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FROM THE COVER

Connecting the sun to flowering in sunflower adaptation

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Abstract

Species living in seasonal environments often adaptively time their reproduction in response to photoperiod cues. We characterized the expression of genes in the flowering-time regulatory network across wild populations of the common sunflower, *Helianthus annuus*, that we found to be adaptively differentiated for photoperiod response. The observed clinal variation was associated with changes at multiple hierarchical levels in multiple pathways. Paralogue-specific changes in *FT* homologue expression and tissue-specific changes in *SOC1* homologue expression were associated with loss and reversal of plasticity, respectively, suggesting that redundancy and modularity are gene network characteristics easily exploited by natural selection to produce evolutionary innovation. Distinct genetic mechanisms contribute to convergent evolution of photoperiod responses within sunflower, suggesting regulatory network architecture does not impose strong constraints on the evolution of phenotypic plasticity.

Keywords: flowering time, gene network evolution, *Helianthus annuus*, phenotypic plasticity, photoperiod response, sunflower

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Introduction

The timing and course of development can be highly plastic. Many traits change in response to environmental cues, and these plastic responses may provide important adaptations to changing environmental conditions (Schlichting & Pigliucci 1998). As species expand their ranges, colonize novel habitats, or face anthropogenic changes, phenotypic plasticity may be gained, lost or otherwise altered by natural selection such that phenotypes adapted to these environments continue to be produced under locally predictive combinations of cues (Moczek & Nijhout 2003; Bradshaw & Holzapfel 2008). Complex gene regulatory networks sense and integrate various environmental signals to produce appropriate phenotypic responses. However, few studies have leveraged knowledge of these pathways to explore the mechanisms underlying evolutionary changes in plasticity in natural populations (Abouheif & Wray 2002).

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In plants, a complex gene network regulates phenotypic plasticity in reproductive timing by integrating information from diverse cues including photoperiod so that flowering occurs during favourable conditions (Wilczek *et al.* 2010). Notably, rewiring of a conserved pathway can reverse photoperiod response. In *Arabidopsis thaliana*, a long-day plant that flowers earlier when day length exceeds a particular threshold, circadian and light signals interact so that *CONSTANS* (*CO*) only promotes expression of the mobile floral signal *FLOWERING LOCUS T (FT)* in long days (Valverde *et al.* 2004; Corbesier *et al.* 2007). In short-day sensitive rice, where flowering occurs earlier in day lengths below a threshold, *CO* represses *FT* in long days but promotes *FT* in short days (Hayama *et al.* 2003). Geographic clines in flowering time and photoperiod plasticity are often reported, but the adaptive nature and genetic causes of this variation are rarely determined (Bohlenius *et al.* 2006; Zhang *et al.* 2008; Takahashi *et al.* 2009). Consequently, the degree to which network structure predicts what genetic changes can yield such evolutionary shifts in plasticity is unknown (Erwin & Davidson 2009; Stern & Orgogozo 2009). Here, we cir-

cumscribe components of the flowering-time gene regulatory network likely contributing to adaptive evolution of flowering time plasticity in the common sunflower, *Helianthus annuus*, and discuss what these findings reveal about the interplay between natural selection and aspects of network structure that confer evolutionary flexibility or constraint.

Materials and methods

Phenology characterization

Six populations along a latitudinal transect (Fig. 1a)—Manitoba (MB), South Dakota (SD), Nebraska (NE), Kansas (KS), Oklahoma (OK) and Texas (TX)—were chosen on the basis of availability of seeds with family structure, absence of recent nearby sunflower cultivation, and previous use in genetic crosses (Table S1, Supporting information). Seeds from five heads per population were scarified, germinated in the dark on moistened filter paper for 5 days, and exposed to a single light-dark cycle to allow greening before sowing. Maternal half-sibs were evenly distributed among photoperiod treatments. TX seed with family structure was unavailable; seeds of unknown family structure were provided by the United States Department of Agriculture (PI494567). Seedlings were sown in 5″ pots containing a 1:1 mixture by weight of sand and Metro Mix (Sun Gro Horticulture). Growth chambers were maintained at 25.5 °C and 8-h light:16-h dark, 12-h light:12-h dark or 16-h light:8-h dark. Light intensity was adjusted so plants in different photoperiods received equivalent daily photosynthetically active radiation. Days to budding (R1 stage; Schneiter & Miller 1981) and anthesis were measured for one plant per maternal half-sib family in each chamber. Plant positions were randomized biweekly. Log-transformed data were analysed by balanced ANOVA in Minitab specifying photoperiod treatment, latitude, and their interaction as fixed effects.

