



Original Article

Genome-Wide Association Mapping of Floral Traits in Cultivated Sunflower (*Helianthus annuus*)

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Abstract

Floral morphology and pigmentation are both charismatic and economically relevant traits associated with cultivated sunflower (*Helianthus annuus* L.). Recent work has linked floral morphology and pigmentation to pollinator efficiency and seed yield. Understanding the genetic architecture of such traits is essential for crop improvement, and gives insight into the role of genetic constraints in shaping floral diversity. A diversity panel of 288 sunflower genotypes was phenotyped for a variety of morphological, phenological, and color traits in both a greenhouse and a field setting. Association mapping was performed using 5788 SNP markers using a mixed linear model approach. Several dozen markers across 10 linkage groups were significantly associated with variation in morphological and color trait variation. Substantial trait plasticity was observed between greenhouse and field phenotyping, and associations differed between environments. Color traits mapped more strongly than morphology in both settings, with markers together explaining 16% of petal carotenoid content in the greenhouse, and 17% and 24% of variation in disc anthocyanin presence in the field and greenhouse, respectively. Morphological traits like disc size mapped more strongly in the field, with markers together explaining up to 19% of disc size variation. Loci identified here through association mapping within cultivated germplasm differ from those identified through biparental crosses between modern cultivated sunflower and either its wild progenitor or domesticated landraces. Several loci lie within genomic regions involved in domestication. Differences between phenotype expression under greenhouse and field conditions highlight the importance of plasticity in determining floral morphology and pigmentation.

Key words: anthocyanins, carotenoids, color, GWAS, morphology, pigment

Floral traits have long been acknowledged for their major role in plant-pollinator coevolution and a major driver of plant diversity on Earth (Specht and Bartlett 2009; Van der Niet 2014; Gómez et al.

2015). The Asteraceae is the largest family of flowering plants, and members possess a distinctive inflorescence type variously referred to as a capitulum, pseudanthium, or composite head (Barkley et al.

2006; Funk et al. 2009). These inflorescences are composed of many distinct florets, and typically contain both zygomorphic ray florets bearing petals and actinomorphic disc florets bearing anthers, stigmas, and ovules (Gillies et al. 2002; Barkley et al. 2006). The Asteraceae contains an enormous diversity in floral size, morphology, and pigmentation (Funk et al. 2009), although to date studies of the genetic architecture of such traits have explored very few traits in very few taxa within the family (e.g., Deyue et al. 1999; Laitinen et al. 2005; Yue et al. 2008; Chapman et al., 2012).

This study examines the genetic architecture of floral diversity in cultivated sunflower (*Helianthus annuus* L.) using an association mapping approach, with a goal to expand knowledge of floral trait genetics. Describing the genetic architecture of floral traits in sunflower can inform our understanding of the evolution of floral diversity across the diverse wild relatives of cultivated sunflower and serve as a model for floral evolution in the Asteraceae. The genus *Helianthus* contains approximately 50 species, occupying diverse habitats across North America (Heiser et al. 1969). These species exhibit broad diversity in floral phenotypes with high evolutionary lability, for example, composite head size varies 30-fold (between 3.8 and 121.3 cm²), with a concomitant diversity in disc size, ray size, and ray number, as well as pigmentation of discs and rays (Mason et al. 2017). Understanding the genetic architecture of floral traits in *Helianthus* can provide insights into the tempo and mode of floral trait evolution, as well as whether observed evolutionary tradeoffs in floral phenotypes likely arise from genetic constraints (e.g., pleiotropy) or selection on combinations of floral traits (Salz et al. 2017). Recent synthesis has suggested that pleiotropy may be a major driver of floral integration, with select genes controlling multiple different aspects of floral phenotypes (Smith 2016). If the genetic architecture of floral traits exhibits strong pleiotropy (e.g., co-localization of associations consistent with “biological pleiotropy” *sensu* Solovieff et al. 2013), this may serve as a constraint on the phenotypic combinations evolution can easily produce, and may facilitate rapid evolutionary shifts between co-occurring suites of floral traits (Smith 2016). From an applied perspective, floral traits in many crops strongly influence pollination success and thus fruit or seed yield (Andrews et al. 2007; Campbell et al. 2012; Bartomeus et al. 2014), and there are emerging efforts to improve yields in several crops by manipulating floral phenotypes (Palmer et al. 2009; Mallinger and Prasifka 2017; Bailes et al. 2018; Portlas et al. 2018). In cultivated sunflower, the majority of acreage consists of oilseed and confectionary cultivars whose yield and seed traits are highly correlated with many floral traits like head size and floret number (Palmer and Steer 1985; Marinković 1992; Alkio et al. 2003). Among diverse cultivars, disc floret size by itself has been shown to explain 52% of variation in wild bee preference, with minimal reductions in corolla length resulting in a doubling of pollinator activity (Portlas et al. 2018). In addition, pollen presence, nectar sugar content, and other floral traits have been shown to strongly influence both wild and honeybee visitation rates (Mallinger and Prasifka 2017). This suggests that sunflower floral phenotypes warrant further study for the improvement of yield through enhanced pollination success. In addition to seed and oil production, cultivated sunflower is also a major ornamental crop to the cut-flower industry, where floral traits like color and morphology are of direct economic importance (Cvejić et al. 2016, 2017; Short et al. 2017). Given the central role of floral traits in the agricultural and horticultural value of cultivated sunflower, understanding the genetic architecture of these traits is of major potential value for breeding efforts. This study seeks to describe

this architecture using a diverse mapping panel of cultivated sunflower germplasm.

Materials and Methods

Association Mapping Panel

For this study, we used an existing association mapping panel for cultivated sunflower consisting of 288 inbred lines (Mandel et al. 2013). This panel captures approximately 90% of allelic variation across all cultivated sunflower varieties present in the germplasm repositories of the United States Department of Agriculture (USDA) and the French Institut National de la Recherche Agronomique (INRA), and thus allows for robust mapping of phenotypes to the sunflower genome with broad relevance across the crop gene pool (Mandel et al. 2011, 2013). Briefly, each line was genotyped for 5788 single nucleotide polymorphisms (SNPs), with these markers spanning all 17 linkage groups across the 3.6 Gbp cultivated sunflower genome (Mandel et al. 2013; Badouin et al. 2017). This set of markers has been previously used to successfully map the genetic architecture of flowering time and branching architecture within this association mapping panel (Mandel et al. 2013). Locations of markers within the sunflower genome are reported with respect to the genetic map of Bowers et al. (2012).

