötterl & Gershenzon, 2023). Trade-offs may limit resource allocation to functionally similar traits, such as the negative relationship between the abundance of leaf trichomes and toxic leaf glands that both are important for herbivory defense in cotton (*Gossypium* spp.) and related taxa (Rudgers *et al.*, 2004).

Integration can also occur between traits of different modalities, such as reduced floral scent emissions and floral sizes of plant lineages that have evolved autonomous selfing (Herrera *et al.*, 2002; Ferrari *et al.*, 2006; Doubleday *et al.*, 2013; Petré *et al.*, 2021). In truly integrated phenotypes that include strong and causal trait correlations, studying the effects of selection on single traits can be difficult, as such effects are often obscured by selection on correlated traits (Arnold, 2023).

Plant secondary chemical traits are involved in both defense and attraction and can be selected upon by both antagonistic (herbivores, florivores, seed predators, and nectar robbers) and/or mutualistic (pollinators and natural enemies of herbivores) insects. The ecological costs of emitting floral scent, including risking herbivore attraction and/or biosynthetic energy costs, may explain why a transition to self-compatibility is often followed by significantly reduced floral scent signals (Doubleday *et al.*, 2013; Petré *et al.*, 2021). The loss of self-incompatibility may change not only the selective forces acting on plant signaling but also compound composition and/or the levels of trait integration between emission rates of attraction and defense phytochemicals. A previous meta-analysis investigating differences in phenotypic integration of floral morphological traits between selfing and outcrossing plant species revealed that selfing species had higher levels of integration for both whole flower and sexual organs compared with outcrossing species (Fornoni *et al.*, 2015). This pattern likely had a functional basis, as movement of pollen within the selfing flower is an important process favored by correlational selection (Fornoni *et al.*, 2015). If production and emission of phytochemicals is genetically linked to morphological structures in the flower, we could expect a similar pattern to emerge for chemical traits.

Floral scent bouquets and plant defense signals are complex traits, consisting of one to several hundred volatile or nonvolatile compounds produced by several biosynthetic pathways of which a handful are well-documented (Knudsen *et al.*, 2006; Pichersky *et al.*, 2006). For floral scent and foliar defense volatiles, these include the shikimate/phenylalanine pathway (phenylpropanoids and benzenoids), the mevalonic acid pathway (sesquiterpenes), the methylerythritol phosphate pathway (diterpenes, volatile carotenoid derivatives, monoterpenes and hemiterpenes), and the lipoxygenase pathway (methyl jasmonate/green leaf volatiles) (Dudareva *et al.*, 2013). The intricate crosstalk within and between these pathways (i.e. sharing substrates) means that integration could result from developmental links between vegetatively produced foliar defense volatiles and floral scent volatiles (Dudareva *et al.*, 2013). Further, some chemical compounds, such as monoterpenes (e.g. Junker & Blüthgen, 2010; Parachnowitsch *et al.*, 2013), may serve as attractants of interacting species (Theis *et al.*, 2007; Schiestl *et al.*, 2014; Gross *et al.*, 2016; Raguso, 2016; Friberg *et al.*, 2019; Petré *et al.*, 2021) and repellents of others (Tollsten & Bergström, 1988; Geervliet *et al.*, 1997; Kessler & Halitschke, 2009; Kessler *et al.*, 2011; Schiestl, 2014; Moreira *et al.*, 2019; Lackus *et al.*, 2021), thus being developmentally linked but functionally dissimilar. Additionally, in evolutionary time, floral scent compounds were likely derived from herbivore repellents (Pellmyr & Thien, 1986;

Schiestl, 2010; Dudareva *et al.*, 2013), further suggesting that these two functions of plant chemistry may be developmentally connected. Foliar volatiles can, in turn, be biosynthetically linked to tissue-bound defense compounds, such as glucosinolates, targeted at herbivores (Fahey *et al.*, 2001). Upon tissues disruption, the hydrolyzation of glucosinolates creates powerful defense volatiles, isothiocyanates, which if present in the foliar volatile profile could provide information about the defense status of the plant (Fahey *et al.*, 2001). Due to the hydrolyzation step, glucosinolate and isothiocyanate content could, in theory, be correlated (but see Schiestl, 2014). In the presence of correlational selection on functionally similar compounds, this association could extend to other foliar volatiles. Therefore, plant chemotypes could be considered integrated traits (Junker *et al.*, 2018). Yet, intraspecific variation in floral scent (e.g. Gross *et al.*, 2016; Dormont *et al.*, 2019; Friberg *et al.*, 2019; Petré *et al.*, 2021) and defense compound composition (Wurst *et al.*, 2008; Bustos-Segura & Foley, 2018; Benedek *et al.*, 2019; Buckley *et al.*, 2019) have typically been studied in isolation. The potential for integration of chemical traits has been tested once before in a large meta-analysis, where levels of integration within and between floral and foliar plant chemistry were investigated across a large number of plant taxa (Junker *et al.*, 2018). The authors found that overall, floral scent compounds experienced lower levels of integration than foliar volatile compounds. However, this study included only one case where both floral and foliar volatile compound data were obtained from the same species (*Brassica nigra*, Bruinsma *et al.*, 2014). As such, the extent to which species and populations vary in level of chemical integration remains largely unknown but has wide-ranging consequences for the ecology and evolution of these traits.

Here, we investigate the level of phytochemical integration across multiple populations of the same species, a step toward evaluating the evolutionary independence of floral scent and defense chemistry. The herb *Arabidopsis thaliana* L. (Brassicaceae) represents an ideal system for assessing integration in chemistry, as it has ample population-level variation in both floral scent attractants (Petré *et al.*, 2021) and constitutive defense compounds (glucosinolates, Buckley *et al.*, 2019). Floral scent and glucosinolates have been suggested previously as the primary targets of selection, without examining whether these plant chemical traits may be evolving in concert. Indeed, floral volatiles and glucosinolates can share structural compounds, such as indole rings, produced in amino acid metabolism (Halkier & Gershenzon, 2006; Knudsen *et al.*, 2006). Furthermore, European populations of *A. thaliana* can be either self-incompatible (in Greece and Italy) or self-compatible (in western, central, and northern Europe). As such, the functional importance of emitting floral scent signals likely varies among populations (Petré *et al.*, 2021), which may create trade-offs between emitting floral scent and defense compounds.

We combine data on floral scent, glucosinolate tissue-bound defense compounds, and foliar volatile compounds to test hypotheses (Fig. 1) that investigate the extent to which these compound groups are evolutionarily linked. Specifically, we test for pairwise integration of floral scent, foliar volatiles, and

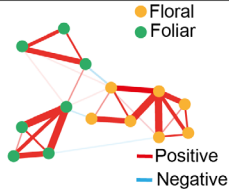
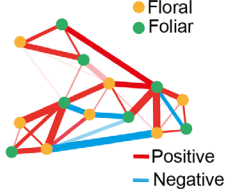
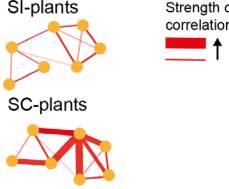
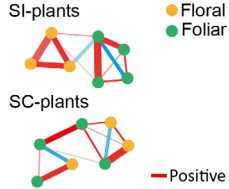
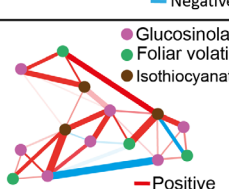
Theme	Hypothesis	Visualization	Index
(a) Integration between floral scent volatiles and biosynthetically related foliar defense volatiles	Floral scent and foliar defense compounds are not integrated and can thus respond to selection or drift independently		High within tissue, low between tissues
	Floral scent and foliar defense compounds are integrated and are thus interdependent. There are correlations between compounds sharing biosynthesis		High between tissues or compounds sharing biosynthesis
(b) Integration between floral scent volatiles in self-compatible and self-incompatible individuals	Self-incompatible plants have low among-compound integration of floral scent due to breaking of correlations between compounds	SI-plants SC-plants 	Low SI index, high SC index
(c) Integration between floral scent volatiles and foliar volatiles in self-compatible and self-incompatible individuals	Self-incompatible plants have lower between-tissue integration due to selection increased benefit of decoupling floral scent from foliar defense in response to selection	SI-plants SC-plants 	Low SI index, high SC index
(d) Integration between foliar volatiles and foliar glucosinolates	Foliar volatiles and foliar glucosinolates have highest correlation between glucosinolates and glucosinolate derived volatile isothiocyanates due to shared biosynthesis		High index within group, moderate between groups

Fig. 1 Conceptual figure describing hypotheses explored in this study on phytochemical integration in *Arabidopsis thaliana*. Themes, hypotheses, and visualization of predictions are described in the different panels.

glucosinolates, as well as integration of all three combined (Fig. 1a,c,d). Furthermore, we test whether pollinator-dependent, self-incompatible plants experience lower levels of chemical trait integration within and/or among compound groups compared with self-compatible populations (Fig. 1b).

Low levels of trait integration in floral scent, foliar volatiles, and glucosinolates among populations would imply that these traits are free to evolve separately in relation to local selection pressures (Fig. 1a). If pollinator-mediated selection has favored the breakage of trait correlations, we predict self-incompatible populations to exhibit a low degree of integration within floral volatiles and between floral volatiles and foliar volatiles, as this would allow for an adaptive response to pollinator-mediated selection (Fig. 1b,c). In self-compatible populations, we expect a higher degree of integration within floral scent volatiles and between floral and foliar volatiles, as relaxed pollinator-mediated selection will maintain developmental links between compounds (Fig. 1b,c). Additionally, we expect a higher degree of integration between functionally similar foliar volatiles and glucosinolates,

because of the glucosinolate–isothiocyanate relationship (Fig. 1d), whereas we expect little integration between floral volatiles and glucosinolates, as *A. thaliana* does not emit nitrogen- or sulfur-bearing compounds in its floral scent (Petren *et al.*, 2021), thereby lowering the chances of shared structures, such as indole rings.

