American Journal of Botany
Celebrating 100 years 1914–2014

American Journal of Botany 101(10): 000–000, 2014.

AJB SPECIAL INVITED PAPER

TRANSITIONS IN PHOTOPERIODIC FLOWERING ARE COMMON AND INVOLVE FEW LOCI IN WILD SUNFLOWERS (*HELIANTHUS*; ASTERACEAE)[1]

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- *Premise of the study:* Evolutionary changes in how flowering time responds to photoperiod cues have been instrumental in expanding the geographic range of agricultural production for many crop species. Locally adaptive natural variation in photoperiod response present in wild relatives of crop plants could be leveraged to further improve the present and future climatic ranges of cultivation or to increase region-specific yields. Previous work has demonstrated ample variability in photoperiod response among wild populations of the common sunflower, *Helianthus annuus*. Here, we characterize patterns of photoperiod response variation throughout the genus and examine the genetic architecture of intraspecific divergence.
- *Methods:* The requirement of short day lengths for floral induction was characterized for a phylogenetically dispersed sample of *Helianthus* species. In addition, flowering time was assessed under short days and long days for a population of F_3 individuals derived from crosses between day-neutral and short-day, wild *H. annuus* parents.
- *Key results:* An obligate requirement for short-day induced flowering has evolved repeatedly in *Helianthus*, and this character was correlated with geographic ranges restricted to the southern United States. Parental flowering times under long days were recovered in high proportion in the F_3 generation.
- *Conclusions:* Together, these findings (1) reveal that substantial variation in the nature of flowering time responses to photoperiod cues has arisen during the evolution of wild sunflowers and (2) suggest these transitions may be largely characterized by simple genetic architectures. Thus, introgression of wild alleles may be a tractable means of genetically tailoring sunflower cultivars for climate-specific production.

Key words: climatic adaptation; crop-wild relatives; *FLOWERING LOCUS T*; flowering time; *Helianthus*; photoperiod; sunflower.

Harnessing the novel sources of genetic and phenotypic diversity present in the wild relatives of crop plants has been heralded as a promising solution for addressing current agronomic challenges (McCouch et al., 2013). The rate of increase in crop yields through conventional breeding has slowed significantly (Hafner, 2003; Brisson et al., 2010; Ray et al., 2012), and maximizing yields for elite cultivars increasingly requires expensive and environmentally problematic chemical inputs (Ladha et al., 2005; Huang, 2009), particularly on the marginal lands that comprise the majority of the area currently cultivated worldwide (Tester and Langridge, 2010). Wild species have many adaptations that allow them to cope with repeated and highly variable biotic and abiotic stresses. This diversity is likely to prove particularly valuable for maintaining or increasing the range and yield of crop production in the face of ongoing climatic changes, soil degradation, and water shortages, as equivalent variation is often absent from cultivated germplasm as a result of selection or genetic bottlenecks during domestication and improvement (e.g., Mandel et al., 2011; Koziol et al., 2012). Although mining wild relatives for useful allelic variation is a longstanding pursuit, this practice has become a subject of renewed interest recently because genome-enabled methods and international investment in germplasm resources have dramatically reduced the associated labor, time, and risk (Hajjar and Hodgkin, 2007; McCouch et al., 2013; Dempewolf et al., 2014).

Crop adaptation to regional climates is often highly dependent on flowering time and its sensitivity to environmental signals. Altering the seasonal timing of reproduction to customize crops for local, marginal, or future environments has been and continues to be a key focus of plant breeding efforts (Jung and Müller, 2009). For instance, changes in the responsiveness of flowering to photoperiod cues have played important roles in expanding the geographic range of cultivation or increasing crop yield in cereals (Turner et al., 2005; Faure et al., 2012; Hung et al., 2012; Zakhrabekova et al., 2012; Yang et al., 2013), beets (Pin et al., 2012), and legumes (Weller et al., 2012). Photoperiod responses may be facultative or obligate, and they are generally classified into three major types. Short-day plants flower earlier (or only) if grown in day lengths below a critical

[1]Manuscript received 9 March 2014; revision accepted 6 July 2014.

