The Role of the Plant Flavonoid Pathway in Adaptation to Elevation

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Dissertation Abstract

Plants produce secondary chemicals to interact with and adapt to their environment. The plant flavonoid metabolic pathway is responsible for several different and important ecological roles, including pigmentation, UV radiation screening, stress amelioration and defense against herbivores and pathogens. The flavonoid pathway is an ideal model for exploring the evolution of biochemical and genetic networks because of its relatively simple biochemical basis and well-characterized phenotypic expression. To explain the flavonoid pathway, I used the widespread plant *Silene vulgaris* ("bladder campion"), which grows across several elevational gradients. I predicted that at high elevations, UV screening would be a constant necessity, so wanted to observe what would happen to plant pigmentation and defense against herbivores at high elevation plants as these traits are linked through shared flavonoid pathway enzymes. I also hypothesized that the flavonoid pathway would play a role in adaptation to the local environment.

First, I found that *S. vulgaris* expresses anthocyanins and at least 15 different flavones in its leaves, petals, and calyces, with significant differentiation among tissues. Plants also express signatures of their geographic origin, with calyces being the most distinct tissue. Second, I found evidence for divergence in the flavonoid pathway along elevational gradients in the French Alps. Plants at higher elevations had more darkly pigmented calyces and petals, and their leaves had higher levels of UV-screening flavones. I did not find evidence for any associations between flavonoid chemicals and herbivory. Third, naturally high- and low-elevation origin plants,reciprocally transplanted into high- and low-elevation gardens, had genetic and environmental influence to calyx color, whereas leaf flavone concentration and diversity was largely environmentally induced. Lastly, I measured flavonoid pathway gene expression in a subset of the reciprocal transplant individuals. Genes that were significantly different in expression were responsible for flavone production and glycosylating flavonoids (thus creating flavonoid diversity). Overall I was able to determine that the flavonoid pathway plays a role in adaptation to elevational environments through both natural selection and environmental inducibility.

Dedication

To:

My parents, whose unwavering support of my pursuit of a PhD (and frequent flier miles) have been the best support a daughter could have.

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Introduction

Many phenotypes are the products of biochemical networks that generate complex patterns of pleiotropy and correlations among traits (Proulx *et al.*, 2005). How network structure affects the evolution of traits, and how selection acts on different components of the pathway are still poorly known (Frank, 1999; Rausher, 2008; Streisfeld & Rausher, 2009). The approach of studying traits as emergent properties of interacting hierarchical biochemical pathways, known as "network thinking", can be contrasted with the classical view of quantitative genetics where a genetic correlation can be statistically estimated between any pair of traits. The architecture of biochemical networks generates an *a priori* prediction about the nature of genetic correlations among a subset of quantitative traits, and how those genetic correlations may evolve as selection acts on that network.

Plant secondary metabolites, also known as specialized metabolites, are synthesized by all plants to interact with the environment. They are frequently targets of natural (and artificial) selection in that they contribute to the adaptation to the plant's ecological niche (Pichersky & Lewinsohn, 2011). Many of the metabolic gene networks responsible for their production have been well characterized, and using the gene pathway framework can be a powerful tool in making ecological and evolutionary hypotheses regarding the function of each subgroup of metabolites (Proulx *et al.*, 2005).

The plant flavonoid pathway is an ideal model for exploring the evolution of biochemical networks and metabolic pathways because of its relatively simple biochemical basis and well-characterized phenotypic expression. Because the flavonoid pathway is expressed in all plants, the pathway is highly conserved and of broad and applied importance (Winkel-Shirley, 2001). The flavonoid pathway forms diverse groups of secondary compounds including the anthocyanin pigments, the proanthocyanidins (condensed tannins), 3-deoxyanthocyanidins (phlobaphene pigments), the non-pigmented flavonols, flavones, and isoflavones. Each of these groups serves a functional role under different developmental or biotic/abiotic conditions including floral color, deterring herbivores, antifungals, antibiotics, protection from UV radiation, antioxidants, nodulation, auxins, and sugar transporters (Winkel-Shirley, 2001; Quattrocchio *et al.*, 2006; Treutter, 2006). Total flavonoid expression is expected to be a complex suite of traits that can combine to form a diverse array of biochemical phenotypes. Thus, the products of the pathway are not only diverse, but they are potentially affected by a variety of selective forces in nature. The interconnected network arrangement of genes is responsible for different products, and so requires regulation of each branch to ensure the appropriate compounds are produced when needed (Quattrocchio *et al.*, 2006). Regulation, and thus production, will be constrained by the fact that genes and enzymes are often shared among different branches of the pathway.

The flavonoid regulatory and structural pathways have been well characterized in several plant species, including *Arabidopsis thaliana, Ipomoea* spp., *Petunia* spp., *Zea mays, Glycine max, Populus* spp., and *Antirrhinum major*. Much is known about the genes and enzymes responsible for the flavonoid structural pathway, e.g. chalcone synthase (CHS) is the first committed step, followed by a small network of modifying genes (Figure 1). Researchers have described the general regulation of the flavonoid pathway; it is influenced by three families of regulatory genes (MYB, bHLH, WD40), is under developmental control (e.g. floral color expression), and is influenced by many environmental factors, such as light, drought, and generalized plant stress (Winkel-Shirley, 2002; Quattrocchio *et al.*, 2006).

Most evolutionary research on the flavonoid pathway has involved the anthocyanins, a terminal branch of the pathway that produces visible plant pigments. The most commonly studied trait is flower color. From a pathway perspective, the evolution of flower color can be influenced by selection and mutation at different points along the pathway, and upstream genes experience more selective constraint than the downstream genes, presumably because variation has potentially larger selective consequences on downstream loci (Rausher *et al.*, 1999, 2008). My dissertation is complementary to previous work in two fundamental respects. First, rather than focus on the evolution of a particular trait, I studied adaptation on multiple flavonoid-associated ecological traits simultaneously. Second, I focused on the entire flavonoid biosynthetic network present in my study species, *Silene vulgaris* (Caryophyllaceae), rather than only one branch (e.g. anthocyanins only). Several different flavonoid traits are expected to be involved in adaptation to the environment in *S. vulgaris*, and their biochemical relationships may constrain (or permit) a coordinated phenotypic response to selection.

Throughout my dissertation, I focused on variation in three main flavonoid-associated traits: floral color, herbivory, and protection against UV-radiation. The reason is that through shared upstream genes, these traits (easily measured in the field) may be set up for correlations and/or trade-offs at the levels of gene expression, chemistry, and physical phenotypes. Several plant species have shown covariance among these traits, such as herbivory and flower color (Irwin *et al.*, 2003; Frey, 2004; Strauss *et al.*, 2004; Tsuchimatsu *et al.*, 2014), and anthocyanin, flavone, and flavonol accumulation and UV protection (Ryan *et al.*, 2001; Steyn *et al.*, 2002; Lu *et al.*, 2009; Gould *et al.*, 2010).

Field observations and manipulations were performed in the French Alps across an elevational gradient. There are two aspects of elevational gradients that can be useful in testing the flavonoid pathway. First: since UV radiation increases along elevational gradients, and UV-screening flavonoids accumulate to absorb UV radiation (especially UV-B), flavonoid composition and the response of flavonoid-associated phenotypes and ecological interactions can be observed at the ends of the spectrum of one important environmental stressor. Here we can use the flavonoid pathway structure to make predictions - if flavones and flavonols are produced at high elevations, does this mean that anthocyanins are up-regulated in concert due to upstream enzyme sharing? Or, might there be a trade-off between the groups due to substrate competition? Second: plants tend to express more pigments, especially anthocyanins, at higher elevations (Billings & Mooney, 1968). Anthocyanins certainly accumulate under general plant stress, and are sensitive to changes in moisture, temperature, and light (Steyn *et al.*, 2002; Winkel-Shirley, 2002; Gould *et al.*, 2010). This aspect also creates a question regarding flavonoid pathway production at high elevations. Do anthocyanins accumulate constitutively, inducibly, or in a combination in floral and leaf tissues at high elevations in *Silene vulgaris*, a widespread plant? How does this affect the rest of the pathway on a within-plant basis, and also across generations through selection?

Study system

Silene vulgaris (Moench) Garcke (= *Silene inflata* Sm., *Silene cucubalis* Wibel, *Cucubalus behen* L.) or "bladder campion" (Caryophyllaceae), is a herbaceous and gynodioecious perennial named for its inflated, bladder-like calyx. It has a broad spatial and elevational range; from its native Eurasia to the introduced region of North America, and elevations from sea-level to upwards of 2000 m in the Alps and Pyrenees. The variety of environments where *S. vulgaris* is found suggests that *S. vulgaris* is capable of persisting in a large range of ecologically variable environments, and the species is known to have undergone both neutral and adaptive evolution along longitudinal gradients (Keller *et al.*, 2009).

Previous research has documented among-population variance in anthocyanin production in *S. vulgaris* calyces, petals, leaves, and stems (Marsden-Jones & Turrill, 1948, 1949, 1950, 1957). Anthocyanins are the main floral pigments in the plant kingdom. In fact, the presence:absence ratio of anthocyanins segregates to a roughly 3:1 ratio (Marsden-Jones & Turrill, 1957). There is also variation in intensity/hue of anthocyanins within and among tissues, including stems, leaves, calyces, petals, androecia and gynoecia (Marsden-Jones & Turrill, 1957). Their data from observations of several families of *S. vulgaris* collected from high-altitude areas such as the Pyrenees, the Bulgarian mountains, and the French Alps suggested a higher incidence of anthocyanin pigments in multiple tissues in the high altitude populations relative to *S. vulgaris* populations they studied in the UK. What can be gleaned from Marsden-Jones and Turrill (1957) is that *S. vulgaris* is truly a widespread plant that grows from seaside to true alpine environments with some genetic differentiation, and that pigmentation is a trait that changes over the range of the species.

The sister species *S. latifolia* has geographically structured flavone expression in flowers (Mastenbroek *et al.*, 1983). A similar pattern can be expected in *S. vulgaris* as it shares similar evolutionary history (Taylor & Keller, 2007), and closely related species often produce similar sets of flavonoids (Harborne, 1977; Markham, 1982). This pattern holds true in reviewing the flavones, flavonols, and C-glycosyl flavonoids produced by the Caryophyllaceae (Richardson, 1978). It should perhaps be mentioned that the Caryophyllales has been a phylogenetic order of interest with regards to the evolution of the betalains, which are visibly colorful, nitrogen-containing pigments. Many families in the Carophyllales produce these pigments, replacing the anthocyanins (Brockington *et al.*, 2011). The Caryophyllaceae, however, is one of the families that produces anthocyanins (Brockington *et al.*, 2011).

Purpose

The purpose of this dissertation is to assess the role of the flavonoid pathway in adaptation to the environment; specifically, along elevational gradients by using genetic, chemical, and field techniques. I hypothesize that since there is upstream enzyme sharing, anthocyanin pigmentation (floral and vegetative), defense against herbivory, and UV protection will be correlated. This main hypothesis is addressed in the following four chapters:

Chapter 1: I determined what flavonoids are present in *Silene vulgaris* leaves, calyces, and petals. I detected differences in flavonoid expression among tissues, as well as among ancestral geographic demes.

Chapter 2: I monitored several elevational transects of natural *S. vulgaris* populations over two years and measured floral color and herbivory. I used F_{ST}-P_{ST} and ANCOVA approaches to detect that calyx color has likely been under divergent selection. Flowers have more floral anthocyanins and more leaf non-anthocyanin flavonoids at high elevations than at low elevations. High amounts of leaf flavonoids at high elevations indicate protection against UV radiation.

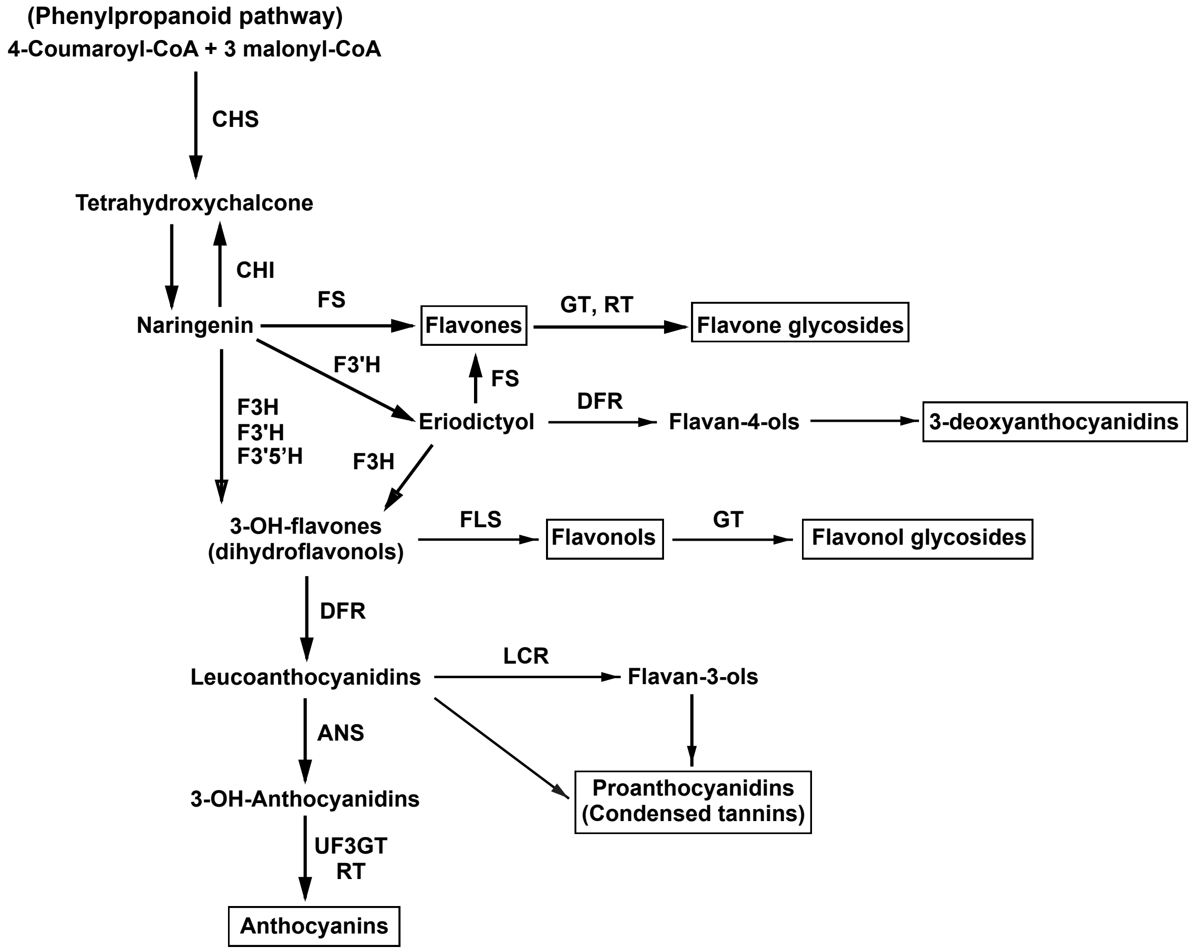
Chapter 3: I took populations of *S. vulgaris* from high and low elevation of origin and arranged them in a reciprocal transplant design in the French Alps with one high elevation and one low elevation garden. I measured herbivory, anthocyanins, and total flavonoids. Nearly all traits showed genetic variance as well as environmental variance among the populations, suggesting that there are both genetic and plastic strategies to flavonoid expression. Most importantly, I found that high elevation populations express higher amounts of anthocyanins in flowers and leaves consistently and constitutively, and that they typically have higher amounts of leaf flavonoids as well. This finding agrees with Chapter 2. Low elevation origin populations had less anthocyanin pigmentation in flowers and leaves, but had a more plastic response once transplanted to the high elevations, where they produced more flavonoids in all tissues. This indicates that life at higher elevations requires more constitutive levels of flavonoids in flowers and in leaves.

Chapter 4: In an attempt to connect the genotype to the phenotype, I used an RNAseq approach to assess gene expression in flowers and leaves in a subset of populations from the reciprocal transplant experiment described in Chapter 3. I found signatures of flavonoid pathway expression similarities by elevation of origin. I also found elevated levels of gene expression responsible for flavone accumulation, especially in the low elevation of origin populations. This result is similar to the chemical findings of Chapter 3, where the low elevation of origin populations appear to change their flavonoid production to fit their environment, and the high elevation of origin populations do not. I also saw high levels of expression of glycosylating genes, which corresponds to my findings of several glycosylated flavones (as opposed to aglycones) in Chapter 1. Lastly, terminal branch genes (those responsible for modifying core flavonoid structures into the subgroups, such as anthocyanins and flavones) were more active than upstream genes.

The flavonoid pathway structure has allowed me to identify ecological roles that may or may not covary. One of the main findings of my dissertation is that anthocyanins and UV-protecting flavones positively covary on a per-plant basis, where each group increases in concentration at high elevations. Notably, this pattern does not necessarily stand for within-tissue patterns; floral pigmentation and leaf flavones are positively correlated, but within flower relationships of anthocyanins and flavones are more elusive. There could be a within-tissue trade-off, but more studies and experimental manipulations need to be done to better describe this relationship. In addition, I was not able to describe any broad relationship through correlation between flavonoid production and herbivory. Thus, in *Silene vulgaris*, flavonoids are not the most effective protectants, or at least the first line of defense, against herbivore damage. The flavonoids produced likely serve as UV-protectants, pollinator attractants, and stress ameliorants, rather than as herbivore antifeedants.

Modern genomics and metabolomics are growing areas of biological research. The combination of these techniques will be important in understanding the evolution of metabolic pathways and phenotypic evolution. My research provides a foot in the door to combining these techniques with field manipulations. My research improves the knowledge of flavonoid evolution by using modern techniques such as RNAseq and metabolomics from the field; most studies to date have relied on laboratory settings.

Figure 1. The flavonoid pathway is a network of genes, flavonoid subgroups, and ecological roles. Arrows signify the direction of the enzymatic steps through the pathway. Genes and their encoded enzymes are abbreviated above or next to arrows. Chemical subgroups and end products are represented in boxes. Note: the isoflavone branch of the flavonoid pathway is not shown since it has a limited phylogenetic distribution and is most frequently found in the Fabaceae (Legume) family. Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FS, flavone synthase; F3H, flavanone-3-hydroxylase; F3’H, flavonoid 3’ hydroxylase; F3’5’H, flavonoid 3’5’ hydroxylase; DFR, dihydroflavonol 4-reductase; FLS, flavonol synthase; ANS, anthocyanidin synthase; LCR, leucoanthocyanidn reductase; UF3GT, UDP-flavonoid glucosyl transferase; GT, glucosyl transferase; RT, rhamnosyl transferase.



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CHAPTER 1:
GEOGRAPHICAL PATTERNS AND DIFFERENTIAL TISSUE EXPRESSION OF FLAVONOIDS IN *SILENE VULGARIS* (CARYOPHYLLACEAE)

This chapter is formatted for the journal Phytochemistry.

Abstract

The genus *Silene* (Caryophyllaceae) differs in flavonoid expression patterns geographically across its range, but there has been a lack of comparison among tissues. We aimed to describe the differences in flavonoid expression of the leaves, calyces, and petals of the bladder campion, *Silene vulgaris*, as well as determine whether there is geographical differentiation in flavonoid expression. We used a combination of LC-UV and LC-MS techniques to identify 16 flavone glycosides in methanolic extracts. Flavonoid phenotypes varied among tissue types, with isoorientin, an unidentified flavone of a mass of 540, and vitexin-2”-O-rhamnoside as the most important flavonoids in distinguishing the flavonoid profiles of tissue types. Combined tissue flavonoid profiles varied among geographical origin, more so than among tissues, and these differences were genetically based. The geographic patterns are similar to those found in other *Silene* species, and may reflect convergent adaptation and or a shared history of post-glacial expansion.

Introduction

The plant flavonoid pathway is an ideal model for exploring the evolution of biochemical networks and metabolic pathways because of its relatively simple biochemical basis, well-characterized phenotypic expression, and its near ubiquity in the plant kingdom (Winkel-Shirley, 2001). Flavonoids are a remarkably diverse group of phenolics that serve a variety of functions in the plant, ranging from pollinator attraction, protection against stress, thermoregulation, defense against pathogens, nodulation, and development of pollen (Harborne & Williams, 2000; Winkel-Shirley, 2001; Brockington *et al.*, 2011). Flavonoids also perform different functions in the various plant tissue types. Leaf flavonoids, for example, are known to play a role in protection against a variety of abiotic (UV, light) and biotic factors (herbivores, pathogens) (Harborne and Williams 2002). Petals are known to play a significant role in pollinator attraction (Stebbins, 1970; Fenster *et al.*, 2004), and often have high concentrations of flavonoids, especially anthocyanins. Calyces likely contribute to the overall floral display due to their size relative to the petals and the presence of pigments. The flavonoid profiles of calyces, however, have been less well studied.

In the Caryophyllaceae the calyx is often fused to produce a tube or spherical bulb, and can be a marked feature of the flower (Rabeler & Hartman, 2005). The genus *Silene* typically has particularly prominent, inflated calyces, specifically that of *Silene vulgaris* - hence the common name, “bladder campion”. *Silene vulgaris* (Moenche) Garcke is a widespread herbaceous plant. Its native range is Eurasia, but it is naturalized in North America (Keller *et al.*, 2009). Petals are typically white, and rarely pink. In *S. vulgaris*, calyces vary markedly in color from veined chlorophyll green to veined anthocyanin crimson (the Caryophyllaceae produces anthocyanins, despite several families in the order Caryophyllales producing betalains (Kamsteeg *et al.*, 1978; Brockington *et al.*, 2011)). Since calyces can photosynthesize (as evidenced by their green pigmentation, chloroplasts, and chlorophyll in extracts; A. Berardi, *personal observation*) and produce flavonoids, we expect the flavonoid composition to be similar to both petals and leaves, with petals and leaves being more distinct from each other due to different ecological roles.

Previous studies describe to the presence of flavone glycosides in *Silene* species, as well as in other species of the Caryophyllaceae (e.g. *Dianthus*, *Gypsophila*) (Mastenbroek *et al.*, 1982, 1983; Brederode & Kooten, 1983; Steyns *et al.*, 1983; Huang *et al.*, 2012; Obmann *et al.*, 2012). Mastenbroek *et al.* (1982, 1983) found evidence for the geographic variation of petal flavone glycosides in a sister taxon, *Silene latifolia* Poiret, white campion, *(*syn. *Silene pratensis* (Rafn.) Godron & Gren, *Silene alba* (Miller) Krause, *Lychnis alba* Mill.). *S. vulgaris* and *S. latifolia* have similar ranges, habitats, as well as a similar history of post-glacial expansion (Taylor & Keller, 2007; Keller *et al.*, 2009). The ancestral genetic demes assigned to samples of the two taxa (Keller & Taylor, 2010) match closely to the geographic groups found by Mastenbroek. We therefore set out to examine the distribution of flavones in *S. vulgaris* across different geographic regions *and* tissue types.

In this study, leaves, calyces, and petals were sampled from *Silene vulgaris* to determine the flavonoid composition of individuals of distinct geographic origin when grown in a common garden in Giles County, Virginia, USA. Our goals were to determine whether tissue types have distinct flavonoid expression patterns, and if they share a geographic expression pattern with their sister taxon, *S. latifolia*. We predicted that there would be distinction between flavonoid production among the tissues due to the different functions each tissue requires and performs, such as UV absorption (all tissues), photosystem protection (mostly leaves, some calyx), and reproductive signaling (mostly petals, some calyx due to its size, color, and distinctive shape in *S. vulgaris*). In addition, we predicted that we would see general flavonoid expression patterns segregated by geographic region of origin, due to genetics, in the common garden.

Results and Discussion

Flavonoids of Silene vulgaris

Sixteen flavones were found regularly in the leaves, calyces, and petals of *S. vulgaris*. We were able to identify eight compounds to their specific structure through chemical standards, mass spectra libraries, and literature references (Table 1). The remaining eight flavonoids were identified to their mass and classified by their UV spectral aglycone pattern and mass spectrometry fragmentation patterns; after consulting the texts of Mabry *et al.*, (1970) and Markham (1982), they were determined to be glycosides of apigenin, luteolin, and scoparin (Table 1). These compounds are hereafter referred to by their flavonoid class and mass, e.g. “Flavone-564”. We also found chlorogenic acid present in several samples, but it was excluded from statistical analyses due to its rarity.

Similar to *S. latifolia,* the predominant flavones present in *S. vulgaris* were mono- and diglycosides of the 8-C-glucosyl flavones orientin, vitexin, and scoparin, as well as their 6-C-glucosyl-flavones isomers (van Brederode *et al.*, 1980). We hypothesize that Flavone-564-1 and Flavone-564-2 are isoorientin-2”-O-arabinose and orientin-2”-O-arabinose, respectively, since they have the correct weight and have been observed in closely related *S. latifolia* (Mastenbroek *et al.*, 1982), but more directed mass spectroscopy and NMR are required to identify these compounds more specifically. Flavone-594 shares the UV spectrum of isoorientin, and the MS fragmentation pattern of a di-glycosylated isoorientin, but we were unable to determine the exact sugar moieties.

While flavones and their glycosides were found in *S. vulgaris* samples, there was no evidence for the presence of flavonols or isoflavones in *S. vulgaris* despite their estimated presence in a previous study without mass spectrometry (van de Staaij *et al.*, 1995a). While we found evidence for the presence of cyanidin compounds when observing the results at 530nm, our extraction method was not optimized for the extraction of stable anthocyanins or their precursor anthocyanidins, but for the extraction of the more stable flavones for geographical and tissue expression pattern analysis.

PCA analysis of flavonoid concentration of leaves, calyces, and tissues

A principal components analysis of flavonoid concentrations (compounds 2-16 for a total of 15 compounds; Table 1) among plants and tissue types resulted in 15 principal components. The first three PCs explained 56.4% of the total variance (Table 2), and were used for further analyses (additional PCs added marginal variance explanation). The low amount of total variance explained by the first three PCs suggests that the flavonoids produced in *S. vulgaris* are generally occur together, but the particular loadings of the PCs describe concentration nuances. PC1 can be described as being influenced by strong (> |0.30| loading) negative concentrations of flavone-564-1, flavone 564-2, isovitexin, vitexin, flavone-770, and flavone-540. PC2 included strong positive loadings of isoorientin and isoscoparin. PC3 included a strong positive loading of saponarin and flavone-740, and a strong negative loading of vitexin-2-O-rhamnoside and flavone 540.

PC1 and PC2 significantly varied among tissue types, while PC3 did not (Table 3). This suggests that tissues vary in their overall flavonoid signature but retain some baseline similarities. A *post-hoc* Tukey test confirmed that there are significant differences between leaves and calyces and petals and calyces for PC1 and PC2, but not petals and leaves in any of the three PCs (Table 3). Visualization of tissue differences of PCs is shown in Figure 1. Petals and leaves tend to cluster with their own tissue type in this figure, and have similar PC values to each other as well, while calyx samples are interspersed in the leaf-petal cluster. Some calyx samples appear to have extreme PC values, especially for PC1 and PC3.

There were significant differences in PC2 and PC3 among ancestral genetic demes (Table 3, Figure 2), though no post-hoc pairwise comparisons were significant. Flavonoid signatures of ancestral demes in *S. vulgaris* is similar to the geographic patterns in flavonoid expression in petals and leaves in *S. latifolia* (Mastenbroek *et al.*, 1982, 1983, 1984).

PC2 varied with tissues and demes, while PC1 only associated with tissues and PC3 only associated with demes. Thus, the chemical differences between PC1 and PC3 may further illustrate the difference between tissues across the range of *S. vulgaris.*

Flavonoid composition of tissues

While principal components analysis is informative, Random Forests (RF) was used to identify particular compounds that distinguish groups in terms of "importance"; then further analyses were completed with those distinguishing compounds. We analyzed the difference of flavone composition in leaves, calyces, and petals using Random Forests (RF) classification. In our RF model, we analyzed all tissue data together to parse out which flavonoids best described the differences among the three tissues, since our replication best suited tissue differentiation rather than for distinguishing demes. The RF model chose a minimum set of three predictor flavonoids necessary to distinguish each tissue type: isoorientin, flavone-540, and vitexin-2"-O-rhamnoside (Figure 4A). Model frequencies are reported in Table 4.

A two-way ANOVA on each of these flavonoids showed that there were concentration differences among tissues for all three predictor flavonoids, and significant differences among demes in flavone-540 and isoorientin, but not vitexin-2"-O-rhamnoside (Table 5). Similar to the prior suggestions of Mastenbroek *et al.* (1983) and Mastenbroek & Van Brederode (1986), there is distinct variation in flavone production across the European distribution, including presence/absence of some flavones. For example, we did not find isoorientin in eastern deme petals or southern deme leaves (Table 6), and neither did we find detectable levels of vitexin-2"-O-rhamnoside in western or southern deme leaves (Table 6).

To further elucidate flavonoid differences of tissue types, we examined the mean decrease in accuracy, which measures importance to the model (Ranganathan & Borges, 2010), of tissue flavonoid composition for leaves, calyces, and petals separately (Figures 3B, 3C, 3D). Calyces and petals have more similar flavonoid importance profiles to each other than to leaves, but the relative importance of saponarin is shared among all three tissues.

The order of importance of each flavonoid in distinguishing tissue types and samples from each other varied among the leaf, calyx, and petal samples; we report out-of-bag (OOB) probability of membership for each tissue class for leaves (Fig. 4), calyces (Fig. 5), and petals (Fig. 6); the x-axis sample identification number is the same in all three figures. The overall OOB estimate of error rate is 27.66%, indicating that a tissue sample would be incorrectly assigned to a tissue at that error rate. At a frequency of 0.67 or above, 55% of leaves were correctly assigned to the leaf class, 53% of calyces were correctly assigned to the calyx class, and 35% of petals were correctly assigned to the petal class (Figure 4A-C). Calyx tissues appeared to be the most distinct with the fewest amount of leaf and petal samples assigned to it (Figure 4B). A larger sample size may be beneficial to more accurately distinguish the particular differences of total flavonoid composition among tissues.

Concordance of PCA and RF flavonoid profiles

Isoorientin, flavone-540, and vitexin-2"-O-rhamnoside were the best predictor variables in the RF models. Each of these flavonoids is weighted heavily in at least one of the top three PCs: isoorientin in PC2 (0.55), flavone-540 in PC1 (-0.40), and vitexin-2"-O-rhamnoside in PC3 (-0.48). In addition, saponarin was consistently a strong loading factor in the principal component analysis, and was marginally less frequent in the RF models than vitexin-2"-O-rhamnoside (Table 4). One particular flavonoid pattern was similar in both PCA and RF methods: the presence or absence of vitexin-2"-O-rhamnoside, singled out in both analyses, plays a large role in determining leaf differences among demes (Table 6), perhaps contributing to the significance of PC3 in explaining differences among demes.

Conclusion

In the Caryophyllaceae, flavonoid glycosides are known to serve a variety of important functions including antifungals (Galeotti *et al.*, 2008) and as modifiers of petal structure (Steyns & van Brederode, 1986). Flavonoids also vary during *Silene* life-history (Steyns *et al.*, 1983), and accumulate under light and UV stress (Niemann, 1984; van de Staaij *et al.*, 1995a; van de Staaij *et al.*, 1995b). Isoorientin in particular appears to be particularly influenced by light regime in *Silene*, where isovitexin is replaced by isoorientin at higher levels of light (van

Brederode *et al.*, 1980); dihydroxylated flavones and flavonols (e.g. flavones orientin, luteolin and flavonol quercetin) have stronger photoprotective capacity than their monohydroxylated counterparts (e.g. flavones vitexin, apigenin and flavonol kaempferol) (Ryan *et al.*, 2002, 2009; Gerhardt *et al.*, 2008). In fact, isoorientin was the most important distinguishing flavonoid (from the RF analyses) among the three tissues we tested. Using PCA, we showed that petals, calyces, and leaves had distinct flavonoid profiles, especially those described by PC1 and PC2, but retain many similarities in flavonoid composition. Flavonoid phenotypes, especially those described by PC2 and PC3, were significantly associated with ancestral demes identified using molecular marker data. Perhaps this is unsurprising since divergence in flavonoid expression phenotypes in this experiment is likely due to genetic differences among samples, as they were grown in a common garden. Flavonoid-deme patterns were similar to *S. latifolia,* which produces flavonoids in a similar geographical pattern (Mastenbroek *et al.*, 1983).

Tissue-specific flavonoid differences are best described by particular concentrations of each of the 15 flavonoids found, rather than the presence or absence of particular compounds. Although there appears to be some variance with presence/absence of isoorientin and vitexin-2"-O-rhamnoside in leaves or petals and saponarin in calyces among ancestral demes, all tissues are able to produce each of the flavonoids found. The visualization of principal components shows that calyces appear to be the most distinct tissue (Figure 1), and the RF analyses were best able to correctly assign calyx tissues to the calyx class compared to leaf and petal assignment (Figure 5), further supporting the fact that calyces produce the most distinct array of flavonoids.

Further research would be necessary to test whether the geographic divergence of flavonoid composition is the result of founder effects during the post-glacial colonization of Europe, adaptive divergence among distinct glacial refugia that colonized Europe, or adaptive divergence associated with the species' geographic spread across the continent.

Experimental

Plant material

Seeds from 22 populations spanning the European range of *S. vulgaris* were selected from the Taylor lab seed collection (Appendix) and germinated in the University of Virginia greenhouse in 2010. We assigned populations to their ancestral deme (western Europe, eastern Europe, southern Europe, or admixed, see Keller & Taylor (2010)) with the goal of of identifying geographically distinct flavotypes. European populations were chosen to reflect a broad geographical range, with three to five maternal families sampled per population and approximately five individuals per family. Once seedlings had reached an adult vegetative stage, individuals were planted in a deer exclosure at Mountain Lake Biological Station (MLBS, Giles County, Virginia) at ~1200 m elevation. Plants were planted in an outdoors common garden so the natural sunlight and biotic interactions would stimulate the production of flavonoids (Koes *et al.*, 1989; Steyn *et al.*, 2002).

Two to three individuals per population were haphazardly selected for flavonoid analysis. One leaf and one fully open flower were collected at the time of first flower, which ranged from July – August 2010. Flowers were separated into calyces and petals. Tissues were stored at -20°C in microcentrifuge tubes.

Flavonoid extraction

Frozen leaves, calyces, and petals were each weighed and homogenized with a micropestle in 1 mL MeOH with 1% HCl (v/v) extraction solvent. Samples were set at 4°C on ice and placed on a gyratory shaker in darkness for 18 hours. After centrifugation at 12000 rpm, the supernatant was removed and samples were dried in a SpeedVac. Samples were stored in dry form at -20°C. We sampled 38 leaves, 30 calyces, and 26 corollas (petals).

Flavonoid profiling by HPLC/PDA/MS

For HPLC/PDA/MS analysis, the dried samples were re-suspended in 1 mL of extraction buffer and vortexed until they dissolved. Samples were further diluted in HPLC Buffer A as 20ul sample: 60ul Buffer A (see below). Fifty microliters of supernatant was applied to an HPLC/PDA/ESI-MS system composed of Agilent HPLC 1100 series equipment (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) operated in negative mode in the range m/z 50-1200. Skimmer voltage, -40 eV; capillary exit voltage, -113.5 eV; capillary voltage, 4,200 V; nebulizer pressure, 35 psi; drying gas, 11 L min-1; gas temperature, 330 °C. HPLC was carried out on a Chromolith Performance RP-18e (100 x 4.6 mm i.d.) column (Merck, Darmstadt, Germany) with a guard column (Phenomenex, 5 µm, 4×4 mm) using a linear binary gradient of H_2O (solvent A) containing 0.2% (v/v) formic acid for HPLC-ESIMS and 0.1% (v/v) TFA for HPLC-DAD and acetonitrile (solvent B) with a flow rate of 1.0 mL per min at 25°C. The linear elution gradient was as follows: 0 min 95% A, 16 min 83% A, 23 min 55% A, 23.1 min 0% A, 26.1 min 95% A, 30 min 95% A. A PDA was used for detection of UV-visible absorption spectra in the range of 200-700 nm.

Flavonoids were identified based on UV visible absorption spectra and mass fragmentation by tandem MS analysis in comparison with the following standard compounds: apigenin, luteolin, vitexin, and chlorogenic acid from Sigma-Aldrich (St Louis, MO, USA);

orientin, isoorientin, isovitexin, saponarin, and cyanidin from Extrasynthese Co., (Genay, France). We also searched mass spectra on the MassBank database (Horai *et al.*, 2010), and used the KNApSAcK metabolite database (Afendi *et al.*, 2012) and literature sources (cited in Table 1) to assist in identifying glycosides without standards.

Each sample was quantified from the LC-UV data by comparing to a series of standard dilutions from purchased chemical standards. Each flavonoid was quantified using the closest-match chemical standard available. Once concentration in µg/mL was obtained for each flavonoid per sample, the amount of flavonoid in µg per gram of tissue sample was determined by multiplying concentration by each sample's wet weight in grams.

Statistical Analyses

Principal component analysis (PCA) implemented in the statistical software program R (R Development Core Team 2013) was used to reduce the dimensionality of the dataset. The first three principal components (PCs) were plotted by tissue type, and ancestral deme assignment to assess differences in flavonoid composition. Univariate ANOVAs were conducted on each of the first three PCs to test for significant differences among tissue types and ancestral deme using R package car (v 2.0-19). Post-hoc Tukey tests (with multiple comparison corrections) were conducted on each ANOVA comparison (R package stats v.3.0.1). To visualize the results, 3D scatterplots were plotted using R package scatterplot3d (v 0.3-33).

Random Forest classification algorithms have been recently used to examine and describe differences in complex traits, such as plant volatile emission, where there are many more variables (compounds) than there are samples. Flavonoid concentrations was used to visualize differences among tissues using Random Forests (RF) with the package VarSelRF in R

(Ranganathan and Borges 2010). Random Forests provided 1) a minimum set of predictor flavonoids necessary to distinguish each tissue type and 2) a ranking of relative importance of each compound in distinguishing among tissues. Two types of RF analyses were conducted. The first comprised the entire dataset with all tissues present, and the grouping variable was tissue type. The second analysis further investigated the variability of flavonoid composition within tissues, so the analysis was restricted to one tissue at a time, and grouped by population of origin, with the goal of assessing the mean decrease in accuracy of distinguishing tissues resulting from removal of specific flavonoids from the models, thus understanding flavonoids by their relative importance in distinguishing each tissue. Each of our analyses used 200 bootstrap iterations, and the mean decrease in accuracy for removal from the models was calculated for each flavonoid compound per tissue, and then from the whole dataset. The probability of a sample belonging to a tissue group (leaf, calyx, petal) or to the whole dataset was calculated using the average "out of the bag" (OOB) probability of membership (the classification error obtained when the unselected (OOB) samples are examined) (Ranganathan & Borges, 2010; Parachnowitsch *et al.*, 2012) with $N_{leaf} = 38$, $N_{calyces} = 30$, $N_{petals} = 26$.

Random Forest algorithms excel at choosing variables that best perform as classifying factors. Lastly, the top three predictor flavonoid variables were chosen from the Random Forest analysis of all three tissues and ran two-way ANOVAs in R package car, with tissue and deme as fixed factors (then results from PCA could be directly compared). We also calculated Pearson's correlations among the three flavonoid variables.

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Figure 1. Principal component analysis of tissue distinction in flavonoid concentration and composition.

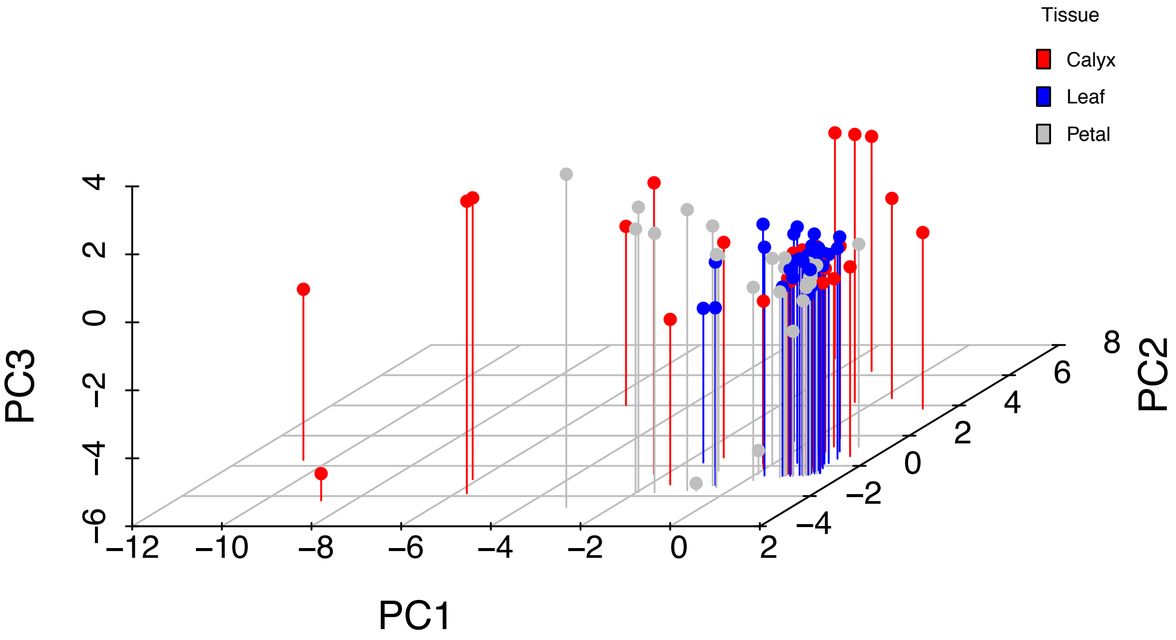


Figure 2. Principal component analysis of ancestral deme (from Keller & Taylor (2010)) and flavonoid concentration and composition.