Materials and Methods

Study plant and populations

Alpine rock-cress (*Arabidopsis alpina* L.; Brassicaceae) is a white-flowered, perennial herb of small stature that is widely distributed across alpine-arctic habitats in the Northern Hemisphere. It prefers wet, open gravel, or rocky habitats, often with calcareous bedrock, and forms basal rosettes from which a 20- to 30-cm-tall flower stem with sparse clusters of flowers form (Koch *et al.*, 2006; Mossberg & Stenberg, 2010). The species originated in Asia Minor and spread to Europe by multiple migration events

of a single lineage (Koch *et al.*, 2006). In this study, we used 28 European *A. alpina* populations from Northern Scandinavia, France, Spain, Italy, Switzerland, and Greece (Table 1) cultivated in a glasshouse common garden at Lund University, Sweden.

Previous work on 17 of these populations (Petrén *et al.*, 2021) showed how scent composition, emission rate, and flower size are highly variable among populations. Similarly, another study documented among-population variation in glucosinolate content using nine Swiss populations (of which three are included for glucosinolates and nine for foliar and floral volatiles in this study), which was related to changing exposure to invertebrate herbivores with elevation (Buckley *et al.*, 2019). Furthermore, mating system varies among the 28 populations, where individuals from Italy and Greece are largely self-incompatible (SI) and individuals from Northern Scandinavia are self-compatible (SC) and to a large extent autonomously selfing (Ansell *et al.*, 2008; Toräng *et al.*, 2017; Petrén *et al.*, 2021). Populations in France, Spain, and Switzerland are self-compatible but require insect vectors for pollen transfer within flowers (Tedder *et al.*, 2011, 2015;

Toräng *et al.*, 2017). The mating system variation among European populations is reflected in nectar production and floral scent, with SI populations typically producing larger volumes of nectar per flower (Eisen *et al.*, 2023) and generally emitting substantially more floral scent, with the aromatic compound benzaldehyde dominating the scent profiles of some SI populations (Petrén *et al.*, 2021). In comparison, SC populations emit less scent overall, and some populations are almost scentless. Generally, the scent profiles of SC populations are dominated by three compounds (phenylacetaldehyde, phenylethyl alcohol, and phenylethyl acetate). These compounds are also emitted at high rates in some SI populations but are not present in the scent profiles in other SI populations. Thus, there is extensive among-population variation also within mating systems (Petrén *et al.*, 2021).

Experimental setup

Field-collected seeds from 28 populations (Table 1), 15 seed families per population, and 10 seeds per seed family were sown

Table 1 List of *Arabidopsis thaliana* populations used in the experiment, with sample size (*n*) representing the individuals used for floral, foliar, and glucosinolate phytochemical extractions, mating system (self-compatible (SC) or self-incompatible (SI)) and pollinator dependence (mixed – capable of self-fertilization but require insect vectors to some extent, complete – fully outcrossing, none – capable of self-fertilization without insect vectors), region, coordinates, and elevation (meters above sea level: m asl).

Population	Floral (<i>n</i>)	Foliar (<i>n</i>)	Glucosinolates (<i>n</i>)	Mating system (insect dependence)	Region	Lat	Lon	Elevation (m asl)
Aal 04**	15	5	8	SC (mixed)	Switzerland	47°25'48.4"N	8°01'27.8"E	816
Aal 12**	18	5	na	SC (mixed)	Switzerland	47°10'34.3"N	7°07'30.0"E	1050
Aal 29**	10	5	na	SC (mixed)	Switzerland	46°29'07.6"N	9°46'22.0"E	2850
Aal 34c**	16	5	na	SC (mixed)	Switzerland	47°05'04.9"N	9°01'00.4"E	988
Aal 36**	13	5	na	SC (mixed)	Switzerland	47°09'15.7"N	9°18'24.3"E	2288
Aal DMB**	12	5	8	SC (mixed)	Switzerland	46°12'37.2"N	7°04'14.7"E	2618
Aal FC**	17	5	na	SC (mixed)	Switzerland	46°52'49.3"N	9°15'52.0"E	2659
Aal PB**	12	5	na	SC (mixed)	Switzerland	46°58'34.8"N	8°15'11.3"E	1879
Aal SFH**	13	5	8	SC (mixed)	Switzerland	46°50'28.0"N	7°58'46.2"E	1735
E3*	NA	NA	8	SC (mixed)	Spain	43°14'N	5°56'W	1171
Fr1*	17	4	8	SC (mixed)	France	45°03'N	6°24'E	2196
Fr2*	10	5	8	SC (mixed)	France	44°57'N	6°36'E	1983
Fr3	14	5	7	SC (mixed)	France	44°03'N	7°30'E	2227
Fr4	13	5	na	SC (mixed)	France	44°06'N	7°31'E	1787
G4	NA	NA	8	SI (complete)	Greece	39°30'54"N	21°01'59"E	1176
IT10	30	5	8	SI (complete)	Italy	42°14'17"N	13°21'50"E	1710
IT13	13	6	na	SI (complete)	Italy	42°05'06"N	13°34'50"E	980
IT15	21	5	8	SI (complete)	Italy	42°12'43"N	13°31'26"E	1285
IT16	11	5	8	SI (complete)	Italy	41°56'31"N	13°21'43"E	1547
IT17	18	5	8	SI (complete)	Italy	42°15'14"N	13°29'24"E	1410
IT18	9	5	8	SI (complete)	Italy	41°42'29"N	15°57'11"E	813
IT2*	18	5	8	SI (complete)	Italy	42°30'N	13°35'E	1635
IT4*	NA	NA	8	SI (complete)	Italy	42°15'N	13°19'E	1192
IT5*	13	5	8	SI (complete)	Italy	41°58'N	13°33'E	656
IT6*	12	5	8	SI (complete)	Italy	41°50'N	13°56'E	1542
IT8*	NA	NA	8	SI (complete)	Italy	44°08'N	10°15'E	850
IT9*	15	5	8	SI (complete)	Italy	44°05'N	10°19'E	1002
S1*	NA	NA	8	SC (none)	Sweden	68°24'N	18°19'E	769
Total	340	115	159					

Populations were found at varying altitudes (Aal29 highest: 2850 m asl; IT5 lowest: 656 m asl). Populations marked with a single asterisk were used in a previous study on intraspecific variation in floral scent (Petrén *et al.*, 2021). Populations marked with two asterisks were used in a previous study on variation in defense traits (Buckley *et al.*, 2019). All data collected in this study is novel and not used in previous publications; na, no individuals were available for sampling in that category.

in wells onto a soil mixture containing two-third potting soil ('YrkesPlantjord'; Weibulls Horto AB, Hammenhög, Sweden) and one-third 2–6 mm LECA (Saint-Gobain Byggprodukter AB, Sollentuna, Sweden), with a top layer of moist sowing soil ('Plugg och Säjord'; Weibulls Horto AB). Five seeds were sown in each well and were subsequently stratified in a dark 4–6°C room for 7–10 d before being moved to a 20°C (day) and 16°C (night) glasshouse chamber with 16 h 165 $\mu\text{mol m}^{-2} \text{s}^{-1}$ artificial light and additional natural light. As seedlings started to emerge, they were transplanted into individual pots (7 × 7 × 7 cm) containing the soil mixture described above. Seedlings were then allowed to grow for 6 wk before being transferred into a room maintaining 8-h days (25–30 $\text{m}^{-2} \text{s}^{-1}$, 7°C) and 16-h nights (5°C) for vernalization. After 15 wk, the plants were returned to the glasshouse chamber maintaining 16 h, 165 $\mu\text{mol m}^{-2} \text{s}^{-1}$ artificial light and additional natural light days and 8-h nights to induce flowering. The plants were watered regularly and nutrients (3‰ SW Horto 'SW Bouyant' RIKA S 7-1-5 + Mikro) were added once a week throughout the flowering period. Because of low flowering rate in one population (IT4), a subset of plants was allowed a second season of flowering to enable additional data collection. The growth regime of these plants was identical to that described above, and floral scent emissions were unlikely to be affected by this second flowering season (H. E. Thosteman *et al.*, unpublished data).

Floral and foliar volatile collections

Floral scent volatiles were collected in 3-h bouts starting between 08:00 and 10:00 h in the morning, using dynamic headspace sampling (Raguso & Pellmyr, 1998; Petré *et al.*, 2021). One fully developed inflorescence of each individual was enclosed in a thin plastic bag (15 × 10 cm) (ICA AB 'Stekpåsar' and Top-pits® 'Stekpåsar 2 in 1'), and the number of fully open flowers enclosed were counted. A narrow Teflon tube containing a scent trap of 10 mg Tenax GR filter was put inside the bag together with the flowers and connected to a plastic tube leading to a flowmeter (Cole-Parmer 65 mm direct-reading, Vernon Hills, IL, USA) and then either to a custom-built air pump (Grotech, Ahorn, Germany) or to a Micro Air Sampler pump (Spectrex Inc., Redwood City, CA, USA). Air was drawn from the bag and through the filter at a constant rate of 200 ml min^{-1} for three consecutive hours. For every scent collection bout, we added an ambient air control (empty bag), which was handled in the same way as the scent samples.

Constitutive (undamaged leaves) foliar volatiles (hereafter 'foliar volatiles') were collected using the exact same procedure as described for floral scent sampling. Using dynamic headspace sampling, four undamaged leaves still attached to the plant were enclosed in a sampling bag. After sampling, the leaves were removed and dried for weighing.

VOC extractions and GC-MS analyses

After sampling, volatiles were eluted from the traps into glass vials using 300 μl ultrapure, gas chromatography/mass spectrometry (GC-MS)-quality hexane and stored in a –18°C freezer

until analysis. In preparation of GC-MS-analysis, each sample was concentrated to 50 μl by evaporating hexane through a moderate flow of nitrogen gas. Samples were analyzed using GC-MS on a Finnigan TRACE GC Ultra 2000 gas chromatograph equipped with a 30 m × 0.250 mm × 0.25 μm DB-Wax column (Agilent Technologies, Santa Clara, CA, USA) with ultra-high purity (99.999%) helium as a carrier gas at a constant velocity of 1 ml min^{-1} . The GC was linked to a Finnigan TRACE DSQ mass spectrometer (ThermoFisher, Waltham, MA, USA). Column temperature was initially maintained at 50°C for 3 min before increasing to 250°C at a rate of 10°C min^{-1} for 20 min, where it was then held constant for 7 min until the end of the program.