The authors thank D. Rasmussen, E. Brown, R. Chen, K. Abbott, E. Patterson, S. Andersen, C. Kitrinos, I. Jameel, W. Poehlman, B. Campbell, K. Aracena, J. Yu, A. Mensah, M. Slomka, A. Greenlee, and W. Crannage for assistance with plant husbandry; R. Timme for sharing her phylogenetic tree files for *Helianthus*; S. Smith, M. Claibourn, and C. Ford for expert guidance with data analyses; and members of the Blackman laboratory group for their comments on the manuscript. This work was supported by a graduate fellowship from the Jefferson Scholars Foundation to R.H.B.W. as well as funds from the University of Virginia and the National Science Foundation Plant Genome Research Program (IOS-1238040) to B.K.B.

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doi:10.3732/ajb.1400097

maximum threshold than if grown in day lengths above the maximum. In contrast, long-day plants flower earlier (or only) if grown in day lengths above a critical minimum threshold than if grown in day lengths below the minimum. Finally, day-neutral plants flower at the same time under all day length conditions. The molecular basis of photoperiodic flowering has received substantial attention in many species. Although some species-specific regulators have been described, a core pathway integrated by homologs of the floral inducer *FLOWERING LOCUS T* (*FT*) is largely conserved across species (Amasino, 2010; Andres and Coupland, 2012), and genes in this pathway have been frequently associated with domestication, geographic differentiation, and heterosis in crop plants (e.g., Zhang et al., 2008; Takahashi et al., 2009; Krieger et al., 2010; Blackman et al., 2011b; Meng et al., 2011).

An understanding of the genetics of photoperiod response variation in wild relatives of crop plants can inform introgression efforts in several ways (Jung and Müller, 2009). First, variation in photoperiod response can be locally adaptive, allowing individuals to flower during favorable seasonal conditions and also to optimize allocation to growth and reproduction. Targeted phenotypic introgression by selecting for markers linked to this variation may accelerate the production of varieties that allow for further expansion of the climatic range conducive to cultivation or for increased region-specific yields. Alternatively, when breeders endeavor to transfer other desirable phenotypes from wild relatives into crops, photoperiod response loci from the wild relatives can oppose progress by extending generation times while they segregate in hybrid progeny (Jung and Müller, 2009). A capacity to rapidly eliminate these loci by marker-assisted selection would thus be of great value for improving breeding efficiency. Similarly, when increased phenotypic stability across environments (i.e., reduced plasticity) is the primary breeding goal, then characterizing which flowering time loci have effects dependent on or insensitive to day length is vital. The efficiency with which breeders can manipulate or preserve photoperiod response and flowering time during introgression depends on two factors: (1) the extent of variability for this trait in wild relatives and (2) the underlying genetic architecture. Marker-assisted selection will be greatly facilitated if widespread variability exists and these differences derive from changes at a few loci of major effect.

Introgression of alleles from wild relatives has been a frequent source of genetic raw material for agricultural innovation in the common sunflower, *Helianthus annuus*, and new genomics resources promise to accelerate similar efforts (Seiler, 1992; Kane et al., 2011; Mandel et al., 2013). Wild relatives of this staple oil and confectionary seed crop species are native to diverse environments throughout North America (Heiser et al., 1969). The majority of the 51 species in the genus can or have been leveraged as sources of novel allelic variation; barriers to interspecies crosses are incomplete or can be overcome through embryo culture or chromosomal doubling (Fick and Miller, 1997). Breeding efforts have successfully introduced wild alleles that confer cytoplasmic male sterility (e.g., Leclercq, 1969; Whelan, 1981; Christov, 1990) or disease resistance (Seiler, 1992) into cultivated germplasm. Because some *Helianthus* species inhabit diverse environments over broad geographic ranges and others are specialists endemic to habitats characterized by high temperature, water, or salt stress (Heiser et al., 1969; Seiler, 1992), wild sunflowers are prime sources to mine for alleles that confer higher yield in new or marginal abiotic environmental conditions (Khoury et al., 2013).

