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Gene expression divergence between locally adapted inland annual and coastal perennial ecotypes of *Mimulus guttatus* across developmental stages

Jason D. Olsen,[a,b] Billie A Gould,[a] Yani Chen,[a] and David B. Lowry[a,b,c,*]
[a]Department of Plant Biology, Michigan State University, East Lansing, MI, USA 48824
[b]Ecology, Evolution, and Behavior, Michigan State University, East Lansing, MI, USA 48824
[c]Plant Resilience Institute, Michigan State University, East Lansing, MI, USA 48824
*Correspondence should be addressed to D.B.L. (dlowry@msu.edu)

The action of natural selection across heterogeneous natural landscapes drives local adaptation and the formation of plant ecotypes, the precursors to new species. Plant ecotypes typically differ greatly in morphology, physiology, and development, yet we have a poor understanding of the underlying genetic basis of this divergence. Despite their importance, studies of the molecular underpinnings of ecotypic divergence through developmental stages are rare. In this study, we compared gene expression at different developmental time points between two locally adapted populations derived from the coastal perennial and inland annual ecotypes of the yellow monkeyflower, *Mimulus guttatus*. We conducted gene co-expression network analyses to identify putative genetic and molecular mechanisms driving ecotypic divergence and evaluated gene ontology enrichment. Overall, we found significant global expression differences between the ecotypes and among developmental stages. We also found evidence to support the hypothesis that interacting hormone pathways, especially the gibberellin and jasmonic acid pathways, may play a key role in annual versus perennial ecotype divergence. Further, genes within known chromosomal inversions were more likely to be differentially expressed than genes in colinear regions, but only in leaves and not for floral buds. Collectively, our results reinforce our existing hypotheses about how ecotype formation has occurred in this system through shifts in hormone biosynthesis/signaling and help advance our understanding of the evolution of annuality versus perenniality in plants.

Introduction

Differential natural selection across heterogenous landscapes drives the evolution of local adaptation (Clausen et al. 1948; Kawecki & Ebert 2004; Leimu & Fischer 2008; VanWallendael et al. 2019). Numerous reciprocal transplant studies between plant ecotypes have found a home-site fitness advantage for the local genotype over foreign transplants (Leimu & Fischer 2008; Hereford 2008; Agren et al. 2013; Agrawal 2020). Over time, local adaptation can lead to an accumulation of reproductive isolating barriers and the evolution of divergent ecotypes (Rieseberg & Willis 2007; Lowry 2012).

Since ecotypes can show vast intraspecific divergence in many traits, including morphology, life-history, and ontogeny, the expression of genotypic differences through development can result in evolutionary trade-offs (Walter et al. 2018). For example, trade-offs between growth, reproduction, and herbivore defense are common in plants and can cause differences in allocation of resources to vegetative tissue versus reproductive structures (Herms & Mattson 1992; Hahn & Maron 2016; Monson et al. 2022). Current theories regarding these trade-offs have expanded the concept to allow for dynamic or optimal allocation of resources across time or environmental gradients (Kooyers et al. 2017; VanWallendael et al. 2019; Agrawal 2020; Monson et al. 2022). Despite extensive theory and empirical observations, there is still a limited understanding of the molecular mechanisms that underlie the evolution of trade-offs associated with resource allocation and life-history variation (Friedman 2020; Lundgren & Des Marais 2020; Li et al. 2022). To better understand potential molecular mechanisms of trade-offs among growth, reproduction, and defense, our study explores gene expression divergence during development between locally-adapted annual and perennial ecotypes of the yellow monkeyflower, *Mimulus guttatus*.

M. guttatus is a model system for plant evolutionary ecology and genetics that occurs over an extensive geographic range across western North America. *M. guttatus* populations are adapted to numerous different environments, from sea level to the alpine zone, including novelties like hot springs, copper mine tailings, and serpentine outcrops (reviewed in Wu et al. 2008 and Twyford et al. 2015). Across this geographic range, *M. guttatus* occurs as a mosaic of morphologically divergent annual and perennial populations (Van Kleunen 2007; Oneal et al. 2014; DeMarche et al. 2016), depending on availability of soil water during dry summer months. Over the past two decades, extensive research has identified genetic loci underlying the morphological divergence of the annual and perennial ecotypes (Hall et al. 2006; Lowry & Willis 2010; Gould et al. 2017). The most important locus for divergence between annual and perennial populations is a large chromosomal inversion polymorphism on chromosome 8, which has opposite orientations that are

highly associated with wetter versus drier habitats (Lowry & Willis 2008; Oneal et al. 2014; Twyford & Friedman 2015). In contrast to this chromosomal inversion polymorphism, there is a high level of shared alleles at loci across the rest of the genome for annual and perennial populations. For inland regions of the species' range, population structure analyses have revealed partitioning of neutral genetic variation into northern and southern clusters with no correspondence to life-history (Twyford & Friedman 2015; Twyford et al. 2020). Exceptions to this pattern are coastal populations of *Mimulus guttatus* from California, Oregon, and Washington (Lowry et al. 2008; Twyford & Friedman 2015), which represent a distinctly structured set of perennial populations that collectively comprise what we will refer to in the remainder of this paper as the coastal perennial ecotype (Lowry 2012). Based on the morphological distinctness of the coastal perennial ecotype, some taxonomists have raised it to the species level, naming it the magnificent monkeyflower, *Mimulus grandis* (syn. *Erythranthe grandis*; Nesom 2012). However, given the high level of allele sharing between coastal perennial and inland annual groups of populations (only four fixed nucleotide differences out of >29 million SNPs; Gould et al. 2017), we more cautiously treat each group as an ecotype instead of a species. While we don't consider the coastal perennial and inland annual ecotypes as distinct species, we do recognize that they are on the speciation continuum, as they have acquired a significant level of ecologically-based reproductive isolation (Lowry et al. 2008; Lowry & Willis 2010).