Greenhouse Phenotyping

For each of the 288 lines, 3–4 replicate plants were grown under environmentally controlled conditions at the University of Georgia Plant Biology Greenhouses in Athens, GA. Given the logistical infeasibility of phenotyping so many plants simultaneously, the full panel was randomly divided into 8 groups (hereafter referred to as “stagers”) grown in rapid succession from early June through late December 2014. Within each stagger, 2 genotypes (RHA280 [PI 552943] and RHA801 [PI 599768]) were grown in triplicate and used as phytometers to investigate any effects of stagger on phenotypes. These 2 genotypes were selected to represent the 2 major market classes of cultivated sunflower: RHA280 is a large-seeded confectionary variety (Fick 1974), while RHA801 is an oilseed variety (Roath 1981). For each stagger, seeds were scarified and germinated in petri dishes on wet filter paper and then transferred to seedling trays, where they were stored in a randomized spatial design in a plant growth chamber (Convicon, Winnipeg, Canada). Growth chamber conditions were held at 25 °C with 14-h daylength. Upon the emergence of true leaves, seedlings were transplanted to 6-inch azalea pots (~1.3 L) filled with pure river sand mixed with 5 mL of Osmocote Plus 15-9-12 slow-release fertilizer with micronutrients (Scotts, Marysville, OH). Plants were hand-watered daily to field capacity and fertigated weekly to field capacity with a dilute solution (5 mL-in-3 L-water) of Jack’s 20-10-20 liquid fertilizer (JR Peters, Inc., Allentown, PA). Once juvenile plants were well-established (i.e., had reached 4–6 leaf pairs), they were moved to a randomized design on benches in a glass greenhouse. In the greenhouse, the stagers were exposed to ambient light conditions as well as supplemental metal-halide lighting to maintain daylength at 14 h (just under the summer maximum daylength for Athens, GA). Hand-watering was replaced with an automated irrigation system which watered plants to field capacity daily, and weekly hand-fertigation was continued. Greenhouse temperature control was set to 25 °C and did not fall significantly below this level, though summer daily high temperatures varied up to 5 °C above this set point on particularly hot sunny days due to limits of the greenhouse evaporative cooling system.

Fourteen traits were phenotyped on all plants, as further described below: number of leaves at bud, number of leaves at flower, height at flower, disc diameter, ray length, ray width, number of rays, ray density, flower diameter, flower area, disc area, petal area, petal area fraction, petal carotenoid content, and presence or absence of anthocyanins in the floral disc (Figure 1). Phenotyping was standardized by ontogenetic stage using the standard stage scoring system for cultivated sunflower (Schneiter et al., 1981). Plants were assessed for reproductive phenology 3 times per week and measured when at the appropriate stage for each trait, with floral traits assessed on the primary inflorescence. At stage R1, where an immature inflorescence is visible in place of a new pair of leaves at the terminal bud, the number of true leaves was counted (yielding “number of leaves at bud”). True leaves were defined as having a length of at least 4 cm (Schneiter et al. 1981). Once plants reached anthesis (stage R5), plant height and the number of true leaves were recorded (yielding “number of leaves at flower” and “height at flower”). Between the stages of R5.3 and R5.7 (30% and 70% of the disc florets having opened, respectively) the remaining floral traits were measured. These included disc diameter, measured as the diameter of the largely flat circular aggregate of disc florets (hereafter “disc”) or the average of 2 perpendicular diameters

if the disc was elliptical; ray length and width, measured on a representative ray floret ligule (hereafter “ray” or “ray floret petal”); number of rays, assessed by counting all rays present; and the presence of anthocyanin pigments in the disc, visually assessed as dark coloration of either corollas or stigmas and binarized as presence/absence (Figure 1). Lines exhibiting variable presence and absence of disc anthocyanins across replicates were given a value of 0.5. At this time, 3–5 ray floret petals were sampled, snap-frozen, and stored at -80°C for subsequent analysis of petal carotenoid content. From the morphological data collected, several additional traits were derived. Flower diameter was calculated as the sum of the disc diameter and 2 times the ray length. Disc area was calculated from the disc diameter to represent the 2-dimensional elliptical area occupied by the disc florets. Ray area was calculated as the ellipse of a single ray (using ray width and length), multiplied by the number of rays. Flower area was calculated from addition of the disc area and ray area. Petal area fraction was calculated as the ratio of the total ray floret petal area to the entire flower area and serves as a metric of relative investment in ray floret petal area versus disc area. Ray density was calculated as the number of rays divided by the circumference of the disc and serves as a metric of the compactness of rays on the sunflower head.



Figure 1. Floral traits assessed in the field and greenhouse mapping studies. Top left: disc diameter assessed on longest and orthogonal axes in the field on a head with light disc corollas and stigmas. Top right: field growth setting showing a line with dark discs in the foreground. Bottom 3 panels: a small slice of variation in color and morphology observed among varieties grown in the greenhouse.

For carotenoid content, sampled frozen petals were processed according to the methods of Barrell et al. (2010) and Torres et al. (2014), where pigments were extracted with methanol and the absorbance of carotenoids was recorded at 436 nm via visible spectrometry and converted to mg g⁻¹ equivalents of β -carotene (CAS# 7235-40-7) with a standard curve under the Beer–Lambert law.

Field Phenotyping

Alongside our greenhouse phenotyping, previously unpublished data on a small set of floral traits from the field phenotyping study of Mandel et al. (2013) was also included in our analyses to compare genomic associations derived from greenhouse and field phenotyping for a subset of traits. Briefly, the same 288 lines were planted in replicate under agricultural field conditions at the Plant Sciences Farm in Watkinsville, GA in spring of 2011. At the R5 stage, 2 traits were assessed—disc diameter and the presence of anthocyanins in discs. For disc diameter, the entirety of the disc was measured at the longest axis (e.g., vertical) and the corresponding orthogonal axis (e.g., horizontal). From these measurements, disc area was calculated as an ellipse using the vertical and corresponding horizontal diameters. All measured data gathered was roughly normally distributed and homoscedastic (Supplementary Figure S1). Presence of anthocyanins was scored visually for both disc floret corollas and stigmas separately. These data were binarized to presence/absence, with lines exhibiting variable presence and absence among replicates given a value of 0.5. To align data sets between greenhouse and field phenotyping, anthocyanins were considered present in the discs overall as a composite trait if anthocyanins were scored as present in either the disc corollas or stigmas in the field.