The resulting chromatograms were manually integrated in Xcalibur Qual Browser v.1.4 (Thermo Electron Corp., 1998–2003, Waltham, MA, USA), and peaks were identified using library suggestions (NIST MS Search 2.0, 2008). Compounds were verified using standardized retention indices obtained from the literature (Supporting Information Table S1) and occasionally by synthetic standards (Table S1). Petré *et al.* (2021) identified 14 floral scent compounds to be responsible for > 99% of the observed intraspecific variation. Visual analyses of our chromatograms confirmed this observation, and we therefore focused on these 14 compounds when analyzing floral scent. For both floral scent and foliar volatiles, compounds with emission rates lower than three times the corresponding control sample (ambient air) were excluded from further analysis (cf. Eisen *et al.*, 2022). All compounds present in samples from the foliar headspace were verified based on library suggestions and Kovats retention indices, resulting in a final dataset of 19 foliar volatile compounds, and 14 floral volatile compounds (Table S1). Finally, all emission rates were converted to nanograms per hour per flower (floral volatiles) or dry mass (foliar volatiles) (Svensson *et al.*, 2005). The floral scent unit was chosen to agree with previous research on floral scent in this species (Petré *et al.*, 2021; Eisen *et al.*, 2023), while the unit for leaf volatiles was based on similar experiments in other systems (e.g. Glauser *et al.*, 2008; Kant *et al.*, 2008). After filtering, 340 floral scent samples representing 340 unique individuals (9–30 per population, 23 populations; Table 1) and 115 foliar volatile samples representing 115 individuals (4–6 per population, 23 populations; Table 1) were used for further analysis.

Glucosinolate extraction and LC-MS analyses

Leaf tissue from 160 individuals from 20 populations (Table 1) was sampled and flash-frozen in Lund, before being freeze-dried and transported to ETH Zürich (Switzerland). Leaves were ground to a fine powder for 30 s at 1750 rpm in a Geno/Grinder 2010 (SPEX sample prep, Metuchen, NJ, USA) with two 0.3-mm steel grinding balls. Approximately 20 mg of dried tissue was weighed out and used for extraction; the mass of tissue used was recorded and used to estimate final glucosinolate amounts. Samples were extracted in two batches of 80 randomly ordered samples with two sinigrin standard curves used per batch to quantify glucosinolate compounds. Glucosinolate extractions

were performed as described in a recent HPLC protocol (Grosser & Van Dam, 2017), with the following minor modifications. To denature the myrosinase enzyme, samples were suspended in 1 ml of 70% methanol and heated to 85°C for 15 min. Columns were prepared using DEAE Sephadex A25 (Sigma-Aldrich), and extracted samples were loaded onto columns and incubated overnight with sulfatase (Sigma-Aldrich) to generate desulfo-glucosinolates, which were eluted with 1.4 ml ultrapure MilliQ water (Merck, Darmstadt, Germany). Eluted samples were dried down on a Savant Speed Vac Concentrator SPP1010 (Thermo Scientific, Reinach, Switzerland) and resuspended in 140 µl ultrapure MilliQ water (Merck) in glass GC vials. Samples were run on an Agilent 6550 iFunnel Q-TOF LC-MS equipped with an Eclipse Plus C8 column (RRHB 1.8 µm, 2.1 × 100 mm, 95 Å) using a water (with 5 mM ammonium formate) to acetonitrile gradient. The mobile phase conditions consisted of 98% water for 0.8 min, then a gradient to 65% water over 14 min, and followed by a rapid gradient to 0.5% water over 3.2 min at a flow rate of 0.4 ml min⁻¹.

Glucosinolate annotation and quantification

Desulfo-glucosinolates were annotated using MS spectra and UV profiles: Identification of putative desulfo-glucosinolates was based on the fragmentation pattern due to the loss of a hexose-derivative from a parent aglycone, demonstrated by a mass shift of 162 amu, and through formula matches with Agilent MASSHUNTER qualitative software; ultraviolet profiles were used to distinguish aliphatic and indole glucosinolates. Peaks for glucosinolate compounds in the 229 nm UV spectrum were automatically integrated by the MASSHUNTER quantitative software, and manually inspected and corrected where necessary. Compounds that could not be reliably integrated across samples were excluded from the final dataset, resulting in a preliminary dataset of 26 glucosinolates (Table S2). Glucosinolates were quantified through integrating the 229 nm UV spectrum based on a comparison to a sinigrin concentration curve (averaged across the four standard curves used) and published response factors (as described in Grosser & Van Dam, 2017). Amounts of desulfo-glucosinolates were converted to µmol g⁻¹ dry tissue weight (DW). Eight glucosinolates in this dataset were found in fewer than 5% of all samples and were excluded from further analysis, leaving 17 quantified glucosinolates, though one represents two coeluted compounds, 4MSOB and R2OH3B. Due to an error in the extraction process, one sample (Fr3_16b) was excluded from the final dataset, leaving a total of 159 samples (Table 1).

Statistical analyses

All statistical analyses were performed using R (v.4.3.1) and RSTUDIO (v.2023.06.2) (R Core Team, 2024).

Total compound emission rate or content was estimated as mean population log(ng/flower/hour) for floral volatiles and log(ng/dry tissue weight/hour) for foliar volatiles, and µmol g⁻¹ DW for glucosinolates. Differences in total compound content

or emission rate between mating systems were tested using Wilcoxon signed-rank tests. To examine among-population variation in compound composition, we performed PERMANOVAs based on zero-adjusted Bray–Curtis dissimilarity matrices for each compound group (floral scent volatiles, foliar volatiles, and foliar glucosinolates) using the *adonis2* function in the VEGAN package (Oksanen *et al.*, 2022) (9999 permutations). To visualize differences in compound composition between populations and mating systems, we used nonmetric multidimensional scaling plots (NMDS) (*metaMDS* function, VEGAN package, $k = 3$, 500 tries) with either population or mating system as fixed factor.

To test for the degree of integration among the three compound groups (floral scent volatiles, foliar constitutive volatiles, and foliar glucosinolates), we calculated integration indices within each compound group, between pairs of compound groups, and for all three groups (whole plant integration). Our calculations followed Junker *et al.* (2018), where Pearson's correlation r was determined for all pairs of compounds (in absolute amounts) produced by individuals in one population. We treated every population or group (i.e. floral volatiles, foliar volatiles, and glucosinolates) separately. We then calculated eigenvalues and their variances, where the raw eigenvalue indicates magnitude of explained variation by each principal component, and a higher variance of the eigenvalue indicates a higher phenotypic integration. The nonstandardized *integration index* spans from 0 to n (n = number of compounds analyzed). To correct for varying sample sizes across populations, we then standardized the integration index (between 0 and n) by subtracting the expected value of integration under the assumption of random covariation (number of compounds emitted by the population – 1/number of samples). We then divided the result by the potential maximum value of phenotypic integration in the dataset (number of compounds emitted by the population). Finally, we multiplied by 100 to obtain percentage integration. This standardization allowed us to compare phenotypic integration across populations or groups of varying sample size and chemical richness. The resulting integration index should be interpreted in a statistical framework considering only statistical correlations among compounds. These do not disentangle genetic, functional, selective, or developmental linkage as mechanistic drivers of the trait correlations (Armbruster *et al.*, 2014). Floral scent and foliar volatiles were sometimes sampled from the same individual, while glucosinolates were never sampled from the same individual as floral scent or foliar volatiles. Therefore, calculations comparing two or more compound groups were done using population mean compound content, whereas calculations including samples from only one compound group were performed using individuals as the data unit. To compare floral, foliar, and glucosinolate integration indices, we performed a Kruskal–Wallis test to identify significant differences between the three groups. Further, we used a Wilcoxon signed-rank test to compare floral volatile integration between SC and SI populations. Differences in floral × foliar integration between SC and SI populations were examined using bootstrapped data ($n = 10\,000$), as these datasets were based on population mean compound content. We calculated the probability of the observed difference between the integration indices

being smaller or larger than the bootstrapped differences (null-hypothesis) in a two-tailed *t*-test. Due to the structure of our data, statistical testing of differences between intragroup indices (floral, foliar, and glucosinolate indices) and intergroup indices (e.g. floral \times foliar indices) through bootstrapping was not possible. Instead, we compared levels of correlation between groups, and performed a Kruskal–Wallis test using interaction (floral volatile interacting with floral volatile: FVfV; floral volatile interacting with leaf volatile: FVLV; leaf volatile interacting with leaf volatile: LVLV; leaf volatile interacting with glucosinolate: LVGL; glucosinolate interacting with glucosinolate: GLGL; and floral volatile interacting with glucosinolate: FVGL) as grouping factor and correlation coefficient (*r*) as dependent variable, followed by Dunn's test coupled with Bonferroni corrections for multiple tests, to identify significant differences (0.95 confidence interval). Further, we compared correlation coefficients between mating system (SI or SC) using Mann–Whitney *U* tests. For all statistical comparisons of correlation coefficients, we used correlation matrices with threshold = 0 to capture all levels of correlations. Because of limitations in the data (i.e. all three compound groups were not always collected from the same individuals), the correlation coefficients were calculated using mean compound content for each population, testing an overarching evolutionary phenotypic correlation, without accounting for within-population variation in correlation coefficients.

Correlation analyses were visualized in networks using CYTOSCAPE (v.3.10.1; Cytoscape Consortium, 2018) and were based on dissimilarity matrices calculated from the Pearson's *r* matrices. To create network input data, we used the dissimilarity matrices to create dendrograms in hierarchical cluster analyses (threshold: *r* = 0.3) using the *hclust* function, where branches (compounds) that split early occur independent of each other and branches that split later co-occur more often. The weight and direction of the interaction between compounds were then used as input data to CYTOSCAPE to graphically represent the extent of compound integration.