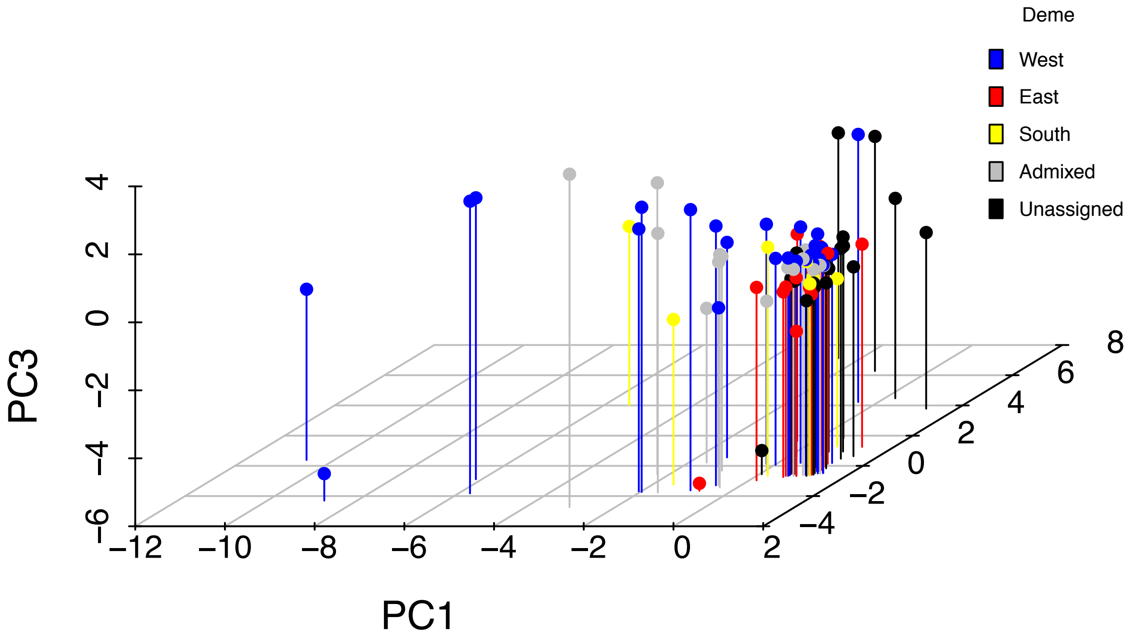
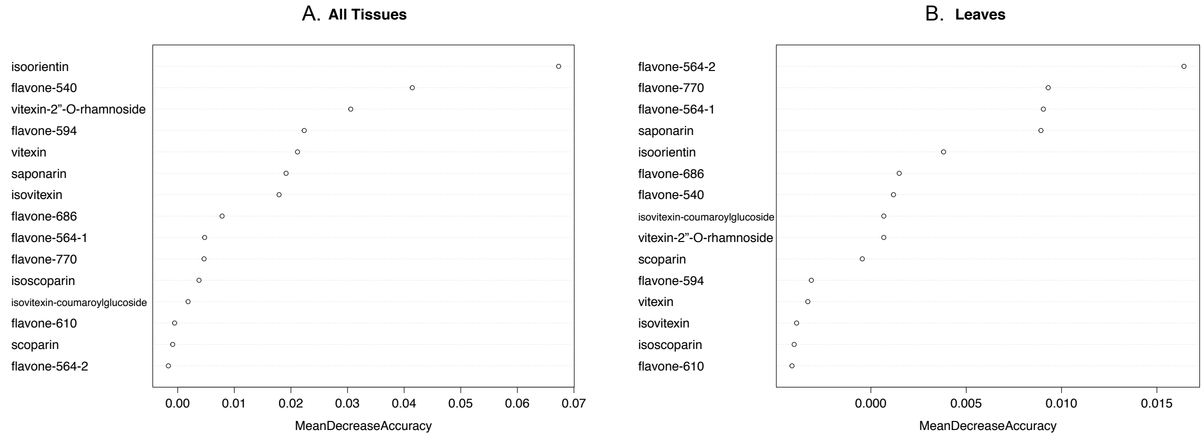


Figure 3. Plots of mean decrease in accuracy for removal from the models for each flavonoid compound for (A) the entire dataset reflecting *S. vulgaris* as a whole, (B) leaves, (C) calyces, and (D) petals.



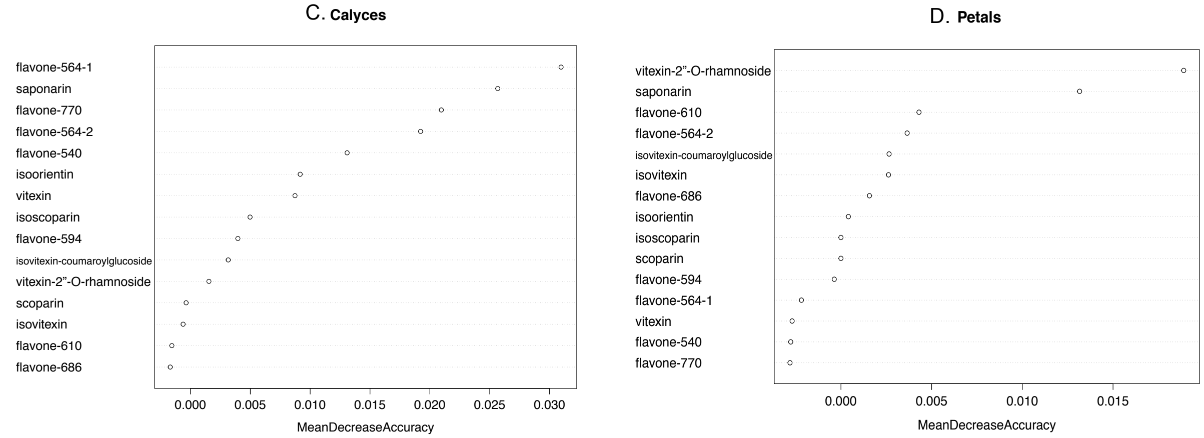
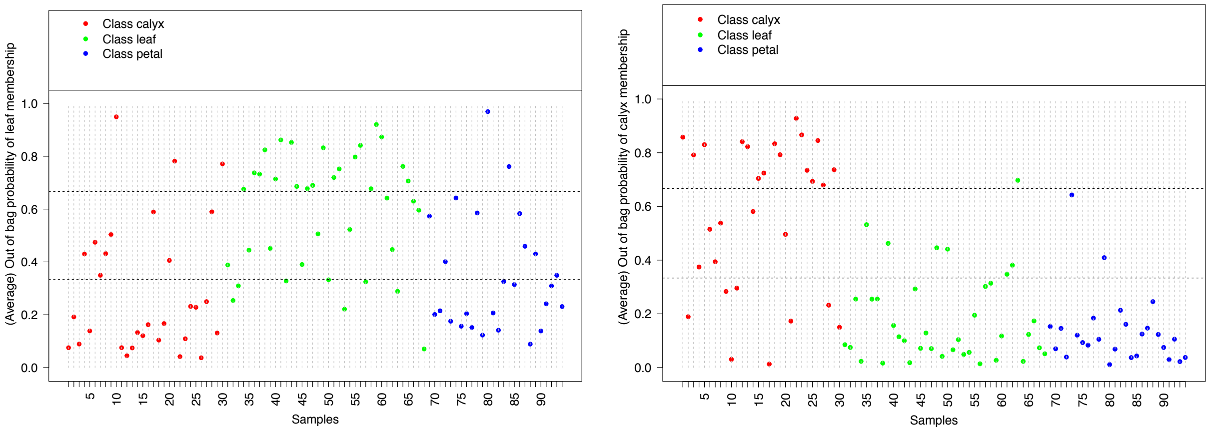


Figure 4. Comparisons of flavonoid concentration and composition by tissue type from samples of *S. vulgaris* using the 'out of the bag' (OOB) probability of membership. Each tissue was compared to the other two in a separate analysis: (A) leaf, (B) calyx, (C) petal. Each point represents the same individual plant sample (numbered on the x-axis) throughout the three panels, and is colored by its true tissue identification. If each sample was identified as the correct tissue, all of the samples from the actual tissue would be above the dotted horizontal line representing 0.67 (e.g. all green-colored leaf samples in panel A, all red-colored calyx samples in panel B, and all blue-colored petal samples in panel C would be above the line at y=0.67).



A. Leaf OOB classification

B. Calyx OOB classification

C. Petal OOB classification

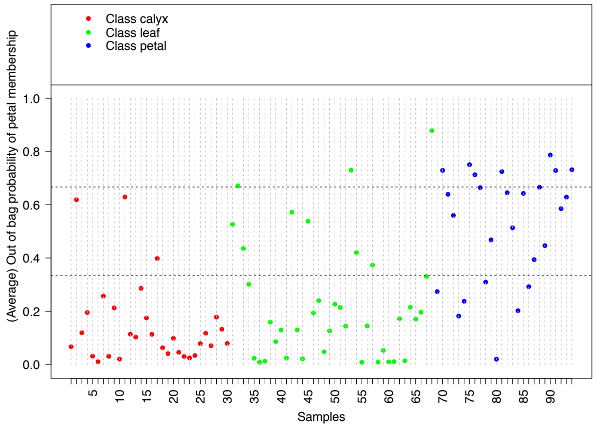


Table 1. The flavonoid profiles in acidic MeOH extracts of Silene vulgaris, HPLC-ESI-MS. Concentrations are mg flavonoid per gram of wet tissue. $N_{leaves} = 38$, $N_{calyces} = 30$, $N_{petals} = 26$.

Peak no.[a]	Rt (min) LC-MS	ID	ESI-MS (m/z)	Fragmenta (m/z; MS2, MS3)[b]	Leaf concentration mean	Leaf concentration standard deviation	Calyx concentration mean	Calyx concentration standard deviation	Petal concentration mean	Petal concentration standard deviation	Reference
1	*8.7*	Chlorogenic acid	353 $[H]^-$		--	--	--	--	--	--	chemical standard
2	*12.9*	Flavone-610; iso/orientin di-glycoside	609 $[H]^-$		0.87	1.91	7.16	19.53	0.87	1.91	
3	*15.1*	Saponarin	593 $[H]^-$	311, 283	105.54	144.62	45.34	102.84	105.54	144.62	chemical standard
4	*15.4*	Flavone-594, orientin di-glycoside	593 $[H]^-$	353, 297	2.02	5.2	39.64	82.28	2.02	5.2	
5	*15.8*	Flavone-564-1, isoorientin-glycoside	563 $[H]^-$		1.94	3.49	10.85	20.66	1.94	3.49	
6	*15.9*	Flavone-564-2, orientin-like	563 $[H]^-$		9.35	10.87	22.55	47.56	9.35	10.87	
7a, 7b[c]	*16.0*	Isoorientin, orientin	447 $[H]^-$	285, 243	0.36	1.37	38.74	58.08	0.36	1.37	chemical standard
8	*17.5*	Isovitexin	431 $[H]^-$	311, 283	0.58	1.28	4.99	7.46	0.58	1.28	chemical standard
9	*18.5*	Vitexin	431 $[H]^-$		2.68	4.27	7.51	9.08	2.68	4.27	chemical standard
10	*18.6*	Vitexin-2"-O-rhamnoside	577 $[H]^-$	413, 293	13.81	34.96	7.21	10.63	13.81	34.96	Obmann et al. 2011; Massbank PR100821
11	*19.3*	Isoscoparin	461 $[H]^-$		0.23	1.17	1.76	2.95	0.23	1.17	Bollina et al. 2010
12	*19.7*	Scoparin	461 $[H]^-$	341, 298	0.09	0.46	0.65	2.48	0.09	0.46	Bollina et al. 2010
13	*20.1*	Flavone-770, iso/vitexin diglycoside	769 $[H]^-$		4.39	8.27	9.99	20.34	4.39	8.27	
14	*20.4*	Isovitexin-coumaroylglucoside	739 $[H]^-$		3.97	7.68	2.01	5.89	3.97	7.68	Caasi-Lit et al. 2007
15	*21.1*	Flavone-686, unknown flavone glycoside	685 $[H]^-$		6.31	13.93	2.04	4.82	6.31	13.93	
16	*21.7*	Flavone-540, unknown flavone glycoside	539 $[H]^-$		10.84	13.27	16.34	24.22	10.84	13.27	

[a] Peaks 1-16 UV spectra read at 270nm, 17 read at 530nm

[b] MS negative

[c] 7a and 7b co-chromatograph in LC-MS, but LC-UV separate by 0.2 min

Table 2. Loadings of the first three principal components of the *S. vulgaris* leaf, calyx, and petal dataset.

	PC1	**PC2**	**PC3**
Flavone-610	-0.0642	0.1779	0.1706
Saponarin	-0.2333	-0.2253	0.4261
Orientin diglycoside	0.0485	0.2637	-0.1111
Flavone-564-1	-0.3862	-0.0841	-0.2582
Flavone-564-2	-0.3309	-0.1240	-0.2790
Isoorientin	-0.0663	0.5545	0.0696
Isovitexin	-0.3279	0.2645	0.0155
Vitexin	-0.4077	0.1982	0.0364
Vitexin-2-O-rhamnoside	-0.0539	-0.0113	-0.4810
Isoscoparin	-0.0289	0.5501	0.0466
Scoparin	-0.1099	0.1827	-0.0501
Flavone-770	-0.4048	-0.0454	0.1068
Flavone-740	-0.2418	-0.2317	0.3603
Flavone-686	-0.0634	-0.1042	-0.4970
Flavone-540	-0.4001	-0.0780	-0.0425
Proportion of Variance	0.2834	0.1619	0.1193
Cumulative Proportion of Variance	0.2834	0.4453	0.5646

Table 3. Univariate ANOVAs testing for differences in tissues (leaves, calyces, petals, with Tukey *post-hoc* comparisons), and ancestral deme.

	Tissues			**Tissue Tukey *post-hoc* comparison**			**Ancestral Deme**		
	F	df	*p*	*leaf-calyx*	*petal-calyx*	*petal-leaf*	*F*	df	*p*
PC1	7.8041	2, 91	***	***	*	NS	1.6351	4, 89	NS
PC2	13.226	2, 91	***	**	***	NS	5.2151	4, 89	***
PC3	0.1403	2, 91	NS	NS	NS	NS	3.1719	4, 89	*

*** = p<0.001, **= p<0.01, *= p<0.05, NS = not significant.

Table 4. The Random Forest model predictor flavonoids that distinguish leaves, calyces, and petals from each other when grown in a common garden, with their model frequencies. Three flavonoids were distinguished as the minimum flavonoids necessary for tissue distinction, marked with an "x". Flavonoids rejected from model: Isovitexin-coumaroylglucoside, orientin, scoparin, flavone-540.

Predictor Flavonoids	**Model Frequency**	**Variables used in model**
Isoorientin	1	x
Flavone-540	0.945	x
Vitexin-2"-O-rhamnoside	0.575	x
Saponarin	0.565	
Vitexin	0.495	
Flavone-594	0.4	
Isovitexin	0.35	
Flavone-564-2	0.215	
Flavone-770	0.08	
Flavone-564-1	0.07	
Flavone-686	0.03	
Flavone-610	0.015	
Isoscoparin	0.01	

Table 5. Two-way ANOVAs testing for differences in tissues (leaves, calyces, petals) and ancestral deme with the three predictor flavonoids chosen by the RF model, as well as saponarin due to its high model frequency and high concentrations

	Isoorientin			**Flavone-540**			**Vitexin-2”-O-rhamnoside**			**Saponarin**		
	F	df	*p*	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
Deme	3.40	4, 79	*	4.36	4, 79	**	1.46	4, 79	NS	3.78	4, 79	**
Tissue	8.45	2, 79	***	12.29	2, 79	***	3.00	2, 79	*	7.47	2, 79	**
Deme*Tissue	1.31	8, 79	NS	2.50	8, 79	*	2.86	8, 79	**	1.49	8, 79	NS

*** = $p<0.001$, **= $p<0.01$, *= $p<0.05$, NS = not significant.

Table 6. Means and standard deviations of the top three RF model predictor flavone concentrations by tissue type and ancestral deme: isoorientin, flavone-540, and vitexin-2”-O-rhamnoside, and saponarin due to its high model frequency and high concentrations. The unit of flavonoid measurement is mg flavonoid per g fresh weight of tissue.

		Isoorientin		**Flavone-540**		**Vitexin-2"-O-rhamnoside**		**Saponarin**		
	Deme	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	N
Calyces	West	29.08	45.32	38.50	33.63	5.38	15.22	91.58	133.26	8
	East	14.24	12.31	6.18	8.74	5.40	4.15	0.00	0.00	2
	South	13.12	9.11	21.47	24.68	16.04	11.24	38.65	60.58	4
	Admixed	7.35	6.31	4.11	4.59	7.04	9.66	92.99	170.85	5
	Unassigned	73.80	77.36	5.76	9.43	5.73	7.17	0.74	1.75	11
Leaves	West	4.94	6.12	2.00	2.72	0.00	0.00	9.12	18.22	16
	East	4.81	8.37	0.63	1.18	1.95	5.12	6.65	12.33	8
	South	0.00	0.00	6.93	1.84	0.00	0.00	92.75	64.30	2
	Admixed	0.82	1.59	2.18	4.35	8.67	18.22	33.72	49.75	7
	Unassigned	9.22	11.36	0.55	1.23	1.61	3.59	0.00	0.00	5
Petals	West	0.92	2.28	17.09	18.44	0.27	0.81	169.90	166.40	9
	East	0.00	0.00	11.19	11.40	16.14	18.01	23.21	48.00	6
	South	0.44	0.62	1.64	2.32	12.19	1.41	22.52	8.25	2
	Admixed	0.04	0.11	6.64	7.96	8.05	10.17	171.62	166.53	6
	Unassigned	0.00	0.00	5.96	2.48	62.40	98.60	0.31	0.53	3

Chapter 1 Appendix

Location of seed collections, elevation classification, and ancestral deme classification from Keller and Taylor (2010).

Population	Country	Latitude	Longitude	Elevation (m)	Ancestral Deme
ALN4	France	48.3966	0.0934	148	West
BAD1	Germany	50.9519	14.2473	348	East
BOL	France	44.2792	4.7516	60	South
CAT2	UK	54.3802	-1.6289	73	West/South
CON	Switzerland	46.8465	6.7131	437	Unassigned
CRE3	France	44.7744	-0.3483	104	West
CRO	UK	52.9338	1.2900	41	West
EGR	Hungary	47.9032	20.3731	182	East
GAS	Switzerland	46.7582	8.1351	1050	West/East/South
LAR1	Spain	42.8016	-5.6290	961	West/East/South
LSZ	Switzerland	46.6586	6.5192	506	Unassigned
NBS	Lebanon	33.9432	35.8298	1454	South
OSE	Spain	43.1382	-5.0367	757	West/East/South
PCO1	Spain	42.6188	-0.1693	1232	West
PRG4	Czech Rep.	50.1302	14.3991	192	East/South
SVL2	Austria	48.1654	14.5296	277	East
VDM3	Portugal	41.6351	-8.1418	427	East/West

CHAPTER 2:
CLINAL VARIATION IN FLORAL COLOR AND FLAVONOIDS ALONG ALPINE ELEVATION GRADIENTS IN *SILENE VULGARIS*

This chapter is formatted for the Journal of Evolutionary Biology

Abstract

Spatial environment heterogeneity can lead to divergent selection in populations, often leading to phenotypic differentiation and local adaptation. The plant flavonoid biosynthetic pathway is responsive to both selection and environmental stressors; its pathway structure allows us to make predictions about phenotypic divergence, which may co-vary across environmental gradients. To determine whether there has been divergence in flavonoid pathway expression along elevational gradients, we monitored populations of the widespread bladder campion, *Silene vulgaris* (Caryophyllaceae), spanning 700-2100 m in the French Alps for two years, measuring traits associated with the flavonoid pathway: floral color, floral and foliar flavonoids, and herbivory. We predicted that with increasing elevation, more non-anthocyanin flavonoids would be produced as a response to increased UV radiation, plants would be better defended against herbivores, and that anthocyanins would accumulate to ameliorate stress associated with high elevation environments. We found evidence for adaptive divergence of floral color across elevational transects, where pairwise $P_{ST} > F_{ST}$. Anthocyanin-based floral color increased with

elevation, as did flavonoids in leaves. However, there were no associations between the increase in flavonoid production with herbivory. Our results indicate that floral color and leaf flavonoid production are linked by means of shared upstream enzymes, and that selection likely acted on the flavonoid pathway, reinforcing the relationship.

Introduction

Plants may encounter and adapt to different ecological conditions along environmental gradients via habitat-associated natural selection. Selection from environmental heterogeneity over an evolutionary timescale is expected to result in local adaptation in populations (Felsenstein, 1976). Adaptation across an environmental gradient may result in phenotypic clines, although clines can also form as a result of drift and founder effects during migration (Keller *et al.*, 2009). There is abundant evidence for adaptation of plant populations to environmental gradients such as latitude, temperature and moisture, resulting in phenotypic clines (Clausen *et al.*, 1948; Antonovics & Bradshaw, 1970; Lacey, 1988; Weber & Schmid, 1998; Stinchcombe *et al.*, 2004; Méndez-Vigo *et al.*, 2011). Plant flavonoid pigments are known to be responsive to natural selection, and have diverged across environmental gradients, such as soil type and pollinator guild composition, in several plant species (Schemske & Bierzychudek, 2007; Hopkins & Rausher, 2011). In this study, we observe flavonoid pigments across an environmental gradient to look for patterns of local adaptation.

The plant flavonoid pathway has a relatively simple biochemical basis and has well-characterized phenotypic expression in both model and non-model plant species. Because flavonoids are nearly ubiquitous in the plant kingdom, the pathway is highly conserved and of broad applied importance (Winkel-Shirley, 2001). The flavonoid pathway forms diverse groups of secondary compounds including the visible anthocyanins, proanthocyanidins (condensed tannins), and phlobaphene pigments, and the UV-absorbing flavonols, flavones, and isoflavones. Each of these groups serves a functional role under different environmental, ecological, and developmental conditions including floral color, vegetative "reddening", defense against herbivores, antifungals, antibiotics, protection from UV radiation, antioxidants, and sugar transporters (Winkel-Shirley, 2001; Quattrocchio *et al.*, 2006; Treutter, 2006). Thus, the products of the pathway are both diverse and can be affected by a variety of selective forces in nature. The interconnected network of genes is responsible for different products, and so requires regulation of each branch to ensure the appropriate compounds are produced when needed (Quattrocchio *et al.*, 2006).

Studies that have explored clinal variation in flavonoids have mainly focused on visible, typically anthocyanin, pigmentation in flowers (Schemske & Bierzychudek, 2007; Smith & Rausher, 2008; Arnold *et al.*, 2009; Hopkins & Rausher, 2011, 2012; *but see* Mastenbroek *et al.*, 1982, 1983). Anthocyanins are of particular interest because they are known to attract pollinators (Faegri & van der Pijl, 1979), accumulate under stress (Steyn *et al.*, 2002), and are one of the terminal branches of the flavonoid pathway. Fewer studies, however, have focused specifically on non-anthocyanin flavonoids, or flavonoid-trait specific relationships, and whether the observed phenotypic divergence is adaptive. Given their involvement in a plant's ecology, several different flavonoid traits are expected to be involved in adaptation and their biochemical relationships may constrain (or permit) a coordinated phenotypic response to selection. In addition, flavonoid production patterns are heritable and frequently influence fitness (Irwin *et al.*, 2003; Connor *et al.*, 2005; Johnson *et al.*, 2009; Hopkins & Rausher, 2012), which allows us to test for patterns of selection and adaptation on flavonoids.

The flavonoid literature lacks field-based studies encompassing a broader view of the biosynthetic pathway that may generate correlations among multiple traits. This network thinking generates testable hypotheses as to how the structure of the pathway influences the ecological roles associated with the production of flavonoid groups and patterns of genetic divergence. The pairing of floral color and herbivory defense has been researched, establishing both positive and negative correlations (trade-offs) between floral anthocyanin color and herbivory (Irwin *et al.*, 2003; Frey, 2004; Gould, 2004; Johnson *et al.*, 2008; Tsuchimatsu *et al.*, 2014). With regard to pathway structure, pleiotropic relationships between the floral color and herbivory roles have been observed (Simms & Bucher, 1996; Fineblum & Rausher, 1997), suggesting that the flavonoid pathway structure, through the sharing of upstream enzymes, may constrain adaptation. In addition to controlling floral color and defense against herbivores (in some systems), the flavonoid pathway controls UV protection; it remains to be seen if there are relationships between the three flavonoid subgroups and ecological roles.

Elevational gradients are a particularly powerful system in which to evaluate the evolution of the flavonoid pathway because this type of gradient includes different suites of selective conditions, including shifts in herbivores, pollinators, and UV radiation (Arnold *et al.*, 2009). In fact, UV-B levels can increase from 10-16% per 1000m in elevation (Schmucki & Philipona, 2002). UV-B radiation can influence multiple branches of the flavonoid pathway (Treutter, 2006); up-regulation of the pleiotropic upstream genes may also up-regulate all or some of the downstream flavonoid subgroups so that increased levels of flavonoids responsible for UV absorption (flavonols or flavones, depending on the plant species) are not the only change stimulated by UV-B radiation. Specifically, if there is an increase in UV-B radiation, flavonoid production is expected to increase (Jordan et al. 1994, Treutter 2006). It is unknown how the up-regulation of flavonols or flavones will percolate across the pathway to affect different flavonoid-related traits under different selective pressures. With high elevation populations experiencing higher levels of UV-B radiation, and low elevation populations experiencing lower levels of UV-B radiation, it is possible that portions of the pathway responsible for flavonol or flavone production have been involved in repeated episodes of adaptive evolution.

The question that remains, however, is how the other flavonoid-based ecological roles will react to such a perturbation in the pathway. If the UV-absorbing groups are up-regulated, do other groups like floral pigmentation or compounds for herbivory defense decrease in concentration, or are they up-regulated in concert? Several studies have documented an increase in flavonoid production in plants with increased UV-B radiation introduced through experimental manipulations (e.g. (Jordan *et al.*, 1994; Bieza & Lois, 2001; Nybakken *et al.*, 2004; Treutter, 2006). These traits likely confer variance in fitness and have the potential to change along a gradient of increased UV radiation, therefore producing suites of selective conditions.

We chose to investigate our questions with the widespread plant *Silene vulgaris* (Caryophyllaceae), a small perennial herb that has a broad spatial and elevational range (sea level to ~2400 m) across its native Eurasia and the introduced region of North America. The variety of environments experienced by extant populations shows that *S. vulgaris* is capable of persisting in a large range of ecologically variable environments, and a recent study demonstrated that *S. vulgaris* has undergone both neutral and adaptive evolution along latitudinal gradients (Keller *et al.*, 2009). Earlier work in *S. vulgaris* has documented flavonoids present (Darmograi, 1977). There has been local adaptation of an Austrian highland (1600 m) and Dutch lowland (sea level) population to their local UV-B regimes (van de Staaij *et al.*, 1997), including an increase in foliar compounds absorbing in UV-B wavelengths from the high-elevation Austrian population (van de Staaij *et al.*, 1995). *S. vulgaris* also varies among populations in visible anthocyanin production, intensity, and hue in calyces, petals, leaves, and stems (Marsden-Jones & Turrill, 1948, 1957). Observations of *S. vulgaris* collected from high-elevation areas such as the Pyrenees, the Bulgarian mountains, and the French Alps suggests a higher incidence of anthocyanin pigments in multiple tissues in the high elevation populations relative to lowland *S. vulgaris* populations (Marsden-Jones & Turrill, 1948, 1957). Based upon Marsden-Jones & Turrill (1957), one might reasonably conclude that *S. vulgaris*'s wide geographical and ecological range will include adaptive variation in pigmentation. Indeed, *S. vulgaris* does show geographical differentiation in flavonoids among leaf, calyx, and petal tissues (Chapter 1), though the question remains as to whether flavonoid differentiation occurs along an elevational gradient.

Here, we examine *S. vulgaris* populations along an elevational gradient to determine the role of the flavonoid pathway in adaptation to elevation, with a specific focus on anthocyanin-based floral color. First we will determine whether there is evidence for a floral color cline along elevational transects. Second, we will determine whether there has been spatially divergent selection on floral color, accounting for neutral genetic processes using modified F_{ST}-Q_{ST} comparisons (Spitze, 1993). Third, we will use the flavonoid pathway structure to make and test predictions regarding flavonoid-related ecological roles across elevational gradients. Specifically, we predict that floral anthocyanins will increase with elevation due to the more extreme environmental factors that typically induce anthocyanin accumulation (cold temperature, increased oxidative stress). Since anthocyanins, flavonols, and flavones (hereafter simply flavonoids) share upstream genes and resources, we predict that concentrations of anthocyanins and flavonoids will be correlated. We hypothesize that since the need for UV protection is greater at high elevations, that we will see a positive correlation between anthocyanins and flavonoids in floral and foliar tissue. We also investigate concentrations of the remaining non-visible flavonoid pigments as well as herbivory in natural populations in order to shed light on potential ecological relationships of the flavonoid pathway.

Methods

Population and phenotypic sampling

Silene vulgaris (Caryophyllaceae) is a gynodioecious species that grows abundantly in the French Alps in agricultural fields, waste areas, and roadsides. We identified 21 natural populations along four transects of approximately 20-25 km each in the Parc National des Écrins (Figure 1, Table 1). When populations consisted of more than 10 individuals or were particularly dense, we sampled one plant per meter along 10m transects. Otherwise we sampled the entire population. Each plant was measured for calyx color, petal color, sex (female or hermaphrodite), number of stems, height of longest stem, and proportion leaf and floral herbivory. The height of the longest stem was measured in 2011 in the Berarde and Allemond transects only, and the number of stems was measured for 2012 and 2013 in all transects. As a proxy for anthocyanin pigment concentration, we used an index of calyx color which was recorded by binning individuals into one of four color classes; 1 representing no pigmentation to 4 representing dark anthocyanin pigmentation (Figure 2). Petal color was measured as a binary trait: white or pink petals. Herbivory was assessed by estimating the average amount of damage across all leaves or flowers on each plant and classifying the proportion of damage as 0%, 25%, 50%, 75%, or

100%. Each plant was also inspected for potential herbivores and the type of herbivory present (puncture wounds, leaf miner trails, bites, or other specific browsing patterns). We also preserved one leaf from each sampled individual in a microcentrifuge tube with silica beads for DNA extraction.

Phenotypes correlated with elevation

We assumed that elevation was a suitable proxy for the environmental variables associated with each population. To test this assumption, we extracted 19 bioclimatic variables ("Bioclim") from the WorldClim dataset of interpolated global climate (http://www.worldclim.org/bioclim) (Hijmans *et al.*, 2005) from the GPS coordinates for each of our sampling locations. We used the analytical software R (version 3.0.1) package raster (v2.1-49) to assemble the climatic data and used principal components analysis in R to reduce the dimensionality of the resulting Bioclim dataset. We then used Spearman rank correlations to evaluate whether the elevation of each population was associated with the principal components. We also extracted monthly values for solar insolation for each population over the summer months (May-August) using the Area Solar Radiation tool in ArcGIS-10.1 from two 90m DEM files (strm_38_03, strm_38_04; http://srtm.csi.cgiar.org/). In ArcGIS-10.1, solar radiation (WH/m^2) represents the total amount of incoming solar insolation (direct + diffuse).

The 16 Bioclim variables were summarized by the first two principal components, which explained 96% of the total variance. Large values of Prin1 and Prin2 represent cooler temperatures and greater precipitation (respectively), and smaller values are warmer temperatures and less precipitation. Elevation was highly correlated with the first principal component (Spearman rank correlation, r = 0.85, $p < 0.0001$), thus we proceeded with further analyses using elevation as a proxy for temperature and precipitation.

To determine whether the field-measured phenotypes (population means) were correlated with elevation and summer solar radiation we calculated Pearson's correlation coefficients, as implemented within the R package Hmisc (v3.12-2). Statistical significance was determined using a sequential Holm-Bonferroni (Holm, 1979) correction for multiple comparisons ($\alpha = 0.05$; calculated with package stats (v. 3.0.1)). We ln-transformed the number of stems to improve normality. We analyzed phenotypic variables that significantly correlated with elevation with a regression to establish a causal relationship (PROC REG). We also used a logistic regression to examine whether petal color (white or pink) was explained by elevation (PROC LOGISTIC, SAS 9.3).

Floral color and foliar flavonoid measurements

In order to validate our method of using discrete calyx color classes as a proxy for calyx anthocyanin concentration, and to estimate the concentration of other floral flavonoids, we sub-sampled plants from two high-elevation populations located on different mountain peaks (Population 206, elevation 2501, N 45.060902°, E 6.40915°, N=12; Population 1031 (N=11), Table 1) and two corresponding low-elevation populations (Population 925 (N=10), Population 1001 (N=10), Table 1). Plants were sampled randomly and represented all four of the floral color classes. One open flower per plant was placed in a microcentrifuge tube for chemical analysis and transported on ice back to the Station Alpine Joseph Fourier (Col du Lautaret, Hautes-Alpes, France). Flowers were weighed, ground in liquid nitrogen with clean micropestles, and were immediately extracted with 1 mL 1% HCl in MeOH (v/v) (Harborne, 1998). After gentle shaking on ice for 30 min, samples were vortexed and centrifuged at 14000 rpm for 3 minutes. The supernatant was removed, diluted to 1/10x and 1/100x with the extraction solution, and transferred to a cuvette for spectral analysis with a Beckman DU-64 Spectrophotometer. We took the absorption value from the dilution that fell within the linear range (where absorption, A, < 1). All steps were completed as quickly as possible, and on ice, to prevent degradation.

Anthocyanins in the supernatants were measured at 530nm with a Beckman DU-64 Spectrophotometer and expressed as mg of cyanidin-3-O-glucoside (a representative anthocyanidin) per g fresh weight (Harborne & Baxter, 1999; Mato *et al.*, 2000), based on an extinction coefficient (ε) of 34300 in MeOH-HCl (Giusti & Wrolstad, 2001) and 465.1 molecular weight (MW). We also estimated non-anthocyanin flavonoids expressed as mg of quercetin equivalents ($\varepsilon = 23166$, MW= 302.236 g/mol) from absorbance values read at 372nm (Mazza *et al.*, 1999). We estimated $\varepsilon_{\text{quercetin}}$ by performing a serial dilution in 1% HCl and MeOH and by using the Beer-Lambert Equation: $A = \varepsilon \times c \times d$, where A= absorbance, c= concentration in moles per liter, and d = path length of cell (cm) (Markham, 1982). To estimate the amount of flavonoid in mg per mL, we used a modified Beer-Lambert equation: $(A \times DF \times MW \times 1000/(\varepsilon \times d))$, where DF is the dilution factor and MW is the molecular weight of the standard compound (Berardi *et al.*, 2013). We used the weight of the samples to convert the concentration in mg/mL to milligrams of flavonoid per g fresh tissue. Using this data, we performed a one-way ANOVA (PROC MIXED; SAS 9.3) and a *post-hoc* Tukey test to determine whether our calyx color bins contained significantly different anthocyanin concentrations. We also used an ANOVA to determine whether the anthocyanin and flavone concentrations, respectively, differed between high and low elevation populations with population as a random factor and elevation (high or low) as a fixed factor; SAS PROC MIXED).

To estimate the amount of foliar non-anthocyanin flavonoids in the field, we used a Dualex 4 Flavonoid and Chlorophyll Meter (hereafter “Dualex”; Force-A, Orsay, France) to estimate the total flavonoid content of one leaf on the second node below the inflorescence. The Dualex measures the amount of *in vivo* leaf epidermal flavonoids at 375 nm using the Chlorophyll fluorescence screening method (Cerovic *et al.*, 2012). Briefly, it produces an index that is the log of the ratio of near-infrared fluorescence of chlorophyll to the near-infrared fluorescence of flavonoids at their UV-A band, from which the amount of flavonoids is calculated. We performed three replicate measurements on different locations on the same leaf and took the mean of the replicates. Leaf flavonoid content was correlated with elevation, calyx color, and/or the proportion of vegetative herbivory of that individual (R package Hmisc, Pearson’s correlation coefficients). Leaves were sampled in July 2011 in the Berarde and Allemond transects (N=102).

Analyses of Phenotypic Population Variation

To examine the distribution of phenotypic variance, we first used SAS to analyze among-population variation in our measured traits. Since our experiment was conducted over several years, we first tested whether phenotypic traits (calyx color, petal color, the number of stems, stem height, proportion vegetative and floral herbivory) varied significantly between the first and second years of observation. We performed individual repeated-measures ANOVAs on population means per year using PROC MIXED with population nested in transect as the repeated subject. A ln-transformation was applied to floral herbivory and the number of stems to improve normality. We then pooled observations across years per population and tested whether each phenotypic trait varied among populations nested within transects using PROC MIXED.

To examine whether phenotypes differed between elevational areas of the transects, we delineated populations as high- or low-elevation based on previous work on *S. vulgaris* in the French Alps (Abbate & Antonovics, *in press*; Abbate, 2012); populations were assigned to the "high" class and "low" class if they were located above or below than 1300 m in elevation, respectively. We then tested for differences between high and low elevation populations in calyx color, petal color, stem number (ln transformed), stem height, and the proportion of vegetative and floral herbivory with a MANOVA to control for multiple tests since some of the traits are correlated. We used PROC GLM for the MANOVA and then separate one-way ANOVAs to further explore traits that showed significant differences between high and low elevations.

S. vulgaris individuals are either hermaphrodite or male-sterile (female) and can show sex-specific phenotypic tradeoffs and trait plasticity (Dykstra *et al.*, 2009). Since many of the populations observed were biased in their sex ratios, some consisting entirely of hermaphrodites, we tested for sex differences of the measured phenotypic traits using the same methodology above, but included sex as a covariate. However, there was never a significant effect of sex, so it was removed from further analyses.

To test specifically for clinal variation across elevation in calyx pigmentation, we first calculated the elevational distance for each population relative to the population at the lowest elevation (738m) at Le Bourg d'Oisans, Isère, France (thus removing transect as a potential nesting factor). We then used the elevational distance index as a covariate in an ANCOVA with population as a fixed factor and with mean population calyx pigmentation as the response variable.

Molecular analyses

DNA was extracted from leaves dried on silica using a standard CTAB protocol (Doyle & Doyle, 1987) for 19 out of 21 of the observed populations (N=227 plants). Samples were genotyped at eight co-dominant microsatellite markers derived from a closely related species, *S. latifolia* (SL_eSSR04, SL_eSSR05, SL_eSSR012, SL_eSSR016, SL_eSSR017, SL_eSSR020, SL_eSSR022, SL_eSSR028; Moccia *et al.*, (2009)). The molecular markers used for the present study cross-amplified in a number of *Silene* species, including *S. vulgaris*, with no evidence of null alleles or locus duplication (Moccia *et al.*, 2009). Microsatellite amplifications were performed according to Keller *et al.*, (2014) and sized on an ABI 3130xl sequencer. We used GENEMAPPER v3.0 (Applied Biosystems) to automatically score allelic sizes. After manual verification of allele size, we binned alleles using TANDEM (Matschiner & Salzburger, 2009).

Analyses of Population Structure

All population genetic parameters were calculated using the program GenoDive v2.0b24 (Meirmans & Van Tienderen, 2004). Multi-locus observed (H_o) and expected heterozygosity (H_E) were calculated for each population (Table 1), and each population was tested for Hardy-Weinberg equilibrium. To test for variation in allelic frequencies among populations, we computed pairwise F_{ST}s for all populations and for all pairwise transects. We also calculated a single global F_{ST}.

Finally, we tested for isolation-by-distance and/or isolation-by-elevation using Mantel tests with 1000 permutations and $\alpha = 0.05$. Three matrices were created for the Mantel tests: population pairwise F_{ST}, population pairwise elevational distance, and population pairwise geographic distance (measured as Euclidean distance in km with function earth.dist in R package fossil (v.0.3.7)). Neutral genetic differentiation (pairwise F_{ST}) was plotted and regressed against geographic and elevational distance between populations. Significance of the regressions was tested using nonparametric Mantel tests with 1000 bootstrap iterations (Antoniazza *et al.*, 2010). We also used partial Mantel tests with 1000 bootstrap iterations to determine the contribution of geographic and elevational distance to the variation observed in genetic distance. Partial Mantel tests allow for comparisons among one dependent variable matrix (genetic distance) and the two independent variable matrices (geographic and elevational) (Saether *et al.*, 2007; Antoniazza *et al.*, 2010; Hangartner *et al.*, 2012). All Mantel-type tests were performed with R package vegan (v2.0-8).

Comparing floral pigmentation and neutral genetic variation to detect selection

To properly study phenotypic clines, gene flow must be taken into account. First, populations within a species may diverge due to their distance and limited gene flow (Wright, 1943). Second, the effects of gene flow can erode the effects of local adaptation (Whitlock, 2008). Demographic history and population subdivision can also influence the relative effect of genetic drift, and both genotypic and phenotypic divergence may occur from the influence of drift (Wright, 1943; Whitlock, 2008). To test whether phenotypic clines in floral (calyx) color were the result of selective or neutral processes, we compared calyx color variation among our populations to a genetic index describing neutral processes of evolution (such as population history and demography). This approach, known as the F_{ST} - Q_{ST} comparison, is commonly used to study spatial variation in selection and local adaptation among populations. The Q_{ST} index estimates among-population variation of a standardized quantitative trait relative to that of neutral allelic variation (Spitze, 1993; for reviews see Merilä & Crnokrak, 2001; McKay & Latta, 2002; Leinonen *et al.*, 2008; Whitlock, 2008; Whitlock & Guillaume, 2009). While our trait of interest, calyx color, is measured in discrete bins, we found that the bins were normally distributed and that anthocyanin variation is continuous (data not shown). The components of Q_{ST} are calculated by using additive genetic variance of a trait within a population ($V_{A, within}$) and the genetic variance among populations ($V_{G, among}$); these variables are typically estimated from reciprocal transplants, controlled breeding designs, or cross-fostering experiments, following Whitlock (2008):

$$Q_{ST} = \frac{V_{G,among}}{V_{G,among} + 2V_{A,within}}$$

The premise of the comparison is that if the local environments of the measured populations differ enough to allow selection to act upon the additive genetic variation (V_A) of the trait, there will be phenotypic divergence greater than that of neutral genetic divergence ($F_{ST} < Q_{ST}$), especially if populations remain interconnected by gene flow such as in cline models (Antoniazza *et al.*, 2010). If $F_{ST} > Q_{ST}$, stabilizing selection favors the same phenotype in different populations. If $F_{ST} \cong Q_{ST}$, phenotypic differentiation is the same as could be expected from the influence of genetic drift alone.