Phytometer Analyses

While logistically necessary for greenhouse phenotyping, separating lines into temporal staggerers may introduce phenotypic variation. Conditions were kept as similar as possible through engineering controls in both the growth chambers and greenhouse bay, but environmental conditions nonetheless likely varied somewhat among staggerers. To investigate the effects of staggerers on greenhouse phenotyping, we implemented a 2-way ANOVA approach on the phenotypic data collected for the 2 phytometer lines. We treated genotype and stagger as factors, and phenotype recorded as the dependent variable. In 10 of the 14 traits, there was a significant effect of stagger ($P < 0.05$) (Supplementary Figure S3). To correct for effects of stagger on each trait, the overall least squares mean of the phytometers across staggerers was used as a correction term for each stagger. Least squares means were calculated with the R package LSmeans (Højsgaard and Halekoh, 2018). Two correction types were utilized: an additive correction and a multiplicative correction. For the additive correction, the deviation (positive or negative) between the overall least squares mean of the phytometers across staggerers and the mean of the phytometers in a given stagger was added to line means of plants grown in that stagger. Similarly, for the multiplicative correction, a correction term was defined as the ratio of the overall least squares mean of the phytometers across staggerers to the mean of the phytometers in a given stagger. Each line mean within a given stagger was then corrected by multiplying by the correction term. Both additively and multiplicatively corrected line means were used for subsequent association mapping. With and without correction, line means were roughly normally distributed and homoscedastic (Supplementary Figures S3–S5). These 2 correction types represent alternate approaches to removing trait variation arising from environmental variation among staggerers, and reducing the degree to which environmental variation might bias the mapping of phenotypes.

Association Mapping

Association mapping analysis was performed utilizing a compressed mixed linear model (Zhang et al. 2010) implemented with the R package GAPIT (Lipka et al. 2012). Only SNPs with a minor allele frequency (MAF) ≥ 0.05 were used. Four different association models were run per trait. The models used account for family relatedness or kinship (K model), kinship plus population structure as identified by principal component analysis (P + K model), kinship plus population structure (Q + K model) as defined previously in Mandel et al. (2013) using Bayesian clustering implemented in the program STRUCTURE (Pritchard et al. 2000), and a naïve model that does not incorporate kinship or population structure. To assess mixed linear model performance, quantile-quantile plots (q-q plots) were used. Observed P values were plotted against the expected probability distribution to identify where the model may have produced a higher number of significant results than due to chance (Supplementary Figures S7–S10). To reduce bias due to linkage disequilibrium, we elected to use the method of Gao et al. (2008) for multiple testing correction to identify a conservative threshold level of statistical significance. Briefly, the method employs a dimension reduction approach to control for correlation among linked markers. The number of dimensions that adequately explain 99.5% of the variation in SNPs is considered the effective number of tests. In a Bonferroni framework, the alpha (0.05) is then divided by the number of effective tests, creating the statistical threshold of significance (Gao et al. 2008, 2009). Absolute marker effects (proportion of variance explained; PVE) were expressed as a global effect size, whereby the single allele effect size estimate (in trait units) was expressed relative to the range of phenotypic variation observed for each trait. Cohen's f^2 was also calculated to determine local effect size of all SNPs above the Gao threshold (Cohen 1988; Selya et al. 2012). This statistic reflects the proportion of phenotypic variance uniquely explained by the SNP of interest independent of fixed effects (i.e., inclusion of kinship and/or population structure), normalized to the proportion of unexplained variance (Cohen 1988). For example, given a SNP of interest from the Q + K model, the proportion of variance of an SNP can be calculated as the R^2 of the full model (Q + K + SNP) minus the R^2 of the model including only the fixed effects (Q + K) divided by the proportion of variance left unexplained by the full model (Q + K + SNP) (Selya et al. 2012). Narrow-sense heritability was calculated for all traits (including all correction schemes for traits assessed in the greenhouse) as the proportion of phenotypic variance explained by additive genetic factors (Supplementary Table S3). The methods of Endelman and Jannink (2012) were used to calculate the heritability for each trait under each model. Given that the mixed-linear model uses the kinship matrix to estimate genetic variance and the kinship matrix estimates additive genetic relationships, the resulting genetic variance is the additive genetic variance at the basis of our heritability estimation (Endelman and Jannink 2012). In practice, we divided the total additive genetic variance by the combination of the residual and additive genetic variance. All analyses were conducted within the R environment (R Core Team 2016).

Results

Greenhouse Trait Variation

Plants in the greenhouse exhibited substantial phenotypic variation for floral morphology and pigmentation. Petal carotenoid content varied from 0.79 to 16.67 mg g⁻¹ among lines, with an average of 6.34 mg g⁻¹ (± 0.19 SE, Supplementary Table S1). Seventeen lines within the full panel displayed dark discs possessing visible

anthocyanins. Height at flower varied over ten-fold among lines, from 13.1 to 146.05 cm, with number of leaves at bud and flower both varying around 3-fold (Supplementary Table S1). Disc diameter varied from 2.1 to 7.67 cm, ray length from 1.4 to 7.1 cm, and overall flower diameter therefore from 4.9 to 20.6 cm among lines (Supplementary Table S1). Ray width varied from 0.5 to 4.6 cm among lines, the number of rays present varied from 16 to 49.3, and ray density from 0.39 to 4.55 rays per cm of disc circumference (Supplementary Table S1). This variation in ray size and number resulted in a petal area fraction ranging between 0.76 and 0.95 among lines (Supplementary Table S1). In the greenhouse, approximately 70% of measured trait combinations were significantly correlated using genotype means (Pearson's r , $P < 0.05$), regardless of correction used (Supplementary Figures S2–S4). Height at flower was positively correlated with many traits, including the number of leaves at bud and flower, disc and flower diameter, number of rays and overall petal area, but negatively correlated with petal area fraction (indicating that larger plants invested relatively more in discs). Disc diameter was positively correlated with all other morphological traits with the exception of ray density and petal area fraction, with which it was negatively correlated (Supplementary Figures S3–S5).