Results

Composition of phytochemicals

We analyzed variation in 14 floral scent compounds (after Petré et al., 2021), 19 foliar volatile compounds, and 17 glucosinolates. Among floral compounds, there were 13 aromatics and one terpenoid (Table S1). Among foliar compounds, there were six aromatics, three fatty acid derivatives, two isothiocyanates, seven terpenoids, and one compound of unknown origin (Table S1). Finally, among glucosinolates, there were two fatty acyls, 12 amino acids and glycosides, and three unknown compounds (Table S2). Among these compounds, we identified five volatiles emitted by both flowers and leaves: benzaldehyde, acetophenone, methyl salicylate, benzyl benzoate, and phenylacetaldehyde. There was among-population variation in compound composition in all three compound groups, as well as differences in composition between self-compatible and self-incompatible

populations (Figs S1, S2). Total compound content and emission rate also varied among populations and differed between the two mating systems (Fig. S3). Self-incompatible populations emitted more floral scent per flower per hour compared with self-compatible populations, in accordance with previous research. Conversely, self-compatible populations emitted more foliar volatiles than self-incompatible populations. There was no difference in total glucosinolate content between plants with mating systems, but self-compatible populations exhibited greater variation than self-incompatible populations (Fig. S3).

Integration within compound groups

Floral scent had the lowest standardized integration index across populations (mean 18), followed by foliar volatiles (20.8), and lastly glucosinolates (21.7). The glucosinolate dataset contained an outlier population, G4 (index > 80), which was not present in the floral scent or foliar volatile dataset. When removed, the mean integration index for glucosinolates became substantially lower (mean 18.5). The integration indices for floral, foliar, and glucosinolate compounds did not differ significantly from each other (With population G4: Kruskal–Wallis $\chi^2 = 0.67$, *P* = 0.71; without G4: Kruskal–Wallis $\chi^2 = 0.49$, *P* = 0.78).

The correlation analysis revealed positive interactions of varying strength within each compound group. Results for floral scent mirrored the slightly lower integration index observed (Fig. 2a). Three compounds were separated from the correlation network (methyl salicylate, benzyl benzoate, and acetophenone), because the correlation between each of these compounds and any other compound in the scent bouquet was lower than *r* = 0.3. Within foliar volatiles (Fig. 2b), two compounds (phenylacetaldehyde and butyl isothiocyanate) showed correlation coefficients below 0.3 with any other compound, and two compounds (*cis*- and *trans*-beta-ocimene) were only correlated with each other. All glucosinolates were connected in the network (all *r* > 0.3) (Fig. 2c). When correlation coefficients (*r*) were calculated within each group of compounds (see Fig. 3; FVfV, LVLV, and GLGL), there were no differences in these correlations between compound groups (Dunn's Kruskal–Wallis for multiple comparisons: *P* = 1 for all comparisons).

Integration between compound groups

Across populations, the level of integration between floral scent and foliar volatiles (Fig. 4a) was relatively low (integration index: 4.18; range 0–100), indicating little to no integration. Similarly, floral scent and glucosinolate compounds had an integration index of 4.24, while glucosinolates and foliar constitutive volatiles had a slightly higher index value of 5.27 (Fig. 4b). The three two-tailed *t*-tests performed using bootstrapped data revealed these intergroup indices to be of similar magnitude (*P* > 0.48 for all comparisons). When investigating integration among all compound groups (across floral scent, foliar volatiles, and glucosinolates together), we observed even lower levels of integration with an index value of 3.5 across populations. For floral and foliar volatiles, the level of correlation between compound groups was lower than

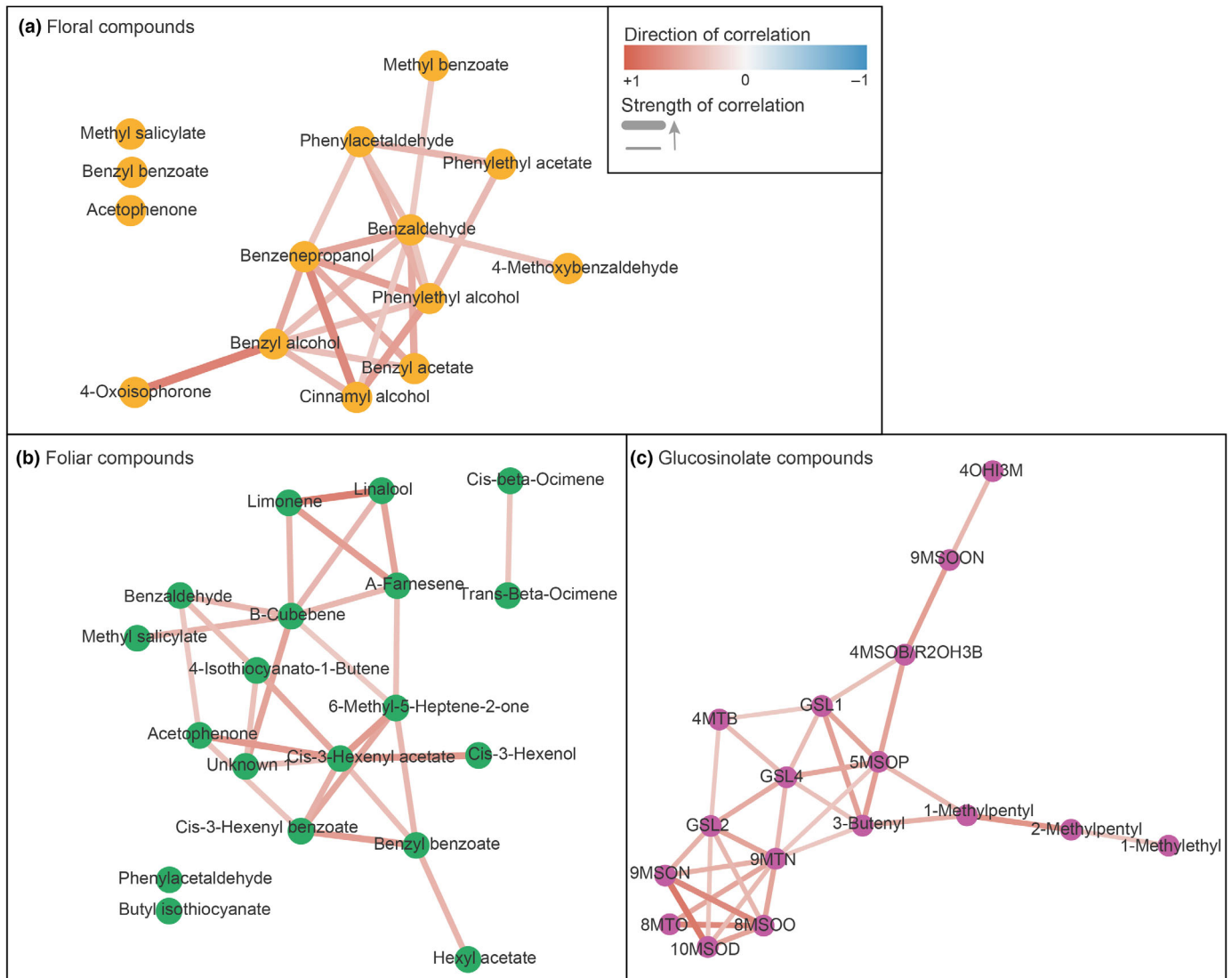


Fig. 2 Correlation networks describing interactions within compound groups across all *Arabis alpina* populations surveyed ($n = 23$ for floral and foliar volatiles, $n = 20$ for glucosinolates). All networks are based on Pearson's correlations and only interactions above threshold $r = 0.3$ are included. (a) Floral volatile compounds. Integration index mean 18, (b) Foliar volatile compounds. Integration index mean 20.8, (c) Glucosinolate compounds. Integration index mean 21.7 (18.5 without G4). Full names for glucosinolates are available in Supporting Information Table S2.

the level of correlation within compound groups ($P < 0.01$ for all comparisons, Fig. 3; FVLV, and LVGL compared with FVFV, LVLV, and GLGL). We found no differences in correlation coefficients between floral volatile \times leaf volatile interactions and leaf volatile \times glucosinolate interactions (FVLV vs LVGL; $P = 1$). Floral volatiles \times glucosinolates (FVGL, Fig. 3) did not differ significantly from any of the other comparisons ($P > 0.1$ for all comparisons), but there was a trend toward lower correlation coefficient values in the FVGL coefficients compared with FVFV and GLGL.

The network analysis (Fig. 4a) describing correlations between floral and foliar volatiles across all populations showed that the majority of correlations were positive, and with a few exceptions, that strongest correlations were present within compound groups. There were no strong negative correlations between compounds,

neither within nor among compound groups. Two floral volatiles, 4-oxoisophorone and benzyl alcohol, showed stronger positive correlations to foliar volatiles than to floral volatiles. Similarly, foliar phenylacetaldehyde and α -farnesene were positively correlated with floral acetophenone. Interestingly, correlations between the production of the same compound in different tissues was not always strong, exemplified by a very weak negative correlation between foliar and floral benzaldehyde, and the nonexistent link between foliar and floral methyl salicylate (Fig. 4a).