There are large differences in a wide swath of traits between coastal perennial and nearby (<100km) inland annual ecotypes (Abrams 1951; Hitchcock and Cronquit 1973; Vickery 1978; Lowry et al. 2008). These ecotypes diverge in traits such as growth rate, morphology, phenology, and resistance to herbivory, and are locally adaptative in reciprocal transplant experiments (Hall and Willis 2006; Van Kleunen 2007; Lowry et al. 2008; Hall et al. 2010; Lowry et al. 2019). In general, the inland annual ecotype flowers early to avoid seasonal drought and produce fewer true leaves before developing floral buds. The coastal perennial flowers later and produces more leaf pairs before budding. In addition to differing in the frequency of the adaptive chromosomal inversion on chromosome 8 (Lowry & Willis 2010; Twyford & Friedman 2015), there is recent evidence of another inversion on chromosome 5 being involved in adaptive divergence between coastal perennial and inland annual populations (Holeski et al. 2014; Gould et al. 2017, 2018; Monnahan & Kelly 2017; Kollar et al. 2024). Despite circumstantial population genetic evidence suggesting a role for the chromosome 5 inversion in local adaptation, only the chromosome 8 inversion has been linked to the phenotypic divergence between coastal perennial and inland annual ecotypes

The primary motivation for this study was to identify gene networks and pathways that underlie the developmental divergence of the coastal perennial and inland annual ecotypes of *M. guttatus.* To accomplish this goal, we analyzed gene expression throughout development, from the production of the first pair of true leaves to the emergence of the first floral buds. We hypothesized that there would be interactions between ecotype and developmental stage for gene expression. This would mean that the magnitude of differential gene expression between developmental stages would significantly differ depending on the plant ecotype in question. Further, based on a previous physiological study (Lowry et al. 2019) that implicated a role of the gibberellin growth hormone in ecotype divergence, we hypothesized that we would see an enrichment of differential expression between ecotypes in genes involved in that pathway. We additionally predicted the enrichment of genes in the herbivore defensive jasmonic acid pathway, as there is thought to be a trade-off of this pathway with the gibberellin pathway (Wasternack 2017; Züst & Agrawal 2017; Guo et al. 2018). Finally, we hypothesized that genes in inversions would be disproportionately differentially expressed between ecotypes relative to the rest of the genome, as was previously found for these ecotypes for one leaf tissue in the field (Gould et al. 2018). While gene expression analyses typically have an exploratory element, here we aimed to address specific pre-existing hypotheses about developmental evolution in this system concerning the evolution of tradeoffs among growth, reproduction, and defense during the evolution of local adaptation. We aimed to refine these hypotheses and develop new ones based on our results.

Methods

Plant material

To identify patterns of gene expression divergence between the ecotypes, we selected one coastal perennial (SWB-4) and one inland annual (LMC-17) accession for a detailed study throughout development. The coastal perennial SWB population (N 39.02.159, W 123.41.428) is located near Manchester, CA, while the inland annual population LMC (N 38.51.839, W 123.05.035) is located near Boonville, CA. Seeds from each accession consisted of self-fertilized families that were two generations inbred in the laboratory before the experiments. To eliminate potential maternal effects, seeds were grown for one additional generation at Michigan State University (MSU, East Lansing, MI) in the four months before the growth chamber experiment.

Plant growth conditions

Surface-sterilized *Mimulus guttatus* seeds were placed on 60 mm diameter x 15mm height Petri dishes containing half-strength Linsmaier and Skoog (LS) medium: 1.5% w/v sucrose and 0.25% Phytagel (P8169, Sigma, St. Louis, MO). Seeds were then stratified at 4°C in the dark for 14 days. After cold treatment, plants were grown at 20°C under a 16 h 100 mE light / 8 h dark cycle for 5 to 14 days in a Percival (Percival Scientific, Perry, IA) chamber. Cotyledon-stage seedlings were transferred to 3.5-inch square pots containing SureMix Perlite (Michigan Grower Products, Galesburg, MI) and grown in a BioChambers FXC-19 flex growth chamber (BioChambers, Winnipeg, Manitoba) in the same conditions as in the Percival chamber. Three key steps were taken to minimize the effects of environmental heterogeneity

within the growth chamber. First, the locations of plants were fully randomized across trays within the chamber. Second, the tray position within the chamber was haphazardly rotated daily. Third, the developmental stage at which each plant was sampled was assigned randomly. Leaf pairs and floral buds were collected in a time series. Leaves were collected at the point in development when they were nearing maximal expansion. The floral buds were collected from plants just prior to anthesis. Only one tissue was collected per plant to avoid induced responses and repeated measures. Each tissue collection was harvested in a fixed time window (14:00 to 14:30) during the simulated daylight period. Tissue was immediately flash frozen on liquid nitrogen and stored in a -80° C freezer. RNA was later extracted from samples using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). We collected 10 biological replicates for each developmental stage x ecotype combination ($N = 80$ total tissue samples). For the inland annual plants, we sampled at the two-leaf, four-leaf, and floral bud stages because plants often flowered after producing two pairs of true leaves. Because the coastal perennial plants flowered at a later developmental stage, we sampled at the two-leaf, four-leaf, six-leaf, eight-leaf, and floral bud stages. All tissue samples were collected before anthesis.

Library preparation and sequencing

We prepared genomic libraries using a 3'-Tag-Seq procedure, which restricts sequencing to the 3' end of mRNAs and dramatically reduces the cost for RNA-seq studies (Meyer et al. 2011; Lohman et al. 2016). Each sample was assigned a fully randomized position across four pools of 20 samples before library construction. The MSU Genomics Core Facility conducted sequencing in Illumina HiSeq 2500 Rapid Run flow cells (v2) in the 1x100bp conformation. Sequencing of the four pooled libraries was conducted across three different flow cells, with two pools being sequenced in two separate flow cells and the other pools only being sequenced on a single flow cell each.