Trait Variation Observed in the Field Versus the Greenhouse

As plants were grown in pots in the greenhouse and in-ground in the field, plants achieved a larger average size in the field both qualitatively and as evidenced by the quantitative differences in head size observed (Supplementary Table S1). Whether grown under greenhouse or field conditions, however, lines exhibited substantial phenotypic variation in disc morphology. Disc diameter exhibited more variation among lines under field conditions, from 3.45 to 13.40 cm, and the disc area, of course, exhibited a similar pattern (Supplementary Table S1). In the field, disc diameter averaged 7.92 cm (± 0.11 SE) across lines, much larger than the greenhouse mean of 4.94 cm (± 0.06 SE). However, the line means for disc diameter and disc area were significantly positively correlated between field and greenhouse conditions, with correlation coefficients ranging from 0.38 to 0.48 among correction types (Supplementary Figures S10 and S11). For anthocyanin-based disc pigmentation, 10 lines exhibited dark disc corollas in the field, while 86 lines exhibited dark stigmas. This was far more than the 17 lines that exhibited dark discs overall in the greenhouse, which may suggest that the expression of anthocyanins was reduced in the greenhouse relative to the field. One explanation for this is that coated glass greenhouse panels filter at least some of the incoming ultraviolet radiation, which is known to be related to anthocyanin expression given the photoprotective role of anthocyanins in plants (Stapleton 1992; Guo et al. 2008). Differences observed in both morphological and pigmentation traits between the greenhouse and field suggest that sunflower floral phenotypes exhibit substantial environmental plasticity, but the correlated trait values suggest that genotype drives a substantial portion of floral phenotype as well.

Comparison of Mixed Models

For each dataset, 4 mixed models were considered: no inclusion of population structure or kinship (naïve), kinship (K), population structure as measured by principal coordinate analysis including kinship (P + K), and population structure as measured by STRUCTURE including kinship (Q + K). Quantile-quantile (q-q)

plots of the observed P values versus the expected P values for each model, were used to assess model accuracy (Supplementary Figures S6–S9). The distribution of observed P values in the model that accounts for Bayesian-inferred population structure and kinship (Q + K model) generally deviated least from the expected distribution, indicating that this model should produce the fewest false positives (Supplementary Figure S6–S9). The Q + K model was thus the primary model considered for marker examination across traits. In several of the traits assessed, 2 models (P + K and Q + K) both produced fewer significant results than expected by chance suggesting that these models may be overly conservative (Wang 2012).

Mapping of Morphology in the Greenhouse

In the greenhouse, our association mapping panel exhibited substantial phenotypic variation across all morphological traits measured, with narrow-sense heritabilities ranging from 0.17 to 0.78 under the Q + K model (Supplementary Table S3). As mentioned previously, there was a significant effect of stagger on phytometer measurements for most traits ($P < 0.05$). Uncorrected, additive, and multiplicative corrections were run in each trait association mapping analysis (Supplementary Figures S12–S15). For multiplicatively corrected data, there were significant markers detected for 3 of the 13 morphological traits measured (ray density, height at flower, and number of leaves at bud) when population structure and kinship were accounted for (Q + K model; Supplementary Figure S14). The local effect sizes (Cohen's f^2) of all identified markers ranged from 5.7% to 7.6%, representing small effect sizes (Selya et al. 2012). For ray density, our analyses identified 2 significant associations on LG3 (24.85 cM) explaining approximately 4% of phenotypic variance and corresponding with local effect sizes ranging from 6.6% to 7.6%, respectively (Figure 2, Table 1). These 2 markers were located within 1 cM of each other and are likely linked to the same causal variant. Height at flower was associated with 1 marker on LG6 (92.20 cM) with a local effect size of 5.7%, explaining approximately 5% of phenotypic variance (Supplementary Figure S14, Table 1). Number of leaves at bud was associated with two markers, one on LG5 (63.51 cM) and another on LG10 (48.10 cM), with local effect sizes of 6.2% and 6%, respectively, explaining approximately 6% of phenotypic variance each (Supplementary Figure S14, Table 1).

Additive correction was considered to be less conservative than multiplicative correction based on the number of associations obtained. All markers identified with multiplicative correction were recovered though additive correction (Supplementary Table S2), and the additively corrected data additionally identified a significant marker for height at flower on LG2 that was not identified with multiplicative correction (Supplementary Figure S13, Supplementary Table S2).

Mapping of Morphology in the Field

All morphological traits measured in the field were significantly associated with one or more markers and exhibited higher narrow-sense heritabilities than the same traits assessed in the greenhouse (Table 1, Supplementary Table S1). Local effect sizes for these traits ranging from 5.8% to 9.5%, representing small effect sizes (Selya et al. 2012; Supplementary Figure S15). Disc diameter of the longest axis was significantly associated with 4 markers (Figure 3). Two of these markers were located on LG6 (37.24 cM, 37.29 cM) explaining 5% and 4% of phenotypic variance, respectively, and one

marker each was located on LG12 (24.47 cM) and LG16 (39.09 cM) explaining 5% and 4% of phenotypic variance, respectively (Table 1). The 2 markers on LG6 had local effect sizes of 6.5% and 6.8%, while associated markers on LG12 and LG16 had local effect sizes of 8.5% and 5.9%, respectively. Markers for the orthogonal axis were largely the same, though one of the markers on LG6 (37.24 cM) was not significantly associated and a novel marker on LG14 (22.83 cM) was identified with a local effect size of 6.1%, corresponding to 6% of phenotypic variance (Table 1). Similarly for flower area, the same 3 markers associated with the longest and orthogonal axes on LG6 (37.29 cM), LG12 (24.47 cM), and LG16 (39.09 cM) were also found to be significantly associated with local effect sizes of 6.2%, 8.1%, and 6%, respectively, and similar proportions of phenotypic variance explained (Table 1). An additional marker was also identified on LG10 (48.09 cM) with a local effect size of 5.8%, explaining 5% of phenotypic variance (Table 1).