For foliar volatiles and glucosinolates, the network was more complex (Fig. 4b). Several glucosinolate compounds showed positive correlations to foliar volatiles and vice versa, but there were also some weak negative associations, especially between the glucosinolates 10MSOD and 9MSOON and several foliar volatiles. Foliar volatiles and glucosinolates were slightly more

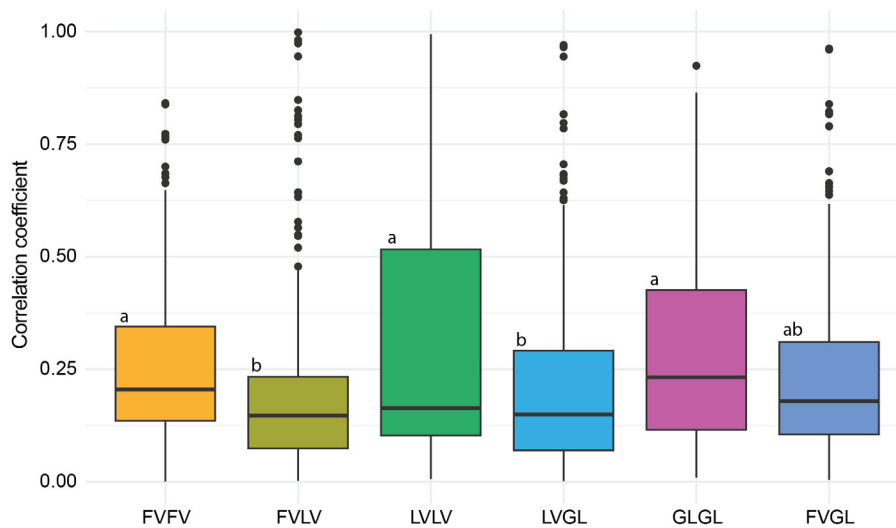


Fig. 3 Comparison of correlation coefficient (r) values between types of compound interactions in *Arabis alpina* plants. FV, floral volatile; GL, glucosinolate; LV, leaf (foliar) volatile. The correlation coefficient (interaction) between two compound groups is denoted for example FV FV (floral volatile interacting with floral volatile) or FV LV (floral volatile interacting with leaf (foliar) volatile). Letters above boxes indicate significant differences. All correlation coefficients are based on Pearson's correlations (r) with threshold = 0. Boxplot showing the distribution of the data. The central box represents the interquartile range (IQR), with the bottom and top edges indicating the first quartile (Q1) and third quartile (Q3), respectively. The line inside the box represents the median. The whiskers extend to the smallest and largest values within 1.5 times the IQR from Q1 and Q3. Data points outside this range are considered outliers and are plotted individually.

integrated (mean 5.27 across populations) than the FV LV comparison (cf. Fig. 4a,b), but this difference was not significant (two-tailed t -test, $P = 0.55$), and the correlation coefficients were not stronger in any direction ($P = 1$, Fig. 3). The corresponding network for floral volatiles and glucosinolates revealed a complex pattern (Fig. S4). In particular, the floral volatiles methyl salicylate and 4-oxoisophorone had positive correlations to several glucosinolates.

Integration in self-incompatible and self-compatible populations

When focusing on floral scent, we found similar levels of integration in self-incompatible and self-compatible populations (Fig. 5, 18.20_{SI} and 17.86_{SC}: Wilcoxon signed-rank test, $W = 61$, $P = 0.8$). In self-incompatible plants, all compounds but two (methyl salicylate and methyl benzoate) were included in the network. For self-compatible plants, three compounds (benzyl benzoate, 4-methoxybenzaldehyde, and methyl salicylate) could not be integrated in the network ($r < 0.3$), and four compounds were only correlated with one other compound in the dataset, indicating a less coherent network. However, when correlations were present, we discovered significantly stronger correlations among compounds in self-incompatible than self-compatible populations ($W = 3238$, $P = 0.01$, Fig. 6).

On average, self-compatible populations (5.47) had a slightly higher level of integration between floral and foliar volatiles than self-incompatible populations (3.67). This pattern was visible in the network analyses, where self-compatible plants (Fig. 7a) had more interconnections between floral and foliar volatiles than

self-incompatible plants (Fig. 7b), but the difference in integration was not significant (two-tailed t -test, $P = 0.44$). The latter group had a stronger separation between floral and foliar volatiles, and most strong correlations were found within each compound group. However, we found no significant difference in floral–foliar correlation coefficients between SC and SI populations ($W = 23\ 672$, $P = 0.45$).

Discussion

Our results indicate little to no interdependence among floral scent, foliar volatiles, and glucosinolates, suggesting that each group of compounds is free to evolve independently in response to local selection or genetic drift. Thus, our results support previous studies of phytochemical diversification in *A. alpina* and other systems (cf. Buckley *et al.*, 2019; Petré *et al.*, 2021) that most often focus on a particular chemical group and its relationship to a particular ecological interaction (e.g. herbivore deterrence or pollinator attraction). Relatedly, a dramatic reduction in one aspect of plant chemistry – the floral scent emission in self-compatible populations of *A. alpina* – was not correlated with major shifts in foliar volatile composition or emission rates. The overall low levels of integration, both within and across compound groups, are somewhat surprising, especially considering that foliar and floral volatiles are generated through the same biosynthetic pathways and that some compounds are emitted from both the floral and foliar tissue (Armbruster & Schwaegerle, 1996; Dudareva *et al.*, 2013; Junker *et al.*, 2018). Here, we discuss potential explanations for the observed patterns and propose directions for future research.

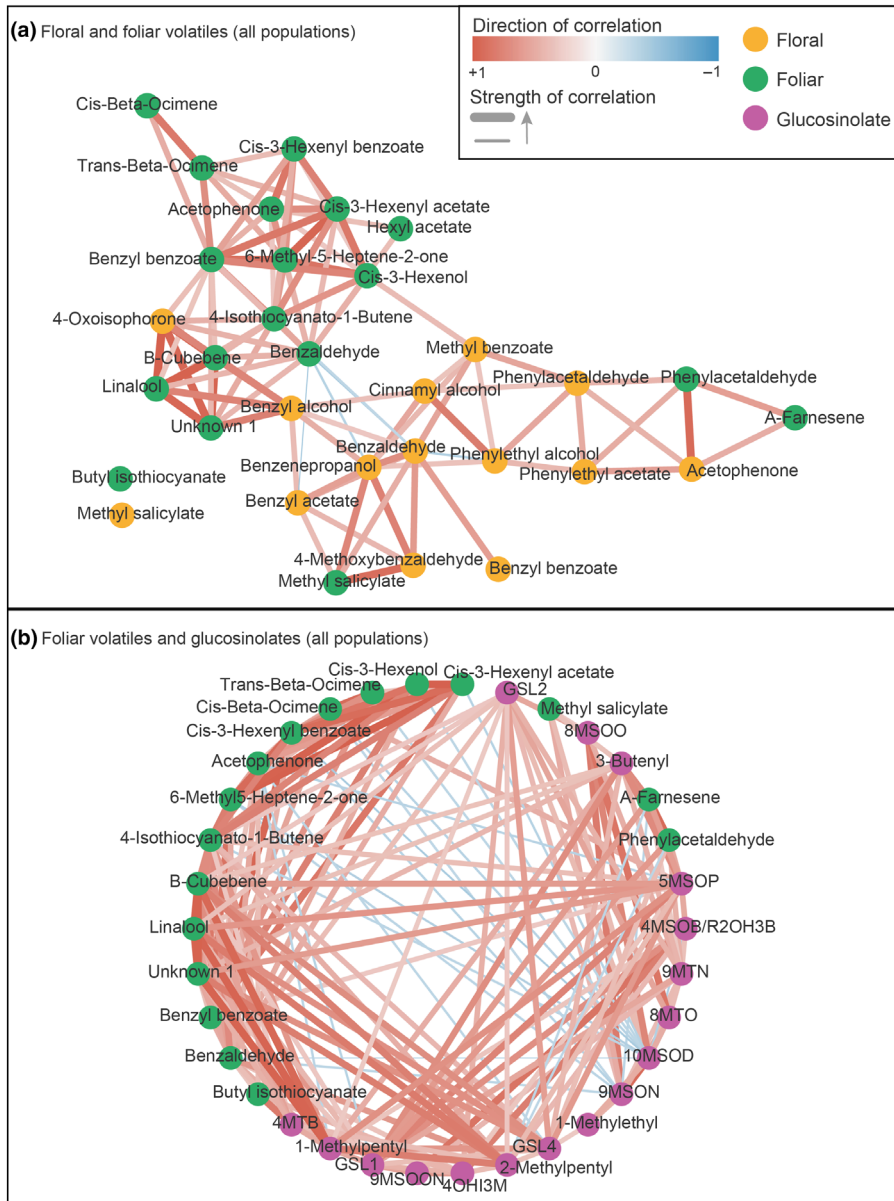


Fig. 4 Correlation networks based on Pearson's correlations with threshold = 0.3 of (a) interactions between floral and foliar volatiles across all populations, and (b) foliar volatiles and glucosinolates across all populations of *Arabis alpina*.

Our large-scale sampling of 28 European populations of the emerging ecological and evolutionary model system *A. alpina* (Wötzel *et al.*, 2022), shows that this species is chemically highly variable not only for floral scent (cf. Petré *et al.*, 2021) but also for foliar volatiles, and glucosinolate defense compounds (cf. Buckley *et al.*, 2019) (Figs S1, S3). The differences are both quantitative (the emission rate of volatiles or concentration of glucosinolates), and qualitative, as different populations are dominated by different major compounds. Interestingly, the results of our comprehensive study of chemical variation within the same plant species are consistent with a meta-analysis that examined integration at a larger biological scale (Junker *et al.*, 2018). Specifically, Junker *et al.* (2018) found low, but variable, levels of integration across taxa, and higher integration within functionally similar compound groups, when analyzing

floral and foliar compounds available from different plant species. Floral volatiles in *A. alpina* are likely more integrated (mean integration index of 18) relative to the average integration observed across 12 systems (mean integration index of 12; Junker *et al.*, 2018). Foliar volatiles and glucosinolates showed a trend toward higher integration compared with floral scent in *A. alpina*, which follows the results of Junker *et al.* (2018), though this trend was not statistically significant. This could reflect the fact that our study investigates a single species where genetic relatedness, and thus correlation of metabolic function, is likely stronger.