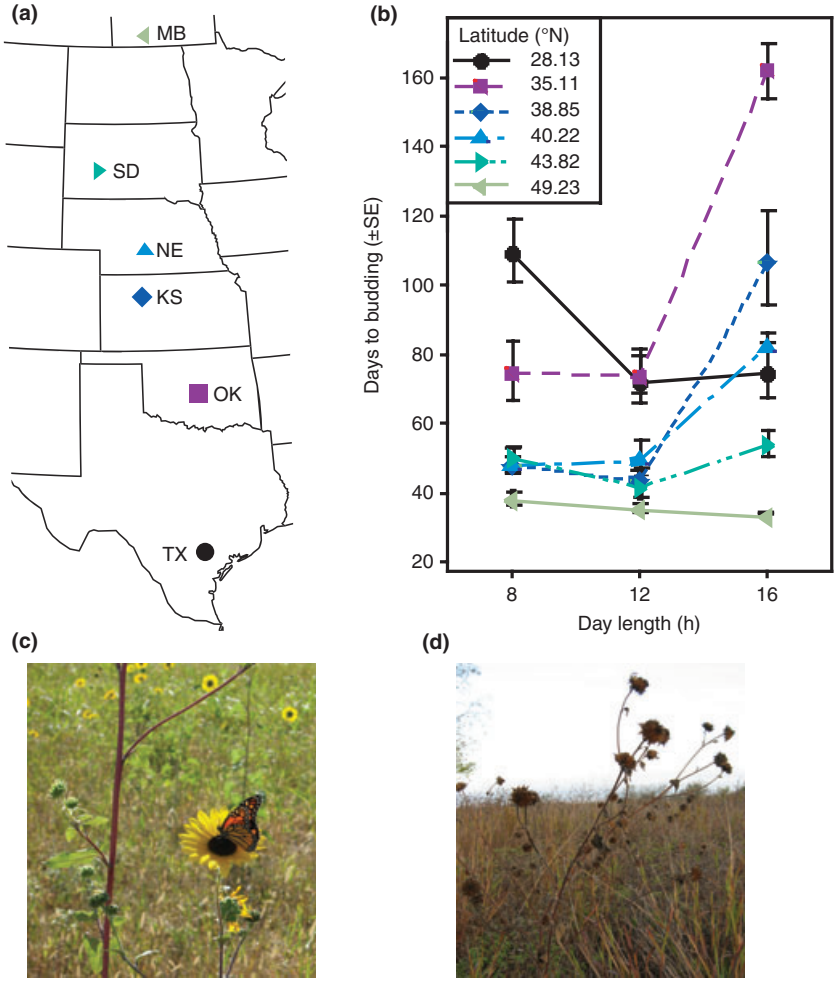

Fig. 1 Clinal differentiation of flowering and changes in photoperiod response in wild sunflower. (a) Locations of the populations sampled. (b) Flowering responses to three photoperiod conditions (mean ± SE). (c) Sunflowers near Norman, OK at peak flowering on September 19, 2006. (d) Senescent sunflowers near Austin, TX on September 21, 2006.

Q_{st} measurement

Seeds from ten maternal half-sib families per population were germinated. OK was excluded due to insufficient germination; too few seeds per family remained from the previous collection, and newly collected seeds were immature as the population had just reached peak flowering at collection (Fig. 1c). TX families came from new collections. Five seedlings per family were sown into 6″ pots and grown in 16-h days in the greenhouse under natural light supplemented by artificial lights. Plants were distributed one individual per family per block over five blocks. Every 7–10 days, plant positions within blocks and block locations in the greenhouse were randomized.

Days to budding and days to anthesis were measured with germination date as day 0. Leaf traits were measured at 28 days after sowing on mature leaves subtending the third node. Floral traits were measured at anthesis. Leaf area was measured using a LI-COR LI-3000A Portable Area Meter. Leaf wet weights and dry weights were measured on a scientific balance, and specific leaf area was calculated as area/dry weight. Succulence was calculated as (wet weight – dry weight)/area. Ray, bract and leaf shapes were calculated as the ratio of maximum length to maximum width of that structure. An outlier for specific leaf area was removed from the analysis as it was very aberrant and strongly affected the results. Bract shape, ray shape, ray number and specific leaf area were log-transformed to improve normality of the residuals.

Q_{st} values were calculated from variance components obtained using a restricted maximum likelihood mixed-linear model implemented with the lme function in R (The R Project for Statistical Computing) specifying block as a fixed effect and population and family nested within population as random effects (Pinheiro & Bates 2000). Variance components were extracted with the *varcomp* function. Q_{st} was calculated as $V_P/(V_P + 2 * (4 * V_F))$ where V_P is the variance between populations, and V_F is the variance among maternal half-sib families within populations. Multiplying V_F by four yields the additive genetic variance (Lynch & Walsh 1998). Theory predicts dominance and epistasis only reduce Q_{st} relative to F_{st} (Whitlock 2008). Thus, if these factors impact the variation observed, they would only reduce our power to detect $Q_{st} >> F_{st}$. Since we used a maternal half-sib design, our model cannot account for maternal effects. Confidence intervals were estimated by simulation (O'Hara & Merila 2005) using the maximum likelihood estimates for the fitted values and dispersion matrices obtained from the model. Q_{st} values were calculated for 1000 simulations. Upper and lower percentile cutoffs for 95% confidence intervals were obtained using the BCa method (Efron & Tibshirani 1993).