For testing phenotypic clines and geographically variable selection using F_{ST} - Q_{ST} methods, contrasting the slopes of the regressions of F_{ST} and Q_{ST} with geographic distance between all pairwise populations constitutes a more robust test than by comparing single composite values of Q_{ST} and F_{ST} (Rousset, 1997; Antoniazza *et al.*, 2010). Thus, selection is inferred if there is a significant difference between the slopes of the regressions, with divergent selection occurring among populations with increasing geographic distance over the effect of neutral divergence resulting from drift and limited gene flow as a result of population isolation. In other words, comparing the regression slopes of F_{ST} and Q_{ST} to geographic distance, respectively, allows us to decouple the Fst/Qst comparison from the absolute magnitudes of phenotypic and genetic differentiation (Antoniazza *et al.*, 2010).

There is no prior information regarding the genetic variance of calyx color in *Silene vulgaris*, although it has been suggested that the trait is likely heritable (Marsden-Jones & Turrill, 1950, 1957). Heritability of calyx color is also indicated by the fact that populations that have deeply colored flowers in the field retain much of this color when grown in the greenhouse (A. Berardi, personal observation). We were able to estimate broad-sense heritability and among-population genetic variance in floral color from a reciprocal transplant experiment (see Chapter 3) in the vicinity of the natural populations studied in this manuscript (Station Alpine Joseph Fourier, Col du Lautaret, Haute-Alpes, France, and Le Bourg d'Oisans, Isere, France). A total of 204 maternal half-sib families (with an average of three siblings each) from 12 populations across Europe and eight French alpine populations from the current study were planted in these gardens. Since we used maternal half-sibs, we were unable to fully isolate additive genetic variance from the total phenotypic variance due to maternal effects, dominance, and epistasis (Lynch & Walsh, 1998). We therefore estimated broad-sense heritability (H^2) using the following equation based on Lynch and Walsh (1998) and using SAS PROC VARCOMP on calyx color measurements in the two experimental gardens:

$$H^2 \approx \frac{V_G}{V_P} \approx \frac{V_{dam(pop)}}{V_{pop} + V_{garden} + V_{dam(pop)} + V_{error}}$$

Because we could not fully estimate V_A, we instead used the phenotypic-Q_{ST} index known as P_{ST} (Saether *et al.*, 2007). P_{ST} is based on the same principle as Q_{ST}, but instead relies on phenotypic variance rather than additive genetic variance. We used the following equation to calculate pairwise P_{ST}, where σ^2_w and σ^2_b are the within- and between-population phenotypic variances, h^2 is heritability (for which we substituted H^2), and g is the proportion of the among-population phenotypic variation due to additive genetic effects (Spitze, 1993; Antoniazza *et al.*, 2010):

$$P_{ST} = \frac{g\sigma_b^2}{g\sigma_b^2 + 2h^2\sigma_w^2}$$

We used the mean squares (MS) from a one-way ANOVA with the fixed factor of population on calyx color to estimate within- and between-population phenotypic variances. We used the MS_w for the within-population phenotypic variances; between-population variance (σ_b^2) were estimated as:

$$\sigma_b^2 = \frac{MS_b - MS_W}{n_0}$$

where n_0 is the weighted average of sample size for each pairwise population comparison (Storz, 2002; Antoniazza *et al.*, 2010). We then determined the effect of geographic distance and elevation on calyx color differentiation by regressing the two distance factors individually with P_{ST}. We tested the significance of these matrix comparisons using Mantel tests with 1000 permutations in R (package vegan v2.0-8). We performed a sensitivity analysis on our P_{ST} measures as described in Storz (2002) and Saether *et al.* (2007) with varying values of h^2 (0.1, 0.5, 1.0) and g (0.01, 0.10, 1.00).

Lastly, we investigated whether natural selection was involved in the evolution of a calyx color cline in the French Alps. We used the method described in Antoniazza *et al.* (2010) to test whether neutral genetic variation and population history (represented by F_{ST}) alone accounted for the phenotypic differentiation (represented by P_{ST}) along elevational transects, or whether the observed phenotypic differentiation persists while controlling for neutral genetic effects. Instead of calculating partial Mantel tests among P_{ST} as a response matrix and explanatory matrices of

Fst and geographic distance (Storz, 2002; Saether *et al.*, 2007; Wilson *et al.*, 2013), a new matrix was calculated as the differences between the P_{ST} and F_{ST} matrices: P_{ST}-F_{ST}. Because P_{ST} and F_{ST} are identical under the null hypothesis in the absence of selection on the trait of interest or traits genetically correlated, the resulting P_{ST}-F_{ST} matrix is correlated with a matrix of geographic distances using a Mantel test (with 1000 permutations) (Antoniazza *et al.*, 2010). A resulting positive correlation would indicate a strong signal of selection on the phenotypic trait of calyx color over space.

Results

Patterns in floral and foliar flavonoids

Calyx anthocyanin content varied from 0.079-0.854 mg/g fresh tissue with a mean of 0.304 (±0.025 SE) mg/g fresh tissue. The concentration of anthocyanins per gram of fresh tissue varied significantly among the calyx color bins we used in the field ($F_{(3,38)} = 11.87$, $p < 0.001$). A *post-hoc* Tukey test showed that all color bins were significantly different from one another ($p<0.05$) except for the third and fourth color bins. The concentration of anthocyanins was greater as floral color got darker.

In the four populations measured in 2011 for floral anthocyanins and floral non-anthocyanin flavonoids, floral anthocyanins and non-anthocyanin flavonoids were positively associated with each other (Pearson's r=0.294, p=0.06, n=42). The two high elevation populations had more floral anthocyanins than the two low elevation populations ($F_{(1,39)} = 4.55$, $p = 0.039$). Mean anthocyanin concentration for high elevation individuals was 0.36 ± 0.18 (SD) mg per g fresh tissue, and for low elevation individuals it was 0.24 ± 0.12 (SD) mg per g fresh tissue. Floral non-anthocyanin flavonoids did not differ between the high or low elevation populations ($F_{(1,39)} = 2.50$, $p = 0.122$), with the mean non-anthocyanin flavonoid concentration for high elevation individuals at 1.89 ± 0.51 (SD) mg per g fresh tissue and low elevation individuals at 2.24 ± 0.49 (SD) mg per g fresh tissue.

We measured non-anthocyanin leaf flavonoids with the Dualex in 2011 only, so calculated phenotypic correlations between leaf flavonoids, elevation, calyx color, and leaf herbivory for this subset of plants (two transects, 10 populations: Berarde and Allemond); results are reported in Table 2. Leaf flavonoids increased with elevation (Pearson's $r = 0.40$, $n = 102$, Table 2) and with calyx color (measured as color bins, $r = 0.39$, $n = 102$). Leaf flavonoids were notably not statistically associated with the proportion of vegetative herbivory experienced by other leaves on the same plant (Table 2) as predicted.

Environmental correlations

We then determined whether phenotypes varied with the environmental proxy of elevation and with summer solar radiation in the larger dataset of four transects. Several phenotypic measures were correlated with the two environmental variables (elevation and radiation), described in Table 3. Summer solar radiation was positively correlated with elevation, negatively correlated with the number of stems, and weakly negatively correlated with herbivory (Table 3). Elevation was positively correlated with calyx color. While more traits were correlated with solar radiation than elevation, we continued with elevation as our proxy for the environment, due to its high correlation with Bioclim variables, as well as positive correlation with summer solar radiation ($r = 0.26$, $p < 0.0001$).

Phenotypic Population Variation

Calyx color, petal color, stem height, and the proportion of vegetative and floral herbivory did not significantly vary between the years each population was observed, but all traits varied among populations, as well as among transects (with the exception of petal color) (Table 4).

A MANOVA on population means testing for broad differences between transects and year showed significant across-transect variation in the proportion of vegetative herbivory ($F_{(3,16)} = 6.87$, $p = 0.0035$) and floral herbivory ($F_{(3,16)} = 16.11$, $p < 0.0001$). We observed both generalist and specialist herbivores in each transect, but we often could not find all recorded herbivore species in each transect, or at all elevations. For example, we only found *Cassida azurea* (Coleoptera: Chrysomelidae), a Caryophyllaceae specialist (Maw & Steinhausen, 1980; Peschken *et al.*, 1997) in the Berarde transect. We saw 24-spotted ladybird beetle larvae (*Subcoccinella vigintiquattuorpunctata*), which inflicted significant damage on plants from mid-elevation to high-elevations in all transects. Slugs were only seen at lower elevations. Grasshoppers, caterpillars, aphids, and thrips were seen at all elevations and transects.

A MANOVA on the phenotypic traits testing for differences between high and low elevation populations suggested that high- and low-elevation populations exhibit significant phenotypic differences (Wilk's λ, $F_{(6, 97)} = 3.34$, p=0.0049). In subsequent one-way ANOVAs, significant effects of elevation were found for calyx color ($F_{(1,103)} = 8.55$, $p = 0.0043$) and the proportion of floral herbivory ($F_{(1,103)}$=4.90, p=0.0291). High-elevation populations had darker calyces (2.85 ±0.100 SE) than low-elevation populations (2.31 ±0.143 SE), and more proportional floral herbivory (0.268% ± 0.023 SE) than low populations (0.176% ± 0.034 SE). Thus, high elevation phenotypes across all transects were marked by darker calyces with higher herbivory, and low elevation phenotypes were marked by lighter calyces and less floral herbivory.

Phenotypic associations along elevational gradients and clinal variation

Plant traits tended to correlate with each other, but especially along the elevational gradients. Our focal phenotype, calyx color, was positively correlated with the number of stems and negatively correlated with stem height, suggesting that plants with darker calyces tend to have many short stems and plants with lighter calyces have fewer long stems.

To better visualize whether there were any associations of phenotypic covariance with elevation, we plotted mean population trait values along elevation (Figure 3). Of the traits measured, anthocyanin-based traits were the only ones to significantly associate with elevation (Table 3, Figure 3). Pink petals were rare, but the frequency of pigmented petals (as a binary trait) increases significantly with elevation (PROC LOGISTIC, SAS 9.3, Wald's $X^2 = 7.897$, $p = 0.005$). The population mean calyx color increased with elevation (Figure 4). We then tested for the presence of a phenotypic cline along elevation in mean population calyx color using a traditional ANCOVA approach, $F_{(18,31)}=3.18$, $p = 0.0230$ (with elevational distance to the lowest population as the covariate, see Methods). Finding evidence for a phenotypic cline in calyx color, we next evaluated whether there was evidence for divergent selection on this trait using $F_{\mathrm{ST}} - P_{\mathrm{ST}}$.

Population structure

All populations were in Hardy-Weinberg equilibrium (p=0.001), and mean heterozygosity for each population is reported in Table 1. The neutral genetic structure of *S.*

vulgaris populations within the ~25km^2 area observed in the French Alps was low, though significantly different from zero, with F_{ST} = 0.053 (97.5% confidence interval 0.037 - 0.079). We found significant isolation-by-geographic distance (Mantel test R^2 = 0.335, p = 0.006) and significant isolation-by-elevational distance (Mantel test R^2 = 0.213, *p* = 0.023). A partial Mantel test showed that geographic distance contributes significantly to isolation-by-distance even after controlling for elevation (R^2 = 0.3074, *p* = 0.0020). In contrast, elevation did not significantly contribute to isolation-by-distance after geographic distance was taken into account ($\alpha = 0.05$, R^2 = 0.163, p = 0.073), but the near-significance might suggest that elevation may be involved in variation in allele frequencies.

Comparing calyx color patterns and neutral genetic differentiation

After establishing the presence of a phenotypic cline in mean population calyx color, and estimating pairwise Fst, we then tested for divergence in calyx color while controlling for neutral population differentiation. The broad-sense heritability (H^2) for calyx color estimated was 0.181. The relative amount of phenotypic variance among populations due to genetic effects (*g*) was 0.0475 (4.8%). Mean (overall) calyx color P_{ST} = 0.0476 (95% CI 0.04022 - 0.05450) and mean F_{ST} = 0. 0523 (95% CI 0.04850 - 0.0561). A Welch two-sample t-test showed that the overall estimates for P_{ST} and F_{ST} were not significantly different from each other (t = -1.1135, df = 620, *p* = 0.2659), but the range of pairwise P_{ST} (0 - 0.5178, 21 populations) was markedly greater than the range of pairwise F_{ST} (0 - 0.133, 19 populations). So, when comparing the global estimated values for F_{ST} and P_{ST} were not different from each other, indicating that, in general, there is no evidence for divergent selection on calyx color.

We then incorporated geographic and elevational distance among pairwise P_{ST} and F_{ST} values. There was not a significant association of pairwise P_{ST} alone with geographic distance (Mantel $R^2 = 0.0627$, p = 0.234), but there was a strong and positive association between pairwise P_{ST} and elevational distances (Mantel $R^2 = 0.394$, $p < 0.001$), suggesting significant color differentiation in color in elevational space. More importantly, $P_{ST} - F_{ST}$ allows for the comparison of pairwise population differences between ($P_{ST} - F_{ST}$), while also controlling for the effect of population and demographic history. We found, again, a similar pattern of ($P_{ST} - F_{ST}$) with respect to geographic distance and elevational distance: a Mantel test with geographic distance as an explanatory matrix variable was not significant (Mantel $R^2 = -0.035$, p = 0.651), however, a Mantel test with elevation as an explanatory matrix variable was significant and positive (Mantel $R^2 = 0.341$, p=0.002) (Figure 5). Thus, incorporating elevation and population history indicates that $P_{ST} > F_{ST}$ and that divergent selection maintains differences between calyx color at high and low elevations.

Sensitivity analysis

We varied values of H^2 and g in a sensitivity analysis to explore the range of potential P_{ST} values given the phenotypic variance in our populations (Table 5). Overall estimates of P_{ST} increased with larger values of additive genetic variance between populations (g), and increased with decreasing H^2 (proportion of genetic variance to phenotypic variance). This result suggests that increasing phenotypic divergence, especially that due to additive genetic variance, will produce a larger value for P_{ST}. Our current estimate of H^2 produces a conservatively low estimate of P_{ST}; a narrow-sense heritability estimate (h^2) would likely be smaller than H^2 as non-additive genetic effects would be removed, thus increasing the value of P_{ST}.

Discussion

We found a floral color cline in *Silene vulgaris*, with darker calyx color increasing with elevation, across several transects. There were significant differences in calyx color among high and low elevation populations, with elevation as a covariate. Using both an F_{ST}-Q_{ST}/P_{ST} and ANCOVA approach, we found evidence for spatially divergent selection on floral color, with phenotypes diverging more than would be expected under a neutral model. Although global F_{ST} = P_{ST}, indicating that phenotypes have not diverged other than what one would expect from genetic drift, one might expect that measuring the average difference of a diverged phenotype along a gradient to even out. Thus, comparing population pairwise P_{ST} and F_{ST} allows for a more sensitive detection (Antoniazza *et al.*, 2010; Hangartner *et al.*, 2012). Pairwise comparisons of the highest elevation populations and the lowest elevation populations were the most divergent. Incorporating geography into this model with partial Mantel tests controls for isolation-by-distance effects. The pattern of calyx color divergence held across elevational distance, and not geographic distance.

We also predicted that the structure of the flavonoid pathway, responsible for floral pigments as well as flavonoids responsible for UV herbivore defense, would create correlations between anthocyanins and non-anthocyanin flavonoids. Anthocyanins and non-anthocyanin flavonoids were negatively correlated in flowers, suggesting a potential within-tissue trade-off; this interaction was stronger at high elevations where the concentration of anthocyanins was the highest. Thus, our prediction regarding more non-anthocyanin flavonoids at higher elevations due to higher UV radiation was not supported in calyx tissue; suggesting that anthocyanins may play multiple phenotypic roles. However, we also found that leaf flavonoids were positively correlated with calyx color and elevation, suggesting that individuals with darker colored calyces had leaves with more flavonoids. These individuals occur more frequently at higher elevations. This supports our prediction of having more UV-protecting flavonoids at higher elevations. Therefore, non-anthocyanin flavonoids may only protect against harmful UV radiation in leaf tissues. Having high concentrations of leaf flavonoids did not, however, correlate with better protection against herbivores, thus suggesting that leaf flavonoids are not effective defensive compounds against herbivores in *S. vulgaris.*

Variation in the environment including temperature, precipitation, photoperiod, growing season length, and biotic factors are likely to result from differences in geographic position and elevation (Antoniazza *et al.*, 2010; Wilson *et al.*, 2013; McKown *et al.*, 2014). The resulting environmental gradients can exert strong selection, creating phenotypic clines with greater than neutral genetic structure depending on the level of gene flow and population history (Savolainen *et al.*, 2007). Spatially divergent selection and clinal adaptation has been documented in several plant species and morphometric traits (Antonovics & Bradshaw, 1970; Streisfeld & Kohn, 2005; Keller *et al.*, 2009; Méndez-Vigo *et al.*, 2011; McKown *et al.*, 2014) and also in floral color (Streisfeld & Kohn, 2005; Schemske & Bierzychudek, 2007; Hopkins & Rausher, 2012). Clinal variation has been documented in *Silene vulgaris*, where both population history and geographically varying environments contributed to phenotypic divergence across temperature and precipitation clines in North America and Europe (Keller *et al.*, 2009). Certainly, phenotypic covariance between traits and the environment can arise from non-adaptive processes and must be taken into account (Keller *et al.*, 2009); thus the F_{ST}-Q_{ST}/P_{ST} approach is theoretically robust to detecting a phenotypic cline.

The flavonoid biosynthetic pathway is a powerful framework for framing hypotheses regarding the connection between genotype (pathway locus) to phenotype (flavonoid pigmentation) and the ecological roles of flavonoids. Because the flavonoid pathway produces several groups of compounds associated with important ecological roles, including defense against herbivory and protection from harmful UV radiation, we predicted that there may be positive or negative correlations (trade-offs) among traits due to shared activating enzymes and regulatory elements. Specifically, we predicted that there would be a phenotypic correlation between vegetative and floral herbivory and floral anthocyanins. We did not detect any broad patterns that associated the intensity of herbivory or florivory with floral color. Both herbivory and floral herbivory were highly variable both among and within populations and transects, which could have hindered our ability to detect associations between herbivory/florivory and floral color. It is also possible that flavonoid defenses are mostly inducible and difficult to detect with the experimental design that we used. In addition, the chemical compounds produced as a defense against herbivores may not be products of the flavonoid pathway in the this system. *S. vulgaris* is known to produce triterpenoid saponins (Glensk *et al.*, 1999) and ecdysteroids (Mamadalieva, 2012), which may play a more prominent role in defense against herbivores and pathogens.

We found that anthocyanin expression in calyces and petals increased with elevation. The effect of elevational location is particularly clear when population calyx color is compared between the highest and lowest populations. In our tests for the presence of a phenotypic cline, we did observe that there was an isolation-by-distance effect of elevation but not geographic distance on calyx color. This observation is consistent with our expectations, as several abiotic factors varied with elevation, including solar radiation, temperature, and moisture. In addition, many plants tend to adopt darker phenotypes with increasing elevation (Billings & Mooney, 1968).

Given there is more solar radiation at high elevations than at low elevations, we hypothesized that we would observe higher amounts of non-anthocyanin flavonoids present in all tissues as a mechanism to protect individuals from detrimental UV radiation, or sunscreen. Previous work in *S. vulgaris* and in *S. latifolia* demonstrated an increase in flavone accumulation under high light in laboratory settings in petal epidermal cells (Brederode & Kooten, 1983) and in leaf tissue (van de Staaij *et al.*, 1995); no one to date has explored the calyx tissue specifically. Our prediction regarding leaf sun-screening was verified by the increased concentration of leaf flavonoids in high elevation populations. However, we did not see an increase in the same compounds in floral tissue (where whole flowers, dominated by calyx tissue, were analyzed). Rather, we saw a negative correlation of UV-absorbing compounds with elevation. This suggests that there may be a trade-off between floral anthocyanins and non-anthocyanin floral flavonoids at high elevations, suggesting a “flavotype” of average high elevation flavonoid expression compared to average low elevation flavonoid expression. This tradeoff might only be realized under conditions (e.g., high elevations) in which resources are often limited and/or other environmental stressors are more common. Calyces and petals will be separately analyzed in future studies to further tease out the tissue-specific response to high elevation UV-B radiation.

These observations suggest a number of questions concerning how high-elevation *S. vulgaris* flowers defend themselves against strong solar radiation. Foliar flavonoids have been shown to provide protection for the photosystems and tolerance to light and solar radiation (Jansen *et al.*, 1998; Ryan *et al.*, 2001; Gould, 2004; Treutter, 2006). It is also possible that protection against harmful UV radiation by flavonoids is A) not as important in the floral tissues as it is in the photosynthetic leaf tissue, B) performed by another class of compounds entirely, and/or C) is performed by anthocyanins. Anthocyanins do accumulate in times of generalized plant stress including UV radiation (Steyn *et al.*, 2002), but are not specifically known for UV protection. The proposed mode of action of anthocyanins in vegetative tissue is to reduce oxidative pressure, and therefore prevent oxidative damage (Lee & Gould, 2002); this could also occur in floral tissues. Anthocyanins can accumulate in all tissues, including floral tissues, under temperature stress (Stiles *et al.*, 2007; Umbach *et al.*, 2009), and may instead be absorbing light and warming the reproductive organs protected by the calyx (Lacey & Herr, 2005; Lacey *et al.*, 2010). UV-B raditaion, in small amounts, can actually stimulate anthocyanin production in general (Chalker-Scott, 1999) and is even expected in photomorphogenesis in plants (Heijde *et al.*, 2013). Because the amount of visible green pigmentation diminishes in more deeply pigmented flowers, it is possible that photoprotection is left to the leaves and thermoregulation is left to the flowers. Another possibility is that chemicals produced to ameliorate the photo-oxidative stress might be members of other metabolic groups (e.g. glutathione, ascorbate, diterpenes, or other non-flavonoid phenolics; Alonso-Amelot, 2008). Finally, *S. vulgaris* may have adapted in other ways to either tolerate or avoid the UV-B radiation (Alonso-Amelot, 2008). Indirect support for this possibility is provided by the fact that some of the highest elevation populations featured plants with leaves that were comparatively smaller, more succulent, and more pubescent than those at the lowest elevation.

We are compelled to assume that darker calyxes and petal pigmentation at higher elevations results largely from selection via abiotic factors rather than pollinators. This interpretation derives from the expectation of increased environmental stress (temperature fluctuation, high UV radiation) and the observation of an increase in self-compatible hermaphrodites. Further studies estimating the contribution of pollinator-mediated selection to the floral flavotype need to be conducted.

Additional information concerning flavonoid accumulation in flowers and leaves across our transects could be made with HPLC (high-performance liquid chromotography) and potentially with a larger sample size from more populations to determine the exact nature of the hypothesized trade-off between anthocyanins and other flavonoids within and among tissues. Chapter 1 describes the prevalence of the flavone glycosides saponarin, orientin, and vitexin which have been described as epidermal UV screening compounds (Caasi-Lit *et al.*, 2007), in all tissues of *S. vulgaris*. Preliminary HPLC data suggests that flowers at high elevations may have fewer non-anthocyanin flavonoids than at lower elevations.

Concerns have been raised regarding the appropriateness of P_{ST}-based analyses that involve phenotypic traits with significant plasticity and environmental induction (Pujol *et al.*, 2008; Whitlock, 2008). Because our estimates of heritability and genetic variance come from a reciprocal transplant study on the offspring of members of the populations under current study and the general European range of *S. vulgaris* (Chapter 3), and because the referenced experimental gardens are located in the area of study, our estimates should be proportionally correct, with minimal inflation of P_{ST} due to environmental effects. Anthocyanin accumulation has been repeatedly shown to be both a constitutive and an environmentally inducible trait (Irwin *et al.*, 2003; Connor *et al.*, 2005; Stiles *et al.*, 2007; Fournier-Level *et al.*, 2009), and pigmentation in *S. vulgaris* certainly has an environmentally-induced component of its anthocyanin phenotypes (perhaps contributing to a low $H^2 = 0.18$, see Chapter 3); but the present study describes the patterns of coloration due to genetic divergence. In addition, phenotypic clines across elevation are not uncommon in plants and animals as selective pressures can be very strong in high elevation environments due to temperature, sunlight, and other more extreme environmental variables (Clausen *et al.*, 1948; Angert & Schemske, 2005; Wilson *et al.*, 2013).

Our P_{ST} sensitivity analysis showed that with our conservative estimates of additive genetic proportion of variance between populations (g) and heritability (H^2), we still largely see values of P_{ST} that are greater than F_{ST}. Low estimates of *g* and high estimates of H^2 would potentially place the overall P_{ST} as less than F_{ST}, but we know that our current estimate of H^2 is higher than the narrow-sense heritability for calyx color will be because non-additive genetic effects will be removed from an h^2 estimate. Our analysis suggests that a low value of heritability will produce a much higher value for P_{ST} than F_{ST} (0.05).

We show in the present study that calyx color in *S. vulgaris* is the result of divergent selection along an elevational gradient in the presence of relatively large quantities of gene flow. Calyx color phenotypes have diverged more markedly among populations with increasing elevational distance than populations diverge at neutral genetic loci. Trade-offs between flavonoid traits and opposite correlations in tissues, suggest this is a potentially powerful system to study how changes in suites of selection pressures may interact with underlying biochemical associations to influence adaptive divergence.

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Table 1. Population sampling characteristics

Transect	Population	Elevation (m)	Longitude	Latitude	N		Years Observed	H_o	H_e
					Genotyped	Phenotyped [a]			
Allemond	925	789	6.04015	45.131489	9	26	2011, 2013	0.462	0.496
	313	892	6.05594	45.14724	10	10	2011	0.401	0.5
	314	1113	6.06345	45.15235	8	13	2011	0.505	0.569
	316	1326	6.08661	45.16242	10	80	2011, 2013	0.425	0.503
	315	1428	6.09422	45.16886	11	19	2011	0.417	0.498
	1313	2079	6.10378	45.18377	14	34	2013	0.383	0.594
Berarde	1001	738	6.0282	45.048389	10	20	2011	0.45	0.526
	311	846	6.07875	44.99456	10	76	2011, 2013	0.485	0.542
	1026	933	6.1085	44.988029	17	64	2011, 2013	0.421	0.488
	1031	1586	6.20197	44.93494	11	92	2011, 2013	0.521	0.554
	310	1608	6.25663	44.93702	10	24	2011, 2013	0.408	0.534
Lautaret	Clapier	779	6.073554	45.030137	17	17	2013	0.295	0.528
	803	978	6.126083	45.04155	17	74	2012, 2013	0.39	0.585
	Freaux	1380	6.283591	45.044179	16	83	2012, 2013	0.348	0.691
	143	1743	6.343683	45.040283	--	16	2013	--	--
	195	2210	6.398067	45.040501	16	10	2012	0.444	0.626
Valloire	254	824	6.469326	45.210131	10	25	2012, 2013	0.411	0.633
	145	1232	6.46933	45.19779	11	25	2012, 2013	0.438	0.607
	Valloirette	1594	6.419244	45.137145	--	16	2013	--	--
	252	1776	6.4688	45.211117	10	53	2012, 2013	0.466	0.676
	317	2129	6.43048	45.08719	10	37	2012, 2013	0.45	0.474

[a] phenotyped individuals were pooled over the two years of observation

Table 2. Associations between leaf flavonoids from a subset of the data measured with the Dualex meter in two transects, Allemond and Berarde (5 populations each) in 2011.

	Elevation	**Calyx Color**	**Leaf Flavonoids**	**Vegetative Herbivory**
Elevation	--	<0.0001 185	<0.0001 102	NS 150
Calyx Color	0.37	--	<0.0001 102	NS 150
Leaf Flavonoids	0.4	0.39	--	NS 102
Vegetative Herbivory	-0.12	-0.08	0.03	--

Pearson's correlation coefficients (below diagonal), P values (above diagonal), and sample sizes (above diagonal) are shown. Underlined correlation coefficients are those considered statistically significant ($p < 0.05$) following a Holm-Bonferroni correction.

Table 3. Phenotypes correlated with environmental variables in *S. vulgaris* across all four transects and years.

	Elevation	**Solar Radiation**	**Calyx Color**	**Stem Number**	**Stem Height**	**Vegetative Herbivory**	**Floral Herbivory**
Elevation	--	<0.001 859	<0.001 858	NS 522	NS 111	NS 541	NS 540
Solar Radiation	0.26	--	NS 858	<0.001 522	NS 111	<0.001 541	<0.001 540
Calyx Color	0.41	0	--	<0.001 521	<0.001 111	NS 540	NS 539
Stems	0.05	-0.33	0.24	--	NS 111	NS 521	<0.001 520
Stem Height*	-0.16	-0.2	-0.49	-0.16	--	NS 111	NS 111
Vegetative Herbivory	-0.06	-0.15	-0.1	0.15	0.27	--	<0.001 540
Floral Herbivory	-0.06	-0.21	-0.04	0.25	0.04	0.33	--

Pearson's correlation coefficients (below diagonal), P values (above diagonal), and sample sizes (above diagonal) are shown. Underlined correlation coefficients are those considered statistically significant ($p < 0.05$) following a Holm-Bonferroni correction. Elevation was measured in meters, solar radiation in WH/m^{2}, stem number (ln-transformed), stem height measured in cm, vegetative and floral herbivory measured as proportion total damage per plant.

* Stem height was measured in two transects, Berarde and Allemond, in 2011.

Table 4. Results of mixed-model ANOVAs for trait variation among populations. Each model also included year, which was not significant.

	Pop(Transect)			**Transect**		
Trait	*F*	df	*p*	*F*	df	*p*
Calyx color	11.38	17, 732	<0.0001	14.68	3, 18	<0.0001
Petal color	3.97	17, 733	<0.0001	2.20	3, 18	0.0864
Number of Stems*	14.35	16, 433	<0.0001	27.80	3, 10	<0.0001
Stem height	23.58	6, 95	<0.0001	20.74	1, 9	<0.0001
Proportion vegetative herbivory	2.71	17, 429	0.0003	23.47	3, 18	<0.0001
Proportion floral herbivory	2.23	17, 428	0.0034	30.79	3, 18	<0.0001

*ln-transformed

Table 5. Results of P_{ST} sensitivity analysis. Our calculated overall $P_{ST} = 0.0476$.

	$g = 1$	$g = 0.1$	$g = 0.01$
$H^2 = 1$	0.1294	0.0205	0.0022
$H^2 = 0.5$	0.1967	0.038	0.0044
$H^2 = 0.1$	0.396	0.1294	0.0205

Figure 1. Map of transects and population sites in the French Alps. The location of the Station Alpine Joseph Fourier indicated with “*”.

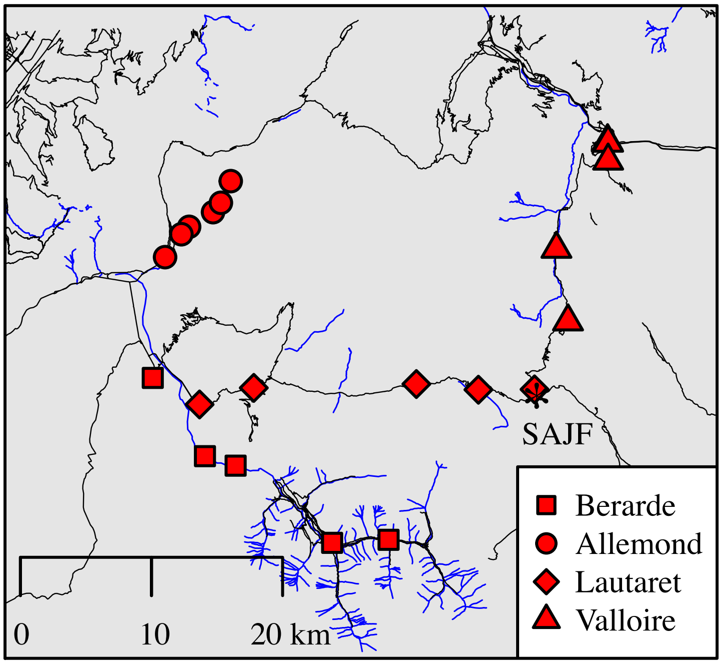


Figure 2. Calyx color classes. Clockwise, starting with upper left: 1, 2, 4, 3.

Figure 3. Plots of population mean phenotypes across elevation (m). Stem height only shows data from one year of observation. Significant correlations (petal color measured as logistic regression) ($p < 0.05$) are denoted with “*”.

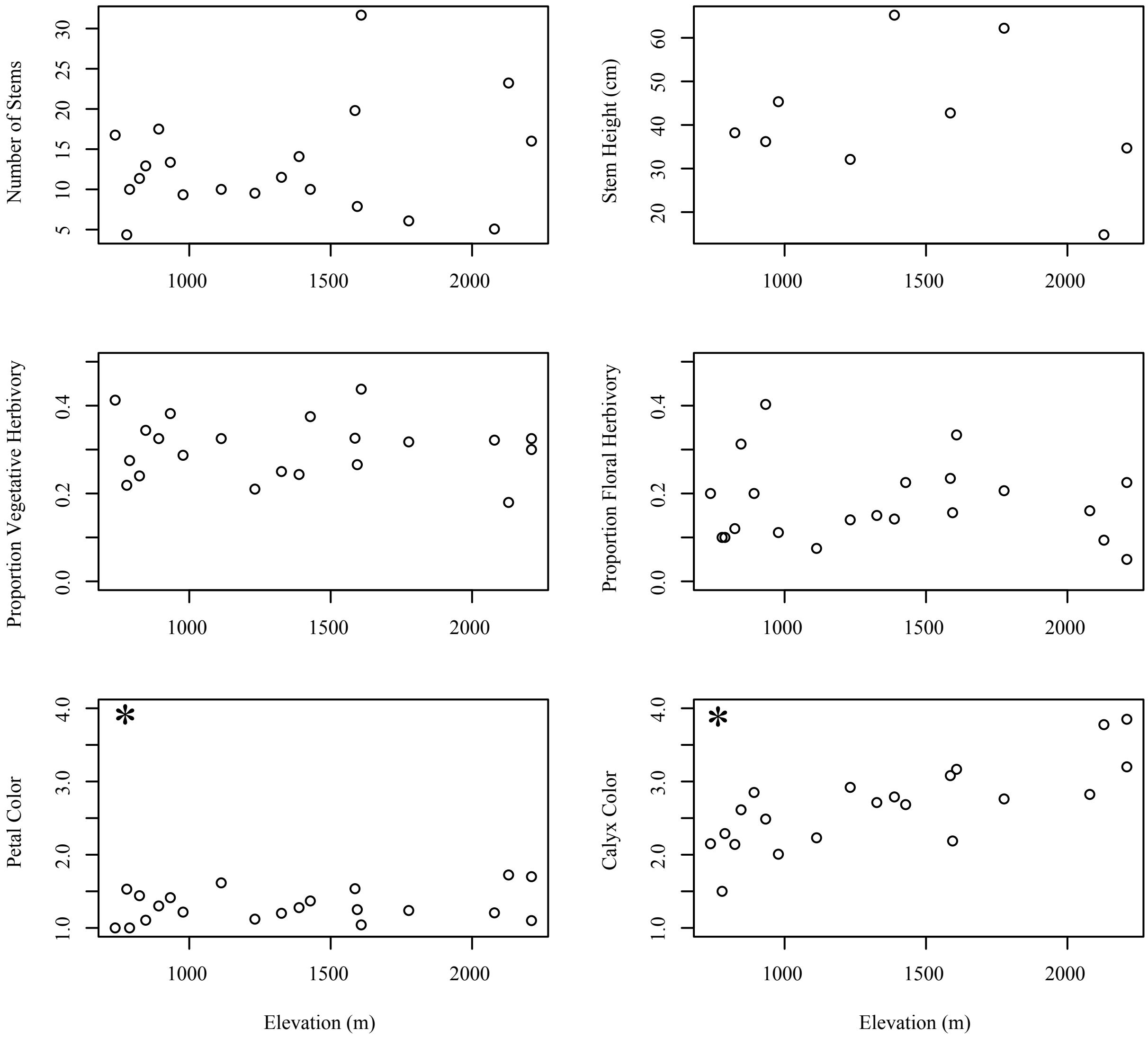


Figure 4. Population means of calyx color (standard error bars) by elevation. Linear regression $R^2 = 0.593$, $p < 0.0001$.

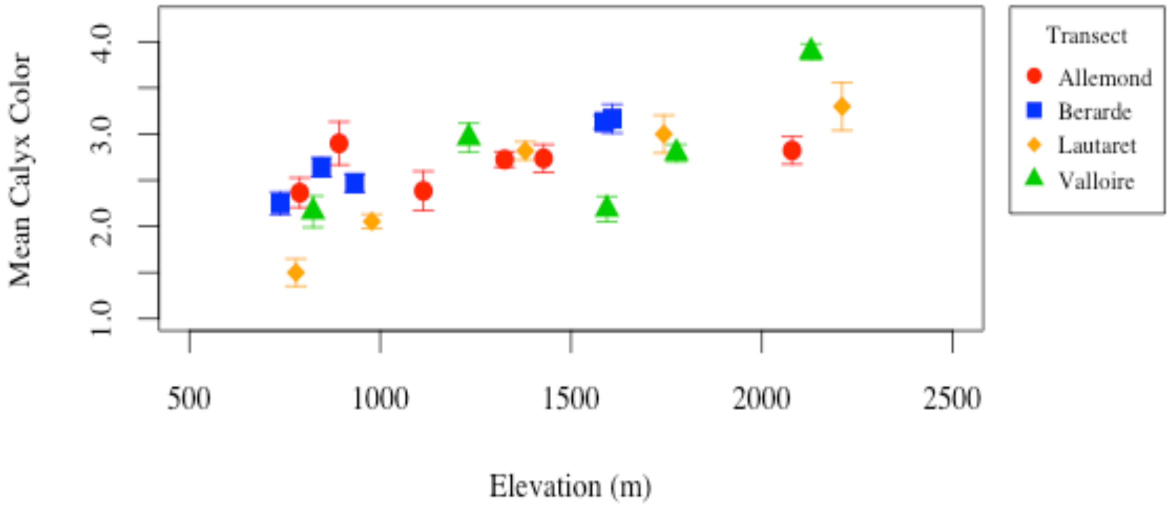
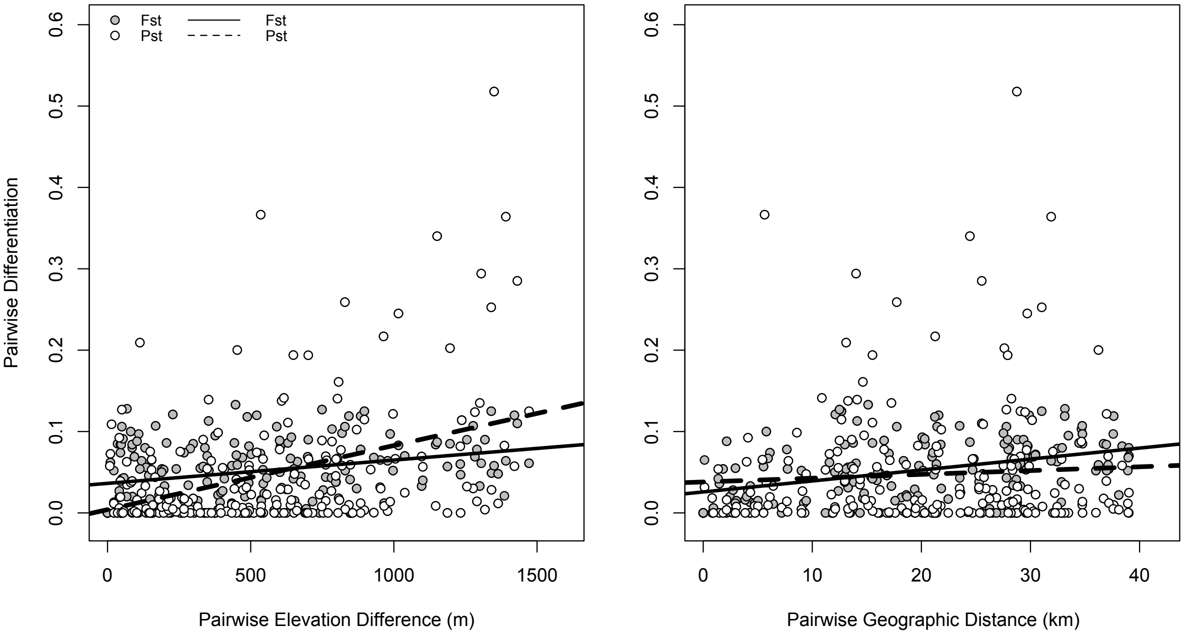


Figure 5. Scatterplots of the (left) pairwise elevation difference, and (right) pairwise geographic distance relative to F_{ST} and P_{ST}. There is a significant difference between P_{ST} - F_{ST}, (or, the slopes of F_{ST} and P_{ST}) for elevation (Mantel $R^2 = 0.341$, $p = 0.002$) but not for geographic distance (Mantel $R^2 = -0.035$, $p = 0.65$), suggesting that phenotypic divergence in calyx color occurs over elevational distance rather than geographic distance.