Mapping of Floral Pigmentation in the Greenhouse and Field

Petal carotenoid content, assessed in the greenhouse, had 12 associated markers on LG15 with local effect sizes ranging from 7.6% to 16.9% each of which explained phenotypic variance ranging from 5% to 9% (Figure 2, Table 1). There was a primary peak around 37.00 cM for carotenoid content markers with 2 secondary peaks around 35.76 and 37.93 cM, respectively. For disc anthocyanin pigmentation, presence/absence was scored separately for disc corollas and stigmas in the field study, but scored only for the disc as a whole in the greenhouse study. In the greenhouse, 2 markers were significantly associated with anthocyanin presence in the disc as a whole, one on LG1 (2.25 cM) and the other on LG11 (9.34 cM) with effect sizes of 9% and 6.5%, increasing likelihood of presence by 14%

and 10% (Figure 2, Table 1). In the field, the same marker on LG1 was also identified as being significantly associated with the presence of anthocyanins in the disc floret corollas, with an effect size of 7.5% and increased the likelihood of presence by 12% (Figure 4, Table 1). Along with that marker, 7 other markers were found to be significantly associated on LG1 with 5 major peaks across the linkage group corresponding to local effect sizes from 6% to 11%, explaining between 1% and 10% of the phenotypic variance (Table 1). Presence of stigma anthocyanins and presence of anthocyanins in the disc overall in field grown individuals were increased by 13% and 17% by the same marker on LG6 (10.62 cM) with a local effect size of 10.1% in both traits, indicating that the presence of stigma anthocyanins drove overall variation in disc anthocyanin presence-absence in the field (Figure 4). Narrow-sense heritabilities for anthocyanin presence-absence in the field were the highest observed for any trait in this study (0.87–0.92), while narrow-sense heritability was somewhat lower in the greenhouse (0.82) though still higher than those observed for morphological traits (Supplementary Table S3).

Discussion

Modern cultivated sunflower is a relatively young crop. Following domestication in eastern North America ca. 4200 ybp (Crites 1993; Blackman et al. 2011), intensive crop improvement only began in the mid-1800s with the development of oilseed varieties and first commercial production in Eastern Europe (Putt 1997). Domestication and improvement generated a substantial genetic bottleneck, leaving modern cultivated sunflower germplasm with less than half of the genetic diversity present in the wild progenitor *H. annuus* (Liu and Burke 2006; Mandel et al. 2011), much more of a bottleneck than the 20–30% reductions in diversity reported for rice, wheat, and maize (Buckler et al. 2001; Liu and Burke 2006). Despite this, we find substantial phenotypic diversity in floral traits across our diversity panel of cultivated germplasm. Most traits vary at least 3-fold among lines (e.g., disc diameter, ray length, ray number, flower diameter), while others vary by over an order of magnitude (e.g., ray density, metrics of disc, petal, and flower area), and petal carotenoid content varies over 20-fold. Compared with an assessment of floral trait evolution across 27 diploid wild *Helianthus* (Mason et al. 2017), cultivated sunflower exhibits larger variation in almost all morphological traits, with only ray density and petal area fraction varying more across wild species than within the crop lineage by virtue of the existence of a completely rayless species of wild sunflower (*H. radula*). With respect to pigmentation, cultivated sunflower exhibits both dark and light discs, similar to the variation seen across wild species (Mason et al. 2017), and a qualitatively similar variation in the degree of ray petal pigmentation from pale yellow to dark orange. This indicates that the process of domestication, landrace diversification, and eventual modern crop improvement has either generated (e.g., via artificial selection or local adaptation), or permitted (e.g., via relaxed selective pressures) more floral trait variation in cultivated sunflower (at least in the traits assessed) than the process of natural diversification in wild sunflowers across diverse North American habitats over millions of years (Heiser et al. 1969; Mason et al. 2015; Mason et al. 2018). Given the degree of phenotypic variation exhibited, cultivated sunflower may be able to provide insights into the genetic architecture of floral diversity in wild *Helianthus* and perhaps the Asteraceae more broadly. For example, the finding here that just a

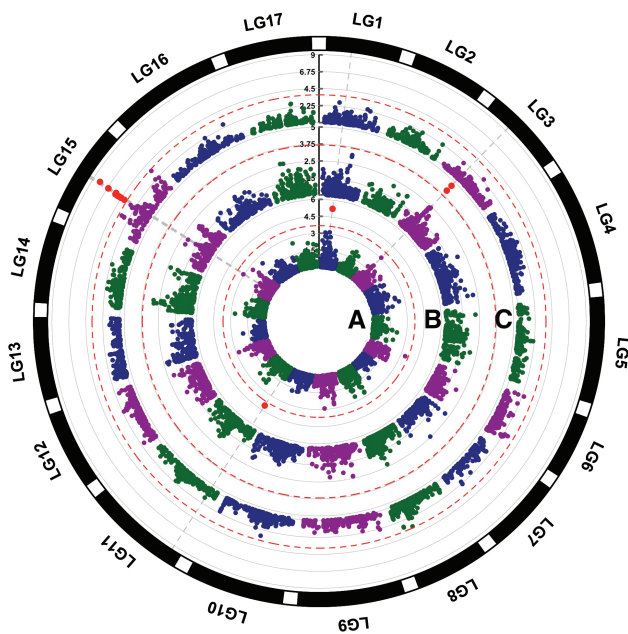


Figure 2. Manhattan plots of genome-wide association mapping results from the Q + K model for multiplicatively corrected traits in the greenhouse for (A) overall disc anthocyanin presence, (B) ray density, and (C) petal carotenoid content. The threshold line refers to Gao's correction at $-\log_{10}(p) = 3.68$. Radial gray-dashed lines occur along the same vector as the associated significant marker, with the same location denoted across plots. Significant markers above each threshold have been enlarged for clarity.