Within compound groups, we found only weak negative correlations between compounds, indicating that negative trade-offs are not constraining chemical variation within functional types. For floral scent, three compounds, all aromatics, failed to meet

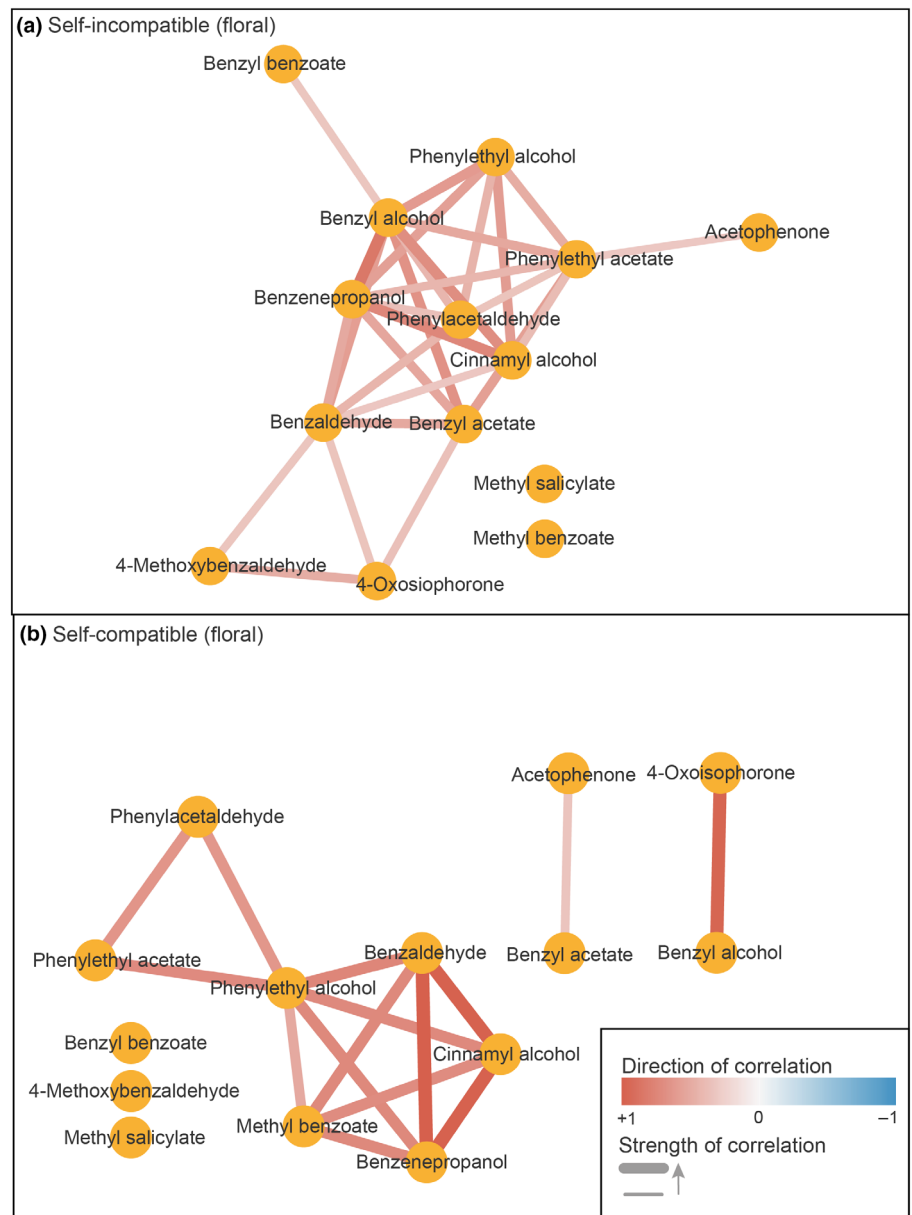


Fig. 5 Correlation networks for floral scent volatiles comparing (a) self-incompatible and (b) self-compatible populations of *Arabis alpina*. The networks are based on Pearson's correlations (r) with threshold = 0.3.

the $r = 0.3$ correlation threshold (Fig. 2a) and were thus not connected into the resulting network. One potential explanation for this pattern is that these compounds are important pollinator attractants and thus need to be present in the bouquet regardless of the other compounds emitted. In particular, acetophenone (present in 37% of samples) was uncorrelated with other compounds despite being present in all populations. The other two compounds were only present in a small subset of samples (benzyl benzoate: 18%, methyl salicylate: 6%) and may be therefore uncorrelated with other compounds due to the low occurrence rate. However, all three compounds have been shown to attract butterflies, moths, and bees to varying degree (Dodson *et al.*, 1969; Raguso & Light, 1998; Huber *et al.*, 2005; Knauer & Schiestl, 2015), suggesting that they may also be important for generalist pollinators of *A. alpina*. Similar explanations can be applied to the compounds outside all other networks presented.

Morphological floral trait covariation is expected to be decoupled from general trait variation present in the vegetative parts of plants in order to preserve flower structures suitable for successful pollination (Armbruster *et al.*, 1999). The low integration and weaker correlation coefficients (Fig. 3) of floral and foliar volatiles detected in *A. alpina* indicate that this phenomenon may extend to the phytochemicals produced in the respective tissues. Indeed, floral scent is a known pollinator attractant in many systems (Raguso, 2008), where it may be part of the broader pollinator attraction module, which also includes flower size, shape, and color (Caruso *et al.*, 2019). Considering that floral scent and foliar volatiles share evolutionary history (Schiestl, 2010) and that our dataset included five compounds present in the emissions from both flowers and leaves (Table S1), it is tempting to draw the same conclusion about the decoupling of reproductive and nonreproductive chemistry, especially as we

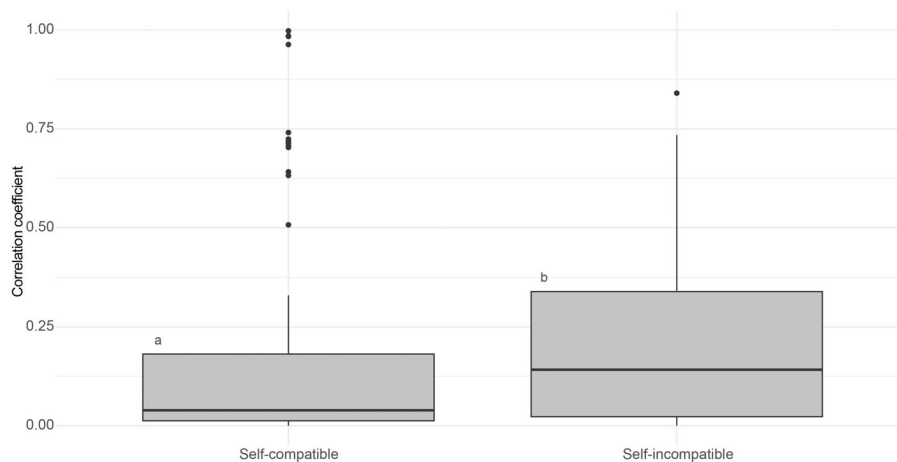


Fig. 6 Comparison of correlation coefficient (r) values for floral scent volatiles between self-compatible and self-incompatible populations of *Arabis alpina*. Letters above boxes indicate significant differences. The correlation coefficients are based on Pearson's correlations (r) with threshold = 0. Boxplot showing the distribution of the data. The central box represents the interquartile range (IQR), with the bottom and top edges indicating the first quartile (Q1) and third quartile (Q3), respectively. The line inside the box represents the median. The whiskers extend to the smallest and largest values within 1.5 times the IQR from Q1 and Q3. Data points outside this range are considered outliers and are plotted individually.

detected very weak or nonexistent links between the same compounds produced in floral or foliar tissues (Fig. 4a). However, even though there were comparatively more connections between foliar volatiles and glucosinolates than between floral and foliar volatiles, integration was low also between these different kinds of leaf chemistry. We found a few instances where certain floral volatiles were correlated with foliar volatiles (Fig. 4a), and further studies are needed to disentangle the functional relationship between these compounds, as not all of them share biosynthesis. Another exception was the relatively strong, positive correlation between the foliar volatiles butyl isothiocyanate and 4-isothiocyanate-1-butene, and the glucosinolates GSL4 and 4MTB, which may be explained by a biosynthetic linkage, since the volatile isothiocyanates are derived from glucosinolates (Fahey *et al.*, 2001). The emission of these foliar volatiles could signal the defense status of a plant individual. By and large, however, the low integration and correlation coefficient values among compound groups agrees with the hypothesis that these three compound groups are functionally separated even within the same taxon (Fig. 3, compare FVLV and LVGL with FVFV, LVLV, and GLGL; Armbruster *et al.*, 1999; Schiestl, 2014; Junker *et al.*, 2018). Interestingly, floral scent compounds were to some extent correlated with glucosinolates (Fig. S4). Despite not originating from the same biosynthetic pathway, methyl salicylate and 4-oxoisophorone showed positive correlations with four glucosinolates; future studies should investigate the functional links between these compounds.

Trait integration patterns varied only moderately between self-compatible and self-incompatible populations. The floral scent profiles of self-incompatible plants were not significantly more integrated yet had significantly stronger correlations between compounds (Fig. 6), indicating that scent compounds emitted by self-incompatible plants experience more interdependence than those emitted by self-compatible plants. This pattern could relate

to variation in pollinator dependence among populations of different mating systems, as self-incompatible populations may experience stronger selection on certain floral compounds that potentially affect pollinator attraction, driving these to co-occur more often. Contrary to our prediction, we found no evidence of weaker connection between floral and foliar volatiles among self-incompatible populations (Fig. 7). Across its distribution, *A. alpina* populations display a wide range of outcrossing rates (Tedder *et al.*, 2011, 2015; Tor ang *et al.*, 2017; Petr en *et al.*, 2023): from autonomously selfing Scandinavian populations to completely self-incompatible populations in the Mediterranean. In the present study, self-incompatible populations were compared with outcrossing populations that have evolved self-compatibility. Therefore, differences in integration due to decoupling of floral scent and foliar defense compounds may not be as pronounced in this comparison, as the self-compatible populations used in this study may still experience selection on floral scent compounds as a distinct group rather than together with foliar defense compounds (Ramos & Schiestl, 2020). Collectively, our results indicate that floral scent volatiles in self-incompatible *A. alpina* populations are generally decoupled from foliar volatiles but have slightly stronger correlations within the floral scent bouquet. A meta-analysis of differences in floral morphological traits between selfing and outcrossing plant species suggested that selfing species had higher integration levels for both whole flower and sexual organs compared with outcrossing species (Fornoni *et al.*, 2015). Our results indicated higher, but not significantly different, integration between floral and foliar volatiles in self-compatible populations compared with self-incompatible populations. This could be caused by the overall higher integration level of floral morphological traits, as volatile production can be tissue specific (Raguso, 2008); however, future studies investigating the role of mating system would benefit from including more populations of high selfing capacity.