F_{st} measurement

DNA was extracted from one seedling from each of 16 families per population—including all families used for Q_{st} measurement—with the Qiagen Plant DNeasy 96 Kit. Individuals were genotyped for 11 microsatellite markers (Table S3, Supporting information) as described previously (Heesacker *et al*. 2008). F_{st} was calculated with Microsatellite Analyzer (Dieringer & Schlötterer 2003), and confidence intervals were obtained by bootstrapping over loci 1000 times.

Transformation

HaCOL1 and *HaCOL2* cDNAs were amplified by PCR from cultivar HA274—*HaCOL1*: forward—CACCATG TTAAATGAAGATCTCACTAG, reverse—TTTGATCCG GAGCATTGCTTAAA; *HaCOL2*: forward—CACCATGT TGGATCACACCGGTACCTTATG, reverse—CGTCTTT AAAACGAGGGTACAATTCC. MB, KS and TX *HaCOL2* cDNA sequences were also obtained with these primers.

Amplified fragments were introduced by Gateway™ cloning (Invitrogen) into vector pMDC32 (Curtis & Grossniklaus 2003). Columbia-0 and *co* mutant (SAIL24H04) *Arabidopsis thaliana* were transformed by the floral dip method (*Agrobacterium tumefaciens* strain GV1301). After 2 days stratification at 4 °C, seeds were germinated at 23 °C on 50 μg/mL hygromycin MS plates. Resistant transformants were transplanted to Metro Mix 5 days later. Successful transformation was confirmed by RT–PCR.

Gene expression

Leaves were collected every 4 h over a single day ~5 weeks after sowing from plants grown alongside the phenotyped plants in the 8- and 16-h treatments. For each time point within each treatment, two biological replicates consisting of leaves pooled from two to three plants from the same population were collected.

To determine the diurnal phase of expression for photoperiod pathway homologues, we germinated intrapopulation F1 seed generated from plants raised for Q_{st} measurement. For OK, field collected seeds were used. Leaf samples were collected at 6, 4, 3, 2 and 1 h before dawn and 2, 4, 5, 6, 7, 8, 10, 12 and 15 h after dawn ~5 weeks after sowing under 8-h days. The periods of most frequent sampling correspond to the periods when *CO*, *GIGANTEA (GI)* and *FLAVIN-BINDING*

KELCH-REPEAT F-BOX 1 (FKF1) paralogue up-regulation occurs. Three biological replicates were collected, and replicate leaves were harvested from different nodes to average out developmental differences. Leaves were never collected from the same plant within a 2-h period.

For shoot apex expression, MB, KS and TX maternal half-sibs were grown under 8- and 16-h days. Three biological replicates consisting of shoot apices pooled from two to three plants were collected ~5 weeks after sowing.

RNA extraction, DNase treatment and quantitative RT–PCR were conducted as previously described (Blackman *et al.* 2010). Primers are listed in Table S3, Supporting information. Two to three technical replicates were performed for each reaction and averaged. Relative expression was calculated as 2^-ΔΔCt—normalizing to *Ha60S rRNA*—and converted to a percentage of the highest expression value measured in either light treatment. For several genes, transcript abundance was assayed by RT–PCR and visualized by ethidium bromide staining on an agarose gel (28 cycles for *Ha60S rRNA*; 30 cycles for all other genes).

Regressions of total daily gene expression on latitude or mean flowering time on mean gene expression were completed in Minitab. Total daily gene expression was calculated as the sum of expression means for the biological replicates at the six time points assayed. For the diurnal phase grow-out, hourly transcript abundances were interpolated into intervals not sampled at that frequency, and total gene expression was calculated over the time course's duration.