Table 1. Significantly associated markers from the Q + K model for greenhouse measured traits (multiplicative correction) and field measured traits, using the Gao threshold for significance: $-\log_{10}(p) > 3.68$

Location	Trait	Marker	Linkage group	Position (cM)	$-\log_{10}(p)$	PVE	Cohen's f^2	
Greenhouse	Height at flower (cm)	M1799	6	9.22	3.7	0.05	0.057	
		M1742	5	63.51	3.97	0.06	0.062	
	Number of leaves at bud	M3509	10	48.10	3.86	0.06	0.06	
		M775	3	24.85	4.64	0.04	0.076	
	Ray density (petals cm^{-1})	M776	3	24.85	4.14	0.04	0.066	
		M4925	15	35.67	4.78	0.07	0.085	
	Carotenoid content (mg g^{-1})	M4926	15	35.76	4.21	0.06	0.073	
		M4928	15	35.76	4.94	0.06	0.089	
		M4931	15	35.76	4.38	0.06	0.076	
		M4932	15	35.76	4.41	0.06	0.077	
		M4933	15	35.76	4.52	0.06	0.079	
		M4934	15	35.76	4.63	0.07	0.082	
		M4941	15	37.00	6.5	0.08	0.127	
		M4942	15	37.00	5.33	0.07	0.098	
		M4943	15	37.00	6.51	0.08	0.127	
		M4946	15	37.26	5.48	0.07	0.102	
		M4952	15	37.93	8.07	0.09	0.169	
		Overall disc anthocyanin presence	M155	1	22.50	5.24	0.14	0.09
			M3625	11	9.34	4.05	0.10	0.065
	Field	Disc diameter (cm) of the longest axis	M1888	6	37.24	4.04	0.05	0.065
M1890			6	37.29	4.21	0.04	0.068	
M3935			12	24.47	5.03	0.05	0.085	
M5152			16	39.09	3.77	0.04	0.059	
Disc diameter (cm) of the orthogonal axis		M1890	6	37.29	3.93	0.04	0.062	
		M3935	12	24.47	5.46	0.05	0.095	
		M4642	14	22.83	3.83	0.06	0.061	
		M5152	16	39.09	4.26	0.04	0.069	
Disc area (cm^2)		M1890	6	37.29	3.92	0.04	0.062	
		M3497	10	48.09	3.68	0.05	0.058	
		M3935	12	24.47	4.82	0.05	0.081	
		M5152	16	39.09	3.78	0.04	0.06	
Disc stigma anthocyanin presence		M1802	6	10.62	5.75	0.13	0.101	
Disc corolla anthocyanin presence		M15	1	4.13	5.77	0.10	0.102	
		M87	1	11.62	3.88	0.04	0.061	
		M106	1	12.84	5.98	0.07	0.11	
		M133	1	18.16	4.76	0.04	0.08	
		M142	1	18.63	4.09	0.05	0.066	
		M155	1	22.50	4.52	0.12	0.075	
		M178	1	25.40	3.82	0.01	0.06	
	M207	1	26.87	4.52	0.06	0.075		
	M3410	10	41.75	3.95	0.03	0.063		
	Overall disc anthocyanin presence	M1802	6	10.62	5.75	0.17	0.101	

Marker positions are reported with respect to the genetic map of Bowers et al. (2012). PVE, proportion of phenotypic variance explained; this marker effect is reported as absolute effect size per allele relative to the range of trait values observed among lines.

handful of genomic regions together explain at least a quarter of phenotypic variation in disc pigmentation may partially explain how there have been at least 7 back-and-forth transitions between dark and light disc color across the phylogeny of wild diploid *Helianthus* without apparent strong selection from pollinators or the abiotic environment (Mason et al. 2017). For morphological and pigmentation traits that are reasonably conserved across the Asteraceae including aspects of the composite head structure and the presence of anthocyanin and carotenoid-based floral pigmentation, insights from this and further work within sunflower may inform patterns of floral trait evolution across the family as a whole, especially if combined with data from other genomically enabled Asteraceae like lettuce and artichoke.

The results of this study highlight 2 important themes concerning the genetic architecture of floral phenotypes in cultivated sunflower. First, floral traits were found to exhibit high phenotypic plasticity with environment, including morphological traits that scale with plant size but also other traits like pigmentation. Agronomic work examining effects of field conditions like plant density on reproductive trait plasticity have demonstrated strong effects on floret number and thus yield (e.g., Pereira and Hall 2019), but our findings go beyond this sort of plant size-dependent floral trait plasticity. The differences in phenotypes observed between greenhouse and field growth conditions indicate that conditions constraining overall plant size unsurprisingly play a significant role in floral trait expression within inbred lines, as may other factors like light quality

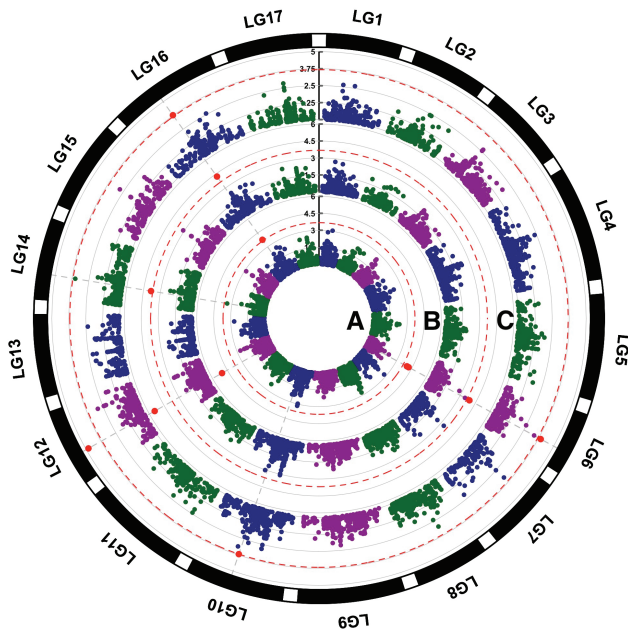


Figure 3. Manhattan plots of genome-wide association mapping results from the Q + K model in the field for (A) disc diameter (cm) of the longest axis, (B) disc diameter (cm) of the orthogonal axes, and (C) disc area (cm²). The threshold line refers to Gao's correction at $-\log_{10}(p) = 3.68$. Radial gray-dashed lines occur along the same vector as the associated significant marker, with the same location denoted across plots. Significant markers above each threshold have been enlarged for clarity.