CHAPTER 3:
GENETIC DIFFERENTIATION AND ENVIRONMENTAL EFFECTS: FLAVONOID PHENOTYPES AND LOCAL ADAPTATION OF SILENE VULGARIS TO DIFFERENT ELEVATIONS

Abstract

The plant flavonoid pathway can contribute to local adaptation, such as through the evolution of flower color, or can have a phenotypically plastic response to environmental variation. To estimate the contribution of the flavonoid pathway to local adaptation along an elevational gradient, I used a reciprocal transplant design, where plants from 10 high- and 10 low-elevation populations of *Silene vulgaris* were grown in high- and low-elevation gardens. To quantify adaptive differentiation in flavonoid production and local adaptation, I measured floral color, herbivory, growth, fitness components, and the concentrations of both leaf and floral flavonoid compounds. Populations derived from high elevations had high concentrations of anthocyanins in their flowers and leaves with higher flavone concentrations with a strong genetic component. However, populations from low elevations had a strong plastic response, increasing their floral color and leaf flavones when grown at higher elevations. Other aspects of the flavonoid pathway showed plasticity, such as leaf anthocyanins. There was evidence for local adaptation to elevation with the proportion of flowering individuals being higher when plants were grown in their native sites. Overall, the expression of some traits in the flavonoid pathway suggested a strong genetic influence, while the majority of the phenotypes I measured had an even stronger influence of plasticity.

Introduction

Plants are unable to escape the habitat in which they grow, creating a challenge for survival in unfavorable or heterogeneous conditions. However, plants cope within and across generations to the heterogeneous environment by genetically adapting to local conditions and by exhibiting phenotypic plasticity (Sultan, 2000; Agren & Schemske, 2012; Steiner *et al.*, 2012). Adaptation to the local environment through selection leads to increased fitness in that particular habitat (Antonovics & Bradshaw, 1970; Kawecki & Ebert, 2004). However, if the environment changes quickly and the phenotype is no longer ideal, fitness may decline, especially if populations are marginal, inbred, and/or small (Antonovics, 1976; Etterson & Shaw, 2001; Leimu & Fischer, 2008). Plasticity, on the other hand, can be advantageous for reacting to a frequently or rapidly changing environment (Schlichting, 1986; Scheiner, 1993; Sultan, 2000). Plasticity itself can be adaptive, especially for those plant populations found in constantly changing habitats, such as alpine environments (Via *et al.*, 1995; Stöcklin *et al.*, 2009; Auld *et al.*, 2010; Steiner *et al.*, 2012). However, if a trait's plasticity is costly to produce or maintain, there may be limitations to its evolution (DeWitt *et al.*, 1998; Weinig *et al.*, 2004). In addition, specialized genotypes may show the highest plasticity among environments, but the plasticity is not necessarily adaptive: it is a consequence of specialization (Taylor & Aarssen, 1988). Given the benefits and costs of both constitutive and plastic strategies, they are rarely mutually exclusive. For example, Clausen *et al.* (1940) found evidence for both genetic differentiation as well as plasticity in *Potentilla glandulosa* plant height in their classical reciprocal transplant experiment.

The flavonoid biosynthetic pathway produces secondary metabolites in all plants; flavonoid pigments are responsible for important ecological aspects of plant life, such as floral color, anti-feedants to herbivores, antibacterial and antifungals, and as sun-screening ultraviolet (UV) radiation absorbers (Figure 1, Winkel-Shirley, 2001). Several subclasses of flavonoids can perform more than one role: anthocyanins are more commonly known to act as floral pigments, but also demonstrate antioxidant activity and can accumulate in all plant tissues during times of stress (Steyn *et al.*, 2002; Gould *et al.*, 2010). Flavones and flavonols are known to accumulate under UV radiation stress, as well as act in the defense against herbivores and pathogens (Winkel-Shirley, 2001).

Given the substantial role of flavonoids in the way plants respond to their environment, their activity across elevational gradients is of particular interest. Elevational gradients include different suites of selective conditions, including shifts in herbivores, pollinators, and UV radiation, exerting different selective pressures on plant populations over small scales due to steep environmental changes (Arnold *et al.*, 2009; Steiner *et al.*, 2012; Matter *et al.*, 2014). In fact, the Alps are one of the regions where the highest UV levels are measured in Europe, with UV-B levels increasing up to 16% per 1000 m (Schmucki & Philipona, 2002). Many studies have documented increased flavonoid production in various plants species with increased UV-B radiation in experimental settings (e.g. Jordan *et al.*, 1994; Bieza & Lois, 2001; Treutter, 2006).

High elevation environments are characterized by higher UV radiation, lower atmospheric pressure, lower temperatures, more climate fluctuation, snow, and a shorter growing season than at lower elevations (Tranquillini, 1964; Blumthaler, 2007). UV-B (315 – 280 nm) radiation influences expression of multiple branches of the flavonoid pathway; up-regulation of the pleiotropic chalcone synthase (CHS; the first committed step of the flavonoid pathway) may also up-regulate all or some of the downstream flavonoid subgroups, not only those directly involved with UV radiation amelioration (Treutter, 2006; Figure 1). Specifically, elevated UV-B levels have increased flavonols and flavones in vegetative tissue, thought to reduce the amount of oxidative damage by absorption (Jordan *et al.*, 1994; Ryan *et al.*, 2001; Close *et al.*, 2003). Anthocyanins accumulate under a more broad scale of environmental induction, including UV radiation stress, but notably in foliar *and* floral tissues (Steyn *et al.*, 2002; Gould *et al.*, 2010).

Flavones and flavonols can act as defensive compounds within vegetative tissues as protection against herbivores and fungal and bacterial pathogens (Soriano *et al.*, 2004; Slana *et al.*, 2011), but their role in defense against herbivores and pathogens is less clear than their role in protection against UV radiation. Flavonoids are produced constitutively and also induced by infection and browsing, but interactions between specific herbivores and plant flavonoid defenses vary in the literature (reviewed in Treutter *et al.* 2006).

Taken together, I expect high elevation populations to have increased amounts of flavones or flavonols to protect against oxidative damage, but it is unknown if there will be a coordinated change in production of the rest of the flavonoid pathway, such as the anthocyanins, given the need for the increase in production of one particular subgroup. If there is a trade-off between flavonoid subgroup production, will there be observable trade-offs in the related ecological roles? For example, if flavone production is increased in the entire plant, must there be a reduction in floral color, or will the plants be less chemically defended against herbivore attack? Within-tissue patterns of floral color differences in purple and white striped Petunia flowers showed that the anthocyanin-pigmented sections experienced less herbivory than the white (anthocyaninless) sections in a laboratory setting (Johnson *et al.*, 2008). Few studies have attempted to examine trade-offs or correlations in flavonoids in multiple tissues and in ecological roles in the field by specifically measuring chemicals in separate plant organs (*but see* Simms & Bucher, 1996; Fineblum & Rausher, 1997).

Several different flavonoid-related traits are expected to be involved in adaptation to elevation, and their biochemical relationships may constrain (or permit) a coordinated phenotypic response to selection (Proulx *et al.*, 2005; Campbell *et al.*, 2013). I aimed to determine the extent to which the flavonoid phenotypes (in the form of concentration and flavonoid chemical diversity, termed "flavotypes") at high and low elevations result from genetic differentiation, phenotypic plasticity in response to the environment, and GxE interactions. The flavonoid pathway is known for being heritable and having genetic variance (Brown & Clegg, 1984; Johnson *et al.*, 2009), as well as being phenotypically plastic and inducible (Winkel-Shirley, 2002; Soriano *et al.*, 2004; Lu *et al.*, 2009). Populations of *S. vulgaris* have darker flower color, thus higher concentrations of floral anthocyanins at higher elevations (Chapter 2). Higher elevations also have increased UV radiation, thus requiring protection. I therefore predict that the entire pathway is up-regulated in the floral tissues. Since protection against UV radiation is theoretically necessary in all tissues, I also predict that anthocyanins and flavones will increase in leaf tissue with elevation as well. What remains unclear is how herbivores react to differences in flavonoid concentrations in multiple tissues at different elevations, and whether or not there will be any fitness costs or benefits to increased flavonoid production.

Few studies have evaluated the flavonoid pathway in the field, and determined its role in adaptation to different environments (*but see* Johnson *et al.*, 2009). I conducted an experiment to estimate whether local adaptation has occurred at high and low elevations in a widespread plant, and then determine whether flavonoids and their ecological counterparts may contribute to local adaptation by comparing metabolite production to fitness proxies in the field. I used several populations of *Silene vulgaris* to answer the following questions with a reciprocal transplant study in its native range: 1) Is there differential phenotypic expression of the flavonoid pathway between high and low elevations, with special attention to flavonoid subgroups present in *S. vulgaris*, 2) to what extent is the flavonoid phenotype attributed to genetic variation and plasticity, and 3) is there evidence for local adaptation to elevation environments, and is the flavonoid pathway a likely contributor? Reciprocal transplants of the high and low elevation populations into both sites will help to distinguish whether there is a genetic basis to flavonoid variation and not just environmental induction (Kawecki & Ebert, 2004), and whether there has been local adaptation to a high or low elevation *sensu* Clausen et al. (1940). I include growth-related measures to account for plant size, which could influence herbivores and pollinators, and also potentially trade off with the production of secondary metabolites such as flavonoids (Stamp, 2003; Strauss *et al.*, 2004; Hofmann & Jahufer, 2011).

Methods

Study system

The herbaceous perennial *Silene vulgaris* (Moenche) Garcke ("bladder campion", Caryophyllaceae) is widespread in its native Eurasia and introduced North America and is found in agricultural fields and roadsides, and other disturbed areas across a broad elevational range (sea level to ~2400 m) (Taylor & Keller, 2007; Keller *et al.*, 2009). *S. vulgaris* is comprised of self-compatible hermaphrodites and females (male-sterile) with a mixed-mating system (Glaettli *et al.*, 2006). This plant has a well-documented evolutionary history involving post-glacial expansion across Eurasia, and invasion of North America in the late eighteenth century; this history includes repeated episodes of founder effect and both neutral and adaptive genetic differentiation along longitudinal gradients (Taylor and Keller 2007, Keller et al. 2009).

The variety of environments in which *S. vulgaris* grows means that populations may experience dramatically different selective pressures, including traits that may be related to the functions of the flavonoid biosynthetic pathway. Earlier work in *S. vulgaris* has suggested that there are flavonoids present (Darmograi, 1977, Chapter 1); there has been adaptation of highland (1600 m) and lowland (-2 m) populations to their local UV-B regimes in seed production (van de Staaij *et al.*, 1997); and increased in foliar compounds absorbing in UV-B wavelengths from a high-elevation Austrian population (van de Staaij *et al.*, 1995). In addition to patterns in non-visible flavonoid pigments, there is also evidence of among-population and within-plant variation in anthocyanin production, intensity, and hue in *S. vulgaris* calyces, petals, leaves, and stems (Marsden-Jones & Turrill, 1948, 1957). In Chapter 1 I found anthocyanins and flavones present in adult *Silene vulgaris* leaf, calyx, and petal tissues (Figure 1).

Plant material

Twelve naturally collected populations from across the Eurasian landmass were selected from the Taylor lab seed bank, representing six high (>1000 m) and six low (< 1000 m) elevation habitats (Figure 2). The seeds were collected between 2004-2009 (Table 1). In addition, four high elevation and three low elevation populations were collected within 25 km of the Col du Lautaret, Hautes-Alpes, France in 2011 for a total of nineteen populations sampled. Maternal families were planted in Fafard 3B soil mix (Conrad Fafard, Inc.; Agawam, MA) and germinated on a mist bench in March 2012 (French source material) and 2013 (Eurasian source material). After germination, seedlings were grown in the University of Virginia greenhouses with watering every other day, biweekly treatment with 20-20-20 NPK fertilizer and supplemental lighting provided on a 16-h ⁄ 8-h light⁄dark cycle. The French populations were transported as seedlings to two experimental gardens in the Hautes-Alpes region of France: one low elevation garden in Le Bourg d'Oisans (700 m, "BDO") and one high elevation garden 40 km east at the Station Alpine Joseph Fourier in the Col du Lautaret, France (2100 m, "Lautaret"). Twenty French individuals per population were planted in May 2012 directly into the ground into a grid pattern, each 25 cm away from the nearest plant. I used the surviving plants in 2013 in the current experiment (20 individuals at BDO, 258 individuals at Lautaret).

To reduce the influence of maternal provisioning, the Eurasian populations were grown to maturity and random crosses were made within populations. As such, the level of genetic information was at the population level. The resulting seeds were germinated in the greenhouses at the Centre d'Ecologie Fonctionelle et Evolutive (CEFE) in Montpellier, France under the same conditions described above. Adult, vegetative plants were placed at the location of their garden for two to three days to acclimate to the climate, and were then planted directly into the ground in a random pattern at the two experimental gardens in June 14-15, 2013 (BDO garden) and June 26-27, 2013 (Lautaret garden, as soon as the snow melted and no nights with frost). At least 10 individuals were planted per population; 236 individuals were planted at the BDO garden and 261 at the Lautaret garden.

Field methods

Each garden was monitored every other day for newly flowering individuals. At the time of first flowering, one flower from each newly flowering plant was measured for calyx color, petal color, sex (male-sterile or hermaphrodite), calyx height (mm), and calyx width (mm).

Calyx color was measured by binning individuals into four qualitative color classes from 1 representing no pigmentation to 4 representing the most pigmentation (Figure 3; Chapter 2). Petal color was measured as a binary trait: white or pink petals, as well as by color class: white, light pink, very pink. Each garden received supplemental water every two days unless there was sufficient soil moisture.

I conducted three censuses at each experimental garden during the 2013 growing season: the first in the third week of July, the second in the third week of August, and the third in the third week of September (although this census was only to record end of season flower and capsule count). At each census, each plant was re-measured for calyx color, petal color, and sex, and additionally the number of stems, height of longest stem (cm), and proportion leaf and floral herbivory. Herbivory was estimated as the average amount of damage across all leaves or flowers on each plant, and classifying the proportion of damage as 0%, ~25%, ~50%, ~75%, or 100%. The total number of flowers and capsules was summed for each individual, and further analyses on flowering and fruiting data were treated as summaries of total seasonal output of flowering or fruiting individuals. I then calculated the proportion of each population that reached flowering for each garden and elevation of origin combination.

Flavonoid extraction and analysis

I analyzed leaves and calyces for flavones as they were the most visibly variable tissues (Chapter 1). Extraction and chemical analysis of flavones was performed with a method optimized for non-anthocyanin flavonoid extraction due to the accuracy of the qualitative field anthocyanin color classes (Chapter 2); anthocyanins are less stable in solution than the flavone glycosides found in *S. vulgaris* and are best analyzed promptly after extraction, thus decreasing in concentration during the long extraction time optimal for flavones (Harborne, 1998). To assess anthocyanin concentration in the field, I used qualitative anthocyanin color classes (Figure 3; described in Chapter 2).

At the time of first flowering, one mature flower and one leaf, from the second node below the inflorescence, were sampled from each flowering plant in the field. Samples were transported on ice and stored at -20°C. Samples were then lyophilized, weighed, ground with a Qiagen TissueLyser II, and extracted in 1mL MeOH 1% HCl (v/v) on a shaker for 18 hours at 4°C in the darkness. Supernatants were removed, filtered, and diluted to 1:4 in the extraction buffer. Samples were then kept at 4°C in a Shimadzu 20AC autosampler before analysis. I injected 50 ul of each diluted sample into a Shimadzu HPLC-PDA system comprised of two SIL-10AVP pumps, degasser, and PDA, with a Chromolith Performance RP-18e (100 x 4.6 mm i.d.) column (Merck, Darmstadt, Germany) with a guard column (Phenomenex, 5 µm, 4×4 mm). I used a linear binary gradient of H_2O (solvent A) containing 0.1% (v/v) TFA and acetonitrile (solvent B) with a flow rate of 1.0 mL per min at 25°C. The linear elution gradient was as follows: 0 min 95% A, 16 min 83% A, 23 min 55% A, 23.1 min 0% A, 26.1 min 95% A, 30 min 95% A. A PDA was used for detection of UV-visible absorption spectra in the range of 200-700 nm.

Each sample was read at 254 and 372 nm. The number of flavone peaks was counted (all representing various glycosylated vitexin, orientin and scoparin derivatives) as a measure of flavonoid diversity, and peaks were quantitated as saponarin (isovitexin-7-O-glucoside) equivalents for a total concentration measure using Shimadzu LCsolution software. Concentration of total flavones was converted to mg flavone per gram of dry tissue.

Statistical analyses

I used SAS v9.3 (SAS Institute, Cary, NC, USA) to analyze the phenotypic data. For all linear models, I tested for differences between the surviving locally-derived French individuals from 2012 and the other experimental populations. This local vs. non-local factor was not significant, so it was removed from further analyses. I calculated the proportion of individuals that survived to the first census and the proportion that flowered per population, grouped them as either high or low origin (where population is a replicate of high or low origin genotypes), and used a two-way ANOVA to test for effects of elevation of origin, garden, and their interaction. I used a *post-hoc* Tukey test to test for significance of individual comparisons and a Tukey-Kramer adjustment for multiple comparisons. For the floral measurements taken at the time of first flowering, which included calyx color, calyx height and width, petal width, and total flowers, as well as the proportion of individuals per population that survived and flowered, I used PROC MIXED with garden, elevation of origin, and their interaction as fixed effects and sex as a random covariate. Due to the infrequency of pink petals, petal color (as measured by color classes) was analyzed in R with a chi-squared test of independence with garden and elevation of origin as factors. I ran repeated measures two-way MANOVAs using PROC GLM with type III error, using census as the repeated measure and garden and elevation of origin as fixed factors, for the following variables that were measured at the July census and August census: proportion leaf herbivory, proportion floral herbivory, number of shoots, height of tallest shoot, and percent of leaves covered by anthocyanins.

I tested for relationships between flavones and phenotypic measurements on a subset of individuals with HPLC data. I used a two-way ANOVA (PROC MIXED in SAS) to test for effects of elevation of origin, garden, and their interaction on the concentration of leaf and calyx flavones separately. I used a *post-hoc* Tukey test to test for significance of individual comparisons and a Tukey-Kramer adjustment for multiple comparisons. I fit a generalized linear model with a Poisson family error distribution to the number of leaf flavones and the number of calyx flavones in R (package stats) with garden and elevation of origin as fixed factors.

To determine whether there were correlations among phenotypic traits, I used the population means of the field-measured phenotypes (petal color, calyx color, calyx height, calyx width, total flowers, total capsules, length of tallest shoot in July and August, number of shoots in July and August, percent of leaves covered by anthocyanins in July and August, proportion of leaf and floral herbivory in July and August) and used a Box-Cox transformation in R (v3.0.1) package “car” to meet the statistical assumptions of Pearson correlations. I produced Pearson correlation matrices in SAS for the pooled data as well as one for each experimental garden, where the multiple comparison false discovery rate was set to 5% using the Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995).

To determine whether there were any correlations between flavone number (diversity), flavone concentration and other phenotype traits, I took the August census values for the height of the longest shoot, the number of shoots, leaf anthocyanins, leaf hairs, but averaged the leaf and floral herbivory across both censuses. I then used the Hmisc package in R to create a Spearman correlation matrix on untransformed variables with Benjamani-Hochberg multiple comparison adjustment. I also conducted a stepwise multiple regression (SAS PROC REG) to determine whether either the number of flavones or concentration of flavones in leaves or calyces served as predictors for any of the other phenotypic traits measured in this dataset. To reduce collinearity, I chose variables that were not highly correlated with each other: garden, height of the tallest shoot at the August census (as a proxy for plant size), proportion of leaf anthocyanins at the August census, calyx color, calyx width (as a proxy for flower size), Box-Cox transformed proportion of floral and leaf herbivory, and total flowers (to determine whether flavonoids conferred any obvious fitness benefit or cost).

Results

Garden survival and fitness components

There were only 20 surviving individuals from local French populations planted in 2012 at the BDO garden (low elevation garden), and 258 surviving individuals at the Lautaret garden (high elevation garden). There was little rain and consistently high daytime temperatures during the summer of 2012, likely leading to low survival at the BDO garden. I thus used the few individuals that survived to summer 2013 from the French set of plants. The 2013 season survival (hereafter, simply "survival") for the gardens was assessed as the proportion of individuals alive through the final census in September 2013 out of those alive or transplanted at the beginning of the season; BDO had 80% season survival (205/256) and Lautaret had 80% survival (416/519). Both gardens had low rates of flowering with BDO at 27% (56 individuals) and Lautaret with 22% (95 indiviudals). Survival and flowering varied significantly by garden, and there was a significant GxE interaction for the proportion of flowering (Table 2, Figure 4). There were no significant differences between elevations of origin and garden for the total number of flowers or capsules (Table 2, Figure 5).

Leaf and floral anthocyanins

Anthocyanins were more highly expressed at Lautaret in calyces, petals, and mid-season leaves (July census) in both low and high elevation origin plants (Figure 6), illustrating a strong environmental component and inducibility. There was also a strong genetic component to anthocyanin production in the calyces and the average season leaf anthocyanin expression (Table 2). Calyx anthocyanins, as measured by color bins, also exhibited a significant GxE interaction, where individuals of high elevation origin produced consistently darker calyces (with a mean of 3, Table 3) at both gardens and individuals of low elevation origin produced calyces with lower concentrations of anthocyanins at the low garden than at the high garden (Figure 6A). Although pink petals in *S. vulgaris* are somewhat rare, pink petals appeared at the high elevation Lautaret garden and did not appear at the low elevation BDO garden (Figure 6B). There were significant genetic and environmental components to average leaf anthocyanin production, but the effect is likely driven by a marked difference of increased leaf anthocyanins at the Lautaret garden at the first census (Fig 6C). *Post-hoc* Tukey tests confirmed that there were no significant pairwise differences among the high and low origin and garden treatments at the August census (Table 2), but anthocyanin production remained higher in high origin plants compared to low origin plants at both gardens.

Leaf and floral flavones

Leaf flavone patterns were slightly different from those exhibited by the flowers (Figure 7), but all measures indicated genetic and phenotypic variability. There was a significant effect of garden on the concentration of leaf flavones, with leaves at the Lautaret garden producing approximately twice as many different flavones on average than those at the BDO garden (Table 3). Calyx flavone concentration did not vary significantly among the gardens, although the mean flavone concentration for the Lautaret garden was higher than the BDO garden (19.62 mg vs 16.66 mg). The number of flavones in leaf tissue varied significantly by elevation of origin, garden, and their interaction; calyx flavone number varied only by garden (Table 2, Table 3, Figure 7). In both tissues there was more flavone diversity at the Lautaret garden than at the BDO garden, but low-origin individuals notably had a greater increase in diversity than did high-origin individuals at the Lautaret garden than at the BDO garden (Figure 7).

I examined whether any phenotypic variables served as predictors of variance in flavone concentration and flavone number (diversity) per tissue. The stepwise regression model selected calyx color and the proportion of leaf herbivory as explanatory variables for calyx flavone concentration (R^2=0.658, $F_{(2,11)}$=8.66, p=0.008) with partial regression coefficients of 0.43 (calyx color) and -0.58 (leaf herbivory). The proportion of leaf herbivory and leaf anthocyanins were the variables selected in the stepwise selection process for the number of flavones present in calyx tissue, though the model was not quite statistically significant (R^2=0. 4457, $F_{(2,11)}$=8.66, p=0.0703; partial regression coefficients: leaf anthocyanin 0.46, leaf herbivory -0.60).). No phenotypic variables were significantly related to leaf flavone concentration and number of leaf flavone peaks.

Phenotypic means and garden differences

All of the repeated measures linear models had high correlation coefficients (all greater than 0.50) between the two censuses and, with the exception of floral herbivory (r = 0.35, *p*=0.1568), were significant ($p < 0.05$). Several traits were genetically variable, including leaf herbivory, shoot number (Table 2, means reported in Table 3). Most phenotypic traits had a significant environmental (garden) contribution to phenotypic variation, including survival, flowering, calyx width, calyx height, leaf herbivory, shoot height, (Table 2, means reported in

Table 3). Significant GxE interactions were found for proportion of flowering individuals, leaf herbivory, and shoot height.

Some general phenotypic and ecological observations can be made regarding differences between the two gardens. Plant morphology varied between the two gardens, where individuals had taller but fewer shoots at the BDO low elevation garden compared to Lautaret, and higher vegetative herbivory. Interestingly, the group that experienced the most amount of vegetative herbivory was high-origin individuals at the BDO low elevation garden at both the July and August censuses, but floral herbivory did not vary among the gardens (Figure 8, Table 2). Shoot height was particularly different among the high and low elevation of origin individuals at the BDO garden (GxE, p=0.0054) with low origin individuals producing taller shoots (25-26 cm) compared to high origin individuals (18 cm) across the season (Figure 9). The elevation genotypes converged on the same height at the Lautaret garden (20-21 cm). Generally speaking, flowers were larger, more darkly pigmented in their calyces, and more likely to display pink petals at the Lautaret high elevation garden.

Correlations between morphological and flavonoid traits

I calculated phenotypic correlation matrices for the Lautaret and BDO gardens separately (Supplementary figures S1 and S2, respectively) to determine whether there were any correlated changes in phenotype at the different garden environments. I saw similar patterns of morphological correlations at both gardens where larger plants (with either longer stems or more shoots) made more large flowers. Calyx height and calyx width were positively correlated at both gardens. Taller plants were correlated in different directions with leaf herbivory at the two gardens; taller plants at the Lautaret garden experienced more leaf herbivory in August ($r = 0.27$,

$p < 0.05$), whereas taller plants ($r = -0.50$, $p < 0.05$) and plants with more shoots ($r = -0.36$, $p < 0.05$) at the BDO garden were negatively correlated with July leaf herbivory. Leaf anthocyanins were positively correlated with leaf herbivory at the first census, but not in the second census at BDO. Leaf anthocyanins were highly correlated between censuses at the Lautaret garden ($r = 0.50$, $p < 0.05$), but not at the BDO garden. There is also a suggestion of different floral herbivory patterns between the gardens, but no significant statistics to support it.

Figure 10 reflects phenotypic correlations of the entire dataset with the addition of the chemical flavonoid data. Several of the observed correlations from the separate gardens remain, especially regarding plant height and reproductive output. The number of leaf flavones and leaf flavone concentration were highly correlated ($r = 0.65$, $p < 0.05$), and the number of calyx flavones was positively correlated with all other flavone measures (Figure 10). Markedly, there is not a significant correlation between leaf anthocyanins and leaf flavones, neither are there correlations between herbivory and flavonoid traits.

Discussion

The flavonoid pathway is known for being evolutionarily relevant and phenotypically plastic, (Winkel-Shirley, 2002; Soriano *et al.*, 2004; Lu *et al.*, 2009), as well as being heritable and having genetic variance (Caldas & Blair, 2009; Johnson *et al.*, 2009; Koski & Ashman, 2013), but few studies have looked at flavonoid-related traits with a pathway perspective in natural populations. The goal of my experiment was to evaluate whether the flavonoid pathway contributes to adaptation of *Silene vulgaris* to a particularly relevant abiotic gradient of low and high elevations, and to determine the relative contributions of genetics and plasticity to successful phenotypes at each garden. I found evidence for local adaptation to elevation of origin through fitness measurements. Specifically, the proportion of flowering for each population had a significant GxE effect, meeting the expectations of the "home vs. away" definition of local adaptation to habitat (Kawecki & Ebert, 2004; Blanquart *et al.*, 2013). High elevation origin populations flowered the most at their "home" habitat garden and low elevation origin populations flowered the most at their "home" habitat garden. I also found that, in general, flavonoids are present in higher concentrations in flowers and leaves at high elevations. Genotype, as measured by elevation of origin, explained a significant part of anthocyanin variation in all tissues, in addition to the effect of the environment. Flavones showed one of the most significant differences in expression across environments, specifically where leaf flavone concentration was much higher in all genotypes at the high elevation garden, suggesting a role in protection against increased levels of UV radiation and light. Although there was more overall herbivory at the low elevation garden and lower concentrations of flavonoids at the low elevation garden, and conversely less herbivory and more flavonoids at the high elevation garden, there is a distinct lack of support directly correlating herbivory and flavonoids. While not necessarily conclusive, the observed variation is suggestive of the interpretation that flavonoids do not play a direct role in defense against the particular herbivory regime in the target *S. vulgaris* range.

Plant size is known to influence pollinator and herbivore visitation (Crawley, 1983), as well as plant fecundity (larger plants often make more flowers). In addition, measuring plant size or biomass is a classical method to test for trade-offs or "costs" to producing secondary chemicals (Stamp, 2003; Strauss *et al.*, 2004; Hofmann & Jahufer, 2011). Thus, I included plant size in my field observations. I saw no correlation between fitness traits and flavonoid traits, suggesting that there may not be a global cost to their production in all environments. Flowering and fruiting data may be skewed by the fact that so few individuals reached the flowering stage, but to measure a true “cost” of production of flavonoids, one would compare fitness components to flavonoid production.

Insight concerning the inference of the presence of local adaptation depends on several factors, including the rate of gene flow, the scale and magnitude of spatial variation in selection, and the heritability of traits (Agren & Schemske, 2012). Explicit evidence for local adaptation of *S. vulgaris* to elevation environments comes in the form of one of the fitness component: the proportion of individuals who successfully flower per population. The proportion of flowering individuals per population varied significantly by elevation of origin (genotype), garden (environment), and had a significant interaction (GxE). High origin individuals had a higher proportion of flowering at the high garden, and the low origin individuals had a higher proportion of flowering at the low garden. This pattern of best performance in a fitness-related trait at the home site is typically considered to be evidence for local adaptation (Clausen *et al.*, 1940, 1948; Via, 1991; Agren & Schemske, 2012).

One previous study on *S. vulgaris* provides evidence for local adaptation to home-site UV-B environment (seed-bearing fruit, number of seeds) (van de Staaij *et al.*, 1997). In the present study, however, evidence for local adaptation in the other fitness component measures was less clear cut (survival, total flowers, total capsules). Survival was greater at the high elevation than the low elevation, and there were no significant differences detected between genotypes or environments for flowers or capsules. Instead, my evidence may suggest that individuals originating from high elevations have a delayed life-history strategy, investing more in roots and growth in their first season than in reproduction (thus having higher survival), and favoring a perennial lifestyle. Populations also tended to retain growth phenotypes (shoot height and number of shoots) corresponding to the mean phenotype of their elevation of origin, indicating a strong genetic component; local adaptation through growth and reproductive traits to elevation of origin has been found in other plant species (Clausen *et al.*, 1940; Angert & Schemske, 2005). Certainly, other plants are known to produce shorter stems when exposed to UV radiation, which comes with increased elevation (Weinig *et al.*, 2004).

Contrary to the focal traits and populations of these previous studies, there is some evidence for local adaptation to high and low elevations, though extending the experiment another year may reveal more conclusive results, or including populations from the very extreme edges of the *S. vulgaris* range. Local adaptation may manifest itself at only very large scales (Galloway & Fenster, 2000), and its signal can be weak (or nonexistent) within-species (Angert & Schemske, 2005). An alternative strategy would include populations from the highest and lowest elevations, which could produce more clear fitness results; however, such a small fraction of plants reached the flowering stage, and given the potential differences in reproductive phenology at different elevations (e.g. (Haggerty & Galloway, 2011)) another year of study may be just as beneficial.

The effects of local adaptation to elevation in this study, however, are not nearly as strong as the effect of the plastic adjustment of *S. vulgaris*. This may be due to gene flow in the species, where populations are not greatly diverged (the global F_{ST} is 0.03-0.09 in European populations) (Keller *et al.*, 2014); frequent gene flow has the potential to erode the effects of adaptation to a local environment (Stanton & Galen, 1997; Kawecki & Ebert, 2004; Hereford, 2009). I found plasticity in most traits and in most populations, suggesting that phenotypic plasticity is essential to survival at all elevations (Figures 4-9). This finding is supported by several common garden experiments involving plants at different elevations (Heisey *et al.*, 1971; McDougal & Parks, 1984; Angert & Schemske, 2005; Stöcklin *et al.*, 2009; Steiner *et al.*, 2012)..

This may be an artifact of the length of time of the study, because phenotypic plasticity is often a plant's response to a rapidly changing environment, and annual vs. lifetime fitness strategies might be different (Schlichting, 1986; Scheiner, 1993); at the same time, plasticity can also be adaptive and maintained as a permanent trait (Weinig *et al.*, 2004).

I observed several phenotypic changes that had genetic and environmental influences. I saw broad changes in morphology between the two environments, with plants at the high elevation garden having darker flowers, shorter stems, more stems, more leaf anthocyanins, and less herbivory; plants at the low elevation garden had lighter (but still variable) flowers, longer stems, more herbivory, and inconsistent leaf anthocyanins. Diminution in stature is thought to aid many alpine plants in thermal conservation and protection from exposure to cooling winds (Tranquillini, 1964; Theurillat & Guisan, 2001; Klanderud, 2008), and this seems to be a genetically fixed trait among high-elevation plants (no significant change between gardens) whereas low-elevation plants were much slower to grow at the high-elevation garden.

I also found that the flavonoid pathway had a strong response to the environment, (garden) as well evidence for strong influences of elevation of origin on both constitutive and plastic production of its various metabolic products. The evolution of floral color in *S. vulgaris* may play a role in adaptation to elevation, wherein there is a heritable component to having a particular calyx color (Chapter 2). Certainly, a selection analysis for floral color is the logical next step, and will be completed at the end of the experiment in 2014; in Chapter 2, I found evidence for divergent selection for calyx color between high and low elevation populations along elevational gradients. At the same time, some environmental variability influenced the calyx color phenotype; a GxE interaction contributed to increased floral color at the high elevation garden, even for some low-elevation populations that produced less of the pigment overall. In addition, increased production of leaf flavones appears to correlate with higher survival at the high elevations. Leaf flavone diversity (measured as the number of flavones) increased when populations were planted at the unfamiliar elevation garden, perhaps indicating that the flavonoid pathway produces a more diverse array of flavonoids as a response to a new environment. The specific pattern of the reaction norm suggests that this trait may also be an adaptive plasticity.

The difference in floral anthocyanin pigmentation appears to have both genetic and environmental components; populations from higher elevations had more anthocyanic flowers in both environments than low-elevation populations, but several low-elevation populations experienced an induction of floral anthocyanin production at the higher-elevation garden. Patterns regarding calyx anthocyanins and calyx flavones were unclear. High elevations can prove more stressful environments with colder temperatures, less oxygen, increased UV radiation, and more light (Tranquillini, 1964; Nybakken *et al.*, 2008), this may be particularly stressful for plants sampled from low elevations. Anthocyanins can accumulate in all plant tissues during times of environmentally-induced stress (Steyn *et al.*, 2002), especially including temperature stress (Stiles *et al.*, 2007; Umbach *et al.*, 2009), light stress (Close & Beadle, 2003; Hoch *et al.*, 2003), and UV stress (Chalker-Scott, 1999; Albert *et al.*, 2009; Heijde *et al.*, 2013). Potential hypotheses explaining more constitutive levels of floral anthocyanins expression at higher elevations are not mutually exclusive. Darker flowers may be the result of pollinator-mediated selection, thermal regulation and protection of reproductive organs, or also a result of stress amelioration (Winkel-Shirley, 2002; Lacey *et al.*, 2010; Hirota *et al.*, 2013). In addition, flowers are more ephemeral than leaves (flowers typically last for about a week before wilting, *personal observation*), so perhaps it is more cost-effective to make one class of flavonoids that accomplish more than one role during a shorter tissue lifespan, or that flavones may be more inducible because they are more costly to produce (e.g. optimal defense theory, Strauss *et al.*, 2004).

Higher amounts of leaf anthocyanins were the norm at the Lautaret garden, but plants at the BDO garden only had high leaf anthocyanins at the July census. This suggests a more consistent environmental stressor, like UV radiation, at high elevations compared to low elevations. Correspondingly, anthocyanin accumulation in vegetative tissue is a more readily induced trait in high-elevation populations due to the frequent micro-environmental climate changes to ameliorate the damages of generalized stress (e.g. reactive oxygen species) (Close & Beadle, 2003). This may be supported by the fact that there was no significant correlation between leaf anthocyanins and leaf flavones, and there were more leaf flavones at the high elevation than in the low elevation garden. I also found that anthocyanins accumulated in leaves with more herbivory. However, this could simply be an observation of a plant reaction to the stress of herbivory (reviewed in Lee & Gould, 2002), rather than an act of inducible defense; anthocyanins are often well-tolerated in animal diets.

Flavones are typically responsible for epidermal screening and absorption of UV radiation (Ryan *et al.*, 2002; Tegelberg, 2002; Casati & Walbot, 2005), as well as in defense against herbivory (Soriano *et al.*, 2004). While I also saw a pattern of increased leaf herbivory at the BDO garden, I did not find a specific association between leaf flavones and leaf herbivory, despite my prediction that they would be associated. While there were general patterns of increased herbivory at the low elevation gardens, and an increase in flavones at the high elevation garden, there was no clear cause-and-effect relationship between the two. Patterns of herbivory may have differed due to the fact that the major herbivores at each garden were different (low elevation slugs vs. high elevation insect larvae and caterpillars). I therefore suspect that flavones play a larger role in the protection against UV radiation than they do in protection against herbivores. The pattern found with leaves increasing flavonoid concentration and flavonoid diversity at the high elevation supports this hypothesis; UV-induced photoinhibition and light-related stimuli can control the secondary metabolite response in leaf tissue instead of from herbivore pressure (Close *et al.*, 2003)

While relationships between anthocyanins and flavones remain unresolved, my regression model for calyx flavone concentration suggested that there may be more predictable relationships between floral and leaf tissues. The regression model of flavone concentration [calyx flavone concentration = 0.43(calyx anthocyanin color) - 0.58 (proportion leaf herbivory)] suggested that that increased calyx anthocyanin was significantly related to a decrease in leaf herbivory. This pattern is also seen in the BDO correlation matrix, although not statistically significant. Certainly, some plant species with particular floral colors have evolved broad "color morphs" where herbivore defense covaries with floral color (Irwin *et al.*, 2003; Frey, 2004; Berardi *et al.*, 2013). This suggests that there may be a trade-off in resources between flowers and leaves, where resources are directed towards floral chemistry for darker colored morphs, leaving the leaves open to herbivory attack. Further support for a between-tissue trade-off comes from my regression model of phenotypic measures with the calyx flavone diversity, another measure of resource allocation to a particular flavonoid subgroup, where I found that flowers with more calyx flavone diversity produced more leaf anthocyanins, but were less defended against leaf herbivores [calyx flavone diversity = 0.46752(percent leaf anthocyanin) - 0.59785(proportion leaf herbivory)]. However, seeing as leaves at higher elevations had more flavones, the trade-off in flavonoids and defense against herbivory may not occur within the flavonoid pathway itself, but perhaps resources are taken away from another class of defensive chemicals, e.g. saponins, ecdysteroids are present in *Silene*, possibly even genetically linked further upstream (Glensk *et al.*, 1999; Zibareva *et al.*, 2003; Böttger & Melzig, 2011; Mamadalieva, 2012).

Conclusion

The environment varies significantly between different elevations, especially in mountainous regions like the Alps, exerting different selection pressures on plant populations (Clausen *et al.*, 1940; Angert & Schemske, 2005; Matter *et al.*, 2014). Thus, traits that are retained by genotypes in a reciprocal transplant may contribute to local adaptation in the home site. Overall, I found some evidence for local adaptation of *S. vulgaris* to elevation, and evidence that the flavonoid pathway may play an important role in the process via protection against harmful UV radiation by flavones. The increase in anthocyanins at the high elevation garden, and the propensity for populations of high elevation origin to express anthocyanins more constitutively in the flowers suggests that other selective forces may also have played a part in this phenotypic divergence, such as pollinator preference and thermoregulation.

This study will be bolstered by an additional year of observations of phenotypic and chemical data in the summer of 2014. Measures of overwintering success and post-establishment within-season growth and flowering rates will increase sample sizes for viability and fecundity measures, as well as statistical power.

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Figure 1. A schematic of the *S. vulgaris* flavonoid pathway. Sections of the pathway in black are those which have been found in adult tissues of *S. vulgaris* (genes and chemicals; see Chapter 1 and 4), and sections in gray are those that have not been found in adult tissues, but may be present in other life history stages of the plant.

Gene abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; FS, flavone synthase; GT, glycosyl-transferase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, Dihydroflavonol 4-reductase; ANS, anthocyanidin synthase, UF3GT, UDP-glucose:flavonoid 3-O-glucosyltransferase, RT, rhamnosyl transferase; FLS, flavonol synthase; LCR leucoanthocyandin reductase. It should be noted that F3'5'H is not represented as it is not known to be functional in the Caryophyllaceae (Mato *et al.*, 2000; Zuker *et al.*, 2002).