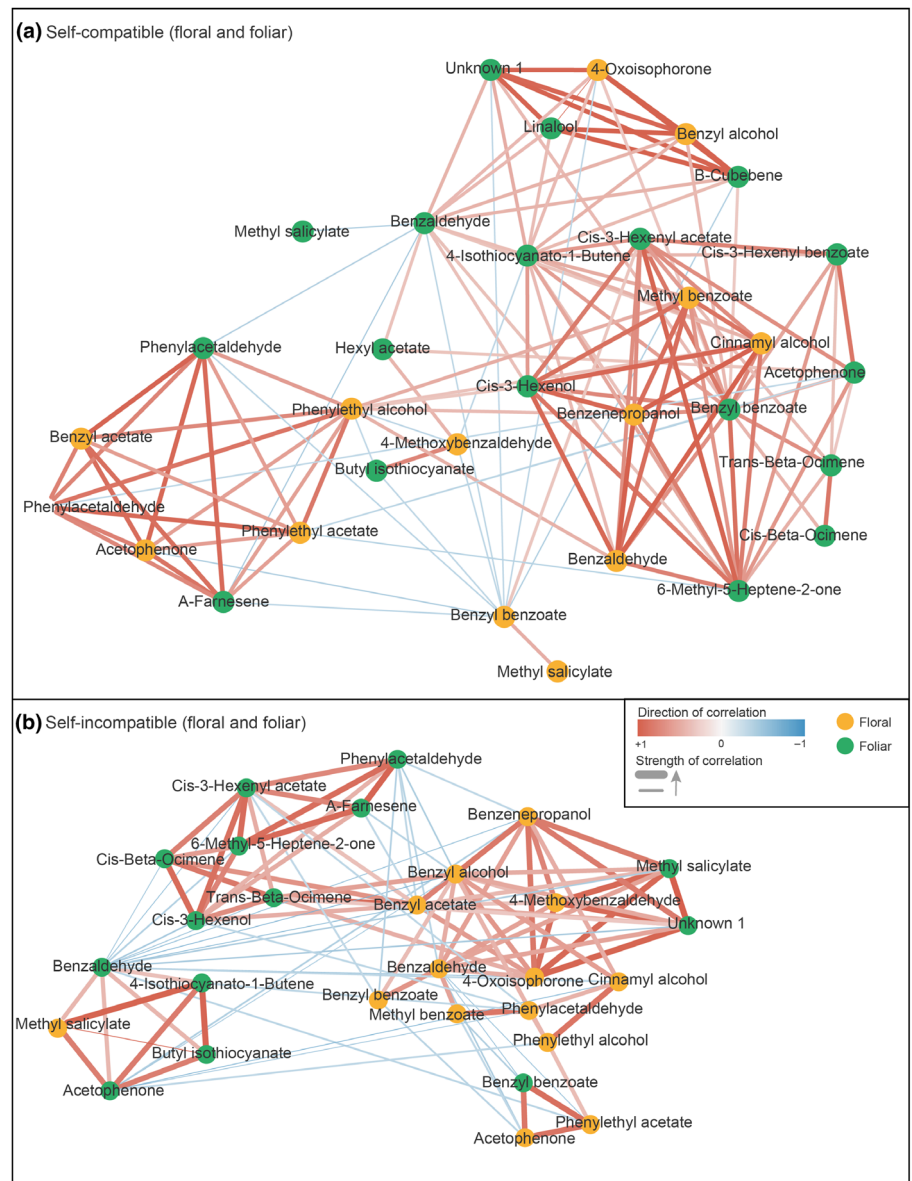


Fig. 7 Correlation networks for floral and foliar volatiles across (a) self-compatible and (b) self-incompatible populations of *Arabis alpina*. The networks are based on Pearson's correlations (r) with threshold = 0.3.

Conclusion

We found no evidence of integration between floral scent (an important component of pollinator attraction in many systems) and the biosynthetically closely related foliar defense compounds, indicating that floral scent can respond independently to potential variation in pollinator-mediated selection across populations. In addition, we found no strong negative correlations between compounds within or among compound groups, suggesting that negative trade-offs between compounds do not constrain phytochemical expression. Our comprehensive large-scale study supports the often assumed but rarely tested idea that the diversification of phytochemistry within species can be caused by independent vectors of selection. Future efforts in this system should focus on a larger sample size of fewer populations representing the full mating system gradient (highly selfing to fully

outcrossing). Furthermore, it is important to investigate functional links between correlated compounds of different biosynthetic origin and explore the bioactive functions of single compounds and compound combinations.

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Competing interests

None declared.

Author contributions

MF and HET designed the experiment. HET, HP, SB, MCM, CMDM, MF, JMH, LP and JB collected data, and HET performed the analyses with support from KE, HP and JB. HET led the writing of the manuscript with substantial contributions from JB, MCM, HP, KE, MF and input and edits from all coauthors.

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Data availability

Raw data can be accessed in a Dryad repository using doi: [10.5061/dryad.15dv41p59](https://doi.org/10.5061/dryad.15dv41p59).

References

- Ansell SW, Grundmann M, Russell SJ, Schneider H, Vogel JC. 2008. Genetic discontinuity, breeding-system change and population history of *Arabis alpina* in the Italian Peninsula and adjacent Alps: phylogeography of *Arabis alpina* in Italy. *Molecular Ecology* 17: 2245–2257.
- Armbruster WS. 1991. Multilevel analysis of morphometric data from natural plant populations: insights into ontogenetic, genetic, and selective correlations in *Dalechampia scandens*. *Evolution* 45: 1229–1244.
- Armbruster WS, Di Stilio VS, Tuxill JD, Flores TC, Vela'squez Runk JL. 1999. Covariance and decoupling of floral and vegetative traits in nine Neotropical plants: a re-evaluation of Berg's correlation-pleiades concept. *American Journal of Botany* 86: 39–55.
- Armbruster WS, Pélabon C, Bolstad GH, Hansen TF. 2014. Integrated phenotypes: understanding trait covariation in plants and animals. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 369: 20130245.
- Armbruster WS, Schwaegerle KE. 1996. Causes of covariation of phenotypic traits among populations. *Journal of Evolutionary Biology* 9: 261–276.
- Arnold SJ. 2023. *Evolutionary quantitative genetics, 1st edn*. New York, NY, USA: Oxford University Press.
- Benedek K, Bálint J, Máthé I, Mara G, Felföldi T, Szabó A, Fazakas C, Albert C, Buchkowski RW, Schmitz OJ *et al*. 2019. Linking intraspecific variation in plant chemical defence with arthropod and soil bacterial community structure and N allocation. *Plant and Soil* 444: 383–397.
- Bruinsma M, Lucas-Barbosa D, Ten Broeke CJM, Van Dam NM, Van Beek TA, Dicke M, Van Loon JJA. 2014. Folivory affects composition of nectar, floral odor and modifies pollinator behavior. *Journal of Chemical Ecology* 40: 39–49.
- Buckley J, Widmer A, Mescher MC, De Moraes CM. 2019. Variation in growth and defence traits among plant populations at different elevations: implications for adaptation to climate change. *Journal of Ecology* 107: 2478–2492.
- Bustos-Segura C, Foley WJ. 2018. Foliar terpene chemotypes and herbivory determine variation in plant volatile emissions. *Journal of Chemical Ecology* 44: 51–61.
- Caruso CM, Eisen KE, Martin RA, Sletvold N. 2019. A meta-analysis of the agents of selection on floral traits. *Evolution* 73: 4–14.
- Cytoscape Consortium. 2018. *Enabling biological research via open software and public resources*. San Diego, CA, USA: Cytoscape Consortium.
- Dodson CH, Dressler RL, Hills HG, Adams RM, Williams NH. 1969. Biologically active compounds in orchid fragrances: function of natural plant products in orchid flower odors and the attraction of specific pollinators are described. *Science* 164: 1243–1249.
- Dormont L, Joffard N, Schatz B. 2019. Intraspecific variation in floral color and odor in orchids. *International Journal of Plant Sciences* 180: 1036–1058.
- Dötterl S, Gershenzon J. 2023. Chemistry, biosynthesis and biology of floral volatiles: roles in pollination and other functions. *Natural Product Reports* 40: 1901–1937.
- Doubleday LAD, Raguso RA, Eckert CG. 2013. Dramatic vestigialization of floral fragrance across a transition from outcrossing to selfing in *Abronia umbellata* (Nyctaginaceae). *American Journal of Botany* 100: 2280–2292.
- Dudareva N, Klempien A, Muhlemann JK, Kaplan I. 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytologist* 198: 16–32.
- Ehrlich PR, Raven PH. 1964. Butterflies and plants: a study in coevolution. *Evolution* 18: 586–608.
- Eisen KE, Boutsi S, Halley JM, Pace L, Petrén H, Thosteman H, Friberg M. 2023. Honest floral signalling traits vary across and within populations in an insect-pollinated plant. *Functional Ecology* 37: 2511–2522.
- Eisen KE, Powers JM, Raguso RA, Campbell DR. 2022. An analytical pipeline to support robust research on the ecology, evolution, and function of floral volatiles. *Frontiers in Ecology and Evolution* 10: 1006416.
- Fahey JW, Zalcmann AT, Talalay P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56: 5–51.
- Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* 35: 375–403.
- Ferrari MJ, Stephenson AG, Mescher MC, De Moraes CM. 2006. Inbreeding effects on blossom volatiles in *Cucurbita pepo* subsp. *texasana* (Cucurbitaceae). *American Journal of Botany* 93: 1768–1774.
- Fornoni J, Ordano M, Pérez-Ishiwara R, Boege K, Domínguez CA. 2015. A comparison of floral integration between selfing and outcrossing species: a meta-analysis. *Annals of Botany* 117: 299–306.
- Friberg M, Schwind C, Guimarães PR, Raguso RA, Thompson JN. 2019. Extreme diversification of floral volatiles within and among species of *Lithophragma* (Saxifragaceae). *Proceedings of the National Academy of Sciences, USA* 116: 4406–4415.
- Futuyma D, Kirkpatrick M. 2018. *Evolution, 4th edn*. New York, NY, USA: Oxford University Press.
- Geervliet JBF, Posthumus MA, Vet LEM, Dicke M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested or uninfested food plants of *Pieris* species. *Journal of Chemical Ecology* 23: 2935–2954.
- Glauser G, Grata E, Dubugnon L, Rudaz S, Farmer EE, Wolfender J-L. 2008. Spatial and temporal dynamics of jasmonate synthesis and accumulation in *Arabidopsis* in response to wounding. *Journal of Biological Chemistry* 283: 16400–16407.