Previous work has documented extensive variation in flowering time and its response to photoperiod in *H. annuus* (Blackman, 2013). Wild populations of *H. annuus* exhibit a latitudinal cline under common garden conditions, with flowering time increasing as latitude decreases (Blackman et al., 2011a). Transitions in photoperiod response also occur at range margins (Blackman et al., 2011a). Northern populations have evolved an early flowering, day-neutral response that differs from the facultative short-day response characteristic of populations throughout the central United States. Moreover, Texan populations, known to have established during a relatively recent southward range expansion (Rieseberg et al., 1990; Whitney et al., 2006), exhibit a facultative long-day response. Similar variation in photoperiod response is also observed among cultivated accessions, and cultivars with additional, more complex responses have been reported as well (Allard and Garner, 1940; Dyer et al., 1959; Goyne and Schneiter, 1987; Leon et al., 2001; Yanez et al., 2005; Blackman et al., 2010). However, with the exception of the Jerusalem artichoke, *H. tuberosus*, another taxon of agronomic interest in the genus (Hackbarth, 1937; Kays and Nottingham, 2008), the diversity of photoperiod response within and between *Helianthus* species has not been extensively surveyed.

Our knowledge of the genetic basis of variation in photoperiod response in the genus *Helianthus* is also limited, even for *H. annuus*. Candidate genomic intervals or genes for flowering time under a single uniform or seasonal environmental treatment have been identified through association mapping studies in cultivated *H. annuus* and quantitative trait locus (QTL) mapping studies conducted in crosses between cultivars or between cultivated and wild lines (Leon et al., 2000; Burke et al., 2002; Bert et al., 2003; Al-Chaarani et al., 2004; Wills and Burke, 2007; Cadic et al., 2013; Mandel et al., 2013). However, these QTLs have not been linked directly to changes in photoperiod response in most instances. In the two cases where the specific impact of flowering time QTLs on photoperiod response has been examined with near-isogenic lines, the loci have moderate to large phenotypic effects depending on the genetic background (Leon et al., 2001; Blackman et al., 2010).

This relatively simple genetic architecture may reflect a history of recent divergence in populations experiencing genetic bottlenecks and strong directional selection, and it is unclear whether similar patterns would emerge for divergence within or between species across spatially and temporally variable natural environments. Many small effect QTLs and multiple significant epistatic interactions affect flowering in a cross between *H. annuus* and *H. petiolaris*; however, whether any of these loci have photoperiod-specific effects is unknown (Rieseberg et al., 2003). Although the genetic changes responsible for the geographic variation in photoperiod response among wild *H. annuus* populations have not been characterized, gene expression correlates have been identified (Blackman et al., 2011a). Specifically, the shift from short-day to day-neutral flowering in the northern range is associated with a gain in expression under long days of *HaFT4*, one of two leaf-expressed sunflower homologs of *FT* (Blackman et al., 2010), indicating the relevant molecular changes likely occur in the *cis*-regulatory region of *HaFT4* itself or affect *trans*-acting regulators. Notably, multiple homologs of *CONSTANS* (*CO*), *GIGANTEA* (*GI*), and additional upstream regulators in the core photoperiod pathway do not exhibit divergent expression patterns correlated with photoperiod response shifts, indicating the causal changes act downstream or in parallel to their transcription (Blackman et al., 2011a).

Here, we report several sets of findings that extend our understanding of the nature and genetic basis of variability in photoperiod response within and between sunflower species, providing insight into the tractability of genetically informed breeding involving this important agronomic trait. Specifically, we (1) examine whether the remarkable variability in photoperiodic flowering observed within *H. annuus* may also be a general characteristic of the genus, (2) determine whether the genetic architecture of a transition in photoperiod response between wild populations can result from changes at a limited number of loci, and (3) test whether variants in *HaFT4* cosegregate with variation in flowering time in controlled crosses. Our findings indicate that regional shifts in species ranges are correlated with evolutionary transitions in photoperiod response across the genus, and these transitions may be characterized by relatively simple genetic architectures.