Raw reads were filtered for quality with FastQC (bioinformatics.babraham.ac.uk/projects/fastqc/) before processing to remove adapter sequences with Cutadapt (Martin 2011). Cutadapt was applied with an adapter mismatch error threshold of 20% of primer length. Primer trimming began with the 5' end (NNNN followed by 3-5 G's with a first step to cut 8 bases and a second step to cut and remaining 5' G's). Trimming of the 3' end was done by removing 15bp of the 3' end of each read to remove the poly-A primer and any readthrough. To retain only high-quality sequences after trimming, any bases on either end with a quality score < 30 were removed and we required the last two bases of each read to match the poly-A primer while discarding any reads <30bp in length. These parameters align with the requirements for the Tag-Seq methodology to remove both adapter sequences and non-template bases created during library preparation (Meyer et al. 2011). Trimmed sequence files were concatenated and aligned to the *Mimulus guttatus* v2.0 reference genome (Hellsten et al. 2013) using the memory-efficient Burrows-Wheeler Aligner (BWA-MEM; Li 2013). Aligned reads were filtered to include only high-quality, primary alignments using Samtools (Li et al. 2009). The total number of aligned reads retained for each sample was calculated with Samtools. Counting the number of reads aligned per gene per sample was done with HT-seq v.0.6.1 (Anders et al. 2015) with "stranded" and "union" parameter options. Count files were filtered to remove genes with < 5 aligned reads to exclude non-expressed genes or alignment artifacts. These count files were then imported into the R environment via DESeq2 (Love et al. 2014) for differential gene expression (DGE) analysis.

Differential gene expression analyses and statistical testing

To prevent downstream complications in data normalization due to low read count outliers, pre-filtering of count files was implemented to remove any transcript with <10 counts summed across all samples. Preliminary data inspection to qualitatively assess sample group separation, clustering, and overall quality was done with a principal component analysis (PCA) performed on variance stabilizing transformed (VST), pre-filtered read count data for visualization (Fig. 1). Differential gene expression analysis was performed in DESeq2. Transcriptome-scale data sets utilize large-scale multiple-hypothesis testing. Therefore, to optimize power while controlling for multiple testing with a false discovery rate (FDR), we used independent hypothesis weighting for our analyses (Ignatiadis et al. 2016). To compare differences across developmental stages within each ecotype and developmental stages between ecotypes, specific contrasts were generated for each grouping combination of ecotype and developmental stage (e.g., Coastal:2-Leaf by Inland:2-Leaf). Whether a gene was significantly differentially expressed was determined by using a threshold-adjusted Q-value of < 0.05.

Although the chromosome 8 inversion of the *M. guttatus* genome is known to have adaptive and life-history significance (Lowry & Willis 2010), less is known about the chromosome 5 inversion. We included the chromosome 5 inversion in our analysis to see whether it has similar expression patterns to the chromosome 8 inversion. To examine whether these inversions were enriched for differentially expressed genes compared with the rest of the genome, we assessed the number of highly differentially expressed (DE) genes using Fisher's exact tests. For sets of genes known to lie within either the chromosome 5 or chromosome 8 inversion, we compared the magnitude of expression difference between ecotypes against the rest of the genome using the absolute value of the $\log_2$ foldchange (LFC) input to Welch's t-tests for unequal variance.

Gene co-expression module analysis

To identify suites of co-expressed genes across ecotypes and within developmental stages, we performed gene co-expression module analysis was using webCEMiTool (https://cemitool.sysbio.tools/), a web interface platform for the CEMiTool R package (Russo et al. 2018; Cardozo et al. 2019). Default parameters were used, except we applied a