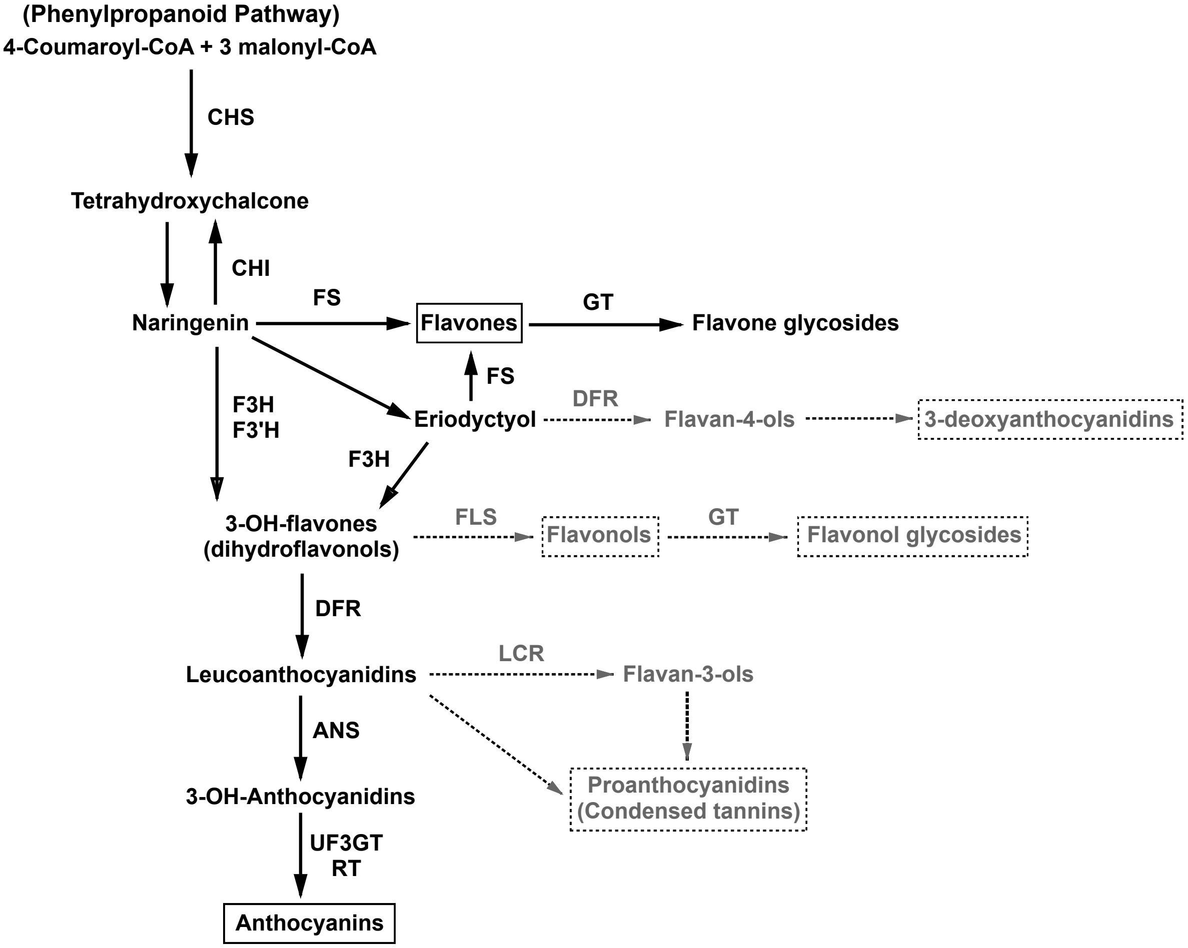


Figure 2. A map of Eurasian source populations for the reciprocal transplant.

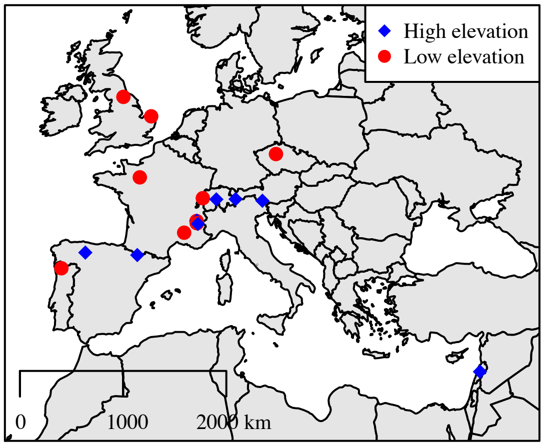
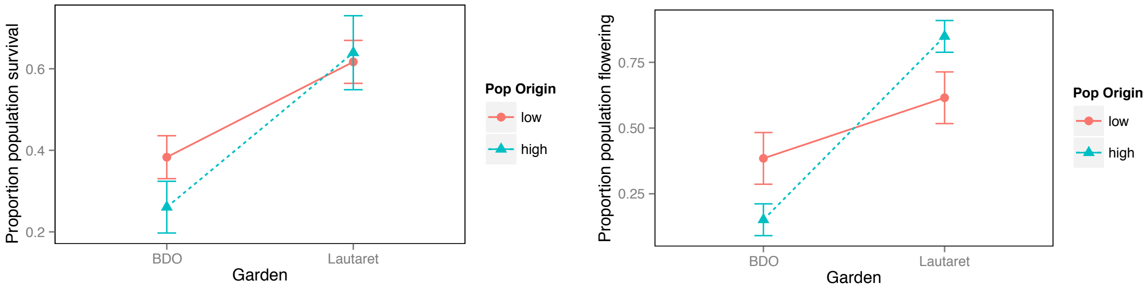


Figure 3. Calyx color classes. Clockwise, starting with upper left: 1, 2, 4, 3.

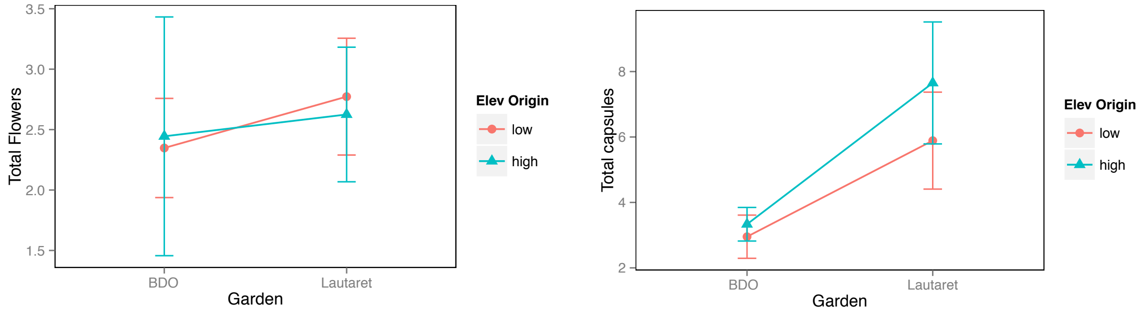
Figure 4. The mean (± SE) proportion of surviving (A) and the mean (± SE) proportion of flowering (B) individuals per population in each garden by elevation of origin (high or low).



A.

B.

Figure 5. Of those individuals that flowered, (A) the mean (± SE) total number of flowers and (B) mean (± SE) capsules per population in each garden by elevation of origin (high or low).



A.

B.

Figure 6. **Anthocyanins:** the population means (± SE) of (A) the mean calyx color, a proxy for anthocyanin concentration, (B) the mean petal color, (C) percent leaf covered by anthocyanins at the July census, and (D) percent leaf covered by anthocyanins at the August census, are shown grouped by the elevation of origin and experimental garden. Petal color was not independent of elevation of origin or garden (X^2 =13.525, df=4, p=0.009).

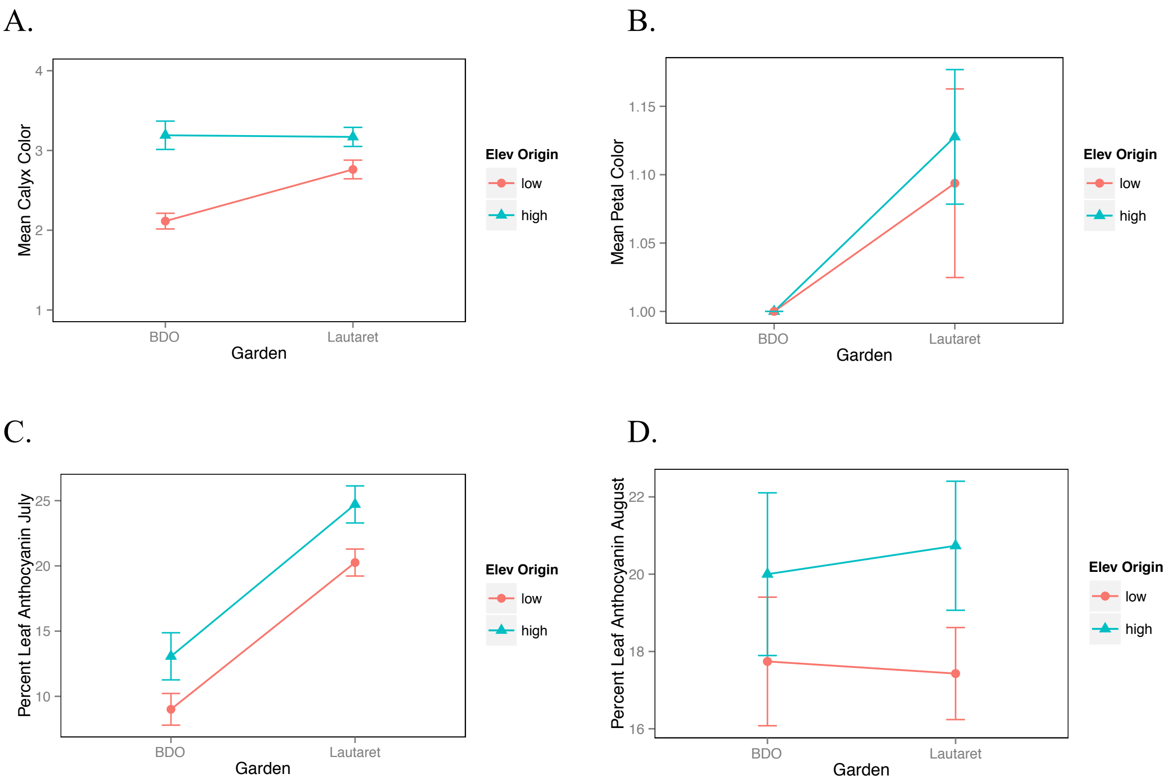


Figure 7. **Flavones:** the means (± SE) of (A) the number of leaf flavones (flavone diversity), (B) the number of calyx flavones (flavone diversity), (C) the leaf flavone concentration (average mg flavones per gram dry tissue) and (D) calyx flavone concentration are shown, grouped by the elevation of origin and experimental garden.

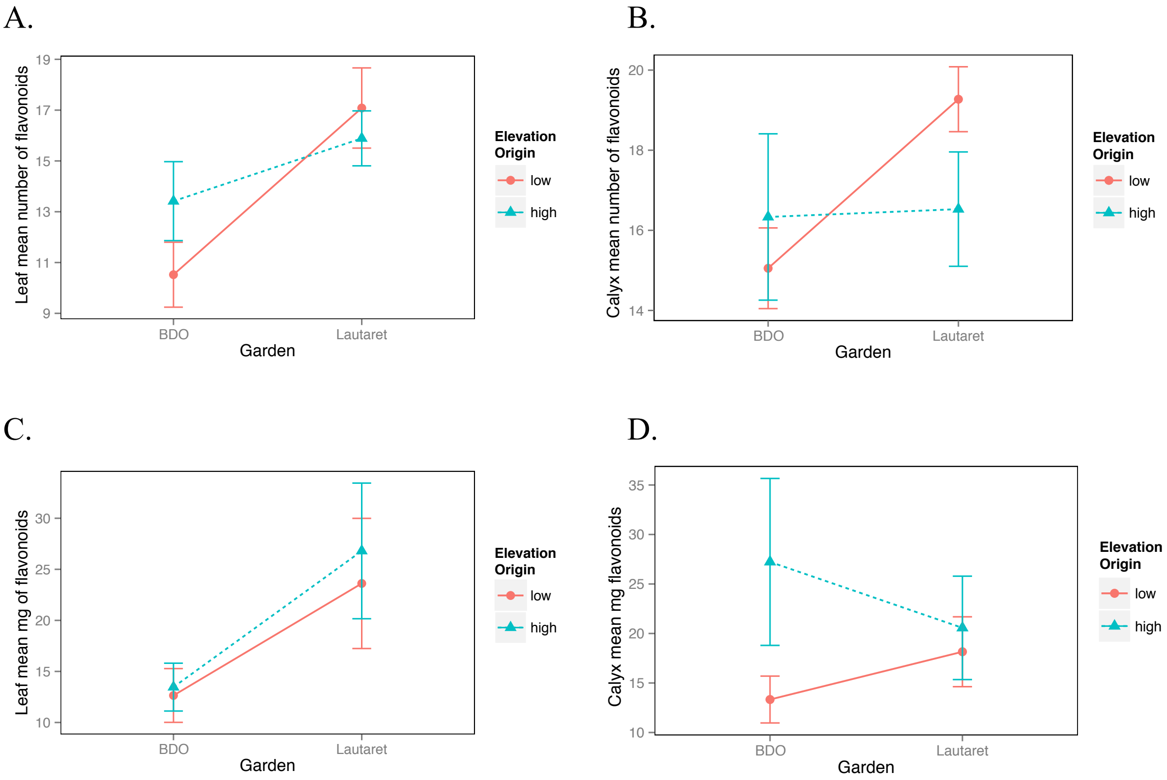


Figure 8. **Herbivory:** the population means (± SE) of the proportion of leaf herbivory in July (A), the proportion of leaf herbivory in August (B), the proportion of floral herbivory in July (C) and proportion of floral herbivory in August (D), are shown grouped by the elevation of origin and experimental garden.

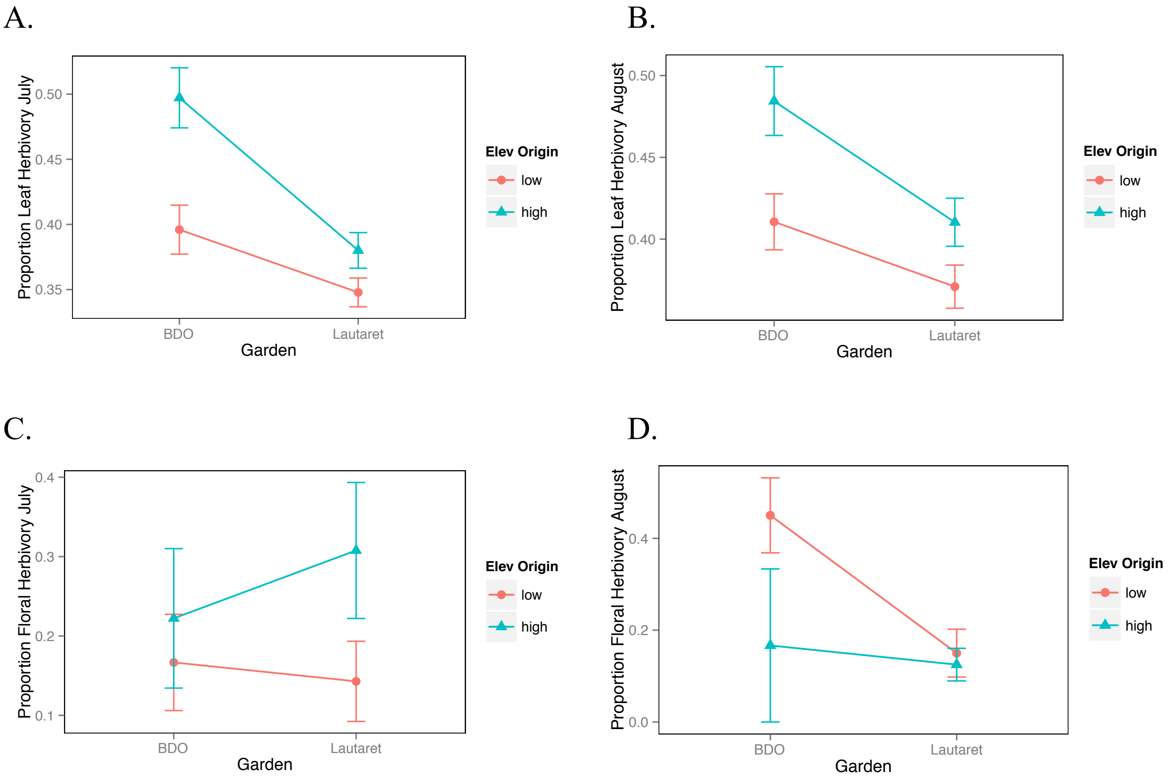


Figure 9. **Plant size:** the population means (± SE) of (A) the height of the tallest shoot in July, (B) the height of the tallest shoot in August, (C) the mean number of shoots in July, and (D) mean number of shoots in August are shown grouped by the elevation of origin and experimental garden.

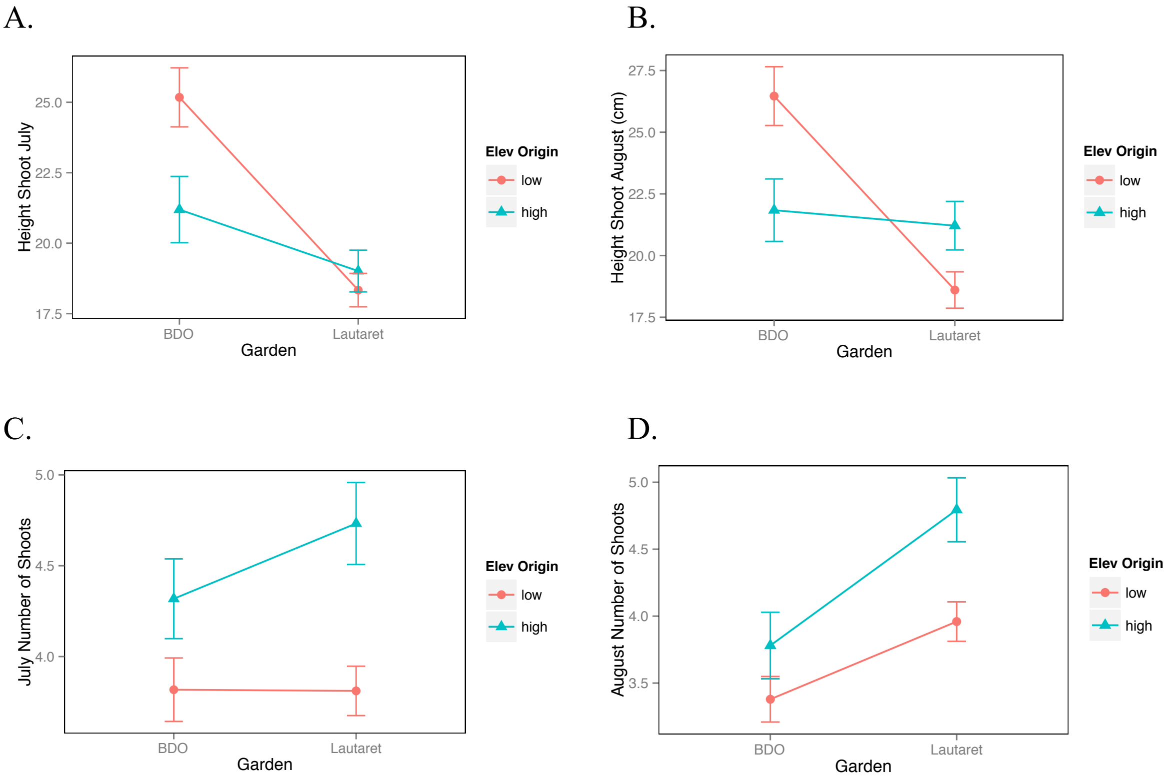
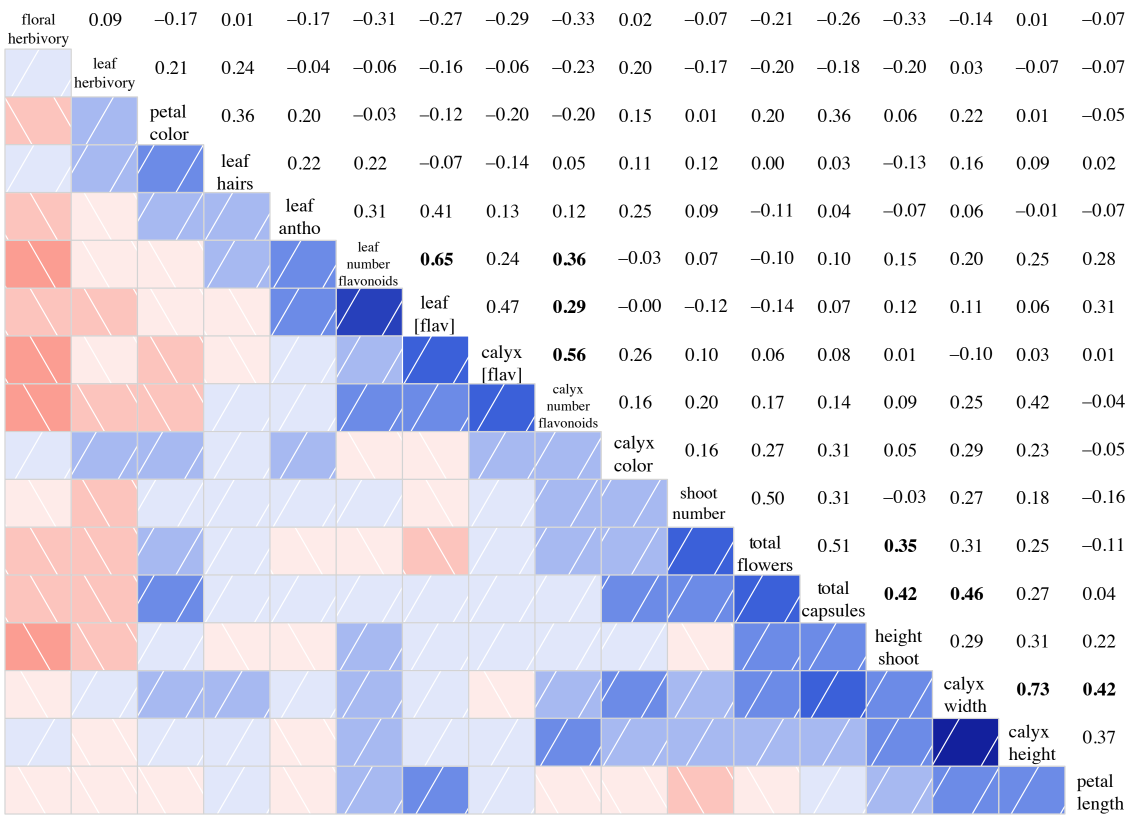


Figure 10. Phenotypic and flavonoid correlation matrix on family means (nested within population, family N = 37-65). Color in the lower diagonal representing the strength of the correlation coefficient, where blue represents +1 and red represents -1. The upper diagonal shows the correlation coefficient r for each comparison, and bolded coefficients are significant at alpha = 0.05 from an (FDR) multiple comparisons correction.



Field phenotype and flavonoid correlations

	leaf herbivory	petal color	leaf hairs	leaf antho	leaf number flavonoids	leaf [flav]	calyx [flav]	calyx number flavonoids	calyx color	shoot number	total flowers	total capsules	height shoot	calyx width	calyx height	petal length
floral herbivory	0.09	−0.17	0.01	−0.17	−0.31	−0.27	−0.29	−0.33	0.02	−0.07	−0.21	−0.26	−0.33	−0.14	0.01	−0.07
leaf herbivory		0.21	0.24	−0.04	−0.06	−0.16	−0.06	−0.23	0.20	−0.17	−0.20	−0.18	−0.20	0.03	−0.07	−0.07
petal color			0.36	0.20	−0.03	−0.12	−0.20	−0.20	0.15	0.01	0.20	0.36	0.06	0.22	0.01	−0.05
leaf hairs				0.22	0.22	−0.07	−0.14	0.05	0.11	0.12	0.00	0.03	−0.13	0.16	0.09	0.02
leaf antho					0.31	0.41	0.13	0.12	0.25	0.09	−0.11	0.04	−0.07	0.06	−0.01	−0.07
leaf number flavonoids						**0.65**	0.24	**0.36**	−0.03	0.07	−0.10	0.10	0.15	0.20	0.25	0.28
leaf [flav]							0.47	**0.29**	−0.00	−0.12	−0.14	0.07	0.12	0.11	0.06	0.31
calyx [flav]								**0.56**	0.26	0.10	0.06	0.08	0.01	−0.10	0.03	0.01
calyx number flavonoids									0.16	0.20	0.17	0.14	0.09	0.25	0.42	−0.04
calyx color										0.16	0.27	0.31	0.05	0.29	0.23	−0.05
shoot number											0.50	0.31	−0.03	0.27	0.18	−0.16
total flowers												0.51	**0.35**	0.31	0.25	−0.11
total capsules													**0.42**	**0.46**	0.27	0.04
height shoot														0.29	0.31	0.22
calyx width															**0.73**	**0.42**
calyx height																0.37

Table 1. Locations of source material used.

Population	Country	Latitude	Longitude	Elevation (m a.s.l)	High/Low
ALN	France	48.39663	0.09341	148	low
BOL	France	44.279206	4.751631	60	low
CAT2	UK	54.38015	-1.6289	73	low
CON	Switzerland	46.846528	6.713072	437	low
CRO	UK	52.93379	1.29002	41	low
GAS	Switzerland	46.75822	8.13511	1050	high
GUA	Switzerland	46.777986	10.15443	1719	high
KAP	Austria	46.6558	12.97578	1026	high
LAR	Spain	42.801591	-5.628978	961	high
NBS	Lebanon	33.943178	35.829781	1454	high
PCO	Spain	42.618814	-0.16931	1232	high
PRG	Czech Rep.	50.13015	14.39913	192	low
VDM3	Portugal	41.635053	-8.141804	427	low
311	France	44.99456	6.07875	846	low
313	France	45.14724	6.05594	892	low
314	France	45.15235	6.06345	1113	high
315	France	45.16886	6.09422	1428	high
318	France	45.15195	6.0636	1095	high
1026	France	44.988029	6.1085	933	low
1031	France	44.93494	6.20197	1586	high

Table 2. Results of statistical tests to determine effects of elevation of origin (high or low elevation of origin), environment (garden), or a GxE effect. Populations were treated as replicates of high or low elevation of origins. Post-hoc Tukey tests were performed on GxE interactions. J and A represent the July and August censuses, respectively, and statistical significance is represented by: * = $p<0.05$, **=$p<0.01$, ***=$p<0.0001$.

									Tukey post-hoc tests for Origin x Garden						Within-subjects test of Census [a]	
			Elevation of Origin		**Garden**		**Origin x Garden**		BH - LH	BH - LL	BL - LH	BL - LL	**Sex covariate**			
Trait type	Trait	DF	*F*	P	*F*	P	*F*	P	P	P	P	P	*F*	P	*F*	P
Flavonoid	Calyx color (anthocyanin)	1, 147	31	**<0.0001**	5.53	**0.02**	19.31	**0.0133**	NS	*	*	*	--	--	--	--
	Percent leaf anthocyanin	1, 608	5.64	**0.0178**	22.12	**<0.0001**	0.02	0.8878	J:***, A: NS	J:**, A: NS	J:***, A: NS	J:***, A: NS	--	--	3.84	**0.0505**
	Leaf flavonoid conc.	1, 61	0.79	0.3764	4.38	**0.0406**	0.13	0.7222	NS	NS	NS	NS	--	--	--	--
	Calyx flavonoid conc.	1, 49	0.86	0.3588	0.09	0.7613	2.61	0.1125	NS	NS	NS	NS	--	--	--	--
	Leaf flavonoid number [b]	1, 64	2.39	**<0.0001**	5.106	**<0.0001**	-2.306	**0.0211**	--	--	--	--	--	--	--	--
	Calyx flavonoid number [b]	1, 52	0.698	0.4854	2.727	**0.0064**	-1.587	0.1126	--	--	--	--	--	--	--	--
Morphological	Floral herbivory	1, 80	0.02	0.8933	0.61	0.4449	0.01	0.9414	NS	NS	NS	NS	--	--	2.24	0.1531
	Leaf herbivory	1, 608	23.02	**<0.0001**	16.95	**<0.0001**	4.05	**0.0445**	J: ***, A:*	J:***, A:*	NS	NS	--	--	12.81	**0.0004**
	Shoot height	1, 610	1.00	0.3183	25.25	**<0.0001**	7.79	**0.0054**	NS	J:*, A: NS	J:***, A:**	J:***, A:***	--	--	1.76	0.1845
	Number of shoots	1, 609	7.3	**0.0071**	1.39	0.2393	0.38	0.5391	J: NS, A:*	NS	J: NS, A:***	NS	--	--	1081.64	**0.0001**
	Calyx width	1, 120	3.56	0.0615	20.88	**<0.0001**	2.04	0.1553	***	***	NS	*	1.42	0.235	--	--
	Calyx height	1, 120	0.02	0.8862	9.81	**0.0022**	0.52	0.4708	NS	**	NS	**	3.65	0.0586	--	--
Fitness	Proportion pop survival	1, 38	0.00	1.00	68.15	**<0.0001**	1.26	0.2689	***	***	***	***	--	--	--	--
	Proportion pop flowering	1, 32	0.00	1.00	21.78	**<0.0001**	8.24	**0.0072**	***	**	**	NS	--	--	--	--
	Total flowers	1, 122	0.05	0.821	1.66	0.1997	0.24	0.6259	NS	NS	NS	NS	0.07	0.7991	--	--
	Total flowers [c]	1, 34	1.5457	0.2223	0.1937	0.6626	0.0845	0.7731	--	--	--	--	--	--	--	--
	Total capsules [c]	1, 34	1.4203	0.2416	0.9504	0.3365	0.3599	0.5525	--	--	--	--	--	--	--	--

[a] Wilk's lambda

[b] For these values the statistic tested was Z, from a generalized linear model with Poisson error distribution

[c] Performed on standardized values

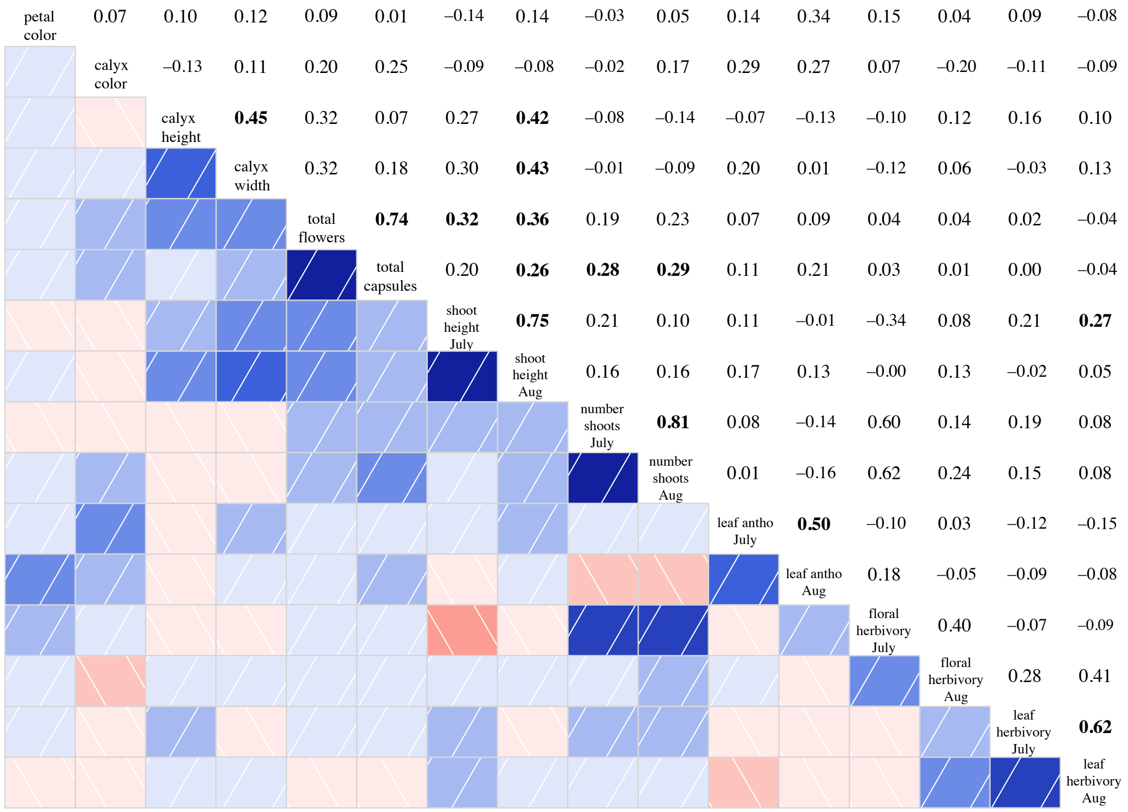
Table 3. Phenotypic means in *Silene vulgaris* experimental gardens.

Trait	N	Grand Mean	Standard Error	Range	BDO mean	BDO Standard Error	Lautaret mean	Lautaret Standard Error
Calyx color (1-4)	151	2.81	0.01	3.00	2.52	0.02	2.99	0.01
Petal color (1-3)	133	1.07	0.00	2.00	1.00	0.00	1.11	0.00
Calyx height (mm)	131	13.34	0.01	10.61	12.67	0.03	13.79	0.02
Calyx width (mm)	131	8.45	0.01	9.74	7.60	0.03	9.02	0.02
Proportion floral herbivory	117	0.22	0.01	0.50	0.30	0.04	0.20	0.01
Proportion vegetative herbivory	651	0.4	0.00	1.00	0.42	0.00	0.37	0.00
Shoot height (cm)	651	21.31	0.02	77.00	24.41	0.06	19.34	0.02
Number of shoots	651	4.05	0.00	16.00	3.86	0.01	4.30	0.01
Leaf anthocyanins (0-100%)	650	18.73	0.03	100.00	14.71	0.06	20.70	0.04
Total flowers	128	0.29	0.00	15.00	0.30	0.00	0.29	0.00
Flowers per individ	65	1.89	0.04	15.00	--	--	--	--
Capsules per flowering individ	65	2.72	0.06	15.00	0.44	0.01	0.56	0.01
Leaf flavonoids (mg/g dry tissue)	65	18.74	0.31	87.79	12.93	0.32	25.53	0.85
Calyx flavonoids (mg/g dry tissue)	53	18.23	0.31	70.31	16.66	0.57	19.62	0.65
Number of leaf flavonoids	65	13.75	0.09	25.00	11.51	0.17	16.37	0.16
Number of calyx flavonoids	53	16.55	0.09	18.00	15.36	0.18	17.61	0.18

Chapter 3 Supplementary Data

Figure S1. Phenotypic correlation matrix for the Lautaret high elevation garden on family means (n = 16-116 families) with color in the lower diagonal representing the strength of the correlation coefficient, where blue represents +1 and red represents -1. The upper diagonal shows the correlation coefficient r for each comparison, and bolded coefficients are significant at alpha = 0.05 from a FDR multiple comparisons correction.

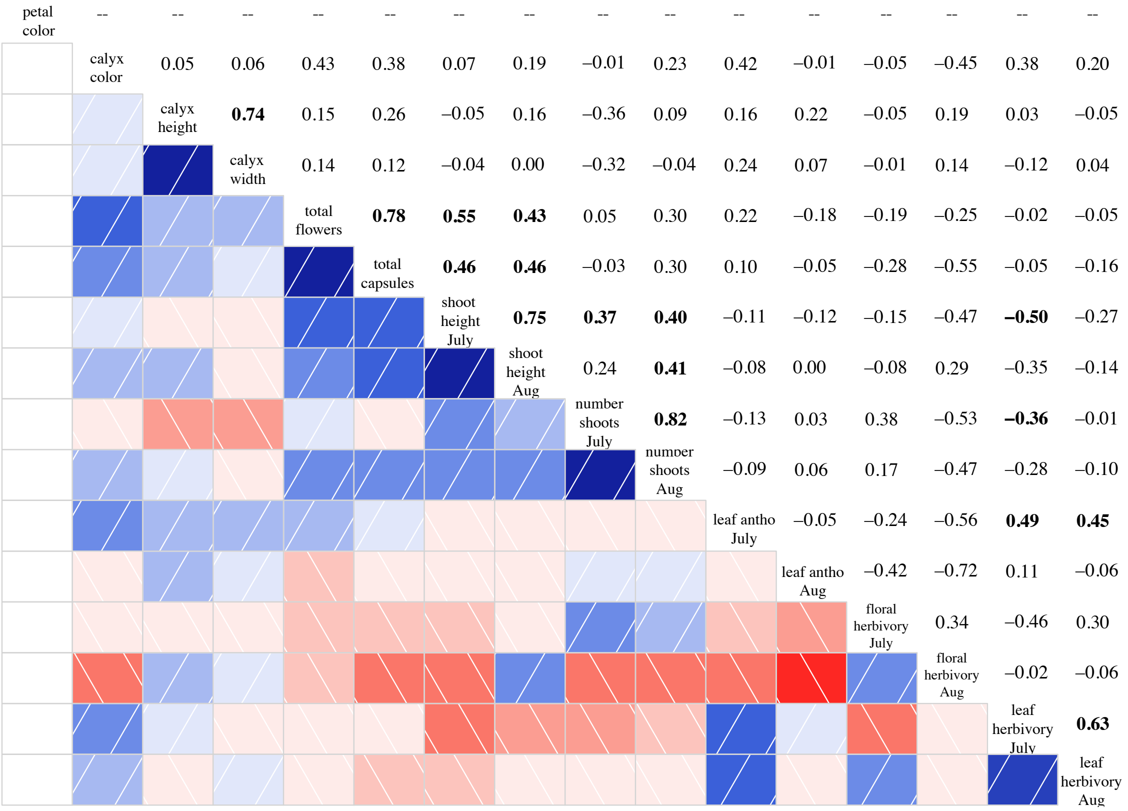
Lautaret (high) garden mean phenotypic correlations



	petal color	calyx color	calyx height	calyx width	total flowers	total capsules	shoot height July	shoot height Aug	number shoots July	number shoots Aug	leaf antho July	leaf antho Aug	floral herbivory July	floral herbivory Aug	leaf herbivory July	leaf herbivory Aug
petal color		0.07	0.10	0.12	0.09	0.01	−0.14	0.14	−0.03	0.05	0.14	0.34	0.15	0.04	0.09	−0.08
calyx color			−0.13	0.11	0.20	0.25	−0.09	−0.08	−0.02	0.17	0.29	0.27	0.07	−0.20	−0.11	−0.09
calyx height				**0.45**	0.32	0.07	0.27	**0.42**	−0.08	−0.14	−0.07	−0.13	−0.10	0.12	0.16	0.10
calyx width					0.32	0.18	0.30	**0.43**	−0.01	−0.09	0.20	0.01	−0.12	0.06	−0.03	0.13
total flowers						**0.74**	**0.32**	**0.36**	0.19	0.23	0.07	0.09	0.04	0.04	0.02	−0.04
total capsules							0.20	**0.26**	**0.28**	**0.29**	0.11	0.21	0.03	0.01	0.00	−0.04
shoot height July								**0.75**	0.21	0.10	0.11	−0.01	−0.34	0.08	0.21	**0.27**
shoot height Aug									0.16	0.16	0.17	0.13	−0.00	0.13	−0.02	0.05
number shoots July										**0.81**	0.08	−0.14	0.60	0.14	0.19	0.08
number shoots Aug											0.01	−0.16	0.62	0.24	0.15	0.08
leaf antho July												**0.50**	−0.10	0.03	−0.12	−0.15
leaf antho Aug													0.18	−0.05	−0.09	−0.08
floral herbivory July														0.40	−0.07	−0.09
floral herbivory Aug															0.28	0.41
leaf herbivory July																**0.62**
leaf herbivory Aug																

Figure S2. Phenotypic correlation matrix for the BDO low elevation garden on family means (n = 8-59 families) with color in the lower diagonal representing the strength of the correlation coefficient, where blue represents +1 and red represents -1. The upper diagonal shows the correlation coefficient r for each comparison, and bolded coefficients are significant at alpha = 0.05 from a FDR multiple comparisons correction. I could not calculate correlations with petal color because there was no variance at BDO; all petals were white.

BDO (low) garden mean phenotypic correlations



	calyx color	calyx height	calyx width	total flowers	total capsules	shoot height July	shoot height Aug	number shoots July	number shoots Aug	leaf antho July	leaf antho Aug	floral herbivory July	floral herbivory Aug	leaf herbivory July	leaf herbivory Aug
petal color	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
calyx color		0.05	0.06	0.43	0.38	0.07	0.19	−0.01	0.23	0.42	−0.01	−0.05	−0.45	0.38	0.20
calyx height			**0.74**	0.15	0.26	−0.05	0.16	−0.36	0.09	0.16	0.22	−0.05	0.19	0.03	−0.05
calyx width				0.14	0.12	−0.04	0.00	−0.32	−0.04	0.24	0.07	−0.01	0.14	−0.12	0.04
total flowers					**0.78**	**0.55**	**0.43**	0.05	0.30	0.22	−0.18	−0.19	−0.25	−0.02	−0.05
total capsules						**0.46**	**0.46**	−0.03	0.30	0.10	−0.05	−0.28	−0.55	−0.05	−0.16
shoot height July							**0.75**	**0.37**	**0.40**	−0.11	−0.12	−0.15	−0.47	**−0.50**	−0.27
shoot height Aug								0.24	**0.41**	−0.08	0.00	−0.08	0.29	−0.35	−0.14
number shoots July									**0.82**	−0.13	0.03	0.38	−0.53	**−0.36**	−0.01
number shoots Aug										−0.09	0.06	0.17	−0.47	−0.28	−0.10
leaf antho July											−0.05	−0.24	−0.56	**0.49**	**0.45**
leaf antho Aug												−0.42	−0.72	0.11	−0.06
floral herbivory July													0.34	−0.46	0.30
floral herbivory Aug														−0.02	−0.06
leaf herbivory July															**0.63**
leaf herbivory Aug															

CHAPTER 4:
CONTRIBUTIONS OF ELEVATION, ORIGIN, AND ENVIRONMENT TO FLAVONOID PATHWAY GENE EXPRESSION

Abstract

The plant flavonoid pathway is instrumental in the response to the environment in the form of phenotypic plasticity and in adaptation. Comparisons between high and low elevation environments provide broad differences in several important factors affecting plant life, such as temperature, moisture, light, and biotic interactions. I identified flavonoid pathway genes in *Silene vulgaris* that would be directly involved in the response to the environment through plasticity or adaptation, and tested their expression in leaf and floral tissue using a reciprocal transplant RNAseq study with high and low elevation plants reciprocally planted at high and low elevation gardens. Flavonoid pathway gene expression was found to be both responsive to the environment on a per-locus basis, as well as having genotypic similarities with populations of the same elevation origin performing similarly. I found more flavonoid gene expression in floral tissue compared to leaf tissue in both experimental gardens. I also found similar expression of FS (flavone synthase) in leaves and flowers by populations of the same elevation origin, with low elevation of origin populations expressing higher amounts of FS at both experimental gardens, suggesting that adaptation to flavone expression has occurred at different elevations. Network analysis showed modularity in flavonoid pathway expression, with the terminal branch genes showing more variability of expression across environments.