Results

Diversity in flowering time and photoperiod response

We investigated the diversity of photoperiodic flowering in wild sunflower, *Helianthus annuus*, because the existence of long-day, short-day and photoperiod insensitive cultivars suggested an opportunity to identify mechanisms through which such variation can evolve within species (Goyne & Schneiter 1987). Seeds from six populations sampled along a latitudinal transect from MB (49.23ºN) to TX (28.13ºN) were germinated and grown under 8-, 12-, or 16-h days (Fig. 1a and Table S1, Supporting information). Day length, latitude, and their interaction explained nearly all the observed variation (balanced ANOVA; budding: $R^2 = 0.84$, $F_{\text{day length }(5,89)} = 45.62$, $F_{\text{latitude }(2,89)} = 30.21$, $F_{\text{interaction }(10,89)} = 8.66$; anthesis: $R^2 = 0.88$, $F_{\text{day length }(5,89)} = 59.46$, $F_{\text{latitude }(2,89)} = 77.68$, $F_{\text{interaction }(10,89)} = 11.75$; $P < 0.001$ for all terms for both traits). The number of days to budding and days to anthesis increased as latitude decreased in all treatments, and photoperiod response transitions occurred at both ends of the cline (Fig. 1b). All three major photoperiod response classes were observed. MB, the northernmost population, was day neutral, flowering earliest and at the same age under all conditions. Mid-latitude populations—SD, NE, KS and OK—were short-day sensitive, flowering ~0.5 to 3 months earlier under 8- or 12-h days than under 16-h days. In contrast, plants from the southernmost population, TX, were long-day sensitive, flowering about a month earlier under 12- and 16-h days than under 8-h conditions.

Variation was also observed in natural habitats. For instance, Oklahoman populations reached peak flowering during the shortening days of early fall when populations in central Texas had already senesced, having flowered months earlier under long days (Fig. 1c, d). This differentiation is likely adaptive. Short growing seasons in Manitoba may impose selection for initiating reproduction prior to experiencing photoperiods that induce short-day populations to flower. In Texas, warm, wet winters and severely dry summers shift the growth season; populations germinate in late February and flower prior to summer drought.

Comparison of Q_{st} and F_{st}

We raised maternal half-sib families from all populations, except OK, under long days and measured phenological traits in addition to leaf, floral, and physiological characters uncorrelated with flowering to obtain estimates of Q_{st}, a measure of quantitative trait differentiation. Populations were also genotyped for 11 microsatellite markers to estimate F_{st}, a measure of neutral genetic differentiation. As neutrally evolving traits and neutral genetic markers should accumulate similar levels of differentiation, if a trait's Q_{st} is much greater than F_{st}, then a hypothesis of neutral evolution can be rejected (Spitze 1993; Whitlock 2008). Q_{st} for flowering-time measures were 4–6 times as high as mean F_{st} and also higher than nearly all other traits (Table 1), consistent with adaptive differentiation in response to geographic variation in selection. Q_{st} for succulence was also high, which we attribute to strong differentiation of TX from other populations and little variation among families within populations.

Gene expression correlates of flowering time variation

We next examined gene expression variation in the photoperiod and gibberellin pathways because these cues have demonstrated effects on flowering in sunflower (Goyne & Schneiter 1987; Almeida & Pereira

Table 1 Quantitative trait differentiation relative to neutral genetic differentiation. Q_{st} is the proportion of the additive genetic variation for a trait partitioned between populations while F_{st} is proportion of the total genetic variation at 11 microsatellite loci partitioned between populations. Confidence intervals were calculated from 1000 simulations of the data. No confidence interval is provided for ray number due to non-normality of the data (Kolmogorov-Smirnov test, $P < 0.001$)

Trait	Q_{st}	95% CI
Succulence	0.756	0.280–1.000
Days to anthesis	0.513	0.136–0.736
Days to budding	0.463	0.106–0.741
Disc diameter	0.363	0.022–0.669
Bract number	0.323	0.036–0.594
Ray number	0.258	
Specific leaf area	0.253	0.001–0.645
Ray shape	0.163	0.000–0.470
Leaf shape	0.120	0.000–0.386
Bract shape	0.077	0.000–0.351
Petiole length	0.025	0.000–0.141
Neutral markers	0.087 (F_{st})	0.062–0.118

1996). Gibberellins promote flowering by antagonizing DELLA-domain proteins, transcriptional repressors that suppress expression of the floral inducers *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) (Achard *et al.* 2004; Ueguchi-Tanaka *et al.* 2005). We drew on these known regulatory pathways by inferring that if gene expression and phenotype were associated, then the changes responsible for both probably act upstream in the network or in *cis*- to expression of that gene. If upstream genes lacked these associations, we inferred the changes responsible for the phenotype act downstream or in a parallel pathway to expression of these genes. Thus, we bracketed network sections likely contributing to the cline and evolutionary shifts between photoperiod response types.