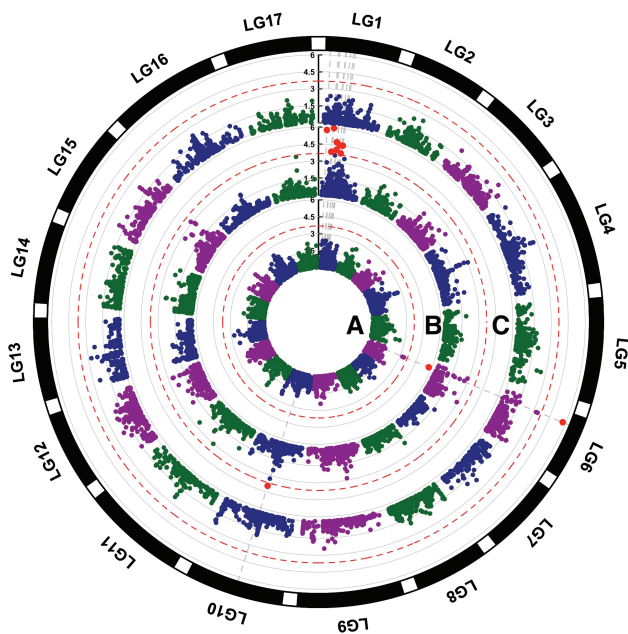


Figure 4. Manhattan plots of genome-wide association mapping results from the Q + K model in the field for anthocyanin presence in (A) stigmas, (B) disc corollas, (C) the disc overall. Red threshold line refers to Gao's correction at $-\log_{10}(p) = 3.68$. The threshold line refers to Gao's correction at $-\log_{10}(p) = 3.68$. Radial gray-dashed lines occur along the same vector as the associated significant marker, with the same location denoted across plots. Significant markers above each threshold have been enlarged for clarity.

given the differences observed in pigmentation. These effects likely explain the generally lower narrow-sense heritabilities observed in

the greenhouse (due to reduced overall phenotypic variation among lines). Perhaps more interesting are the differences observed within phytometer lines across growth staggers under otherwise “controlled” greenhouse conditions, which indicate that the expression of floral traits is quite sensitive to even more subtle differences in environmental conditions that are more difficult to quantify. This large role of plasticity in generating phenotypes likely contributed to the lack of detectable associations for many of the floral traits assessed in this study. Future efforts to describe the genetic architecture of floral traits in sunflower, especially more nuanced or labile phenotypes like odor or chemistry, should, therefore, include careful consideration of growth conditions and ideally replication across different environments for reliable mapping. Second, the genetic architectures uncovered for the subset of traits that were successfully mapped suggest few instances of shared genetic basis among floral traits. This suggests that pleiotropy (or at least “biological pleiotropy” *sensu* Solovieff et al. 2013) is unlikely to strongly limit the combinations of floral phenotypes possible in cultivated sunflower, despite that pleiotropy is recognized as a major mechanism of genetic constraint on floral diversity in many plant systems (Smith 2016). Under such a scenario of minimal pleiotropy, most morphological and pigmentation traits would be free to evolve largely independently of one another. This may be one explanation for the broad diversity of combinations of floral traits observed within cultivated sunflower across breeding pools, market classes, and especially among ornamental cultivars.

Ours is not the first study to consider the genetic architecture of floral traits in cultivated sunflower. Several QTL mapping experiments have used biparental crosses to investigate the genetic basis of floral trait differences between focal germplasm, often as part of a suite of so-called “domestication” or “improvement” traits. An early study using a cross between a wild sunflower (*H. annuus*) accession and a modern cultivar to derive an F₃ mapping population assessed a variety of traits, including disc diameter, ray number, and ray size (Burke et al. 2002). QTL were identified for disc diameter on LG4, LG5, and LG13 explaining 4–6% of phenotypic variation each, ray number on LG1, LG6, LG7, LG9, and LG13 explaining 6–10% of phenotypic variation each, and ray size on LG5, LG6, and LG9 explaining 7–9% of phenotypic variation each (Burke et al. 2002). A follow-up study using recombinant inbred lines produced through single-seed descent from the initial F₃ mapping population identified only one QTL for disc diameter on LG14 and one QTL for ray length on LG9, explaining 9–10% of phenotypic variation each, though the set of lines was grown in 2 geographic locations, and these associations were detected in only one location each (Dechaine et al. 2009). A further study used an F₂ mapping population derived from a cross between the same wild sunflower accession and the domesticated Hopi landrace also assessed disc diameter and ray number (Wills and Burke 2007). QTL were identified for disc diameter on LG1, LG6, LG8, LG9, LG10, LG14, LG15, and LG17 explaining 4–13% of phenotypic variation each, and ray number on LG5, LG8, LG10, LG12, LG15, and LG17 explaining 3–13% of phenotypic variation each (Wills and Burke 2007). Our own results are consistent with this recurring pattern of mapping studies identifying multiple loci of small effect distributed across the genome for morphological traits, with our greenhouse phenotyping finding associations explaining 4–14% of phenotypic variance, and our field phenotyping finding associations explaining 4–17% of phenotypic variance. However, unlike earlier studies based on biparental crosses with wild sunflower, our association mapping approach within cultivated sunflower does not recapitulate associations on the same linkage groups for disc diameter, ray number, or ray length/size with the

sole exception of an association for disc diameter on LG6 (shared with Wills and Burke 2007). This is not totally unexpected, as the genetic architecture of phenotypic differences between 2 plant accessions which span a bottleneck of domestication and/or improvement may be wholly different from the genetic architecture that characterizes variation across a broad collection of cultivated germplasm which all lie on one side of such a bottleneck. Expanding scope to other wild sunflowers, recent work within *H. argophyllus*, the sister species of wild *H. annuus*, finds QTL of moderate effect on disc diameter, flower diameter, and ray length (17–22% of phenotypic variation) on regions of the *H. argophyllus* LG2 which are syntenic with LG6 in *H. annuus* (Moyers et al. 2017). Those identified QTL directly overlap with the QTL identified for disc diameter within the syntenic region on LG6 identified by Wills and Burke (2007) in their Hopi-wild contrast. The disc diameter association identified here on LG6 within cultivated sunflower is just outside the one-LOD interval identified by Wills and Burke (2007), though within 1 cM of a candidate domestication contig at 36.31 cM identified via genomic scans for selection (Baute et al. 2015). That analysis additionally identified two other candidate domestication contigs at 22.86 cM on LG14 and 39.48 cM on LG16 (Baute et al. 2015), both within 1 cM of the significant associations identified here for disc diameter and area (Table 1). As seed yield is heavily driven by disc size (Marinković 1992), these results may indicate that these regions experienced selection by early domesticators.