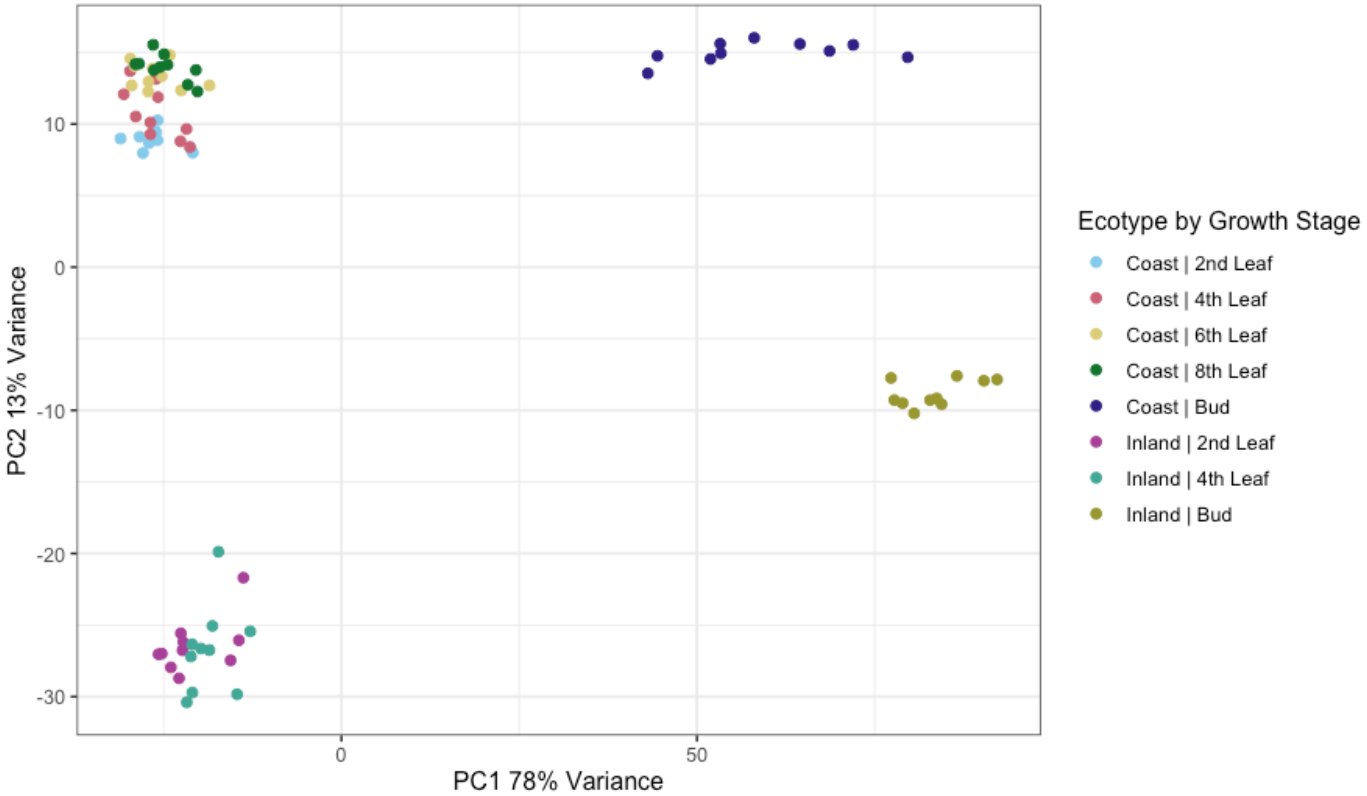

Figure 1: Principal component analysis of pre-filtered, variance stabilizing transformed (VST) transcript count data from RNASeq. Different colors denote all combinations of developmental stages and ecotypes. The first principal axis (PC1) explains 78% of the variance in the dataset and primarily distinguishes differences between leaf (vegetative) and bud (reproductive) stages. The second principal axis explains 13% of the variance primarily distinguishes differences between ecotypes.

variance stabilizing transformation (VST) to the input data. The output of this co-expression analysis is a list of modules (eigengenes) analogous to principal components of the expression dataset. We also used CEMiTool to perform a gene set enrichment analysis (GSEA) to explore the putative biological functions of each module.

Gene ontology enrichment analysis

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Gene ontology enrichment analysis

To elucidate potential mechanisms driving differentiation between coastal perennial and inland annual ecotypes through development, we used Gene Ontology (GO) enrichment analysis. This method groups genes with similar ontological annotation together using a known hierarchy of terms associated with fundamental biological functions/processes and then it conducts FDR-controlled statistical tests for overrepresenting these terms in the data set. Any GO terms that are overrepresented likely characterize biological pathways that contribute to the significant differences in a given contrast. We utilized a GO analysis tool implemented by PlantRegMap (Tian et al. 2020) to find terms overrepresented within the co-expression modules returned by webCEMiTool. The false discovery rate (FDR) for the GO analysis of module 1 was $P < 0.01$. We considered ontology terms with up to $P < 0.10$ for Modules 2 and 3 due to limited numbers of genes (Modules 2 and 3 together had fewer total genes than Module 1) and a willingness to accept a weaker FDR in exchange for potential additional biological insight.

Results

Sequencing and alignment

Overall, sequencing resulted in a total of 343,561,167 raw reads. Across the 80 samples, there was a minimum of 4,271,651 reads and a maximum of 10,858,583 reads (median of 7,403,646 reads per sample). We identified 28,140 unique gene transcripts, which were subsequently filtered, as described above, resulting in 22,501 confidently expressed genes. The PCA (Fig. 1) of the prefiltered, VST normalized expression values for these transcripts revealed a distinct grouping of samples along two major axes. PC1 corresponded most closely to differences between leaf and bud tissue and accounted for 78% of the expression variance. PC2 corresponded to the divergence between the coastal and inland ecotype samples and accounted for 13% of the variance.

Differential gene expression analyses and statistical testing

To compare gene expression between ecotypes, we used DESeq2 to perform differential gene expression analyses and contrast similar developmental stages between inland annual and coastal perennial ecotypes. For each contrast, upregulated and downregulated genes refer to genes in the inland ecotype with respect to the coastal ecotype. For ecotype contrasts at the two-leaf (2-leaf) stage, we identified

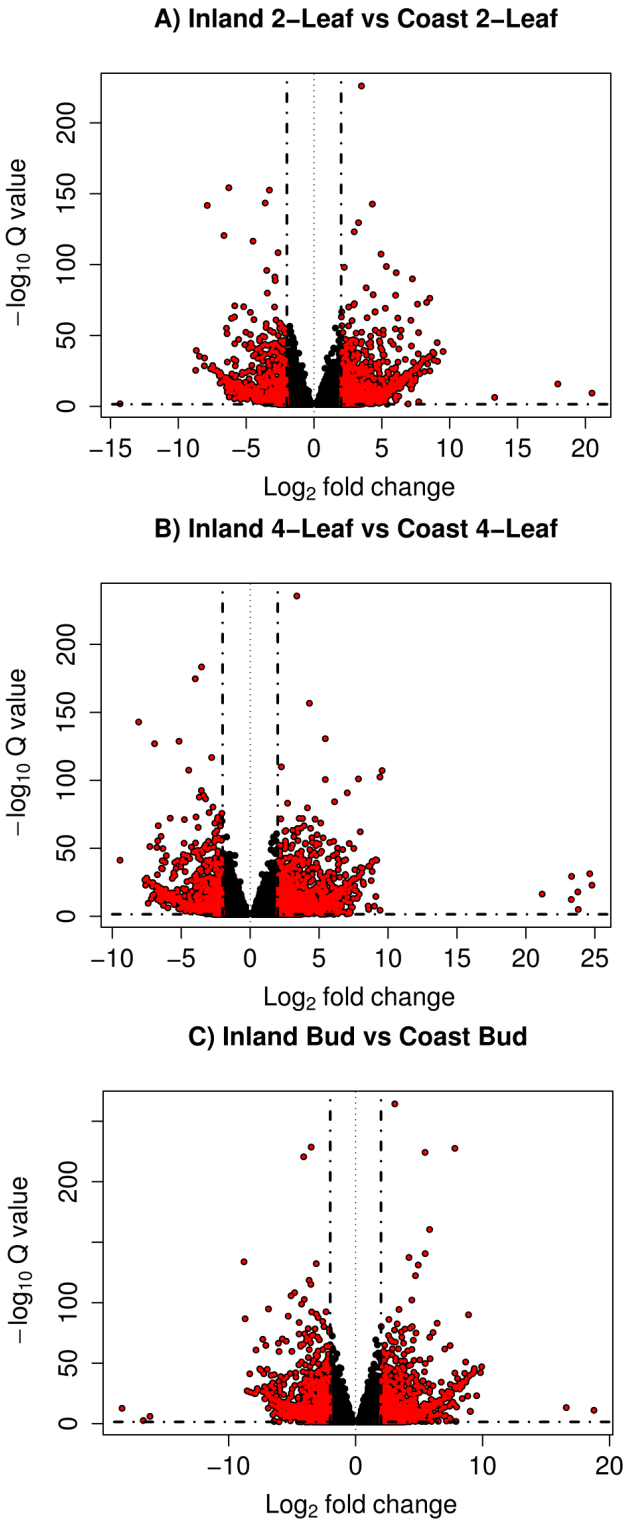

Figure 2: The distributions of differentially expressed genes between ecotypes at each developmental stage. Volcano plots are shown where $\log_2$ foldchange is plotted on the x-axis against significance (-$\log_{10}$ of FDR corrected Q-value) on the y-axis for the **(A)** two-leaf, **(B)** four-leaf, and **(C)** floral bud stages.