Based on their evolutionary conservation among angiosperms, we predicted that the network's gene content and hierarchical structure are conserved in sunflower (Hayama *et al.* 2003; Maizel *et al.* 2005; Tamaki *et al.* 2007; Yasumura *et al.* 2007; Serrano *et al.* 2009). Sunflower homologues of many flowering genes are known, though the genome is not sequenced and additional paralogues may exist (Blackman *et al.* 2011). Even so, functional equivalence of three sunflower *FT* homologues was confirmed by heterologous complementation in *Arabidopsis* (Blackman *et al.* 2010). We also overexpressed two *CO* homologues in wild type and *co* mutant *Arabidopsis thaliana*. Although neither paralogue accelerated flowering in wild-type plants, *HaCOL2* overexpression complemented the *co* mutation while *HaCOL1* overexpression did not (Fig. S1, Supporting information). For functionally uncharacterized paralogue sets, we measured expression of all known copies.

We first assayed transcript levels throughout the diurnal cycle for the downstream floral inducers *FT* and *SOC1* in leaves from sunflowers grown in 8- or 16-h days. Two *FT* homologues exhibited clines in expression under short days. Higher total daily gene expression levels were significantly associated with higher latitude (Fig. 2a, b; regression, *HaFT2*: $R^2 = 79.1\%$, $F_{1,5} = 15.11$, $P = 0.018$; *HaFT4*: $R^2 = 80.4\%$, $F_{1,5} = 16.40$, $P = 0.015$) and were also predictive for earlier flowering (*HaFT2*: $R^2 = 60.8\%$, $F_{1,5} = 6.22$, $P = 0.067$; *HaFT4*: $R^2 = 64.1\%$, $F_{1,5} = 7.13$, $P = 0.056$). In both photoperiods, *HaSOC1A* expression was higher in more northern populations (Fig. 2c; short days: $R^2 = 80.1\%$, $F_{1,5} = 16.07$, $P = 0.016$; long days: $R^2 = 64.4\%$, $F_{1,5} = 7.23$, $P = 0.055$) and positively associated with flowering time (short days: $R^2 = 75.2\%$, $F_{1,5} = 12.13$, $P = 0.025$; long days: $R^2 = 67.7\%$, $F_{1,5} = 8.37$, $P = 0.044$). Similar trends were not observed for *HaSOC1B* (Fig. S2 and Table S2, Supporting information). These patterns suggest that molecular changes contributing to the overall cline in flowering time may involve both the photoperiod and gibberellin pathways and most likely act upstream or in *cis*- to *HaFT2*, *HaFT4* and *HaSOC1A* expression (Fig. 2f).

Although neither *HaFT* paralogue was expressed in long days in most populations, consistent with short-day response, *HaFT4* was expressed in long days in MB, consistent with day-neutrality (Fig. 2b). Therefore, changes upstream or in *cis*- to this *HaFT* paralogue appear to cause a gain of expression likely involved in loss of photoperiod-responsive flowering in *H. annuus*' northern range.

We next examined expression of *CO* and DELLA protein homologues. In long days, one of two leaf-expressed DELLA-like genes was expressed at higher levels in later flowering populations, indicating the genetic changes contributing the overall cline in long days may act upstream or in *cis*- to its expression (Fig. 2e, f and Fig. S3, Supporting information). This finding was surprising and must be treated tentatively as functional consequences of variation in DELLA transcription for flowering have not been reported; however, it is noteworthy that DELLA gene expression in *A. thaliana* responds to a wide variety of cues (Gallego-Bartolomé *et al.* 2010) and transcriptional regulation of DELLA genes is an important mediator of light signalling on germination (Oh *et al.* 2007).

HaCOL2 daily expression abundance was neither associated with latitude (Fig. 2d; short days: $R^2 < 0.1\%$, $F_{1,5} < 0.01$, $P = 0.986$; long days: $R^2 = 31.6\%$, $F_{1,5} = 1.84$, $P = 0.246$) nor associated with flowering time (short days: $R^2 = 23.7\%$, $F_{1,5} = 1.24$ $P = 0.328$; long days: $R^2 = 52.1\%$, $F_{1,5} = 4.36$, $P = 0.105$), and MB expression