Introduction

The plant flavonoid pathway forms distinct groups of compounds responsible for several important roles in plant life history, acting as floral and vegetative pigments, herbivore feeding deterrents, protection against fungal or bacterial infection, antioxidants, and as sunscreens (Winkel-Shirley, 2001). An interconnected and phylogenetically conserved network of genes is responsible for the production of each flavonoid subgroup; coordinated regulation varies to ensure the appropriate compounds are produced when and where needed (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006). Adaptive changes in regulation and natural selection, however, may be constrained by the fact that genes and enzymes are often shared among different branches of the pathway (Rausher *et al.*, 1999; Coberly & Rausher, 2008). There is a paucity of genetic and phenotypic analyses integrating the various flavonoid branches and the phenotypes they produce.

The flavonoid pathway has been well characterized in several plant species (Rausher, 2006). Much is known about the genes and enzymes responsible for the flavonoid structural pathway, e.g. chalcone synthase (CHS) is the first committed step, followed by a small network of modifying genes (Figure 1). In addition, the general regulation of the flavonoid pathway has been characterized; it is influenced by three families of regulatory genes (MYB, bHLH, WD40), is under developmental control (e.g. floral color expression), and is influenced by many environmental factors, such as light, drought, temperature, and generalized plant stress (Quattrocchio et al. 2006, Winkel-Shirley 2002).

Knowledge of the structural flavonoid pathway enables us to make predictions as to how flavonoid subgroups may be correlated in their production. For example, branches sharing genes should be positively correlated, while two branches that share a common precursor may covary positively or negatively depending on which components of the pathway is genetically variable.

The magnitude and direction of genetic correlations among traits may vary depending on which components of the network are genetically variable. Evidence for upstream pleiotropic control has been documented in several studies, where mutations in the upstream genes (i.e. CHS) can turn the entire pathway “off”, or even mutations in genes responsible for permanently inactivating particular branches; this is a common route for the evolution of white flowers (Clegg & Durbin, 2000; Coberly & Rausher, 2008; Smith *et al.*, 2008; Streisfeld & Rausher, 2009b, 2011), and shifts between particular floral color pigments (Hopkins & Rausher, 2011; Smith & Rausher, 2011; Wessinger & Rausher, 2014).

When considering changes in production of the flavonoid pathway in an evolutionary context, it is important to consider both regulatory and structural modifications of the flavonoid pathway components; regulation of the pathway can control variation in flavonoid production, as can structural mutations. Regulation can occur at several points of the pathway, as well as by both cis- and trans-regulation (Quattrocchio *et al.*, 2006; Streisfeld & Rausher, 2009a, 2011). Field studies, such as this one, will be largely examine the effect of regulation of the pathway. For example, as plants experience increased UV-B radiation, they may produce more flavonoids to increase their epidermal protection (Jordan *et al.*, 1994, 1998). This would be due to the response to stress (Winkel-Shirley 2002). Flavonoid response to abiotic and biotic stress is largely due to changes in regulation (Winkel-Shirley 2002). I propose to quantify flavonoid variation in light of gene regulation. Examining flavonoid gene expression will allow us to tie together the complex flavonoid phenotype, and will also describe the link between genotype and phenotype. Measuring the gene expression helps us to understand the flux of the pathway structure (Wright & Rausher, 2010).

Testable hypotheses can be constructed given the layout of the flavonoid pathway and knowledge of the specific roles each branch plays in the ecological life of a plant. I propose to use the widespread plant, *Silene vulgaris* (Caryophyllaceae), to test the bounds of flavonoid expression in experimental gardens. *S. vulgaris* grows at a wide range of elevation, from sea-level to 2400m in the Eurasian landmass. I will use elevation as an axis on variation in which I make predictions of flavonoid pathway expression changes. I utilized a reciprocal transplant design to make predictions regarding the explicit contributions of elevation of origin and experimental garden to flavonoid pathway expression. I expect that the environment will play a large role in the induction of flavonoid expression (Chalker-Scott, 1999; Lu *et al.*, 2009), but also predict that there will be signatures of local adaptation by the retention of some expression signatures by genotype.

First, I predict that FS, encoding flavone synthase, would play a role in adaptation to high environments since the gene encodes for the production of flavones, which can act as sunscreens (Brederode & Kooten, 1983; Staaij *et al.*, 1995; van de Staaij *et al.*, 1995; Caasi-lit *et al.*, 2007; Shaukat *et al.*, 2013). Typically flavonols and FLS (flavonol synthase) have been ascribed to UV protection and plant defensive roles, but there is currently no evidence for their presence in adult tissues of *S. vulgaris* (Chapter 1), and its absence is reflected in Figure 1. Thus, I hypothesize that these roles fall to the flavones, which are close in structure (with the absence of one hydroxyl group) and in UV-spectra and absorbance. Anthocyanins may also increase with elevation due to general accumulation during stress, temperature changes, moisture changes, and UV changes (Steyn *et al.*, 2002; Winkel-Shirley, 2002; Lu *et al.*, 2009). Anthocyanins have clear functions in floral tissues regarding pollination, but their role in the amelioration of stress makes predictions less clear for their role in leaf tissue along an elevational gradient.

Second, I will also utilize network analysis in the form of weighted gene coexpression network analysis to test for significant nodes (genes) and modularity of flavonoid pathway expression. Three groups can be made in the flavonoid pathway: transcription factors, upper structural genes, and lower or branch structural genes. There are different molecular signatures of selection between the upper and lower structural genes of the pathway due to their functional roles; changes in the upper structural genes have pleiotropic effects on the rest of the pathway, whereas there is reduced selective constraint on the lower branches (Rausher *et al.*, 1999, 2008; Lu & Rausher, 2003). I therefore expect the upstream genes in the pathway to be less variably expressed than the downstream genes, due to differential transcription and selection on function (Koes *et al.*, 2005; Quattrocchio *et al.*, 2006; Shan *et al.*, 2009).

Third, I hypothesize that the flavonoid pathway, in its entirety, is generally more transcribed when comparing high elevation plants to low elevation plants. Taking a next-generation sequencing approach is appropriate because it will allow us to appropriately compare flavonoid pathway transcription as compared to general rates of transcription and expression. Not only will this experiment be a genome-wide comparative study of high elevation and low elevation plants, but it will allow us to consider the entire flavonoid pathway at once, rather than focusing on one branch at a time.

Methods

Plant material

S. vulgaris individuals were chosen from two experimental gardens: one high garden at the Station Alpine Joseph Fourier in the Col du Lautaret, France (2100 m elevation), and the other 40 km away in Le Bourg d'Oisans, Hautes-Alpes, France (700 m elevation). Briefly, seeds were collected in the field, brought back to the University of Virginia greenhouse, and populations were randomly mated so as to diminish maternal provisioning effects. The offspring from within-population mating were planted in the field in the France in late May to early June. Supplemental water was provided to all plants to improve survival. Two high elevation origin populations and two low elevation origin populations from this experiment (detailed in Chapter 3) were chosen for analysis. Thus, each elevation origin had two replicate populations: BOL and CON populations from low elevation, and GAS and GUA populations from high elevation origin. Two to three individuals were chosen and pooled per population and per garden (Appendix Tables 1-2) to create a reciprocal transplant design so that each population of interest was sampled at both the high elevation garden and the low elevation garden (N = 16 samples: 8 leaf samples, 8 flower samples).

RNA-seq preparation

One whole flower at sexual maturity (for male-sterile plants: stigmatic receptivity, for hermaphrodites, the second day of anthesis) and either one or two leaves (enough leaf tissue to amount to 1 ug RNA) at the first cauline node below the flowering stalk were sampled per plant between August 3-18, 2013. The BOL sample from the high elevation garden, however, was sampled as a bud due to time constraints. Samples were immediately placed in Ambion RNA-later solution to preserve the integrity of RNA, stored at 4°C overnight, whereupon the supernatant was removed and the preserved tissue stored at -20°C at the Station Alpine Joseph Fourier. Samples were then thawed according to the RNA-later protocol, and ground in liquid nitrogen. Total cellular RNA was then extracted with the Qiagen RNeasy Plant Mini Kit standard protocol, including an on-column DNAse I step. Two to three individuals per flower or leaf sample were pooled into a single library to simulate a “population average” expression sample from each garden (Appendix Table 2). Thus, each high elevation origin population was sampled at the high and low gardens, and each low elevation origin population was also sampled at the high and low gardens. Each reciprocal treatment had two population biological replicates.

Library construction was performed at the UVA Department of Biology Genomics Core Facility: three rounds of selection with Sera-Mag Oligo(dT) Magnetic Particles (Thermo Scientific) were used to enrich for mRNAs. These samples were then used for RNA-seq library construction following standard Illumina protocols with the NEBNext mRNA Library Prep Reagent Set (New England Biolabs) to make 2x100 PE libraries. All 16 libraries (8 flower, 8 leaf) were multiplexed using Illumina barcodes, balanced, and run on two lanes of an Illumina HiSeq2000 by Genewiz, Inc.

Transcriptome assembly and gene expression quantification

A graphical depiction of transcriptome assembly and analysis is depicted in Figure 2. I used the khmer mRNAseq assembly protocol in order to prepare the raw reads from both Illumina lanes for *de novo* assembly (Brown *et al.*, 2013; McDonald & Brown, 2013). khmer is a *in silico* normalization method that allows for a decrease in sampling variation, discarding of redundant data, and the removal of erroneous reads. The khmer protocol has been shown to significantly increase the efficiency and quality of *de novo* assembly of mRNAseq datasets (McDonald & Brown, 2013). Specifically, following removal of adapter sequences by GENEWIZ, Inc., paired reads were interleaved using the khmer script interleave-reads.py. Reads were quality trimmed using fastx-toolkit (Pearson *et al.*, 1997), wherein the fastq_quality_filter utility was used to remove all reads which with a quality score < 30 for greater than 50% of all reads. The remaining paired reads were extracted using the extract-paired-reads.py script, separating orphaned reads into their own fastq file. Reads were subsequently digitally normalized (both paired- and single-end) using the normalize-by-median.py script with a kmer size of 20, a cut-off value of 20, and a hash size of 16GB, with likely erroneous kmers removed using the filter-abund.py script. Paired-end reads were single direction reads using split-paired-reads.py, while the single-ended orphans was added to one of the two sets of previously paired reads. These pre-processing steps improve both the quality of a shotgun *de-novo* assembly and reduces computational time and resources (Brown *et al.*, 2012).

As there is no current reference genome for *Silene vulgaris*, the resulting reads were then assembled *de novo* using default parameters using Trinity v20131110 (Grabherr *et al.*, 2011; Haas *et al.*, 2013). All trimming and assembly operations were performed on Blacklight server (Pittsburgh Supercomputer Center). Gene expression was quantified in the forms of Trinity components (proxy for a gene; hereafter called “genes”; Grabherr *et al.*, 2011) and transcripts using RSEM v.1.2.7 (Li & Dewey, 2011), mapping each sample library back onto the single Trinity assembly.

Gene Annotation

The Trinotate pipeline was used to for the functional annotation the transcriptomes. Briefly, the assembled transcriptome was analyzed with Trinity perl scripts that combines methods for functional annotation including searching for homology to known sequence data (NCBI-BLAST; Top BLASTX hit, Top BLASTP hit), protein domain identification (HMMER/PFAM), protein signal prediction (signalP/tmHMM), and also searching curated annotation databases (EMBL Uniprot eggNOG/GO Pathways databases). Default settings were used throughout the Trinotate pipeline, and the results were placed in an SQLite database. A total of 262,722 unique components (genes) and 617,187 unique transcripts were passed through the Trinotate pipeline. I then used MEGAN5 to illustrate the phylogeny of the BLASTX annotations (Huson *et al.*, 2007).

Finding Flavonoid Genes

Local BLAST+ tools (blastn) were used on the assembled transcriptome to retrieve transcripts and genes with high sequence homology to that of functionally annotated flavonoid pathway genes and the known flavonoid pathway transcription factors from the R2R3-MYB, bHLH, and WD40 protein families (flavonoid pathway gene abbreviations in Table 1). I primarily used gene accessions from *Dianthus* species (a closely related genus in the *Caryophyllaceae*), *Arabidopsis thaliana*, and *Glycine max* (Supplementary Table 1). The gene F3'5'H was not found, and neither was it expected due to its absence in other genera in Caryophyllaceae (Mato *et al.*, 2000; Zuker *et al.*, 2002; Rausher, 2006). The annotation database was searched for flavonoid and anthocyanin pathway annotations, and results were compared to the NCBI Genbank database with BLASTN to confirm homology to the genes of interest. A final list of putative flavonoid pathway genes was then compiled to use downstream in targeted analyses of differential expression on the flavonoid pathway.

Differential Gene Expression Analysis

The RSEM gene expression data was reviewed to determine whether certain genes were expressed by flowers, leaves, or both. Counts of the genes of the structural flavonoid pathway (not including putative transcription factors) were summed by biological sample as a proxy for total flavonoid gene expression. Paired t-tests were used to test the overall difference between flowers and leaves within each garden, and the overall difference between high and low garden flavonoid gene expression within tissues (SAS 9.3). The $\log_2$ transformed expression counts (log base chosen to match with downstream DE analyses) were analyzed by tissue with a two-way ANOVA (SAS 9.3) to test for the effects of elevation of origin, garden, or their interaction, as well as for phenotypic plasticity (variance in garden). Population origin was used as a proxy for elevation of origin (with two replicates of high and low origin each) and the two experimental gardens as were used (high and low common gardens).

All differential expression (DE) analyses were performed on genes versus transcripts to better visualize differences among copies and alleles. The Trinity protocol with biological replicates was used to analyze differentially expressed genes using edgeR v.2.13 (Robinson *et al.*, 2010; Haas *et al.*, 2013) using default settings to identify DE genes among flower samples, leaf samples, all samples from the high elevation garden, and all samples from the low elevation garden. This analysis was to compare all flower pairwise samples and all leaf pairwise samples, respectively. TMM normalization was performed to account for composition and contig length differences among the sample libraries (Robinson & Oshlack, 2010; Haas *et al.*, 2013), after which FPKM (fragments per kilobase of transcript per million mapped reads) values per gene and per sample were calculated.

To analyze the DE genes in a pairwise manner, a false discovery rate (FDR) was set to of 0.01% ($p = 0.0001$) and at least a 4-fold difference in gene expression was required. I used Trinity defaults of Euclidian gene distance and complete gene clustering but adjusted the maximum gene clustering parameter to 16000. Flavonoid pathway genes were then extracted from the centered FPKM results files and their reaction norms were plotted. The centered FPKM values of flavonoid pathway genes were also tested by a two-way ANOVA in SAS (v9.3) for effects of elevation of origin, garden, and their interaction.

Since my experimental design was that of a reciprocal transplant with Garden and Origin as crossed factors, I utilized the standalone version of edgeR (v.2.13) for its GLM function of differential expression analysis (McCarthy *et al.*, 2012; Robinson *et al.*, 2013); the FDR was set to 5%. The RSEM gene counts matrix values were rounded to the nearest integer and samples were normalized by dividing by transcript length. Comparisons were made between population origins and garden locations with likelihood ratio testing, then the top ten DE genes and their annotations were extracted for each comparison. All DE analyses were conducted on the structural flavonoid pathway genes only, and not on the putatively identified regulatory genes.

Network Analysis

Although I analyzed the flavonoid pathway genes on a per-locus basis, it is also important to analyze members of genetic pathways together due to their modularity and their selective and metabolic interdependence (Rausher *et al.*, 1999; Cork & Purugganan, 2004; Hahn *et al.*, 2004; Proulx *et al.*, 2005). The R package WCGNA (Storey *et al.*, 2004; Langfelder & Horvath, 2008) was used to perform a weighted correlation network analysis (WCGNA) for the flavonoid pathway genes (structural and putative regulatory) found for flower and leaf tissues, as well as a consensus analysis of both tissues. A glossary of network analysis terms is provided in the Appendix (Appendix Table 3). WGCNA was used for finding clusters (called modules) of highly correlated flavonoid pathway genes from expression data and for summarizing modules using a module eigengene approach (where the module eigengene is the first principal component of a given module) and intramodular connectivity (which measures how connected or co-expressed a given gene is compared to the other genes in its module) (Langfelder & Horvath, 2008). The same R package was also used for relating the network modules to biological sample traits of individuals from populations of different elevations and grown in different gardens using eigengene network methodology, where gene significance (the absolute value of the correlation between the gene and trait) and module membership (the correlation of the module eigengene and the gene expression profile) were calculated separately for each analysis (leaf, flower, consensus). Genes with the highest gene significance (or, the highest significant correlation) to my traits of interest (garden and origin), were identified in order to pinpoint parts of the flavonoid pathway most greatly contributing to expression divergence between experimental garden and elevation of origin. Lastly, module preservation was calculated for the consensus modules to determine whether they were preserved across the flower and leaf network, and whether the module and network connectivity patterns remained the same when compared among the two gardens and the two elevation origins (Langfelder *et al.*, 2011).

Results

Transcriptome Assembly and Annotation

Two lanes on an Illumina HiSeq2000 produced a total of ~360 million paired-end reads. After khmer processing, Trinity assembled a total of 617,187 contigs with a minimum contig size of 201 bp, a median of 549 bp, and the mean contig size of 915 bp. The overall quality of the reads was good, with the number of N50 contigs at 114592 (N50 length 1518 bp) and N90 contigs at 404289 (N90 length 363). Mortazavi *et al.*, (2008) estimated that if at least 40 million reads are collected from a sample, it should be possible to detect and quantify RNAs from all biologically relevant abundance classes (high to low abundance). Half of the 16 samples (8 flower, 8 leaf) achieved at least 40 million reads, and 14 samples had at least 30 million reads. I am therefore confident that I was able to pick up moderate to strong signals of differential expression.

The results of the Trinotate annotation are described in Table 2. BLASTX was able to assign homology to the most contigs at 41%. The top BLASTX hit report was used by MEGAN5 to produce a phylogeny of the resulting matching accessions in the NBCI annotated protein database (Appendix Figure 1). Several proteins were matched to those of annotated *Silene* genes, with the majority of plant annotations falling under model species with extensive annotation (e.g. *Arabidopsis thaliana*). While many of the proteins were annotated as plant proteins, a fair amount of hits were annotated to other kingdoms. These assignments may reflect various symbiotic relationships that *S. vulgaris* may have with bacteria and fungi; but it may also reflect novel or poorly assembled transcripts.

Flavonoid gene identification

A total of 33 contigs matched flavonoid structural pathway genes and 26 putative contigs matched regulatory flavonoid genes. These contigs were the consensus results from searching through the annotation database (especially through the gene ontology results) and also by homology with BLAST. Multiple contigs were found per gene, with CHS having the most (16) assigned in the structural pathway, and MYBs having the most assigned out of the putative transcription factors (Table 3). While most of the contigs were expressed in both floral and leaf tissue, some were only expressed in floral or leaf samples (Table 3). In addition, about 15% of the contigs found appeared to be “private” contigs (like private alleles); without further testing it cannot be determined whether these private contigs are alleles, splice variants, or homologs.

Total flavonoid pathway expression

The cumulative gene expression count of flower samples between experimental gardens was not significantly different (t = 1.389, df = 2, p = 0.881); neither were leaf samples between gardens (t = -0.137, df = 2, p = 0.449). However, there were differences between populations, with flower samples from BOL, GAS, and GUA increasing from the low to the high garden and CON decreasing (Figure 3); leaf samples from BOL and GAS increasing from the low to high garden, CON decreasing from the low to high garden (Figure 3). Within the high elevation garden, flower and leaf flavonoid gene expression differences bordered on statistical significance (t = -2.670, df = 4, p = 0.0645) with flowers expressing more flavonoid transcripts than leaves. The same pattern was detected in the low elevation garden, but had a much stronger signal (t = -1.909, df = 4, p = 0.0280). In both comparisons of flower to leaf flavonoid gene expression, all populations had lower flavonoid gene expression in leaves than in flowers (Figure 3).

In a two-way ANOVA on total flavonoid gene expression reflecting the experimental design (two population replicates for each origin-garden pair), no significant effects of elevation of origin, garden, or GxE were found for either flowers or leaves (Table S2). However, the interaction of origin and garden on total flavonoid gene expression was nearly significant in flowers ($F_{(1,4)}$ = 6.00, p = 0.0704). Both low-origin populations had lower expression in the low elevation garden, and both high-origin populations had higher levels expression in the high elevation gardens (differences of total flavonoid expression (log2, the analog to fold-change) from high to low: BOL -9.72, CON -30.21, GAS 19.05, GUA 29.74).

Pairwise Population-Garden Differential Expression Analysis

Using Trinity-edgeR pipeline for differential expression (DE) analysis at a minimum four-fold expression change (at $p \leq 0.0001$) on the entire transcriptomes, 15,611 DE features were found in the flower transcriptomes, and 9,578 DE features in the leaf. For the leaf expression data (Figure 4), the clustering dendrogram grouped leaf samples by garden, suggesting a larger effect of the garden environment plants were grown in relative to the elevation they came from. Within each garden grouping, low garden individuals were grouped by elevation origin (low elevation of origin samples were more similar to each other than high elevation of origin samples), while the high garden group did not share the same clustering pattern. The dendrogram clustering pattern was less clear in the flower expression data (Figure 5), where individual samples were clustered by both elevation of origin and experimental garden.

We extracted flavonoid pathway genes from this DE dataset. Flavonoid pathway gene expression values (as centered FPKM) were highly variable among loci, tissues, samples, and gardens. All structural flavonoid loci had DE contigs among tissues except for CHI (Table 3). However, few statistically significant effects of overall elevation of origin, garden, and GxE were found on a per-locus basis, with the exception of two downstream loci, FS and UF3GT. One of the two FS contigs found in the transcriptomes, comp139966_c0, showed a significant effect of elevation of origin in flower tissue ($F_{(1,4)}$ =19.39, $p = 0.0117$; Figure 6A) and in leaf tissue ($F_{(1,4)} = 26.12$, $p = 0.0069$; Figure 6B). A *post-hoc* Tukey test found that the FS gene was expressed at higher levels in the low elevation origin populations than in the high elevation origin populations at both gardens. The single contig found for UF3GT, an anthocyanin glycosylating gene, showed significantly different expression in flower tissues between the two garden environments ($F_{(1,4)}$ =13.02, $p = 0.0226$; Figure 6C), with increased expression in the high elevation garden than in the low elevation garden.

Since I tested the main effect elevation of origin as having two replicate populations for each origin elevation, paired t-tests were calculated for each locus to determine whether there were population-specific effects on gene expression. Loci with significant population-specific effects were those previously detected by the *post-hoc* Tukey tests above, with the addition of two flavonoid contigs. Another FS leaf contig had population-specific effects ($t = 2.26$, df = 4, $p = 0.0429$, Figure 6D) with the low-origin populations decreasing expression at the high elevation garden, GAS high-origin leaves decreasing expression from low to high gardens, but with a less steep reaction norm slope, and GUA high-origin leaves remaining at a very low level of expression at both gardens. A CHS leaf contig showed decreased expression from low to high garden in all populations except for GUA, but retained the same phenotypic ranks with BOL and GAS expressing the most and CON and GUA the least ($t = 2.504$, df = 4, $p = 0.0332$, Figure 6E).

Effects of elevation of origin and experimental garden differential expression analysis

In contrast to testing pairwise population-specific levels of gene expression as above, we leveraged the reciprocal transplant design and performed a GLM-based analysis on all DE genes. The leaf and flower gene counts data were filtered to keep genes with at least one count per million in four samples out of eight, which yielded 24888 unique DE genes for the leaf analysis and 26480 for the flower analysis. The resulting DE genes were extracted and examined for their gene ontology (GO) annotations in hopes of finding flavonoid genes as highly DE.

The general theme of the gene ontology annotations of the top ten DE genes for all comparisons, including both leaves and flowers, was that of primary metabolism and basic plant functions, such as mitochondrial and chloroplast function, ribosomal function, cytochrome b function, and lipid metabolism. There were eight DE genes that were shared among the comparisons of high and low garden, high and low origin, and flowers and leaves; their GO annotations revealed ribosomal, mitochondrial, and glycolysis activity.

When comparing the top 10 DE genes across all comparisons (elevation origin, garden, origin*garden), one putative flavonoid pathway gene was significantly differentially expressed in the comparison of high and low elevation origin flowers at the low elevation garden, comp125066_c0 (annotated as UDP-glycosyltransferase with GO:0080044 molecular function quercetin 7-O-glucosyltransferase activity; Table S3). High-origin individuals down-regulated this gene more than low-origin individuals (the log-fold-change (logFC) of high-origin individuals was -27.074 and -20.284 for low-origin indidivudals at the low garden).

When comparing the top 100 DE genes for all comparisons, four were flavonoid pathway members. The same UDP-glycosyltransferase comp125066_0 was found for an additional comparison between high origin flowers at both gardens. In addition, three other components with flavonoid or anthocyanin biosynthetic pathway GO annotations were found, with annotations as a putative F3H gene, similarity to the *Arabidopsis thaliana* TT12 flavonoid transporter, and a UDP-xylosyltransferase (Table S3).

Given all of the genes transcribed in a plant, the fact that flavonoid genes are present in the top 100 and 10 differentially expressed genes suggests that the flavonoid pathway is an active player in plant life, and perhaps even adaptation to elevation, considering the experimental setup.

Network Analysis

The goal of the network analysis was to determine whether the flavonoid pathway is expressed in a modular way, for example, if upstream genes are regulated and expressed similarly, then they would be grouped into a module. The second goal was then to correlate resulting gene modules to traits of interest, in this case, experimental garden and elevation of origin. The network analyses of the leaf samples and flower samples each yielded three modules of highly correlated genes (as seen in a network eigengene heatmap, where gray is the group of genes not assigned to a module; Figure 7). Interestingly, if I examine the modules of correlated gene expression in flowers or leaves, I found that modules were comprised of genes from all over the pathway (upstream and downstream), including transcription factors. When experimental garden and elevation origin were added to the model and analyzed for adjacency to the eigengene modules (trait-gene expression correlations), no correlations were statistically significant (Figure S2, S3). Despite this result, some patterns can be observed. For the flower analysis: stronger correlation values between the flower modules and garden membership than the gene modules and elevation origin; in addition, the direction of the correlations was generally positive for garden and negative for origin (Figure 8A). The brown and turquoise modules were also more highly correlated with garden, while the blue module more highly correlated with origin. For the leaf analysis, the blue module had the strongest correlation with garden, while the other modules were less strongly correlated (Figure 8B). Table 4 shows a list of genes from these modules that were the most strongly correlated with my traits of interest: garden and elevation of origin. Here, a DFR contig is significantly correlated to low-garden effects (because of the negative correlation) for leaf and flower samples; referencing that gene's expression reaction norm in the Supplementary material shows that there are higher levels of expression at the low-elevation garden (see reaction norm in Supplementary DFR section). A FS contig is strongly correlated with low origin leaves and flowers (again, because of the negative correlation); referencing that gene's expression reaction norm in Figure 6 indicates that there are higher levels of expression at the low-elevation garden. It should be noted that in each of these cases, it is also the same contig (note the comp number), indicating that the same allele is expressed in both tissues. bHLH and MYB transcription factors (but not the same contigs) are also present in this analysis.

The consensus network analysis yielded two modules of highly correlated genes. In this analysis, the modules have the same gene composition and are directly comparable between flowers and leaves, whereas before I kept leaf and flower tissue analyses separate. Some important biological observation is demonstrated by the differences in the gene composition of these two models. The turquoise model is composed of most members of the flavonoid pathway, including several transcription factor contigs. The blue module, however, is comprised of mostly downstream contigs: F3H, FS, DFR, and UF3GT, as well as some transcription factors (MYB and wd40). This indicates that the lower, branching genes of the pathway are separately regulated from the entire pathway.

Module trait-relationships for garden as a trait were not statistically significant for flowers or leaves (Figure S4A and B), although the strength of the correlations were much greater for flowers than they were for leaves, especially with regards to the turquoise module (Figure 9A,D). The blue module correlation strength was close to 0.50 for both flowers and leaves. Network preservation is high when comparing the flower and leaf sets together against experimental garden, especially for the blue module (Figure 9C), which suggests that the modularity, or gene cluster co-expression, acts similarly in leaves as it does in flowers.

Eigengene adjacency for elevation origin as a trait was not statistically significant for flowers or leaves (Figure S4A and B). The correlations for flower-origin and leaf-origin are weak, but the inter-modular correlations for the flower-origin comparison are strong. The network preservation is also high when comparing the flower and leaf sets together against elevation origin (Figure 10).

Table 5 shows the statistically significant members of modules with strong gene significance towards garden or origin for the consensus analysis. Significant F3'H and DFR genes are present in the garden comparisons, indicating that, like the individual leaf and flower analyses, downstream genes have the most gene significance, or trait correlation, for the garden comparisons. The same DFR component that was a significant gene for the garden-leaf and garden-flower individual comparisons was still significant for the garden-flower consensus analysis (and the same pattern holds: higher expression at the low garden).

Both upstream and downstream genes had high gene significance for the elevation of origin consensus analysis, with CHS and ANS contigs being most significant for leaves, although in opposite directions (upstream CHS more significant for high origin and downstream ANS being more significant for low origin). The reaction norm of gene expression for CHS is less than clear, but suggests that low elevation of origin populations tend to have higher expression at the low garden and high elevation of origin populations have, on average slightly higher expression at the high garden. The same FS contig was significant for flower-origin consensus analysis in the same direction as the individual flower analysis (explanation above).

Discussion

I set out to determine whether transcription of the plant flavonoid pathway had a role in local adaptation to existence at different elevations. Specifically, I used four populations of *Silene vulgaris* originating from either high or low elevations in a reciprocal transplant experiment at high and low experimental gardens. I predicted that FS, encoding flavone synthase, would play a role in adaptation to high environments since the gene encodes for the production of flavones, which can act as sunscreens (Brederode & Kooten, 1983; Staaij *et al.*, 1995; van de Staaij *et al.*, 1995; Caasi-lit *et al.*, 2007; Shaukat *et al.*, 2013), and also anthocyanins for their role in and genera accumulation during stress, temperature changes, moisture changes, and UV changes (Steyn *et al.*, 2002; Winkel-Shirley, 2002; Lu *et al.*, 2009).

I identified 33 contigs that were members of the flavonoid structural pathway, and 26 putative transcription factors through homology to related *Dianthus* and other species' flavonoid genes. I expected to find multiple contigs per locus to account for multiple gene copies as well as alleles. Although the exact number of homologs per flavonoid pathway locus is not known in *Silene*, a transcriptome analysis of *Dianthus* found several contigs per flavonoid locus (Tanase *et al.*, 2012).

I identified putative transcription factors from the annotation report, but most homologs of the structural pathway did not have high nucleotide homology to my query sequences. It is, however, the main source from which I found the putative transcription factor contigs. The annotation database, created using the Trinotate pipeline, is thorough for a non-model species, but until there is a well-annotated *Silene* genome, it is not possible to have a well-annotated transcriptome. Regardless, genes of interest identified through the annotation database are candidates for functional testing, as they may very well be members of the *Silene* flavonoid pathway.

One group of flavonoid pathway genes that I was not able to characterize easily was the flavonoid glycosylating genes, which are not typically described in the structural enzymatic pathway, but whose presence is known given the diversity of flavonoid glycosides (Bohm, 1987), and the more recent documentation (Vogt, 2004; Winkel, 2006). Many classes of flavonoids can be further decorated by various sugars (rhamnose, arabinose, glucose, galactose etc.) (Harborne & Williams, 2000; Winkel, 2006). *Silene* is no exception, and these glycosylating genes have been shown to be geographical markers for the related species *S. latifolia* (Mastenbroek *et al.*, 1983). It is therefore likely that I did not identify all auxiliary glycosylating genes; further studies in the form of experimental crosses may be needed to determine their identities.

I expected to see an overall increase in general flavonoid production in all samples at the high elevation garden compared to the low elevation garden because of the increased amount of UV radiation, and the accumulation of anthocyanins as a response to increased UV stress (Jordan *et al.*, 1998; Nybakken *et al.*, 2004; Agati & Tattini, 2010). Rather, I observed a somewhat uniform response in all leaf tissue samples across gardens (Figure 4B). Flower flavonoid expression did not significantly vary among elevation origin or garden, but populations tended to produce more flavonoid transcripts at their home elevation than the other garden. This pattern indicates that total flavonoid expression is plastic, and also suggests that *S. vulgaris* may be locally adapted to their elevation of origin (Chapter 3). Variation in flavonoid plasticity has been shown to be beneficial with regards to adaptation to the local thermal environment, light, herbivory, and defense (Treutter, 2006; Lu *et al.*, 2009; Lacey *et al.*, 2010).

It is also possible that when introduced to the foreign environment, my sampled plants were stressed in their new environment. The low garden experienced very high temperatures in summer 2013, and flavonoids are known to respond to temperature stress (Winkel-Shirley, 2002; Marles *et al.*, 2003; Stiles *et al.*, 2007). Coberly and Rausher (2003) found that a mutant CHS allele in *Ipomoea purpurea* alleviated heat stress and in high temperatures, enhanced fertilization success. It is also possible that the populations observed differ in constitutive and inducible production of flavonoids, and this “snapshot” of RNA expression does not fully define their general reaction to their environment. Whatever the cause, it certainly warrants looking more closely at the pathway loci to really determine what the underlying cause is of this phenomenon.

I also observed a general pattern of increased flavonoid pathway expression in the flower tissues compared to leaf tissues in both gardens, but once again, no significant effects of elevation of origin or garden. This pattern of more flavonoid expression in floral tissue compared to leaf tissue may be expected due to the number of necessary roles of the flavonoid pathway in floral tissue. *Silene vulgaris* flowers are generally more pigmented with anthocyanins than leaves (including stigmas, styles, ovaries, calyces, filaments, and anthers) and also play a role in proper floral development (Winkel-Shirley, 2001; Ageez *et al.*, 2005) and attraction to pollinators (Marsden-Jones & Turrill, 1957; Faegri & van der Pijl, 1979). Genes that did not have many (or any) DE genes among the particular samples, such as CHI and ANS, did still have expression levels in flowers much greater than the leaves, likely contributing to the observed pattern.

When comparing samples in a pairwise fashion per locus for differentially expressed genes, I again found a signature of environmental plasticity across samples and tissues. The significantly differentially expressed flavonoid genes in my samples were those of the terminal branches of the pathway: FS and UF3GT. UF3GT showed increased production in flowers at the high garden when compared to the low garden, thus showing significant plasticity among the environments, but no effect of elevation of origin. This may be due to increased anthocyanin accumulation at higher elevations (see Chapter 2), as it is one of the last steps in anthocyanin production. This finding is particularly interesting, given that in Chapter 3 I found significant variation in flavones (produced by FS) and flavone diversity (presumably controlled by UF3GT). Flavone diversity was at its highest at the high elevation garden, which agrees with the finding at the gene expression level.

FS, or flavone synthase, showed a significant effect of elevation of origin. Both low elevation origin populations had higher expression of flower FS and leaf FS at both gardens compared to the high elevation origin populations (Figure 6A-B). This result may seem counterintuitive, as the need for more epidermal UV protection is thought to be more important at higher elevations, where plants experience more UV radiation and general stress. Although the literature generally agrees with this hypothesis (reviewed in Jansen *et al.*, 1998), and UV radiation (especially UV-B) stimulates flavonoid pigmentation (Jordan *et al.*, 1994; Hofmann, 2000; Ryan *et al.*, 2001) many other studies have been less conclusive, suggesting that UV screening pigments are at their highest concentrations at the seedling stage and decrease with plant age, some suggesting that even the time of day changes transcript levels of CHS (Jordan *et al.*, 1994, 1998; van de Staaij, Ernst, *et al.*, 1995; van de Staaij, Huijsmans, *et al.*, 1995; Nybakken *et al.*, 2008; Qaderi *et al.*, 2010). Thus I cannot rule out the possibility that the high elevation origin populations have a higher constitutive store of UV-screening flavonoid pigments in their vacuoles from local adaptation to higher UV environments compared to low elevation origin populations who may need to manufacture flavonoids more frequently (constitutive production versus induced production) (Treutter, 2006). In Chapter 3 I found clear results of increased levels of flavone pigments in leaf tissue for all populations at the high garden, but floral tissues did not have a clear flavone pattern. More targeted chemical analyses of the same individuals measured for gene expression would clarify this subject.

Several loci had effects of elevation of origin and garden (Figure 6), and two loci showed differences in expression due to population-specific effects. I found population-specific effects in another FS leaf contig as well as a leaf CHS contig. Generally speaking, leaf FS expression of this contig decreased from the low garden to the high garden, but the GAS (high origin) population still showed higher expression at the high garden compared to the other populations (Figure 6). While flavones are suspected to contribute to UV epidermal screening and protection, it is also possible that different herbivory pressure contributed to the differential expression of flavone synthase. Flavones have also been shown to act as feeding deterrents against herbivores and parasites (Winkel-Shirley, 2002; Soriano *et al.*, 2004).

Interestingly, a CHS leaf contig (comp130513_c0, Figure 6) showed an overall effect of garden with decreased expression of CHS at the higher garden (at least 2-fold expression decreases) in all samples except for GUA. CHS is the first committed step of the flavonoid pathway and thus has pleiotropic effects on the rest of the pathway; down-regulation of CHS would therefore reduce flavonoid production (Coberly & Rausher, 2008). However, CHS is known to have several functional and non-functional copies across the plant kingdom (Arioli *et al.*, 1994; Durbin *et al.*, 2000; Dubery & Mienie, 2001; Wang *et al.*, 2007). There are at least five different copies of CHS in *S. vulgaris* (A. Berardi, *unpublished data*) so it is difficult to comment on the overall amount of chalcones across environments based on one copy that is differentially expressed without further experimentation. There is, however, some speculation on the role of redundant CHS in response to UV-A radiation (Zhou *et al.*, 2013).

Notably, no differential expression of the second enzyme in the flavonoid pathway, CHI, was detected. This is not surprising, given CHI has one specific role (in non-legume plants), which is to catalyze the stereospecific isomerization of chalcones into the flavanone naringenin (Naoumkina *et al.*, 2010). Most of the other proteins encoded by the flavonoid pathway interact with several other members of the pathway and compete for substrate and specialize the chemicals further into sub-branches (Koes *et al.*, 2005). One might expect that mutations in CHI to produce variability in expression may be selected against, especially given its important position in the pathway (Rausher *et al.*, 1999).

To test for differentially expressed genes with my factorial design of elevation of origin and experimental garden I used edgeR's glm function, and then I manually searched the Trinotate annotation database I created to examine the annotations for the DE genes. Not surprisingly, the top DE genes from my non-normalized transcriptomes were comprised of "housekeeping" genes related to basic plant function and primary metabolism. I found one putative transcription factor similar to the *Arabidopsis thaliana* TT12 locus, which is largely a flavonoid transporter in seed coats (Winkel-Shirley, 2002; Thompson *et al.*, 2010; Zhao & Dixon, 2010). Similar to the pairwise DE comparisons made, I also found potential flavonoid genes related to the terminal branches of the pathway that were significantly DE. Two of these genes were glycosyl-transferases, which may be members of the population-specific glycosylating genes (Mastenbroek *et al.*, 1982), and also a large family of genes (Li *et al.*, 2001). The putative UDP-xylosyltransferase bore homology to an *A. thaliana* gene that accumulates expression of jasmonic acid-induced anthocyanin production, suggesting anthocyanin accumulation during herbivory, stress, and under UV-light (Shan *et al.*, 2009); the contig with GO annotation as a quercetin 7-O-glycosyltransferase may very well be a glucosyltransferase for flavones in *Silene* (due to the lack of flavonols in adult *S. vulgaris*; Chapter 1) (Lim *et al.*, 2004).

A conclusion that can be drawn from the GLM results is that high-origin individuals in particular appear to have phenotypically labile abilities to glycosylate flavonoids in different environments. Flavonoids are diverse because of the ability to decorate a limited number of shared structures with a nearly unlimited amount of sugars in different patterns (Bohm, 1987). However there have only been a few suggestions as to the role of glycosylating flavonoids to create a diverse “cocktail” compared to a monoculture of flavones (Lavola *et al.*, 1998; Loon *et al.*, 2002; Peck *et al.*, 2006), most suggesting it as a result of stereochemistry and ecological interactions, e.g. fungal symbioses respond to a “cocktail” of flavonoids from root exudates (Peck *et al.* 2006). There may be physical benefits of having different chemical attributes and ratios in UV absorption (Ryan *et al.*, 2002), or in having a diverse array of compounds to fend off generalist herbivores in the hopes that at least one chemical is unpalatable. It is interesting to speculate that the diversity of secondary chemicals may be the result of red-queen dynamics in response to pathogens or herbivores. If true, I would expect to see evidence of positive selection at loci that influence the diversity of products from the flavonoid biosynthetic pathway. The proportional allocation to individual flavonoid compounds in individuals, tissues, and populations likely depends on the specific selective and evolutionary response of plants to both the biotic and abiotic environment (Treutter, 2006; Nybakken *et al.*, 2008).

The goal of the network analysis was to better understand how the flavonoid pathway is expressed in *S. vulgaris* flowers, leaves, and as a whole. I wanted to know if the pathway was expressed in a modular manner, e.g. upstream versus downstream branches, since regulation of each branch is often specific (Quattrocchio *et al.*, 2006; Shan *et al.*, 2009; Hichri *et al.*, 2011). The network was able to group the flavonoid pathway, including putative regulatory genes, into three modules each for leaves and flowers, and two modules for a consensus analysis. Interestingly, the composition of the two consensus modules did vary with one of the modules being comprised of downstream genes, likely reflecting differences in pathway regulation as has been previously shown. Mid- to downstream genes and several transcription factors were identified as having the most gene significance within modules for flowers and leaves (with single tissue analyses and consensus), suggesting that divergence in expression is best characterized by the flavonoid pathway branches. This finding agrees with the expectations of the effect of selection on the flavonoid pathway, where selection is freer to act on downstream genes and constrained in upstream genes due to deleterious pleiotropic effects (Rausher *et al.*, 1999, 2008). All weighted correlation network analyses found that flavonoid pathway modules tended to correlate more strongly with the effect of garden than origin in my samples, reinforcing the finding with overall flavonoid pathway expression being influenced by the environment.