Unlike morphological traits, previous work within cultivated sunflower germplasm suggests that various floral pigmentation traits are controlled by few loci of large effect (Fick 1976; Yue et al. 2008; Fambrini et al. 2009; Cvejić et al. 2016). Early cross-based work using segregation ratios estimated that 2 genes control ray flower color (driven by petal carotenoid content) spanning qualitatively red, orange, yellow, and lemon morphs (Fick 1976). A subsequent study focusing on a cross between 2 lines of the yellow morph recapitulated these results and putatively mapped one of the 2 genes to LG11 (Yue et al. 2008). In addition, cross-based modeling of the inheritance of orange, yellow, and white-cream pollen morphs (driven by pollen carotenoid content) strongly suggest that epistasis between 2 loci drives pollen color variation (Fambrini et al. 2009). Additional work on both ray flower color and disc color using crosses among four ornamental varieties suggests a third gene also controls ray floret color through carotenoid production, and further suggests a single gene controlling the presence or absence of anthocyanins in disc florets determining dark versus yellow coloration (Cvejić et al. 2016). Our own results across the cultivated sunflower germplasm identify a single region associated with petal carotenoid content, though mapped to LG15 rather than LG11. This may be one of the as-yet unmapped genes predicted by segregation ratios, or another gene or genes entirely. The carotenoid biosynthetic pathway in plants contains at least a dozen enzymes (Yoshikazu et al. 2008; Barrell et al. 2010), so it is very likely that different crosses or collections of germplasm may yield different loci of substantial effect, but across our mapping panel the one region on LG15 seems to contribute the only detectable quantitative variation in petal carotenoid content observed within cultivated sunflower. Interestingly, this region overlaps with the QTL of largest effect identified for proportional ultraviolet pigmentation in the syntenic region of *H. argophyllus* (Moyers et al. 2017). Ultraviolet pigmentation is known to be driven primarily by the presence of flavonoids, not carotenoids, but the physical overlap in regions for petal pigmentation controlled by different biosynthetic pathways (if not purely coincidental) might suggest the presence of regulatory genes for petal pigmentation (Yoshikazu et al.

2008). Across our panel, we find several regions associated with disc floret anthocyanins (in stigmas or corollas), on LG1, LG6, LG10, and LG11. This conflicts with the prediction of a single gene for disc coloration by Cvejić et al. (2016), but given the much broader collection of germplasm involved in our study, supports the conclusion of few genes of moderate effect driving variation in disc pigmentation across cultivated sunflower. Anthocyanins, as a product of the same branch of the larger phenylpropanoid pathway as flavonoids (Treutter 2005; Yoshikazu et al. 2008), might be expected to have an overlapping genetic architecture with floral ultraviolet patterning, though to date insufficient phenotypic data exists to dissect the complex tissue-specific phytochemistry that determines overall floral pigmentation. Additionally, one marker on LG1 that we find to be associated with disc anthocyanins lies within 1 cM of a candidate domestication contig at 10.90 cM identified through genome scans (Baute et al. 2015). As anthocyanin-based purple dyes were a known use for sunflower in antiquity (Putt 1997), this could reflect selection by early domesticators for disc pigmentation.

Floral traits are of major importance to the pollination success and yield of modern elite sunflower cultivars. As each disc floret produces one seed when successfully pollinated, head size (driven by disc diameter) is a direct predictor of seed number and size, and thus yield (Marinković 1992). Pollen limitation has been shown to limit yield in many cultivars (e.g., Parker 1981; Chamer et al. 2015), especially in the context of hybrid seed production (e.g., Greenleaf and Kremen 2006), and a wide array of floral traits have been shown to alter visitation and preference of the primary bee pollinators of sunflower (Mallinger and Prasifka 2017; Portlas et al. 2018). In addition to nectar and pollen traits, which provide nutritive rewards to bees, morphology has been shown to modify both attraction (e.g., disc area; Sapir 2009) and handling efficiency (e.g., disc floret size; Portlas et al. 2018). To date, it appears that minimal work has examined the role of pigmentation on pollinator visitation in either cultivated or wild sunflower, despite evidence that pigmentation drives major aspects of pollinator interactions in other Asteraceae (e.g., Niesenbaum et al. 1999; Malerba and Nattero 2012; De Jager et al. 2017). In addition to pollination success, floral traits impact a wide array of other key biotic and abiotic interactions. For instance, it has been demonstrated that larger head sizes in cultivated sunflower increases the attractiveness and oviposition by the American sunflower moth, *Homeosoma electellum*, and decreases the effectiveness of parasitoids, resulting in increased florivory and seed predation (Chen and Welter 2003, 2005, 2006). Work in other species further suggests that floral pigmentation, as driven by concentrations of secondary metabolites like flavonoids and anthocyanins, likely influences pollen viability and rates of spontaneous ovule abortion via susceptibility to ultraviolet damage (Shirley 1996; Koti et al. 2005; Tripathi and Agrawal 2013; Koski and Ashman 2015). These and other classes of phenylpropanoid secondary metabolites have also been shown to influence resistance to bacterial and fungal diseases (e.g., Lattanzio et al. 2006; Treutter 2006; Mierziak et al. 2014; McArt et al. 2013), including floral diseases in cultivated sunflower (e.g., Prats et al. 2006, 2007), as well as reduce insect florivory and seed predation in many plants (e.g., Lattanzio et al. 2006; McArt et al. 2013; Oguro and Sakai 2015), including florivory in cultivated sunflower (e.g., Mullin et al. 1991). As modifications of floral pigmentation and secondary metabolite concentrations are unlikely to directly alter economically constrained traits like seed size, number, and oil quality, these may be straightforward avenues for yield improvement via enhanced pollinator visitation and resistance to biotic and abiotic stresses.

Such improvements, however, will require a more robust understanding of pollinator responses to floral phenotypic variation in cultivated sunflower, as well as a more detailed understanding of the genetic architecture of such traits, many of which involve tissue-specific expression and are often temporally and environmentally labile. Mapping such traits is a more difficult endeavor than mapping morphological or simple color variation, but also more likely to generate applied benefit.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Author Contributions

J.R.M. and J.M.B. designed the field study. C.M.M. and L.A.D. designed the greenhouse study. E.C.R., T.P.P., J.R.M., and C.M.M. collected data. J.A.D., E.C.R., T.P.P., and C.M.M. contributed to data analysis, with final data analysis performed by J.A.D. J.A.D. and C.M.M. wrote the article with input from E.C.R., T.P.P., J.R.M., J.M.B., and L.A.D.

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Data Availability

Trait data are available as a data file in the supporting information and has been submitted to the Dryad digital repository (doi: 10.5061/dryad.26t7cs8).

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