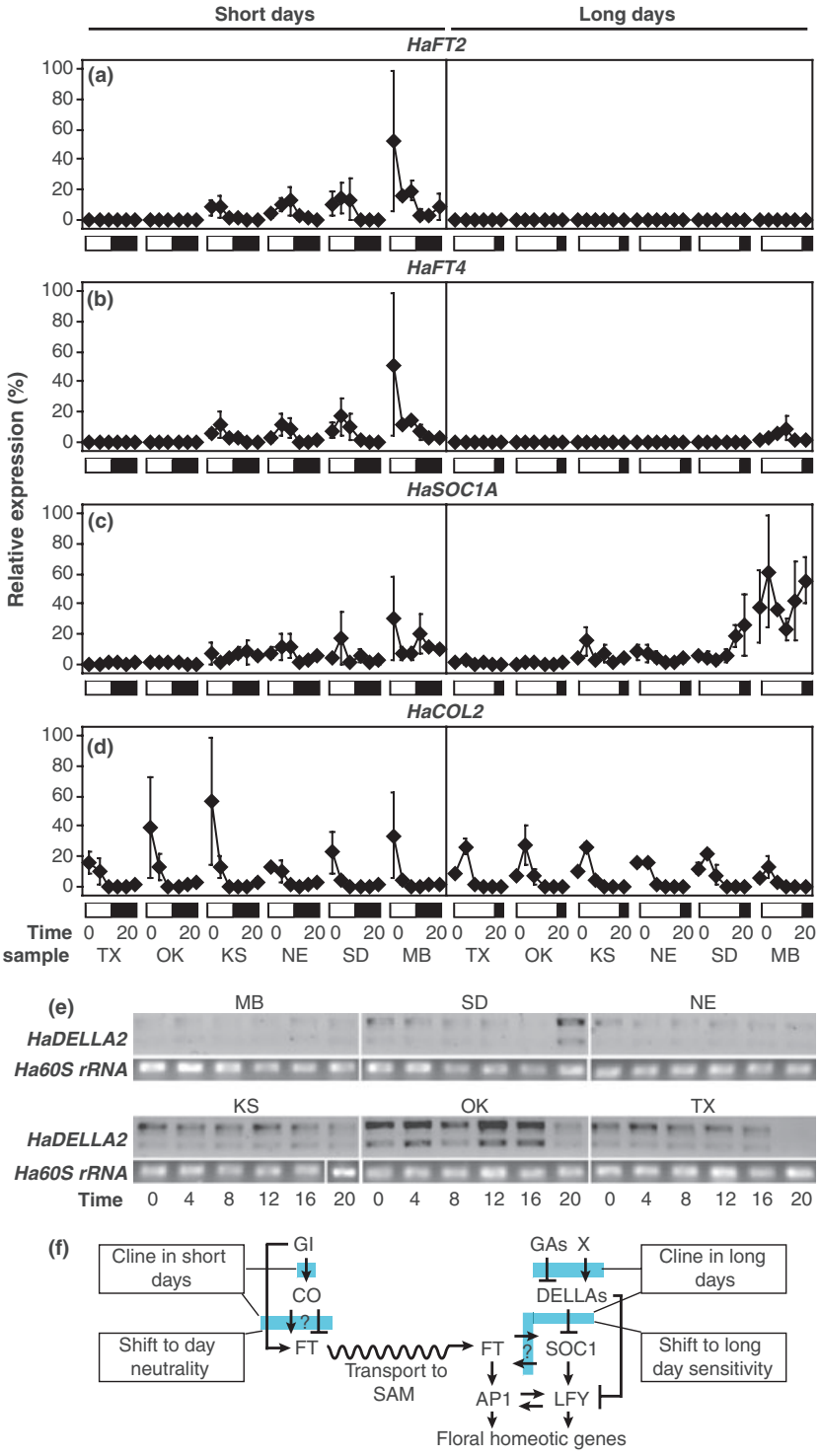

Fig. 2 Natural variation in flowering is attributable to specific portions of the underlying gene regulatory network. Relative expression (mean ± SE) of *HaFT2* (a), *HaFT4* (b), *HaSOC1A* (c) and *HaCOL2* (d) assayed by qRT–PCR in leaves of six wild populations monitored over a diurnal cycle in plants raised in short or long days. Bars along the *x*-axis illustrate periods of light and dark in each condition relative to time (hours after dawn). Expression was normalized to *Ha60S rRNA* expression (see Materials and methods). (e) Expression of *HaDELLA2* and *Ha60S rRNA* in leaves under long days. (f) Inferred regulatory network locations of genetic changes contributing to flowering-time variation in sunflower, highlighted in blue. Question marks note regulatory relationships differing between *Arabidopsis thaliana* and rice. The wavy arrow represents transport of *FT* from the leaf to the shoot apical meristem.

levels did not differ notably. These findings suggested that the changes responsible for the overall cline in short days and the transition to day neutrality lie downstream or act in parallel to *HaCOL2* expression (Fig. 2f). We subsequently assayed *HaCOL2* expression at finer temporal resolution to better characterize its phase of expression relative to daylight, a parameter critical in determining whether *CO* promotes or represses flowering. Unlike northern populations where *HaCOL2* expression peaks before dawn, the peak occurs at dawn in OK and TX (Fig. S4, Supporting information), and this shifted coincidence of *HaCOL2* abundance and light may partly explain the reduced *HaFT* expression in these populations (Fig. 2f). This trend may also be related to the observation that peak *HaFT4* expression occurred later in the day as latitude decreased from MB to KS (Fig. S2a, Supporting information), which may parallel similar findings in poplar (Bohlenius *et al.* 2006); however, we note this trend was only observed in one of our two experiments. No nonsynonymous *HaCOL2* sequence changes are fixed between populations. Expression assays of additional genes homologous to factors acting upstream of *CO*—e.g. *GI* and *FKF1*—or upstream of *FT* and downstream or in parallel to *CO* revealed no clines in transcript abundance or diurnal phase (Figs. S3–S6 and Table S2, Supporting information).