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Table 1. Flavonoid pathway gene abbreviations and names. Genes above the line are members of the structural flavonoid pathway, while the genes below are known transcription factors that regulate the pathway (Koes *et al*., 2005)

Gene Abbreviation	Gene Name
CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase; Flavanone 3b-hydroxylase; Naringenin 3-dioxygenase
F3'H	Flavonoid 3'-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanidin synthase, also known as LDOX
FS	Flavonol or Flavone synthase
UF3GT	UDP-glucose:flavonoid 3-O-glucosyltransferase
MYB	MYB transcription factor
bHLH	basic helix-loop-helix; refers to bHLH-MYC and R2R3-MYB transcription factors
WD40	WD40 repeat protein

Table 2. Gene annotation report showing the number and percentage of contigs assigned to all cDNA libraries combined.

	BLASTX	**prot_id**	**prot_coords**	**BLASTP**	**Pfam**	**SignalP**	**TmHMM**	**eggnog**	**GO**
n	272389	264484	264484	184279	172597	13363	46484	137307	242169
missing	391347	399252	399252	479457	491139	650373	617252	526429	421567
unique	216628	264484	130146	100037	94472	3574	22860	3442	21296
%assigned	41	40	40	28	26	2	7	21	36
% unique assignments	33	40	20	15	14	1	3	1	3

Table 3. Numbers of contigs assigned to flavonoid pathway genes from BLAST and annotation searches. Contigs were determined to be “lowly expressed” if their average RSEM expression count was less than 5 across samples. Contigs were determined to be private if they were only observed in one or two of the populations.

Gene	Number of contigs	Number with low expression	Floral only	Leaf only	Private contig	Number DE floral contigs	Number DE leaf contigs
CHS	16	7	6	1	7	5	7
CHI	2	--	--	--	--	--	--
F3H	4	--	--	--	--	3	2
F3'H	2	--	--	--	--	1	1
FS	4	1	--	--	1	2	2
DFR	3	--	--	--	--	1	1
ANS	1	--	--	--	--	--	1
UF3GT	1	--	--	--	--	1	--
MYB	14	5	2	--	--	NA	NA
bHLH	3	--	--	--	--	NA	NA
WD40	9	2	--	--	1	NA	NA

Table 4. Results from a network analysis on leaves and flowers (separately) that correlated gene expression to my traits of interest, experimental garden and elevation of origin. Only genes with significant correlations to garden and origin from the leaf and flower network analyses are listed. The sign of the correlation reflects to which garden expression is most correlated. Negative correlations reflect more gene significance (or, correlation to the trait of interest) at the low garden or low origin; positive correlations reflect increased gene significance at the high garden or origin. For visual interpretation of high or low expression, each gene's reaction norm plot can be referenced by gene name in the Supplementary material.

Garden					
Leaves			Flowers		
Name	Correlation	*p*	Name	Correlation	*p*
DFR_comp157091_c0	-0.7836	0.0214	DFR_comp157091_c0	-0.8065	0.0156
			MYB_comp136624_c0	0.7603	0.0285

Origin					
Leaves			Flowers		
Name	Correlation	*p*	Name	Correlation	*p*
bHLH_comp159698_c1	0.7105	0.0482	bHLH_comp161389_c0	0.9046	0.0020
FS_comp139966_c0	-0.7341	0.0381	MYB_comp150795_c0	-0.8781	0.0041
			FS_comp139966_c0	-0.7123	0.0474

Table 5. Genes with significant correlations to garden and origin from the consensus network analyses, which described leaf and flower genes with respect to each other, and genes within a consensus model. Negative correlations reflect more gene significance at the low garden or low origin; positive correlations reflect increased gene significance at the high garden or origin

Garden					
Leaves			Flowers		
Name	Correlation	*p*	Name	Correlation	*p*
F3'H_comp144858_c2	-0.7836	0.0214	DFR_comp157091_c0	-0.8065	0.0156
			MYB_comp136624_c0	0.7603	0.0285

Origin					
Leaves			Flowers		
Name	Correlation	*p*	Name	Correlation	*p*
CHS_comp141199_c0	0.7105	0.0482	FS_comp139966_c0	-0.7123	0.0474
ANS_comp151114_c0	-0.7341	0.0381	MYB_comp150795_c0	-0.8781	0.0041
			bHLH_comp161389_c0	0.9046	0.0020

Figure 1. The flavonoid pathway in *Silene vulgaris*. Sections in gray represent potential branches of the flavonoid pathway, but for which there has been no evidence for in adult *S. vulgaris* (Chapter 1), and since no *Silene* species have been observed to display blue flowers, I assume that, like other members of the Caryophyllaceae, the F3'5'H gene is inactivated (Mato *et al.*, 2000; Zuker *et al.*, 2002; Rausher, 2006).

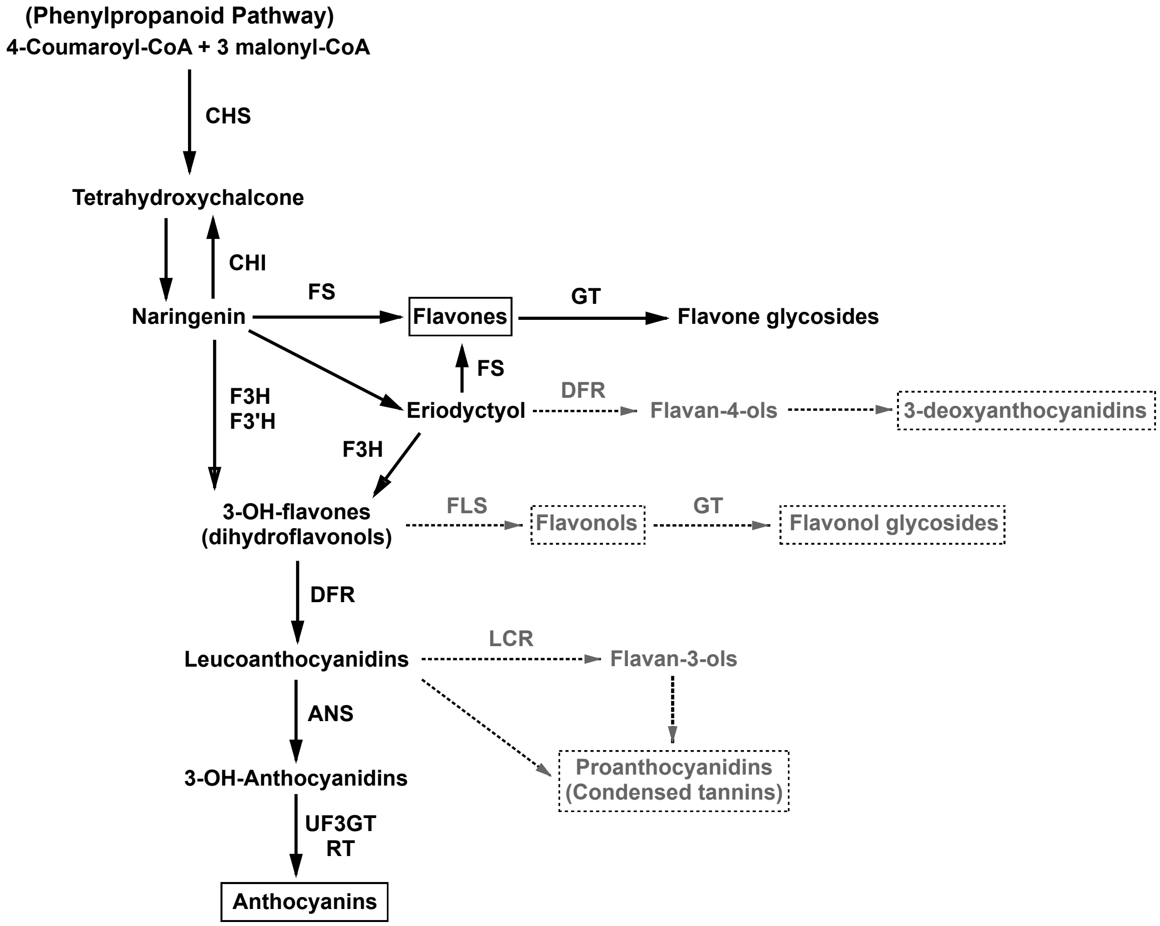


Figure 2. A schematic of the pipeline used for RNA-seq analysis.

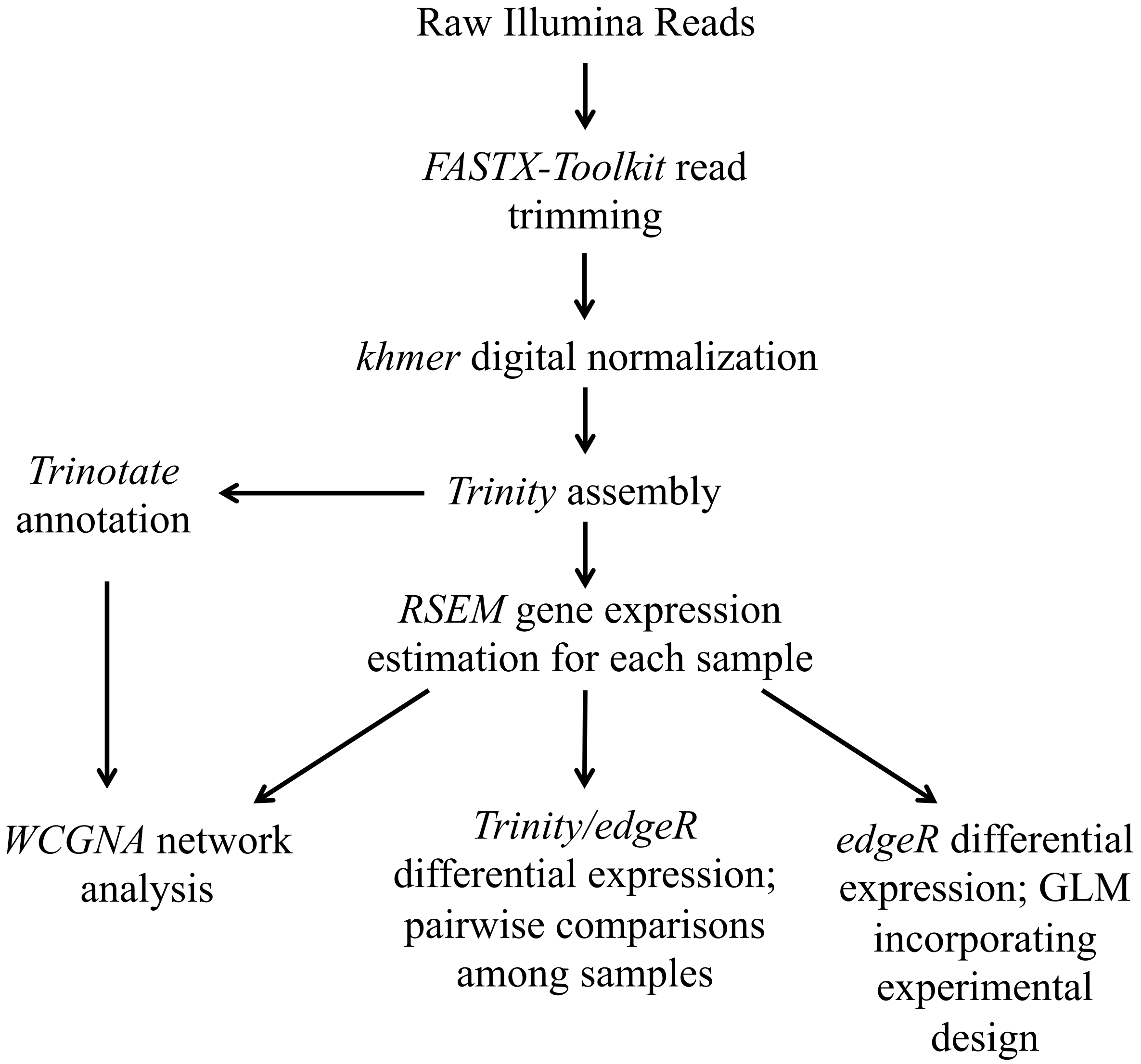


Figure 3. Total flavonoid structural pathway gene expression. Flowers have higher levels of flavonoid pathway gene expression than do leaves.

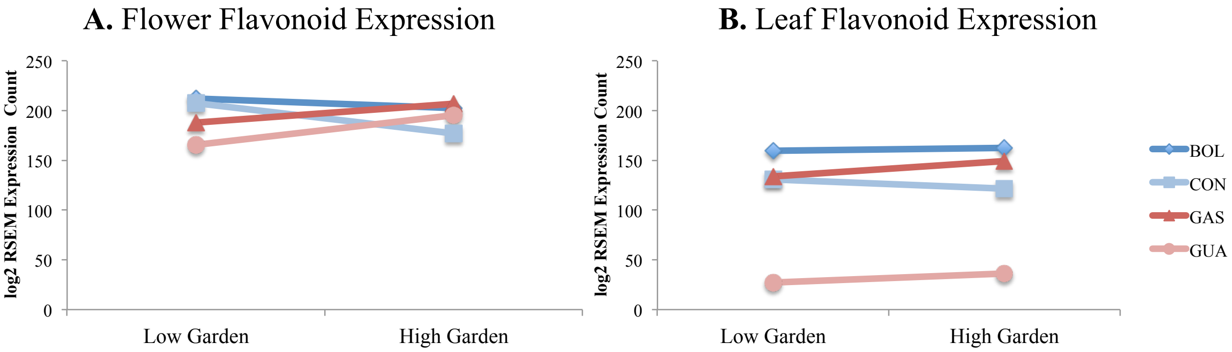


Fig 4. Trinity-edgeR DE analysis showing general differences in gene expression in leaves. The dendrogram to the left groups genes according to their similarity in expression, and each cluster is assigned an arbitrary color. The dendrogram at the top clusters samples based on similarity of gene expression.

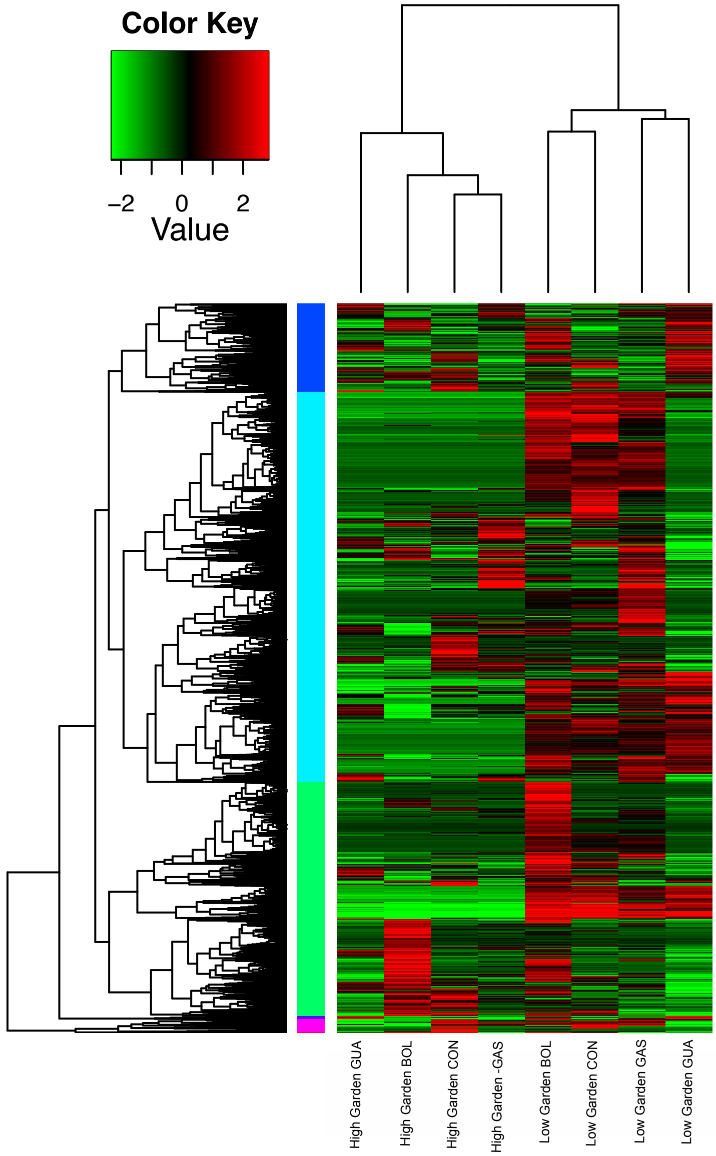


Figure 5. Trinity-edgeR DE analysis showing general differences in gene expression in flowers. The dendrogram to the left groups genes according to their similarity in expression and each cluster is assigned an arbitrary color. The dendrogram at the top clusters samples based on similarity of gene expression.

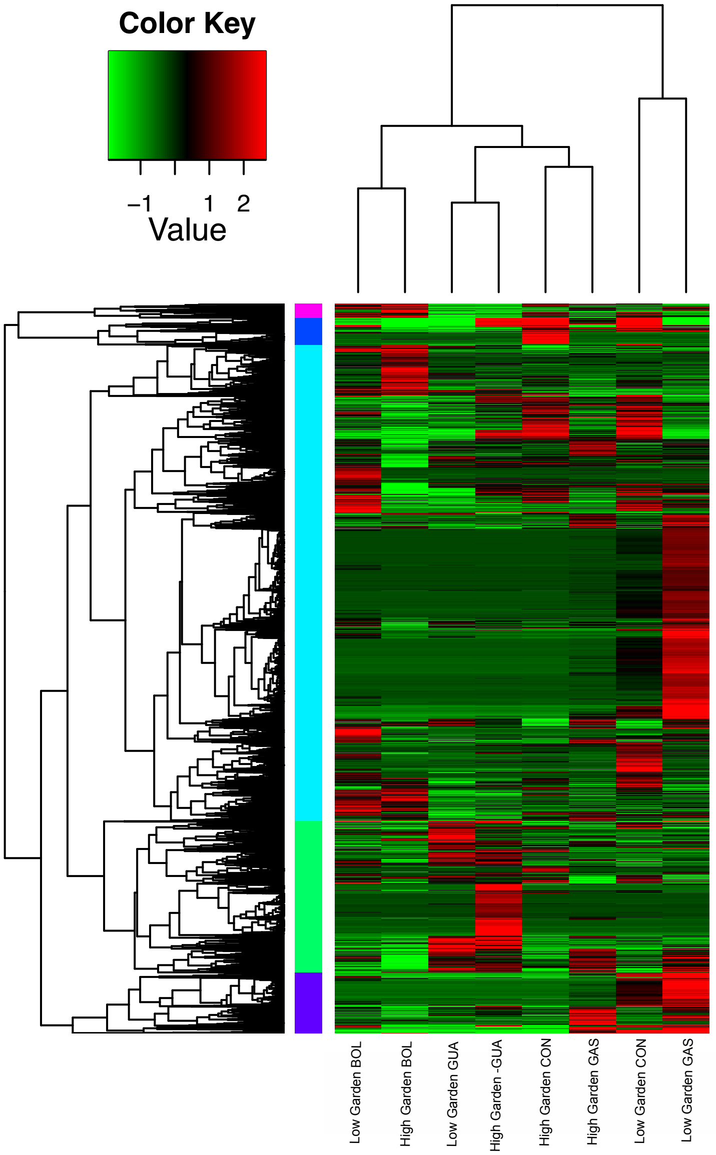
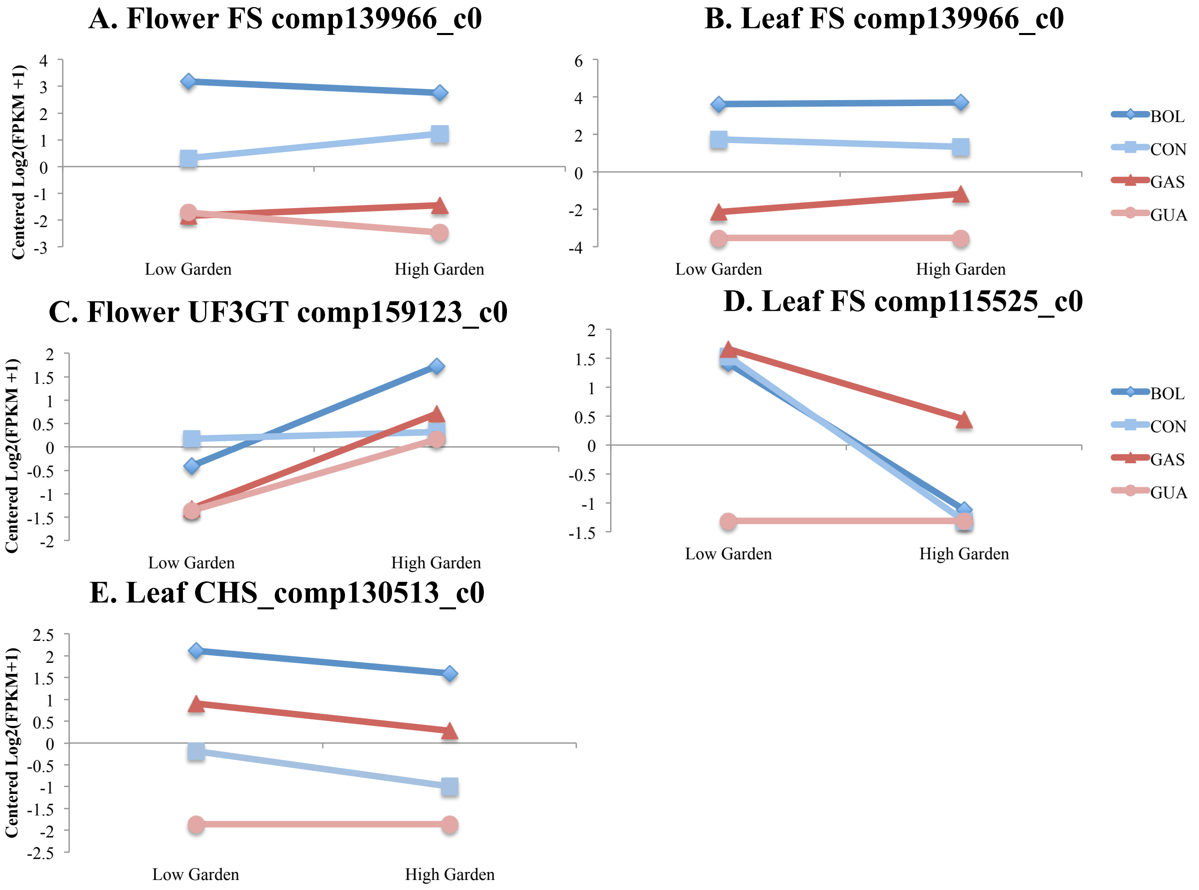


Figure 6. Loci with significant main effects of elevation of origin or garden. Panels A, B, and C represent significant results from a two-way ANOVA; significant effects of elevation of origin: flower FS_comp139966_c0 (6A, $F_{(1,4)}$=19.39, p = 0.012), and leaf FS_comp139966_c0 (6B, $F_{(1,4)}$=26.12, p=0.0069), significant effects of experimental garden: flower UF3GT has a significant effect of experimental garden (6C, $F_{(1,4)}$=13.02, p=0.0226). Panels D and E had a significant effect of population, rather than elevation of origin: leaf FS_comp115525_c0 (6D, t= -2.26, p = 0.043, df=4), leaf CHS_comp130513_c0 (6E, t=-2.50, p=0.033, df=4).


A. Flower FS comp139966_c0

B. Leaf FS comp139966_c0

C. Flower UF3GT comp159123_c0

D. Leaf FS comp115525_c0

E. Leaf CHS_comp130513_c0

Figure 7. Network eigengene heatmaps showing module colors and gene dendrograms for (A) leaves and (B) flowers. Each row and column corresponds to a gene, lighter colors denote low topological overlap, and red denotes higher topological overlap. Darker squares along the diagonal correspond to network modules along the edges of the heatmap, where the grey module represents non-module genes (and is not to be counted as a module) and blue, turquoise, and brown represent modules (Langfelder & Horvath, 2008).

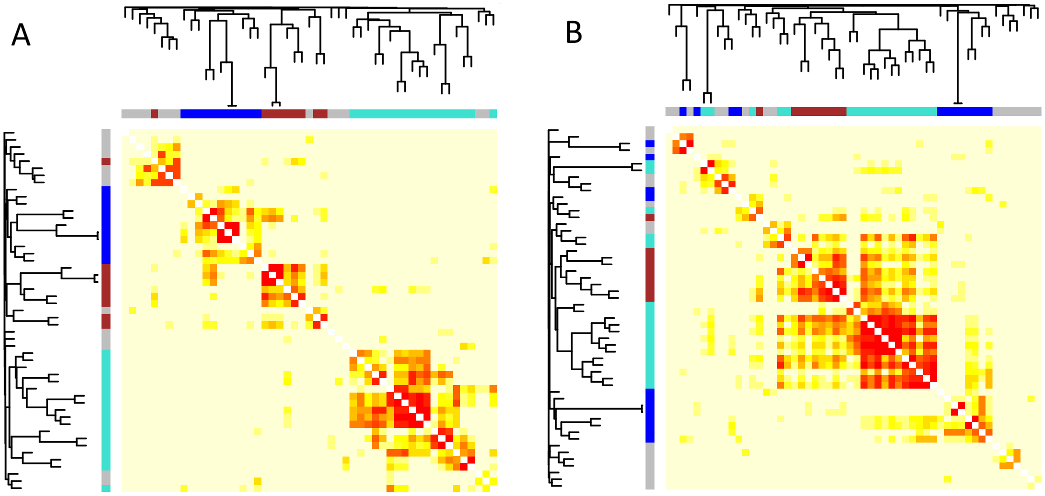


Figure 8. Flower and leaf eigengene adjacency heatmaps. Garden and origin are treated as traits along with clusters of genes grouped into colored modules (modules represented for flowers in Figure 7B and for leaves in Figure 7A). Each module is represented by its eigengene, which are a principal component of gene expression of the module. Correlations were calculated for each pairwise comparison of trait/module, and are represented by a heatmap.

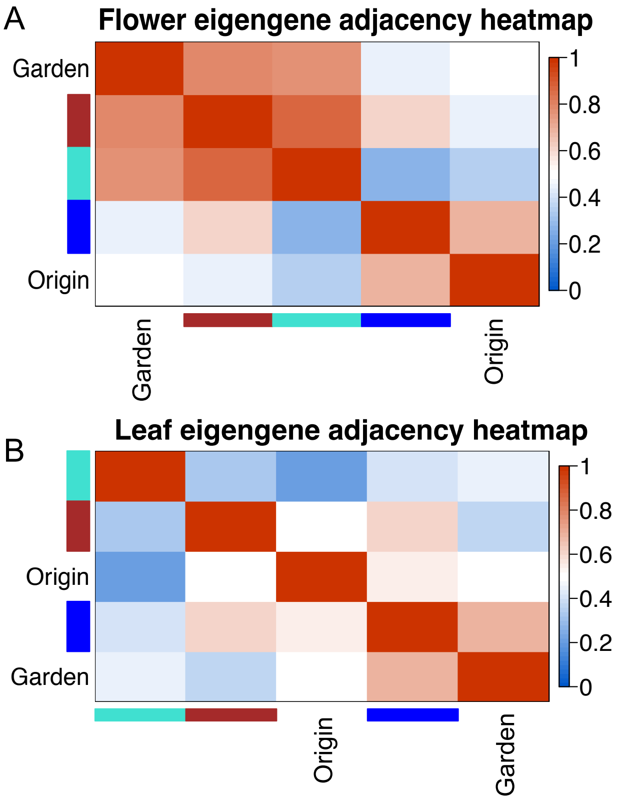


Figure 9. Consensus analysis eigengene adjacency network heatmap for garden as a trait for flowers (A) and leaves (D). Panel B shows a barplot of mean preservation of relationships for each eigengene, with the overall preservation above (D=0.68). Panel C shows the preservation adjacency of flower and leaf samples combined, with large blocks of dark red indicating groups of preserved and correlated eigengenes. This indicates that the inter-module relationships are strongly preserved across the flower and leaf datasets.

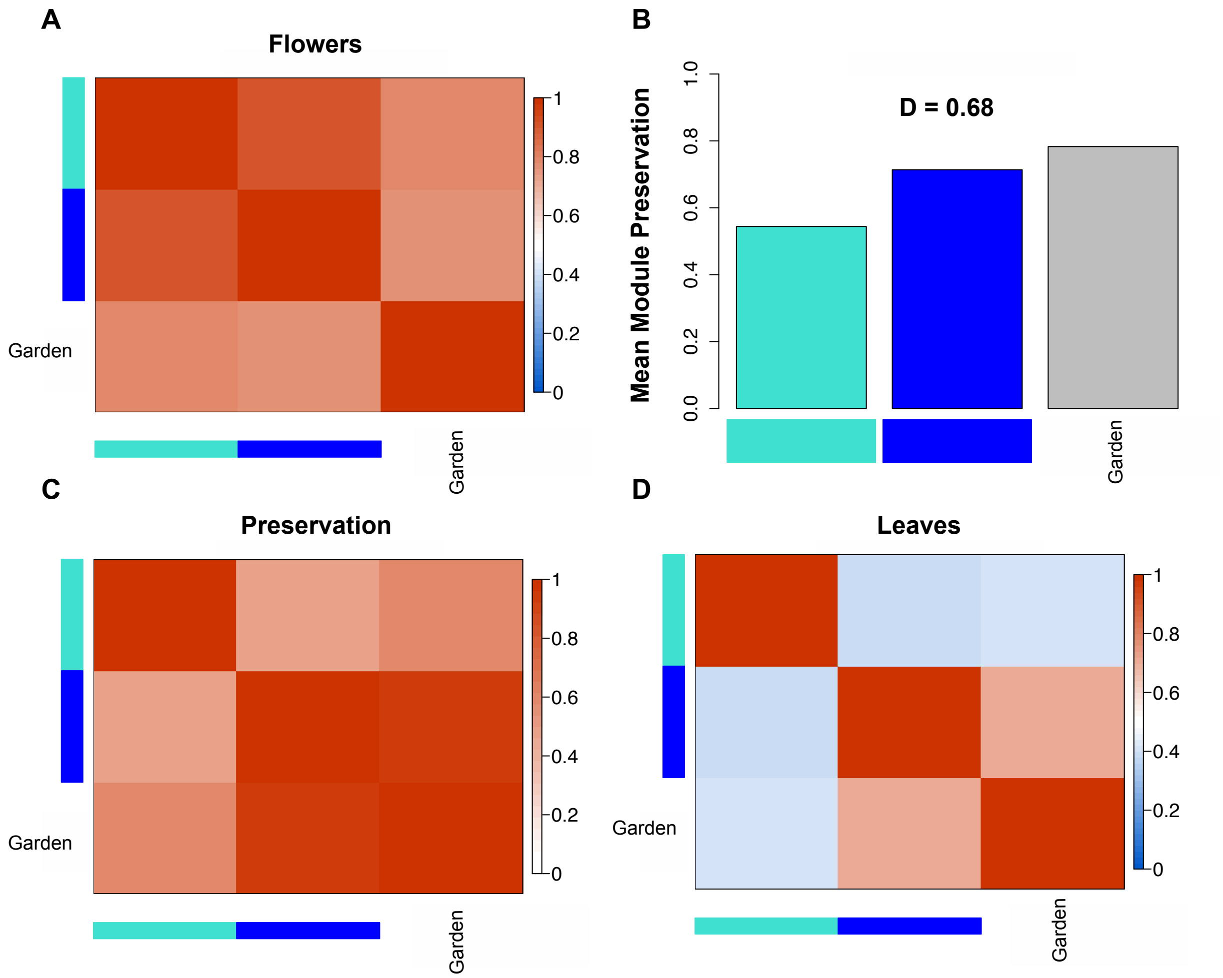
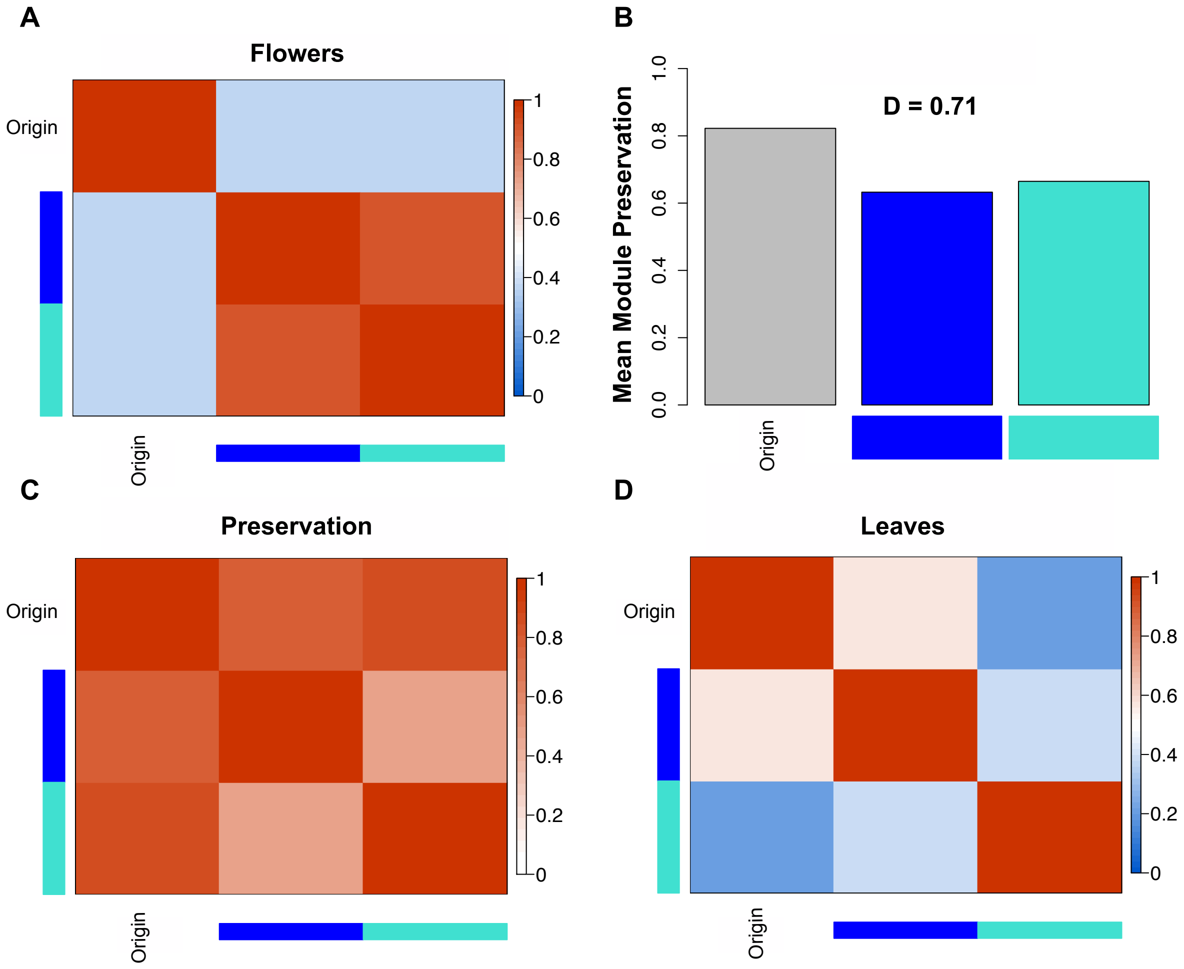


Figure 10. Consensus analysis eigengene adjacency network heatmap for origin as a trait for flowers (A) and leaves (D). Panel B shows a barplot of mean preservation of relationships for each eigengene, with the overall preservation (D =0.71) above. Panel C shows the preservation adjacency of flower and leaf samples combined, with large blocks of dark red indicating groups of preserved and correlated eigengenes. This indicates that the inter-module relationships are strongly preserved across the flower and leaf datasets.



Chapter 4: Appendix

Appendix Table 1. Population locations. Seeds were collected at these locations, grown in the University of Virginia greenhouse, randomly bred within-population, and the resulting progeny planted in two gardens in the French Alps.

Population ID	Country	Latitude	Longitude	Elevation (m)
BOL	France	44.279206	4.751631	60
CON	Switzerland	46.846528	6.713072	437
GAS	Switzerland	46.75822	8.13511	1050
GUA	Switzerland	46.777986	10.15443	1719

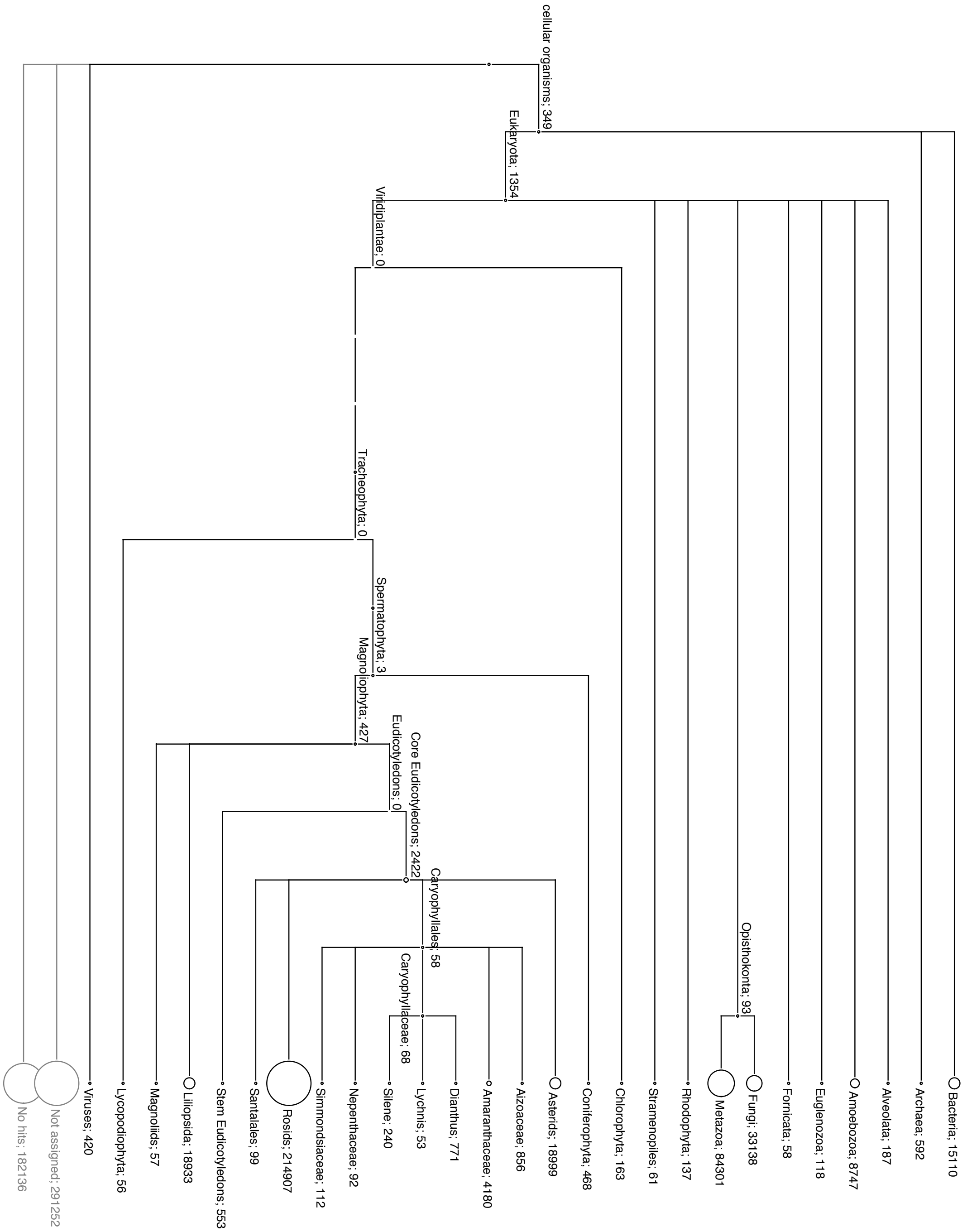
Appendix Table 2. Sample information.

Garden Elevation	Elevation Origin	Population	Sample Individual	Flower Sample	Leaf Sample
Low	Low	BOL	BOL-A3-10	1 hermaphrodite	2 leaves
			BOL-A3-8	1 male-sterile	1 leaf
Low	Low	CON	CON-8-5	1 hermaphrodite	1 leaf
			CON-E4	1 hermaphrodite	2 leaves
Low	High	GAS	GAS-A2	1 male-sterile	1 leaf
			GAS-D2	1 hermaphrodite	1 leaf
			GAS-F2	1 hermaphrodite	1 leaf
Low	High	GUA	GUA-C10	1 hermaphrodite	2 leaves
High	Low	BOL	BOL-A5-17	1 bud, sex unknown	1 leaf
High	Low	CON	CON-A5	1 male-sterile	2 leaves
			CON-A9	1 hermaphrodite	1 leaf
			CON-J3	1 hermaphrodite	2 leaves
High	High	GAS	GAS-A1	1 hermaphrodite	1 leaf
			GAS-E7	1 male-sterile	2 leaves
			GAS-H1	1 male-sterile	1 leaf
High	High	GUA	GUA-B15	1 hermaphrodite	1 leaf
			GUA-C13	1 male-sterile	2 leaves
			GUA-C9	1 male-sterile	1 leaf

Appendix Table 3. A glossary for the most frequent network analysis terms (Langfelder & Horvath, 2008; Langfelder *et al.*, 2011). For more definitions, see Table 1 in Langfelder & Horvath (2008) and Table 2 in Langfelder *et al.* (2011).