2,335 differentially expressed genes, 1,273 of which were upregulated and 1,062 were downregulated, with an average absolute LFC ($\log_2$ fold change) of 1.5 (Fig. 2a). In the comparison of four-leaf (4-leaf) stage between ecotypes, we identified 2,824 differentially expressed genes, 1,721 of which were upregulated and 1,103 were downregulated, with an average LFC of 1.6 (Fig. 2b). For the bud stage, we identified 2,696 differentially expressed genes, 1,297 upregulated and 1,399 downregulated, with an average LFC of 1.5 (Fig. 2c). Results of these contrasts for all genes available in the supplementary information.

Genes within the linkage group 8 chromosomal inversion were overrepresented among differentially expressed genes in both the two-leaf ($P = 0.017$) and four-leaf stage of development ($P = 0.013$), compared to the rest of the genome (Table 1). In contrast, there was no enrichment of differentially expressed genes within the chromosome 8 inversion in the bud stage ($P = 0.119$). Further, the magnitude of expression divergence of the chromosome inversion 8 genes compared to genes across the rest of the genome was not significant for any stage ($P > 0.8$). Just like the chromosome 8 inversion, proportionally more genes within the chromosome 5 inversion were differentially expressed than the rest of the genome for both the two-leaf ($P = 0.020$) and four-leaf ($P = 0.011$) stages of development but not for the floral bud stage ($P = 0.45$). The magnitude of differential expression of inversion 5 genes was not significantly different than the rest of the genome at any stage ($P > 0.1$).

Gene co-expression module analysis

We identified three major co-expressed gene modules with CEMiTool (Fig. 3). The genes in Module 1 corresponded to the transition from leaves to first buds of the plant, irrespective of ecotype. Module 2 correlated with expression differences between coastal perennial and inland annual ecotypes regardless of developmental stage. Module 3 corresponded to genes generally correlated with an interaction term between ecotype and developmental stage. Module 1 contained 1419 genes, Module 2 contained 388 genes, and Module 3 contained 346 genes.

Gene ontology enrichment analysis

Since Module 1 (Fig. 3) likely represents the transition from vegetative to reproductive stages, we expect GO enrichment to return terms related to floral and reproductive development. GO enrichment of Module 1 returned 207 GO terms, of which 112 are biological processes. Notable GO terms included "developmental process involved in reproduction," "floral organ development," and "regulation of pollen tube growth," among others related to reproduction and flowering. We analyzed variation due to ecotype and ecotype-by-developmental stage interaction by combining the gene lists from Module 2 and Module 3 (Fig. 3) before GO enrichment. GO enrichment on this combined set returned 54 terms and 20 biological processes. Notable enriched GO terms included "defense response to insects," "gibberellin metabolism," "regulation of anion transmembrane transport," and "inflorescence development."

Candidate genes identified from modules

We further interrogated modules of differential gene expression to identify potential candidate genes involved in ecotype divergence. There were two notable genes from

Module 3 with ecotype-by-developmental stage interactions: *Gibberellin 3-beta-dioxygenase 1* (*GA3ox1*;Migut.I00834) and Cytochrome P450 94B1 (*CYP94B1*; Migut.D01265) (Fig. 4). *GA3ox1* is a key gene in the gibberellin biosynthesis pathway (Hedden & Phillips 2000). The coastal ecotype had a high transcript abundance of *GA3ox1* in leaves. In contrast, the inland ecotype had a low level of *GA3ox1* in leaves. At the bud stage, both ecotypes had similar transcript abundances of *GA3ox1* (Fig. 4). *CYP94B1* is the first enzyme involved in degrading the bioactive form of jasmonic acid, JA-Ile (Koo et al. 2014). In leaves, the coastal ecotype has a low basal level of *CYP94B1* transcript, while the inland ecotype has expression levels two orders of magnitude higher. At the bud stage, *CYP94B1* transcript abundance was not nearly as diverged between the ecotypes.

Discussion

In this study, we evaluated gene expression divergence between a coastal perennial and an inland annual accession of *M. guttatus* through development. Overall, the results of our study suggest that there are significant divergences in gene expression both between ecotypes and among developmental stages. We found that most of the variation in gene expression is linked to differences between developmental stages of vegetative leaf and reproductive bud, regardless of ecotype identity. However, differences between ecotypes and ecotype x developmental stage interactions are also present and may represent critical changes affecting developmental timing during local adaptation. Among the significant pathways implicated in these differences are the gibberellin and jasmonic acid hormone pathways. These pathways are known to interact (Fujita et al. 2006; Yang et al. 2012; Guo et al. 2018) and play a role in a growth-defense trade-off (Yang et al. 2012; Machado et al. 2017; Major et al. 2017). We also found elevated rates of differential expression between ecotypes within chromosomal inversions. We hypothesize that the divergence in gene expression between ecotypes is driven by local adaptation to coastal versus inland habitats.

Comparing gene expression through development between the coastal perennial and inland annual ecotypes of *M. guttatus* is challenging, as these ecotypes often develop at different rates and put on different numbers of leaves before flowering. Both *M. gutatus* ecotypes go through two-leaf and four-leaf vegetative stages. However, the inland ecotype often flowers at the next node after the 4-leaf stage, while the coastal ecotype transitions through 6-leaf and 8-leaf stages before forming floral buds. Despite this concern, there was a high level of clustering across all leaf stages for both the coastal perennial and the inland annual plants in both PCA (Fig. 1) and co-expression analyses (Fig. 3). The largest contrast in gene expression was in comparisons of the ecotypes and the major developmental stages (leaf vs. bud).