We also examined expression of floral inducers further downstream in the regulatory network that act in the shoot apex. As in leaves, *HaSOC1A* expression in short days was clinal; however, its expression pattern differed dramatically under long days: expression in TX was as high as expression in MB, consistent with long-day response (Fig. 3 and Table S2, Supporting information). *HaSOC1B* exhibited similar expression variation, and likewise, some of this variation is not mirrored in the leaf (Fig. S2 and Table S2, Supporting information). Therefore, tissue-specific regulatory changes acting upstream or in *cis*- to shoot apex expression of *HaSOC1* genes likely contribute to the transition from short-day to long-day response (Fig. 2f). Expression of downstream genes *HaLFY* and *HAM75*, an *APETALA1* (*AP1*) homologue, was consistent with this interpretation (Fig. S7, Supporting information). *HaFT1* and the *FRUITFULL* homologue *HaFUL* exhibited shoot apex expression patterns similar to the *SOC1* homologues, though less robust (Fig. S7, Supporting information). Since conservation of the regulatory hierarchy among these genes is ambiguous, positioning of important evolutionary changes in TX relative to these genes is uncertain. Shoot-apex expression levels of three DELLA paralogues were similar across populations, suggesting that relevant genetic changes act downstream or in parallel to their expression, although upstream factors affecting DELLA protein levels cannot be excluded.

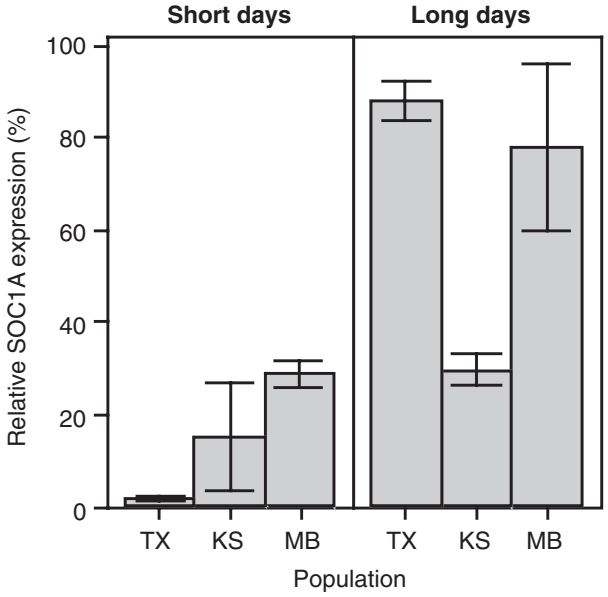

Fig. 3 *HaSOC1A* expression (mean ± SE), assayed by qRT–PCR, in the shoot apex in long days is associated with TX transition to long-day response.

Discussion

Together, these results implicate multiple upstream and downstream changes in both the photoperiod and gibberellin pathways as potential contributors the observed diversity (Fig. 2f), suggesting that natural variation is maintained throughout the network over sunflower's geographic range. Two aspects of gene regulatory network structure, redundancy and modularity, appear particularly important for the evolution of plasticity. In multiple contexts—e.g. *HaFT4* for day neutrality—paralogue-specific expression divergence appears responsible for flowering-time differentiation, indicating that the redundancy afforded by gene duplication makes certain nodes of a gene network more evolutionarily labile. Tissue-specific changes in *SOC1* homologue expression in TX were associated with the transition to long-day response, supporting the idea that modularity in gene networks provided by *cis*- or *trans*-acting factors may foster evolutionary change.

Our understanding of the molecular underpinnings of many plastic traits and the evolutionary conservation of these networks is ever increasing (e.g. germination—Penfield & King 2009; sex-determination—Shoemaker & Crews 2009; Valenzuela 2008; and plant–microbe mutualisms—Heath *et al.* 2010; Kouchi *et al.*

2010). In addition, recent technological innovations now allow gene expression to be measured cost-effectively at tens of loci over many samples from diverse genotypes and treatments (e.g. Wang *et al.* 2011). Therefore, we expect network-informed gene expression approaches to become increasingly more common and productive means of identifying the mechanistic correlates to adaptive natural variation in plasticity.

As noted above, there are several sources of uncertainty with this approach that impact our findings. For instance, while we have assayed expression of all known orthologous copies for many gene families, we cannot exclude the possibility that additional relevant but undiscovered orthologues were not characterized. However, since we find expression patterns correlated with phenotype for one or multiple paralogues in several gene families, and as expression patterns of downstream targets corroborate these findings, we would argue that we have identified multiple relevant readouts and contextualized them appropriately.