- Gross K, Sun M, Schiestl FP. 2016. Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. *PLoS ONE* 11: e0147975.
- Grosser K, Van Dam NM. 2017. A straightforward method for glucosinolate extraction and analysis with high-pressure liquid chromatography (HPLC). *Journal of Visualized Experiments* 121: 55425.
- Halkier BA, Gershenzon J. 2006. Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology* 57: 303–333.
- Herrera CM, Cerda X, Garcia MB, Guitian J, Medrano M, Rey PJ, Sanchez-Lafuente AM. 2002. Floral integration, phenotypic covariance structure and pollinator variation in bumblebee-pollinated *Helleborus foetidus*. *Journal of Evolutionary Biology* 15: 108–121.
- Huber FK, Kaiser R, Sauter W, Schiestl FP. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia* 142: 564–575.
- Junker RR, Blüthgen N. 2010. Floral scents repel facultative flower visitors, but attract obligate ones. *Annals of Botany* 105: 777–782.
- Junker RR, Kuppler J, Amo L, Blande JD, Borges RM, Van Dam NM, Dicke M, Dötterl S, Ehlers BK, Etl F *et al.* 2018. Covariation and phenotypic integration in chemical communication displays: biosynthetic constraints and eco-evolutionary implications. *New Phytologist* 220: 739–749.
- Kant MR, Sabelis MW, Haring MA, Schuurink RC. 2008. Intraspecific variation in a generalist herbivore accounts for differential induction and impact of host plant defences. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 275: 443–452.
- Kessler A, Halitschke R. 2009. Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. *Functional Ecology* 23: 901–912.
- Kessler A, Halitschke R, Poveda K. 2011. Herbivory-mediated pollinator limitation: negative impacts of induced volatiles on plant–pollinator interactions. *Ecology* 92: 1769–1780.
- Kessler A, Kalske A. 2018. Plant secondary metabolite diversity and species interactions. *Annual Review of Ecology, Evolution, and Systematics* 49: 115–138.
- Knauer AC, Schiestl FP. 2015. Bees use honest floral signals as indicators of reward when visiting flowers. *Ecology Letters* 18: 135–143.
- Knudsen JT, Eriksson R, Gershenzon J, Ståhl B. 2006. Diversity and distribution of floral scent. *The Botanical Review* 72: 1–120.
- Koch MA, Kiefer C, Ehrich D, Vogel J, Brochmann C, Mummenhoff K. 2006. Three times out of Asia Minor: the phylogeography of *Arabis alpina* L. (Brassicaceae): phylogeography of *Arabis alpina*. *Molecular Ecology* 15: 825–839.
- Lackus ND, Schmidt A, Gershenzon J, Köllner TG. 2021. A peroxisomal β -oxidative pathway contributes to the formation of C6–C1 aromatic volatiles in poplar. *Plant Physiology* 186: 891–909.
- Moreira X, Castagneyrol B, Abdala-Roberts L, Traveset A. 2019. A meta-analysis of herbivore effects on plant attractiveness to pollinators. *Ecology* 100: e02707.
- Mossberg B, Stenberg L. 2010. *Den nya nordiska floran, 3rd edn*. Stockholm, Sweden: Bonnier Fakta.
- Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHH, Szoecs E *et al.* 2022. *Package "VEGAN"*. [WWW document] URL <https://cran.r-project.org/web/packages/vegan/index.html> [accessed 19 December 2023].
- Parachnowitsch A, Burdon RCF, Raguso RA, Kessler A. 2013. Natural selection on floral volatile production in *Penstemon digitalis*: highlighting the role of linalool. *Plant Signaling & Behavior* 8: e22704.
- Pellmyr O, Thien LB. 1986. Insect reproduction and floral fragrances: keys to the evolution of angiosperms? *Taxon* 35: 76–85.
- Petrén H, Thosteman H, Stift M, Toräng P, Ågren J, Friberg M. 2023. Differences in mating system and predicted parental conflict affect post-pollination reproductive isolation in a flowering plant. *Evolution* 77: 1019–1030.
- Petrén H, Toräng P, Ågren J, Friberg M. 2021. Evolution of floral scent in relation to self-incompatibility and capacity for autonomous self-pollination in the perennial herb *Arabis alpina*. *Annals of Botany* 127: 737–747.
- Pichersky E, Noel JP, Dudareva N. 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* 311: 808–811.
- R Core Team. 2024. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Raguso RA. 2008. Wake up and smell the roses: the ecology and evolution of floral scent. *Annual Review of Ecology, Evolution, and Systematics* 39: 549–569.
- Raguso RA. 2016. More lessons from linalool: insights gained from a ubiquitous floral volatile. *Current Opinion in Plant Biology* 32: 31–36.
- Raguso RA, Light DM. 1998. Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and "green-leaf volatiles". *Entomologia Experimentalis et Applicata* 86: 287–293.
- Raguso RA, Pellmyr O. 1998. Dynamic headspace analysis of floral volatiles: a comparison of methods. *Oikos* 81: 238.
- Ramos SE, Schiestl FP. 2020. Evolution of floral fragrance is compromised by herbivory. *Frontiers in Ecology and Evolution* 8: 30.
- Rudgers JA, Strauss SY, Wendel JF. 2004. Trade-offs among anti-herbivore resistance traits: insights from *Gossypieae* (Malvaceae). *American Journal of Botany* 91: 871–880.
- Schiestl FP. 2010. The evolution of floral scent and insect chemical communication: evolution of floral scent. *Ecology Letters* 13: 643–656.
- Schiestl FP. 2014. Correlation analyses between volatiles and glucosinolates show no evidence for chemical defense signaling in *Brassica rapa*. *Frontiers in Ecology and Evolution* 2: 10.
- Schiestl FP, Kirk H, Bigler L, Cozzolino S, Desurmont GA. 2014. Herbivory and floral signaling: phenotypic plasticity and tradeoffs between reproduction and indirect defense. *New Phytologist* 203: 257–266.
- Sletvold N. 2019. The context dependence of pollinator-mediated selection in natural populations. *International Journal of Plant Sciences* 180: 934–943.
- Svensson GP, Hickman MO, Bartram S, Boland W, Pellmyr O, Raguso RA. 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). *American Journal of Botany* 92: 1624–1631.
- Tedder A, Ansell SW, Lao X, Vogel JC, Mable BK. 2011. Sporophytic self-incompatibility genes and mating system variation in *Arabis alpina*. *Annals of Botany* 108: 699–713.
- Tedder A, Carleial S, Gołębiewska M, Kappel C, Shimizu KK, Stift M. 2015. Evolution of the selfing syndrome in *Arabis alpina* (Brassicaceae). *PLoS ONE* 10: e0126618.
- Theis N, Lerdau M, Raguso RA. 2007. The challenge of attracting pollinators while evading floral herbivores: patterns of fragrance emission in *Cirsium arvense* and *Cirsium repandum* (Asteraceae). *International Journal of Plant Sciences* 168: 587–601.
- Thompson JN. 1999. Specific hypotheses on the geographic mosaic of coevolution. *The American Naturalist* 153: S1–S14.
- Thompson JN. 2005. *The geographic mosaic of coevolution*. Chicago, IL, USA: University Chicago Press.
- Tollsten L, Bergström G. 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *Sinapis*. *Phytochemistry* 27: 2073–2077.
- Toräng P, Vikström L, Wunder J, Wötzel S, Coupland G, Ågren J. 2017. Evolution of the selfing syndrome: anther orientation and herkogamy together determine reproductive assurance in a self-compatible plant. *Evolution* 71: 2206–2218.
- Wötzel S, Andreollo M, Albani MC, Koch MA, Coupland G, Gugerli F. 2022. *Arabis alpina*: a perennial model plant for ecological genomics and life-history evolution. *Molecular Ecology Resources* 22: 468–486.
- Wurst S, Van Dam NM, Monroy F, Biere A, Van der Putten WH. 2008. Intraspecific variation in plant defense alters effects of root herbivores on leaf chemistry and aboveground herbivore damage. *Journal of Chemical Ecology* 34: 1360–1367.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Nonmetric multidimensional scaling plots displaying population variation in floral scent volatile, foliar volatile, and glucosinolate composition.

Fig. S2 Nonmetric multidimensional scaling plots displaying mating system variation in floral scent volatile, foliar volatile, and glucosinolate composition.

Fig. S3 Total compound content or emission rate per population for floral scent volatiles, foliar volatiles, and glucosinolates.

Fig. S4 Floral and glucosinolate interactions across all populations in a correlation network based on Pearson's correlations with a threshold of $r = 0.3$.

Table S1 List of identified compounds present in 23 populations of *Arabis alpina* L. (Brassicaceae) foliar and floral dynamic headspace.

Table S2 Glucosinolate compounds detected in leaf tissue of 20 populations of *Arabis alpina* L. (Brassicaceae).

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