As in a previous study by Gould et al. (2018), gene expression divergence between ecotypes was elevated for chromosome 8 inversion relative to the rest of the genome in our study (Table 1). These differing results across developmental stages are interesting in the context of a recent study by Berdan et al. (2021), who found that the effects of an inversion of global gene expression were more pronounced in adults than larvae of seaweed flies.

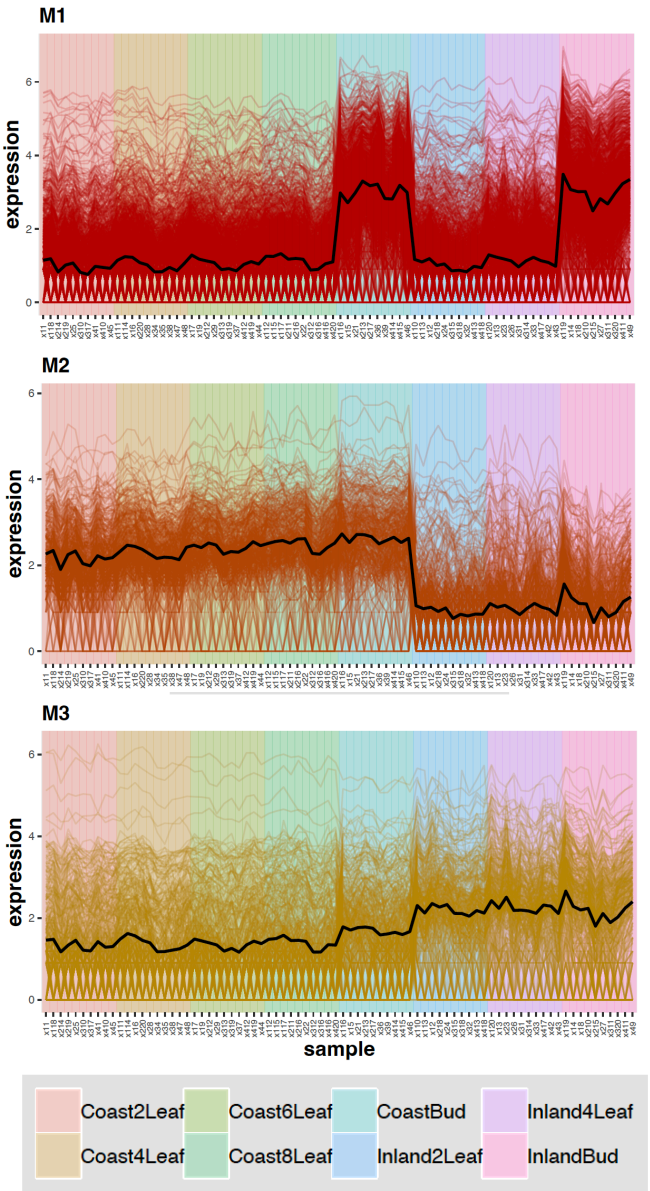

Figure 3: Different gene co-expression modules. Module 1 (M1) contains genes with differential expression between the leaf (vegetative) stage and the bud (reproductive) stage. Module 2 (M2) contains genes with differential expression between the ecotypes (coastal perennial vs. inland annual). Module 3 (M3) loosely groups genes by ecotype but contains genes with ecotype x development interactions.

The overall patterns of gene expression divergence between the plants used in this study support our current hypotheses regarding mechanisms of adaptive divergence between coastal perennial and inland annual ecotypes of *M. guttatus*. Annual *M. guttatus* plants typically allocate more resources to reproduction over vegetative growth and resistance to insect herbivores (Hall & Willis 2006; Lowry et al. 2008; Hall et al. 2010; Baker & Diggle 2011; Baker et al. 2012; Holeski et al. 2013; Lowry et al. 2019). By allocating resources to reproduction, annual plants can successfully escape the terminal end-of-season drought that characterizes summers in western North America. In contrast, perennial *M. guttatus* plants preferentially allocate