The degree to which the flowering time gene regulatory network is conserved between sunflower and other plants is another source of uncertainty. Although the gene content of the gibberellin pathway and the *CO*-dependent and *CO*-independent photoperiod pathways are widely conserved, there are some species-specific differences or unresolved aspects of the flowering time network—e.g. regulatory interactions of *FT*, *SOC1* and *FUL* in the shoot apex, the role of DELLA transcription in flowering time regulation, etc.—that lead to ambiguity in interpreting our results. In addition, we cannot account for the evolution of regulatory pathways specific to the sunflower lineage, as have been observed for the regulation of flowering in rice and maize (Colasanti *et al.* 1998; Doi *et al.* 2004; Itoh *et al.* 2010). If genetic variation is maintained throughout the network, then differential and combinatorial contributions of various upstream factors across populations could also lead to clines in expression of downstream factors without exhibiting clinal expression patterns themselves. Finally, several of the observed clinal gene expression trends are relatively subtle and while worth noting, they must be treated tentatively. Thus, we stress that while our work provides preliminary evidence attributing important evolutionary changes to particular pathways (Fig. 2f) and sufficient data to interpret broad trends, development and application of suitable functional methods will be necessary for confirmation of causal relationships. Nevertheless, we would argue that our conclusion that redundancy and modularity are key properties of gene networks that foster the evolution of plasticity is particularly strongly grounded as it derives solely from findings involving homologues to two broadly conserved downstream floral integrators, *FT* and *SOC1*.

These network properties may also relate to our finding that convergent evolution of long-day response in cultivated and wild sunflower involved different genetic changes. Long-day response evolved independently in the lineage leading to the cultivar CMSHA89, as sunflower was domesticated from short-day populations in Eastern North America (Harter *et al.* 2004). In CMSHA89, changes upstream to *FT* homologue expression in the leaf—rather than the *SOC1* homologue expression changes observed in TX—were associated with the shift to long-day response (Blackman *et al.* 2010). CMSHA89 also flowers earlier and has a higher threshold day length than TX. These phenotypic differences and the distinct mechanisms underlying these short-day to long-day transitions may be consequences of different initial conditions or dissimilar selective pressures during wild *H. annuus*' range expansion southward into Texas vs. modern crop improvement.

Our findings add to the growing variety of mechanisms implicated in flowering-time variation in diverse species (Turner *et al.* 2005; Bohlenius *et al.* 2006; Zhang *et al.* 2008), and they strongly contrast with the repeated involvement of the same genes in evolution of similar phenotypes often observed for more canalized developmental and metabolic traits (Stern & Orgogozo 2009). This evolutionary flexibility may arise from the broader multiplicity of regulatory inputs into the network, which could both increase mutational target size and reduce deterministic developmental constraints. Our findings also suggest that for phenotypically plastic traits, stochastic differences in initial genetic and demographic conditions or differences in the balance of environmental factors exerting selection pressures play more pivotal roles than developmental constraint in evolution (Abouheif & Wray 2002).

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Working with both sunflowers and monkeyflowers, B.K.B.'s research explores the genetic changes and evolutionary forces that contribute to natural diversity in the plasticity of developmental timing. S.D.M. studies the molecular mechanisms through which environmental signals such as vernalization and light quality regulate flowering time in *Arabidopsis thaliana*. L.H.R.'s research focuses on the evolutionary processes that have produced phenotypic, ecological, and taxonomic diversity throughout the Compositae.

Data accessibility

DNA sequences: GenBank accessions HM163571-HM163573. Phenotypic data for sunflowers and *Arabidopsis thaliana* transformants, microsatellite data and gene expression data: DRYAD entry doi:10.5061/dryad.49sk0

Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Geographic information for wild populations surveyed.

Table S2 Associations between gene expression abundance and latitude tested by regression analysis.

Table S3 Primers used for microsatellite amplification and gene expression analysis.

Fig. S1 Overexpression of *HaCOL2* complements *co* mutation in *Arabidopsis thaliana* while overexpression of *HaCOL1* does not.

Fig. S2 Relative expression of *HaFT4* in leaves and *HaSOC1B* in leaves and shoot apices across populations.

Fig. S3 Gene expression of flowering-time gene homologues in leaves across populations.

Fig. S4 Relative expression of *HaCOL1* and *HaCOL2* under short days in leaves of six wild populations.

Fig. S5 Relative expression of *HaFKF1A* and *HaFKF1B* under short days in leaves of six wild populations.

Fig. S6 Relative expression of *HaGI1* and *HaGI2* under short days in leaves of six wild populations.

Fig. S7 Gene expression of flowering time gene homologues in shoot apices across populations.

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