Term	Definition
Module	A group of highly clustered, or associated, genes (in many ways, a sub-network). In my unsigned network, they are specifically clusters of genes with high absolute correlations.
Module color	In WGCNA, modules are given colors; the gray color represents the group of genes that were not assigned to a particular module. These colors represent the different modules throughout the analysis.
Connectivity	The sum of connection strengths with other genes in the network. It measures how correlated a gene is with other genes in the network.
Intramodular connectivity, Module membership	How connected, or co-expressed, a given gene is with respect to the genes of a particular module. This can be considered as a measure of module membership.
Adjacency	The connection strength, or the correlation strength to other network genes
Module Eigengene	The first principal component of a given module, or, a representation of the gene expression profile of a given module
Eigengene significance	The resulting correlation coefficient of a correlation of phenotypic traits to module eigengenes (in this manuscript the "phenotypes" are garden and elevation of origin)
Node	A gene
Cluster	A group of nodes (genes) that are strongly connected.
Preservation (of module, network, etc.)	A quantification of which aspects of within-module or network topology are preserved, or unchanged, between a reference network and a test networks. Loosely termed, it is a type of cluster validation.
Gene significance *GS*	Used when incorporating phenotypic data into the analysis. "Abstractly speaking, the higher the absolute value of GS_i, the more biologically significant is the i-th gene." (Langfelder & Horvath, 2008). In other words, it is roughly the correlation of a gene to a phenotypic trait.
Module significance	The average absolute gene significance measure for all genes in a given module; highly related to the correlations between the module eigengene and a phenotypic trait.

Appendix Figure 1. Taxonomic analysis of assembled contigs by MEGAN5. The circle sizes represent the number of contigs assigned to the taxon based on BLASTX searches against the NCBI nr database. The number of contigs assigned to each taxon is also indicated.



Supplementary Data

Table S1. Reference genes for BLAST+ searches to identify flavonoid pathway genes and flavonoid transcription factors.

Gene	Genbank Accession	Species
ANS	U82432	*Dianthus caryophyllus*
CHI	Z67989	*Dianthus caryophyllus*
CHS	Z67982.1	*Dianthus caryophyllus*
CHS	AF267173	*Dianthus monspessulanus*
DFR	AF291097	*Dianthus gratianopolitanus*
DFR	AF267172	*Dianthus plumarius*
F3'H	AB731559	*Dianthus caryophyllus*
F3H (FHT)	X70378	*Dianthus caryophyllus*
FLS	GI 240256493	*Arabidopsis thaliana*
FS	AM887658	*Arabidopsis thaliana*
FS	AM887656	*Arabidopsis thaliana*
FS	FJ767774	*Glycine max*
UF3GT	AB191245	*Dianthus caryophyllus*
WD40	AJ133743	*Arabidopsis thaliana* (as TTG1)

Table S2. Two-way ANOVA using SAS PROC MIXED on total flavonoid pathway gene expression with elevation of origin and garden as fixed factors and their interaction as a random factor.

	Flowers			Leaves		
Effect	*F*	df	*p*	*F*	df	*p*
Elev. Origin	1.42	1,4	0.2994	1.94	1,4	0.2360
Garden	0.06	1,4	0.8188	0.01	1,4	0.9174
Garden*Elev. Origin	6.00	1,4	0.0704	0.03	1,4	0.8609

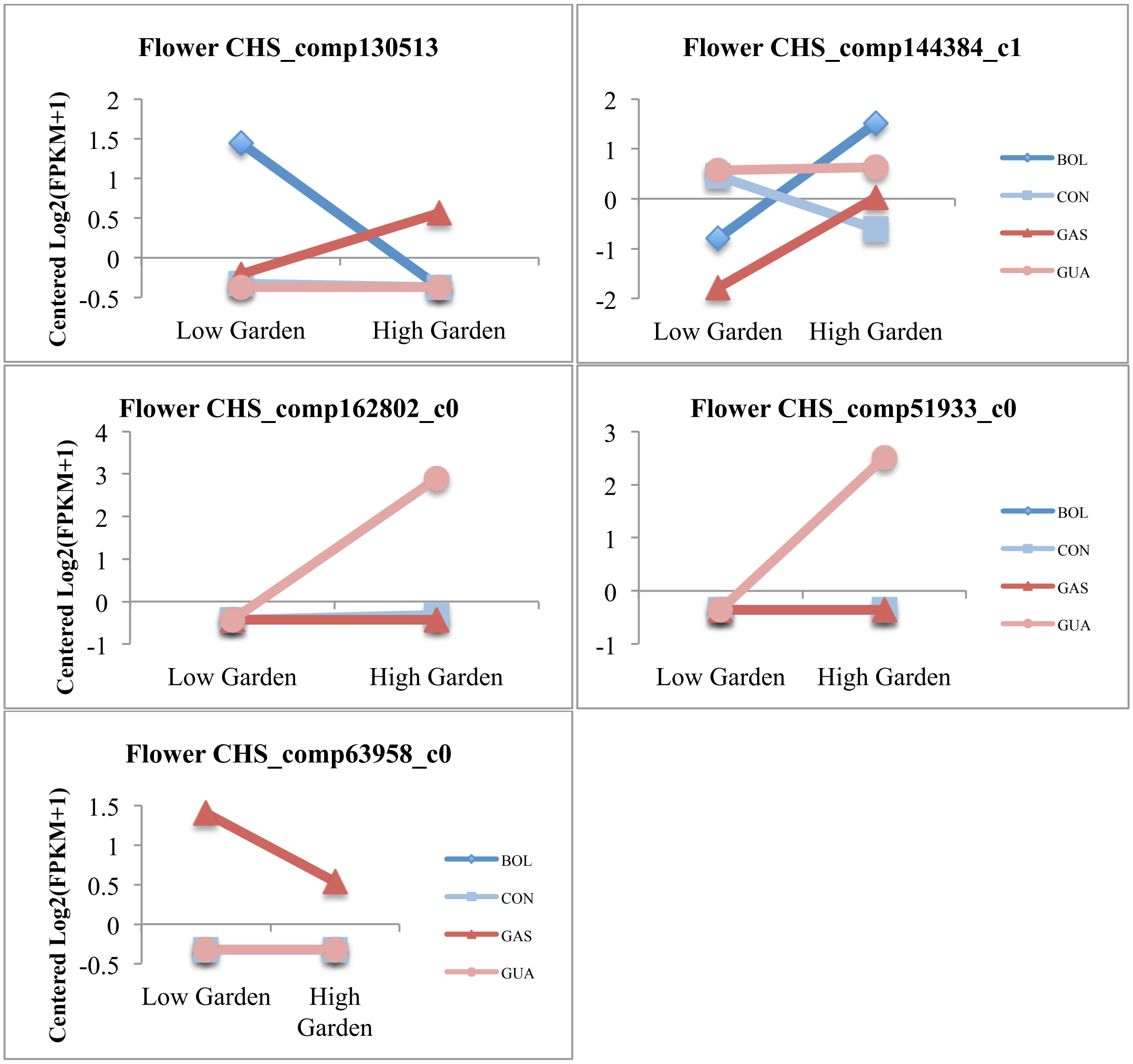
Table S3. A list of the flavonoid pathway annotated contigs found when comparing the top 10 and top 100 DE genes in the experimental design.

Comparison found among:	Gene_id	Top_BLASTX_hit	Top_BLASTP_hit	Pfam	eggnog	Gene_ontology	Putative Flavonoid Role
high garden flowers	comp120900_c0	sp|Q7XZQ7|FL3H_PETCR	sp|Q7XZQ7|FL3H_PETCR	PF14226.1		GO:0031418, GO:0046872, GO:0045486, GO:001670, GO:000981	F3H
low origin flowers	comp120900_c0	sp|Q7XZQ7|FL3H_PETCR	sp|Q7XZQ7|FL3H_PETCR	PF14226.1		GO:0031418, GO:0046872, GO:0045486, GO:001670, GO:000981	F3H
high origin flowers	comp125066_c0	sp|O22182|U84B1_ARATH	sp|O22182|U84B1_ARATH	PF00201.13		GO:0047215, GO:0080044	UDP quercetin 7-O-glucosyltransferase
low garden flowers	comp125066_c0	sp|O22182|U84B1_ARATH	sp|O22182|U84B1_ARATH	PF00201.13		GO:0047215, GO:0080044	UDP quercetin 7-O-glucosyltransferase
high origin flowers	comp131764_c0	sp|Q9LYT3|TT12_ARATH	sp|Q9LYT3|TT12_ARATH	PF01554.13	COG0534	GO:0016021, GO:000970, GO:0015238, GO:0015299, GO:0009813, GO:0010231, GO:0010023	TT12 flavonoid transporter
low garden flowers	comp131764_c0	sp|Q9LYT3|TT12_ARATH	sp|Q9LYT3|TT12_ARATH	PF01554.13	COG0534	GO:0016021, GO:000970, GO:0015238, GO:0015299, GO:0009813, GO:0010231, GO:0010023	TT12 flavonoid transporter
high origin flowers	comp147728_c0	sp|Q9LVW3|U79B1_ARATH	sp|Q9LVW3|U79B1_ARATH	PF00201.13		GO:0016758, GO:0035252, GO:0009718, GO:1901038	UDP-xylosyltransferase

Figure S1. Reaction norm plots for each flavonoid gene locus.

CHS

Flower results


Flower CHS_comp130513

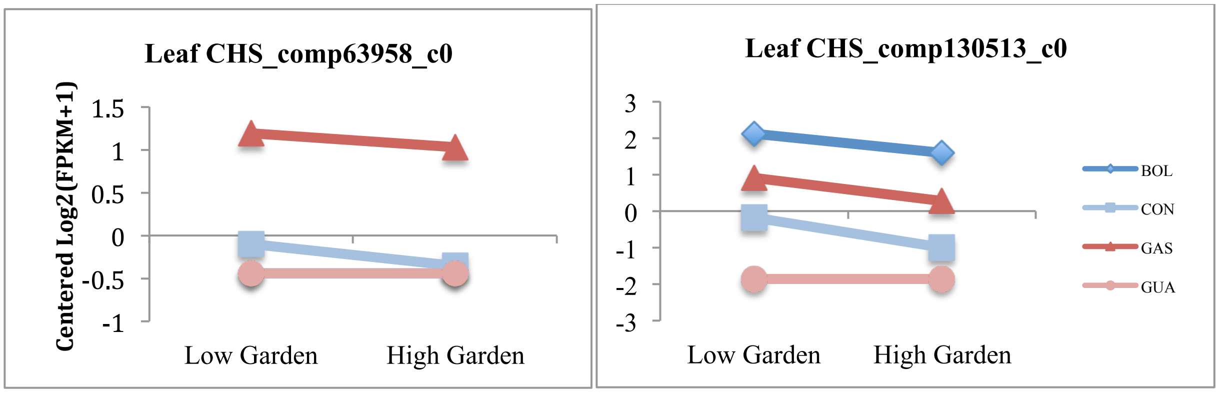
Flower CHS_comp144384_c1

Flower CHS_comp162802_c0

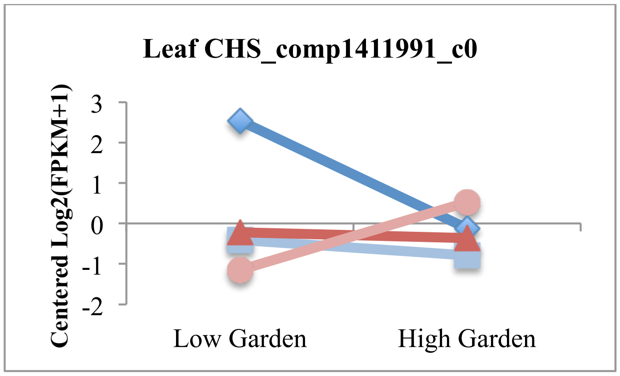
Flower CHS_comp51933_c0

Flower CHS_comp63958_c0

Leaf results


Leaf CHS_comp63958_c0

Leaf CHS_comp130513_c0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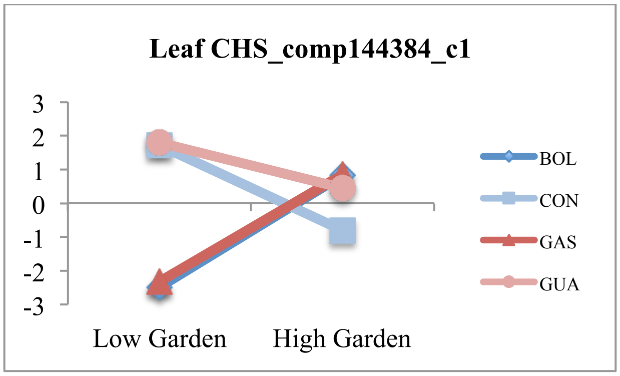
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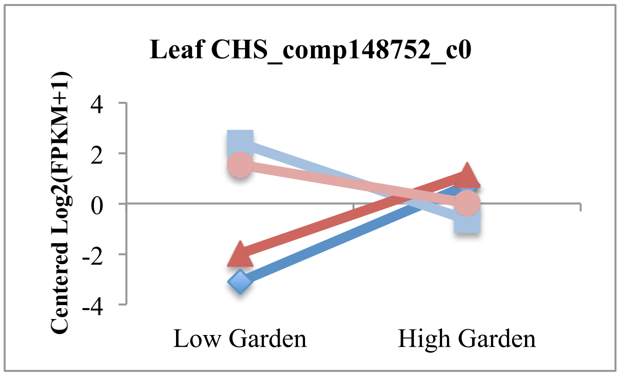
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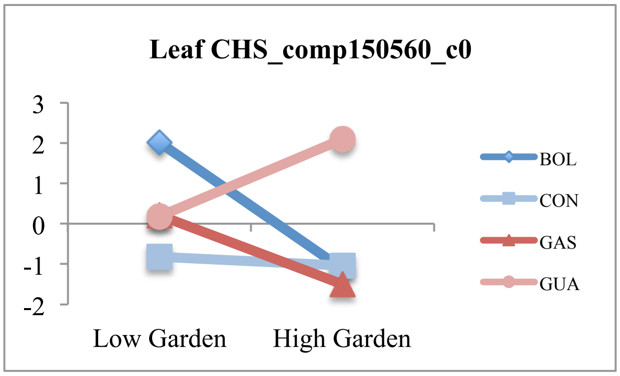
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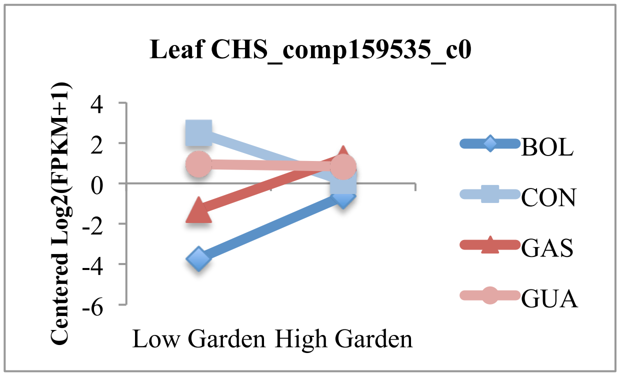
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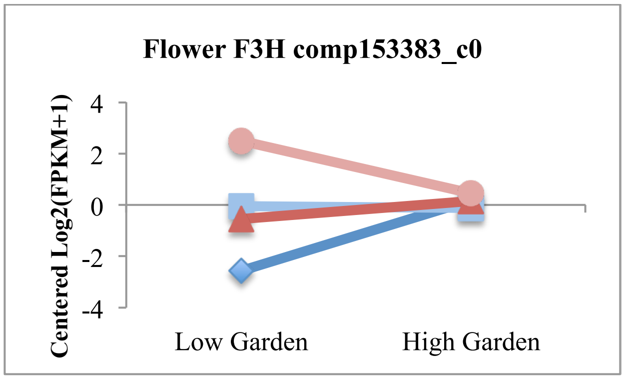
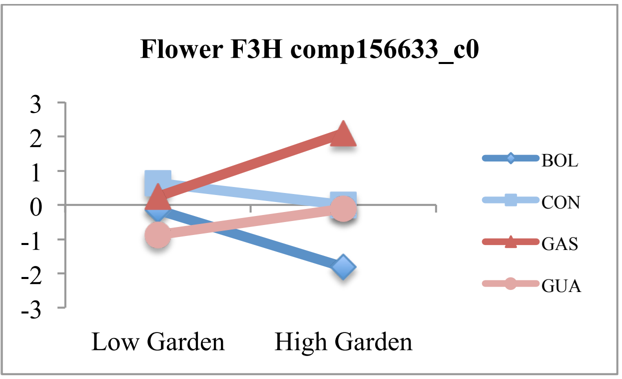
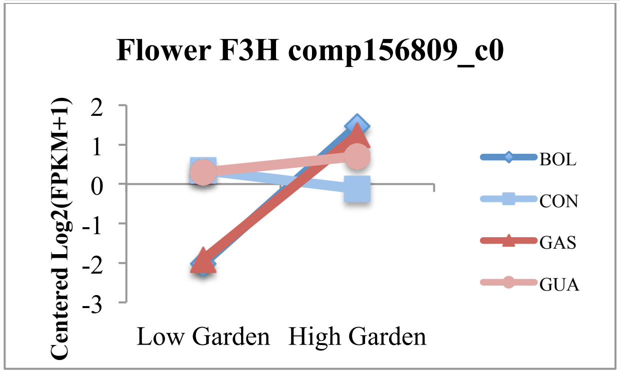
F3H

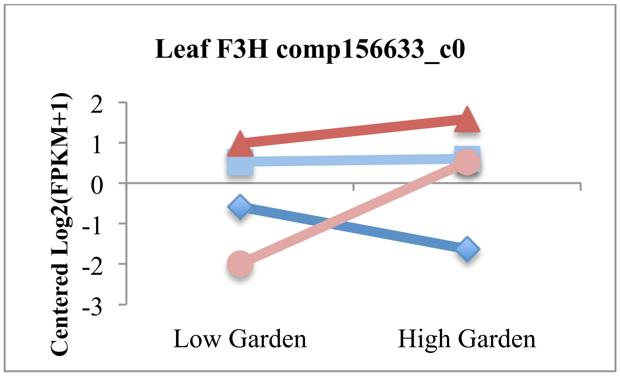
Flower results

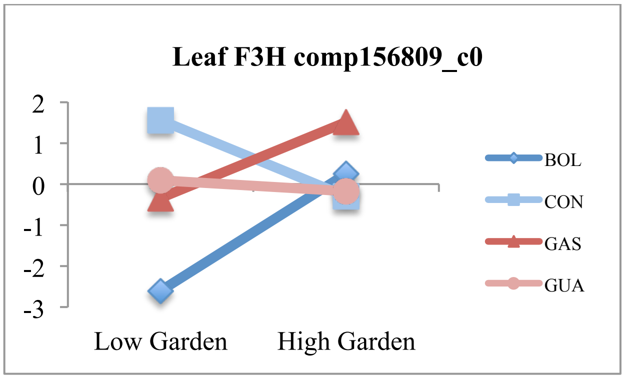

Flower F3H comp153383_c0


Flower F3H comp156633_c0


Flower F3H comp156809_c0

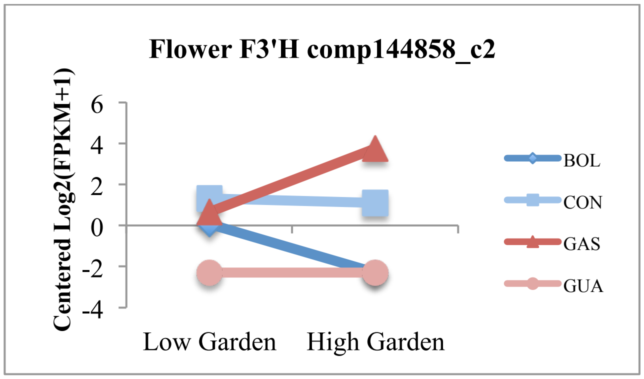
Leaf results


Leaf F3H comp156633_c0

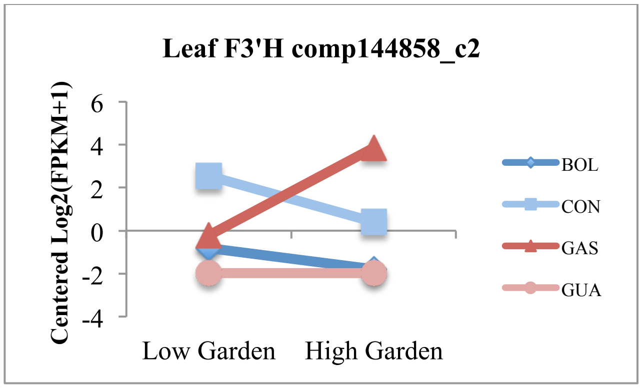

Leaf F3H comp156809_c0

F3’H

Flower results

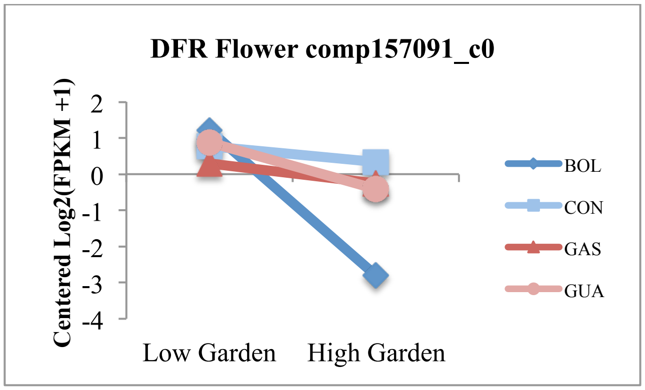


Leaf results

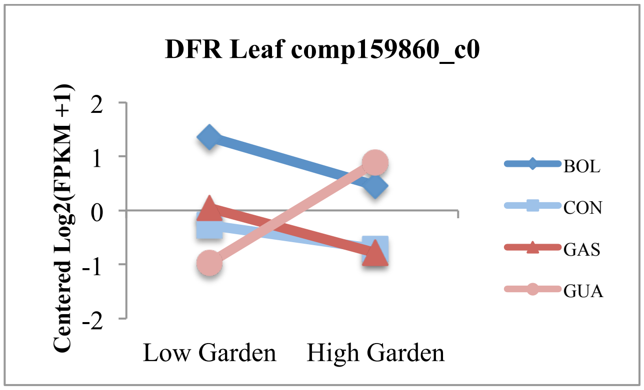


DFR

Flower results

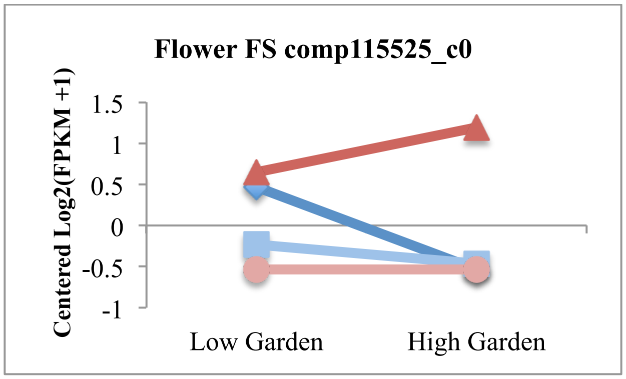


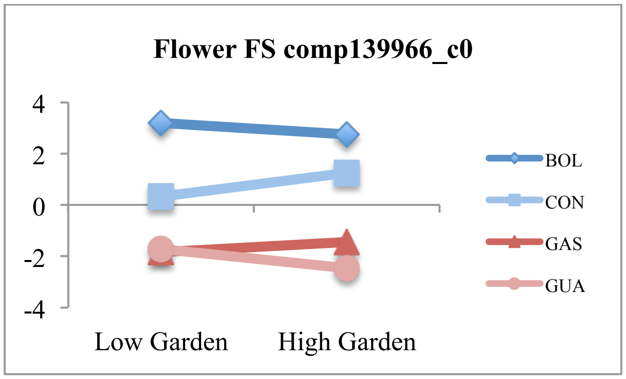
Leaf results



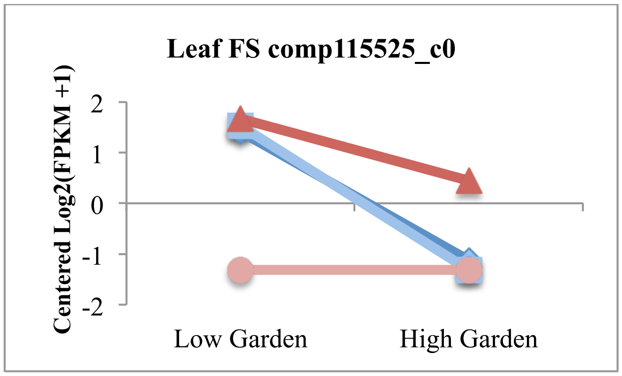
FS

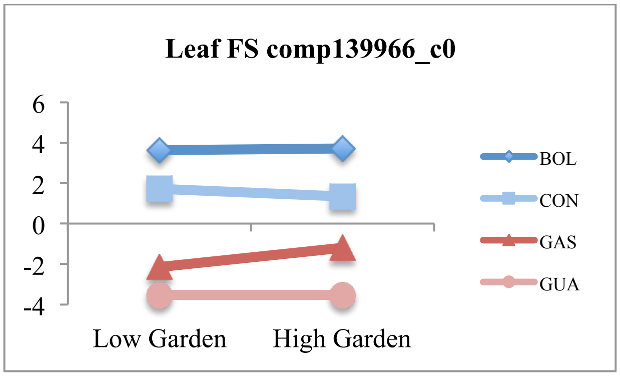
Flower results





Leaf results





UF3GT

Flower results

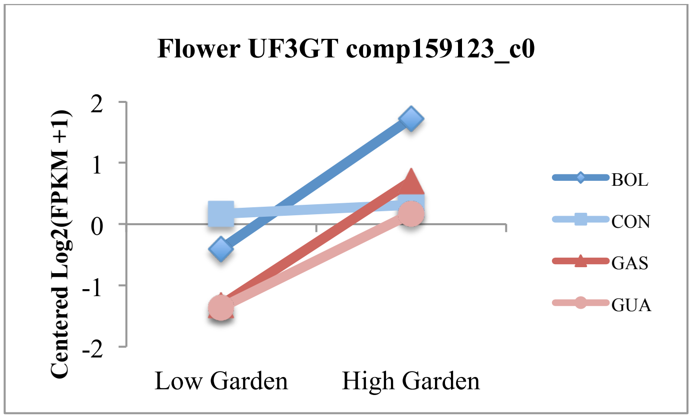


Figure S2. Flower network analysis: Each module, respresented by its eigengene (module eigengene (ME) + color, module colors same as Figures 7-9), was correlated with garden and elevation origin; there were no significant correlations. Correlation coeffecients are represented in each cell and are in a heatmap format. P-values are in parentheses underneath the correlation coefficient.

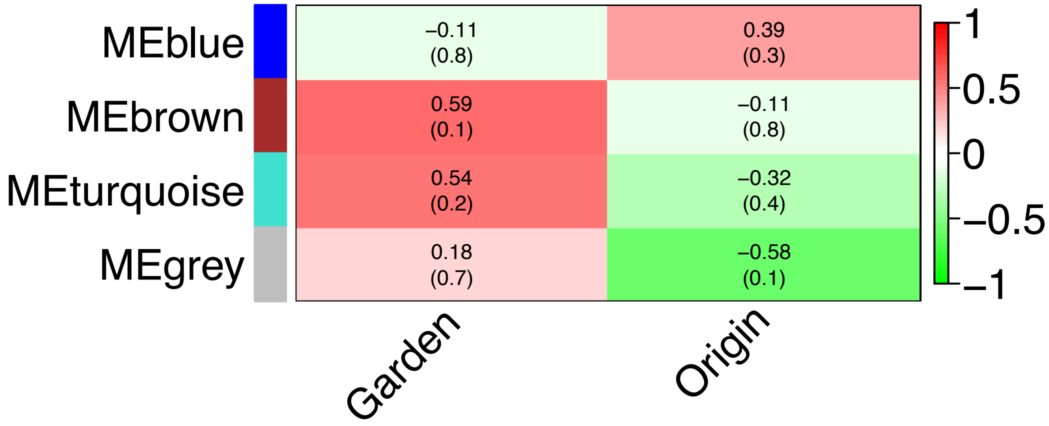


Figure S3. Leaf network analysis: Each module, respresented by its eigengene (module eigengene (ME) + color, module colors same as Figures 7-9), was correlated with garden and elevation origin; there were no significant correlations. Correlation coeffecients are represented in each cell and are in a heatmap format. P-values are in parentheses underneath the correlation coefficient.

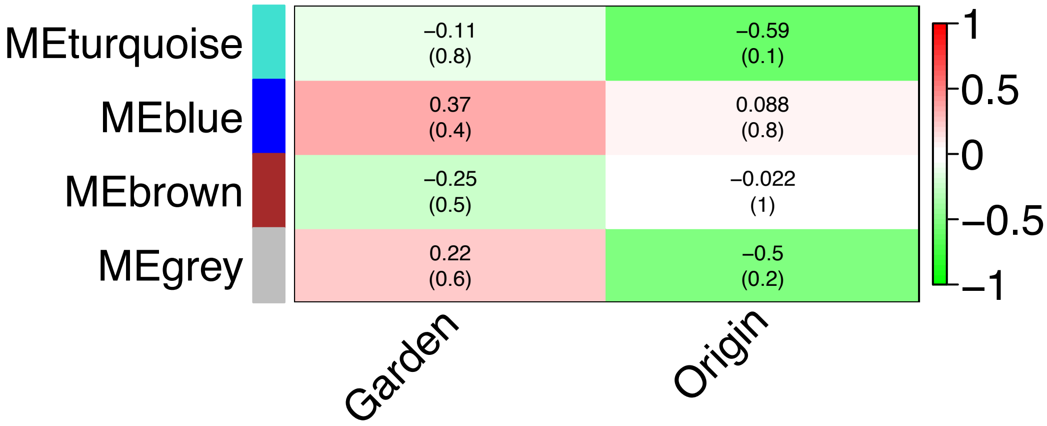
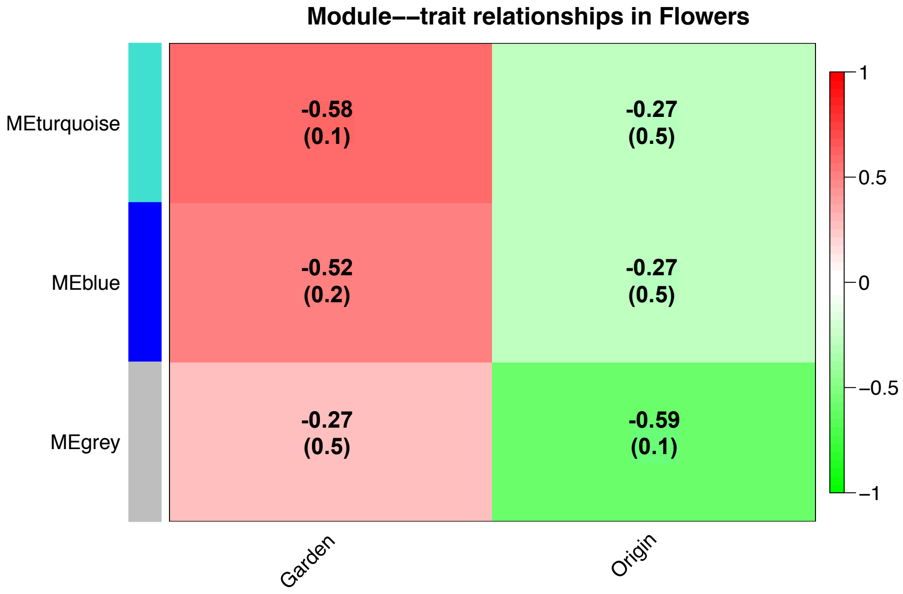
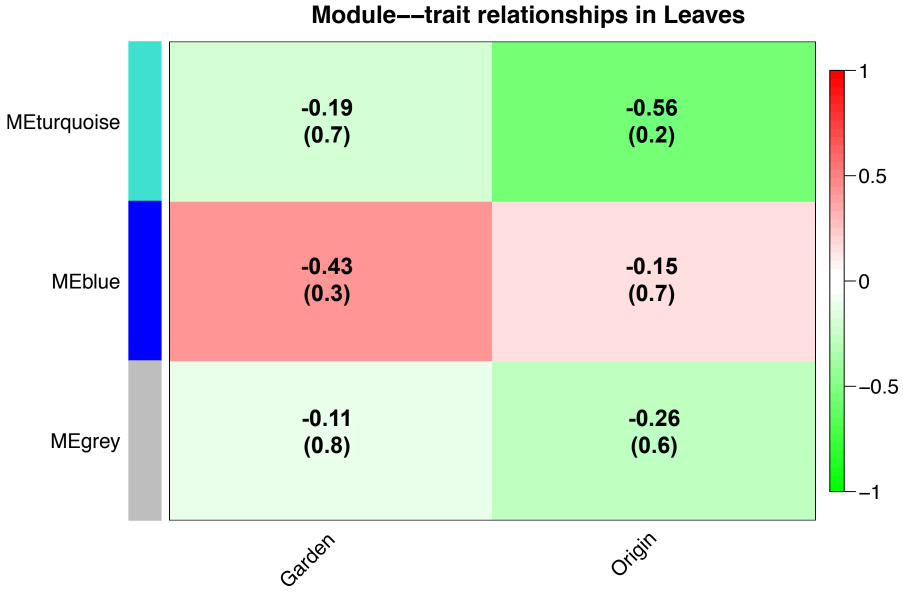


Figure S4. Consensus network analysis. The consensus network analysis created two modules of co-expressed genes. Each module is represented by its module eigengene (ME) and a color (turquoise or blue; gray represents unbinned genes and is not a module). These color modules can be compared between flowers (A) and leaves (B). Correlation coeffecients are represented in each cell and are in a heatmap format. P-values are in parentheses underneath the correlation coefficient.

A.



B.



Supplementary references for Trinotate:

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[SignalP]SignalP 4.0: discriminating signal peptides from transmembrane regions Thomas Nordahl Petersen, Soren Brunak, Gunnar von Heijne & Henrik Nielsen Nature Methods, 8:785-786, 2011

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[eggNOG]eggNOG v3.0: orthologous groups covering 1133 organisms at 41 different taxonomic ranges. Powell S, Szklarczyk D, Trachana K, Roth A, Kuhn M, Muller J, Arnold R, Rattei T, Letunic I, Doerks T, Jensen LJ, von Mering C, Bork P. Nucleic Acids Res. 2012 Jan;40(Database issue):D284-9. Epub 2011 Nov 16.

Dissertation Appendix

A flavonoid primer

There are several excellent guides to flavonoids, but the purpose of this appendix is a brief introduction and a familiarization to chemical shapes and names.

Flavonoids are phenolic and are produced from the general phenylpropanoid pathway. J.B. Harborne, one of the foremost chemical ecologists, diagrams the general relationship of the different phenolic classes (Harborne, 1991):

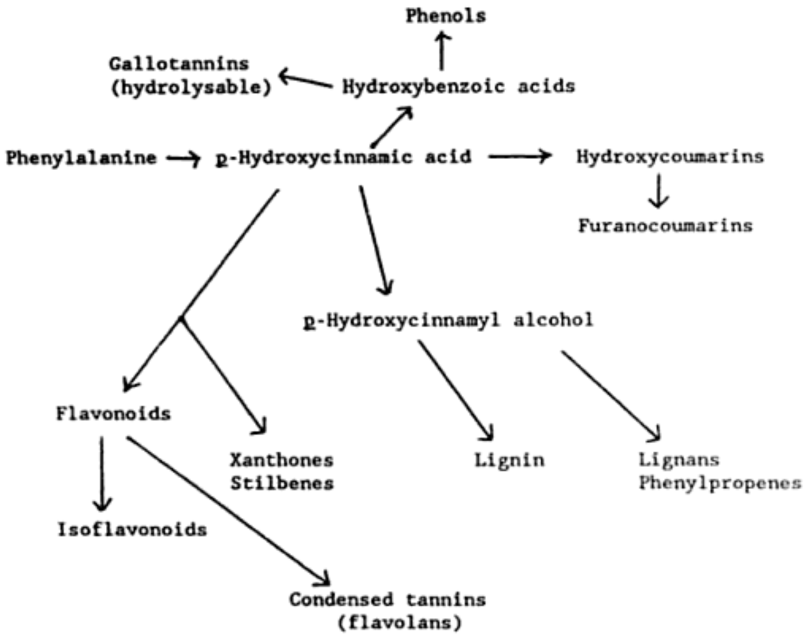
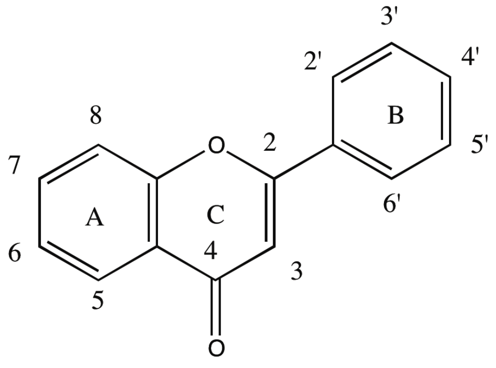


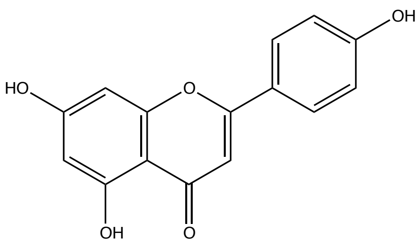
FIGURE 1. Biosynthetic relationships of different phenolic classes.

All flavonoids have a three-ring carbon backbone, and the types of chemical substitutions vary enormously, thus creating thousands of different flavonoid chemicals. Isomers are heavily favored in this group of chemicals as well. Substitutions include (most popularly) hydroxyl groups (OH), sugars (glucose, fructose, arabinose, rhamnose, xylose, rutinose, etc.), carboxyl groups, and carbon chains

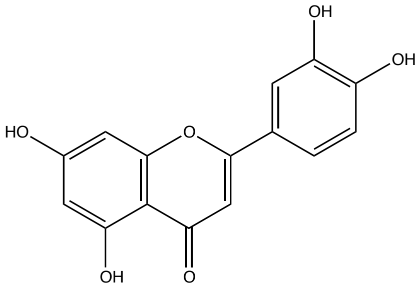


Flavones

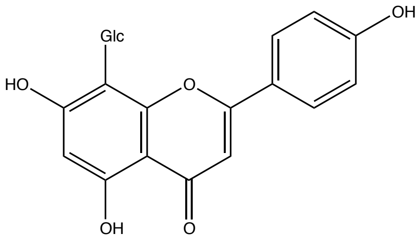
Silene vulgaris produces and stores flavones in the leaves, calyces, and petals. These are typically not visible to the human eye, but within the visible range of most insects. Most of the flavones present are based on the aglycones (the structure without the sugar) apigenin and luteolin. Aglycones are rare in *S. vulgaris*, which tends to favor glycosylation. Most of the flavonoids found in *S. vulgaris* exhibit C-glycosylation, which tells the reader that the substitution is connected via a carbon molecule, as compared to O-glycosylation, which is connected by an oxygen molecule. Thus, the most common "base flavonoids" found in *S. vulgaris* are actually 8- and 6-C-glucosylates of apigenin and luteolin, (iso)vitexin and (iso)orientin respectively (where the base chemical is substituted with glucose at carbon position 8, and the isomer is substituted with glucose at carbon position 6).



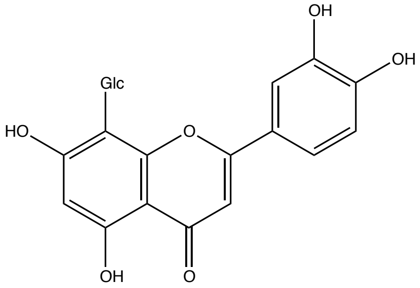
Apigenin



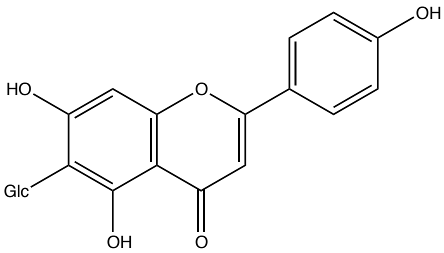
Luteolin



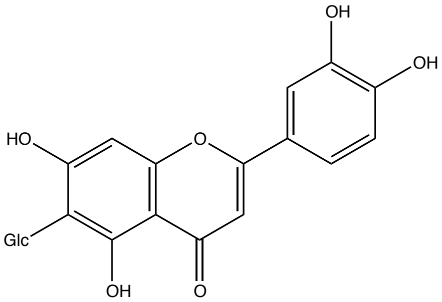
Vitexin



Orientin



Isovitexin

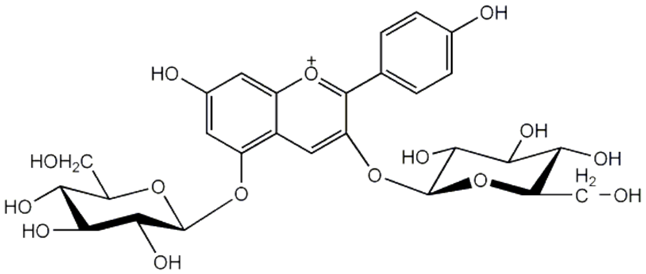


Isoorientin

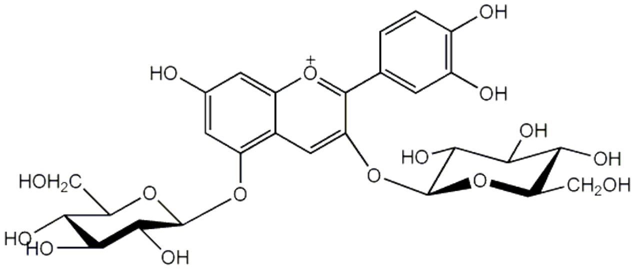
Anthocyanins

Anthocyanins are also present in *Silene vulgaris*. These are visible to the human eye. *Silene* produces cyanin (crimson) and pelargonin (orange/red) compounds (typically glycosylated), but cannot produce delphinin (blue/purple) due to the inactivation of the F3'5'H gene (Rausher, 2006). Roughly speaking, pelargonin shares the same base structure as apigenin, and cyanin with luteolin.
Anthocyanin images from KNApSAcK plant metabolite database (Afendi et al., 2012).

Pelargonin



Cyanin



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