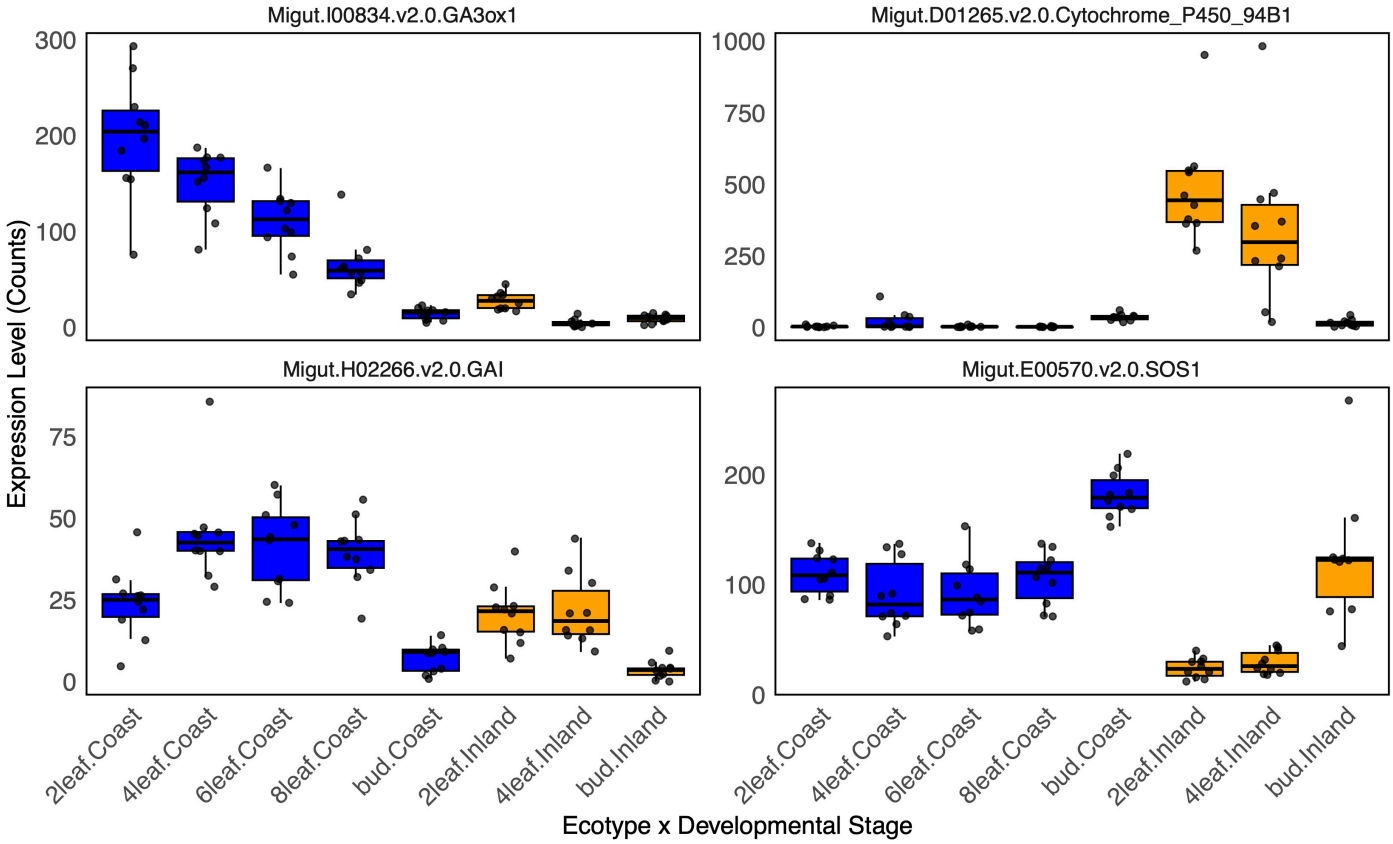

Figure 4: Ecotype x developmental stage interactions in gene expression. Patterns of expression for four candidate genes through developmental stages for which the full two-way factorial models fit with DESeq2 were significant at $P < 0.001$.

resources to vegetative growth and resistance to insect herbivores (Holeski et al. 2013; Lowry et al. 2019; Blanchard et al. 2024). The consistently lower temperatures and summer fog along the Pacific coast of North America (Gilliam 2002; Garcia-Reyes & Largier 2012) provide conditions that facilitate a perennial life history and, thus, this pattern of resource allocation. In this study, we found evidence of differences in gene expression in two key hormone pathways, gibberellin and jasmonic acid, that may underlie previously observed ecotypic differences in resource allocation. Gibberellins promote rapid growth and flowering, while jasmonic acid promotes herbivore defense (Hou et al. 2013; Lowry et al. 2019; Monson et al. 2022).

While trade-offs between rapid growth and reproduction versus herbivore resistance have long been recognized by plant biologists, it is only recently that the underlying molecular mechanisms of such trade-offs have begun to be elucidated. An important recent discovery in this area is that the gibberellin and jasmonic acid hormone pathways directly interact in an antagonistic manner though the DELLA-JAZ signaling node (Karzan & Manners 2012; Qi et al. 2014; Havko et al. 2016; Wasternack 2017). Our finding of GO term enrichment for both the GA and JA pathways in genes with significant ecotype by developmental stage interactions suggests that divergence between the ecotypes resulted from a change in these pathways leading to resource allocation shifts.

Candidate genes for ecotype divergence

A potential role for the gibberellin and jasmonic acid hormone pathways is also illustrated at the individual gene level (Fig. 4), with expression patterns for the key gibberellin biosynthetic gene *GA3ox1* and a gene involved in jasmonic acid degradation (*CYP94B1*). *GA3ox1* had 40x higher counts of transcripts in the early leaf stage of the coastal perennial plants, with a reduction over time to a low level in the bud stage. Inland annual plants had consistently low levels of *GA3ox1* in leaves and floral bud. This may imply that more gibberellin is needed for the allocation of growth to leaf vegetative growth in the coastal perennial ecotype. *CYP94B1* is responsible for the first step of degradation of bioactive jasmonic acid (Koo et al. 2014). The coastal ecotype had a low expression level that was consistent across all developmental stages. In contrast, the inland annual plants had a 40X higher expression at the two-leaf stage, but had a similar level of expression to the coastal ecotype in the floral bud stage (Fig. 4). This pattern of gene expression suggests that jasmonic acid is not degraded across all stages at a high rate in the coastal perennial ecotype while JA may be quickly degraded and recycled in the leaves of the inland ecotype.

Outside of this study, there is limited evidence for the role of these two candidate genes in the divergence between coastal and inland populations. *GA3ox1* occurs on chromosome 9 in a region within a Quantitative Trait Locus (QTL), albeit with a very broad confidence interval, that controls flowering time and stolon production divergence between inland annual and coastal perennial accessions (Friedman et al. 2015). In contrast, *CYP94B1* did not fall within any known QTLs for coast-inland ecotype divergence (Hall et al. 2006; Lowry & Willis 2010; Friedman et al. 2014; Holeski et al. 2014). It should be kept in mind that gene

Table 1: Comparison of count and magnitude of gene expression differences between coastal perennial and inland annual plants for two known chromosome inversions.

Developmental Stage	*Inversion Chr.*	*t-value*	*df*	*95% CI*	*Odds Ratio*	*P-value*
2-Leaf (count)	8	—	—	[1.038, Inf]	1.18	0.0166*
2-Leaf (magnitude)	8	0.13478	341.94	[-0.126, 0.144]	—	0.8929
4-Leaf (count)	8	—	—	[1.049, Inf]	1.19	0.0116*
4-Leaf (magnitude)	8	-0.11721	398.65	[-0.141,0.125]	—	0.9068
Bud (count)	8	—	—	[0.965, Inf]	1.11	0.1186
Bud (magnitude)	8	-0.22847	418.08	[-0.126, 0.10]	—	0.8194
2-Leaf (count)	5	—	—	[1.038, Inf]	1.21	0.0202*
2-Leaf (magnitude)	5	1.5218	230.31	[-0.047, 0.365]	—	0.1294
4-Leaf (count)	5	—	—	[1.061, Inf]	1.24	0.0112*
4-Leaf (magnitude)	5	0.56033	266.31	[-0.168, 0.302]	—	0.5757
Bud (count)	5	—	—	[0.869, Inf]	1.02	0.4534
Bud (magnitude)	5	0.95155	267.52	[-0.091, 0.260]	—	0.3422

Statistical tests of elevated gene expression for chromosome inversions compared to genes genome-wide. Overrepresentation of the number of differentially expressed genes was assessed using Fisher's exact tests. The magnitude of differential expression was compared with Welch's t-test for unequal variance using the absolute value log2 foldchange (LFC). * Significance at $P < 0.05$.

expression patterns are caused by *cis-* and/or *trans-*regulatory effects (Wray et al. 2003). If the expression patterns of *GA3ox1* and *CYP94B1* is the result of trans-regulation, then we would not expect colocalization of phenotypic QTLs and the position of these genes in the genome.

In addition to the possible involvement of *GA3ox1* in coast-inland ecotype divergence, we are currently evaluating the possible role of other gibberellin genes within the large chromosome 8 inversion (Lowry & Willis 2010). The chromosome 8 inversion in *M. guttatus* contains hundreds of genes, including the gibberellin biosynthetic gene *Gibberellin 20-oxidase2* (*GA20ox2*; Kollar et al. 2024). Gibberellin 20-oxidases have frequently been linked to major morphological and developmental evolution in plants (Spielmeyer et al. 2002; Barboza et al. 2013; Duan et al. 2020). Unfortunately, *GA20ox2* is a gene that typically has low transcript abundances and was not expressed at a high enough level in our study to be included in our analyses. In a recent study, Kollar et al. (2024) used qPCR to demonstrate elevated expression of *GA20ox2* in shoot apices for inland annual plants, with lower expression in the same tissue for coastal perennial plants. This expression pattern is the opposite of *GA3ox1* in leaves, which may illustrate the complexity of how the allocation of resources to growth differs spatially across plant subunits for different locally adapted ecotypes. It is also possible that the expression of *GA3ox1* more closely resembles that of *GA20ox2* in the shoot apex, which we did not evaluate in this study.

Even though the expression of *GA3ox1* suggests the possibility of elevated GA production in leaves, this biosynthesis may be counterbalanced by the expression of a critical downstream gene, *Gibberellic Acid Insensitive* (*GAI*), for gibberellin signaling. The expression pattern for *GAI* (Migut.H02266.v.2.0) was elevated in coastal leaves (Fig. 4). *GAI* is the only DELLA domain gene in the *M. guttatus* genome and a critical repressor of growth that is degraded in response to bioactive GA (reviewed in Xue et al. 2022). Future functional studies using recently developed techniques for transformation (Stanley & Lowry 2024) will be necessary to test whether any candidate genes has evolved to cause the adaptive divergence between the ecotypes.

While the overall gene expression patterns suggest the involvement of both the gibberellin and jasmonic acid hormone pathways, gene expression alone cannot definitively identify the ultimate causes of ecotype divergence. That said, recent work by Lowry et al. (2019) found that applying gibberellin (GA3) to coastal perennial plants in the greenhouse leads to a profound change in morphology that resembles the growth form of the inland annual ecotype. In the field, the application of GA3 has the additional impact of increasing susceptibility to damage by oceanic salt spray in coastal perennial plants (Toll et al. 2024). Inland annual plants do not have nearly as dramatic a response to GA3, which indicates that they already produce a high level of endogenous gibberellins and/or have low levels of DELLA-based repression on growth.

In terms of salt spray resilience, we found that the candidate salt tolerance gene Salt Overly-Sensitive 1 (*SOS1*) had higher levels of expression in both coastal perennial plant leaves and floral buds than in these tissues for inland annual plants (Fig. 4). This pattern is consistent with a previous study (Gould et al. 2018) that we conducted only on leaf tissue from the field. However, this study adds the recognition that this gene is expressed in elevated levels in floral buds relative to leaves for both ecotypes.

Towards a general understanding of ecotype divergence

The morphological divergence between coastal and inland ecotypes of *M. guttatus* is similar to coastal vs. inland divergence of ecotypes in many other plant species (Turesson 1922; Gregor 1938, 1939; Clausen et al. 1948;

Lowry 2012). In general, coastal ecotypes flower later and are more prostrate in growth form than inland ecotypes of the same species (reviewed in Lowry 2012).

While the morphological divergence between coastal and inland ecotypes is a common pattern in plants, we still know little about its underlying genetic and physiological mechanisms. The only other system we know where appreciable progress has been made in understanding this type of ecotype divergence is between headland and dune ecotypes of *Senecio lautus*. In *S. lautus*, the headland ecotypes are much more prostrate than the upright growing dune ecotypes (Roda et al. 2013a,b; James et al. 2021a,b). In contrast to our system, auxin has been implicated as the major hormone involved in this transition (James et al. 2021a,b; Wilkinson et al. 2021). However, no gene in either *S. lautus* or *M. guttatus* has been confirmed to be involved in coastal ecotype divergence through functional genetic studies. We are currently conducting transgenic studies to test the involvement of gibberellin genes in the divergence between coastal perennial and inland annual ecotypes of *M. guttatus*.

Overall, we hope that this study, and subsequent studies in this system, will provide a more comprehensive understanding of molecular mechanisms underlying ecotype divergence and the transition between perenniality and annuality in plants (Friedman 2020; Lundgren & Des Marais 2020).

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Data availability

Raw sequence reads and processed count data are publicly available and accessible for reuse through the NCBI Gene Expression Omnibus (GEO) under accession numbers GSE280929 and GSM8607613-GSM8607692.

Supplementary information

The supplementary information for this preprint includes three tables that contrast gene expression for the coastal perennial versus the inland annual ecotypes at the two-leaf, four-leaf, and floral bud stages of development using DESeq2. These data were used to make the volcano plots in Fig. 2.

Author contributions

DBL and YC designed the experiment. YC conducted the experiment, performed RNA extractions, and prepared the genomic libraries. JDO and BAG conducted the analyses. DBL and JDO wrote the manuscript, with input from YC and BAG.

Competing interest statement

The authors declare no competing interests.