MATERIALS AND METHODS

***Photoperiod response diversity survey*—**Variation in the response of flowering to day length across the genus was investigated using seeds from a subset of diploid *Helianthus* species obtained from the U. S. Department of Agriculture Agricultural Research Service (Table 1; http://www.ars-grin.gov). In late May 2013, seeds were surface sterilized in a 2% bleach, 1% Triton-X 100 solution, scarified, and germinated on moist filter paper in the dark for 7–10 d. Seeds were exposed to a single light–dark cycle for 1 d before sowing. Seedlings were sown in a mixture of Fafard 3B soil (Sun Gro Horticulture, Agawam, Massachusetts, USA) and calcined clay (3 : 2 by mass). Seedlings were kept on a mist bench for 2 wk before repotting. Three to five plants per accession were grown for 6 mo under standard greenhouse conditions (16 h light/day with supplemental sodium halide lighting, 21°C days, 16.7°C nights, daily watering). Species that did not flower during this period were transferred to another greenhouse lit by ambient conditions in late November 2013 (day length ≤ 10 h light per day). Plants were then scored for flowering over the following 2 months. All individuals of a given accession exhibited the same behavior, allowing flowering under long days to be scored as a binary trait.

***Character state evolution*—**To examine the number and timing of evolutionary transitions in photoperiod response in *Helianthus*, we conducted ancestral character reconstruction analyses with our dataset using previously published maximum likelihood phylogenies generated with the external transcribed spacer region of the nuclear 18S-26S ribosomal RNA repeat (Timme et al., 2007). Because we phenotyped a diploid accession of *H. decapetalus*, a species of variable ploidy, we conducted our analyses on two trees, one including exclusively diploid taxa and one including all *Helianthus* species (figs. 3 and 4, respectively, from Timme et al., 2007). Phylogenies, obtained directly from R. Timme (U. S. Food and Drug Administration), were converted to ultrametric trees using a semiparametric method based on penalized likelihood (Sanderson, 2002) as implemented in the chronopl {ape} function in the program R (Paradis et al., 2004). Where multiple samples from a single species were present, trees were pruned to include one representative of each diploid *Helianthus* species (Fig. 1; Appendices S1 and S2, see Supplemental Data with the online version of this article) and to best reflect placement in recent phylogenetic studies that have used high-throughput sequencing methods to establish relationships within subsections of the genus (Bock et al., 2014; Renaut et al., 2014). Confirmed and putative homoploid hybrid taxa were excluded. Although a putative *H. exilis* accession was not included in the previous phylogenetic analysis, an *H. bolanderi* sequence was retained for *H. exilis* because recent genomic analyses have found that two species are not reciprocally monophyletic and are equidistant from sister groups (Renaut et al., 2014). Trees produced by alternative pruning choices yielded similar results.

Bayesian stochastic character mapping (Huelsenbeck et al., 2003) was conducted with the software package SIMMAP 1.0 (Bollback, 2006). The outgroup taxon, *Phoebanthus grandiflorus*, was excluded from all analyses. For all simulations, we performed 10 realizations sampled from the priors and 1000 realizations for each tree, using the default prior settings in SIMMAP as well as two sets of randomly selected priors. Because selection of priors had limited influence on statistics for character state transitions, we only report the results for default priors in the main text; full results are reported in Appendix S3 (see online Supplemental Data). Character histories for photoperiod response were simulated with two sets of character states (online Appendix S4). The first data set consisted solely of the flowering phenotypes scored in our greenhouse survey. The second data set was augmented with additional observations in the literature or based on flowering observations recorded in online databases. Specifically, *H. giganteus* and *H. angustifolius* were observed to flower in long days in a classic series of studies on photoperiodic flowering (Allard and Garner, 1940). Because *H. cusickii*, *H. pumilus*, and *H. debilis* subsp. *cucumerifolius* have been reported to flower in natural conditions in late spring by multiple collectors (Heiser et al., 1969; http://www.ars-grin.gov), we tentatively scored them as lacking an obligate short day requirement. Likewise, because *H. floridanus* has been repeatedly observed to reach peak flowering in November and December (http://www.ars-grin.gov), we tentatively scored this species as having an obligate short day requirement.

We tested for correlated evolution between photoperiod response and life history (perennial vs. annual) or geographic range (not limited vs. limited to southern United States) by stochastic mapping in SIMMAP 1.0 (Huelsenbeck et al., 2003; Bollback, 2006). Species were scored as having ranges limited to the southern United States if their reported distributions were confined within the area covered by the states of Texas, South Carolina, North Carolina, Georgia, Alabama, Mississippi, and Florida (Heiser et al., 1969; http://www.efloras.org). This area constitutes a distinct climate zone (http://planthardiness.ars.usda.gov/PHZMWeb/), and many *Helianthus* species occur only in this region. In this approach, posterior distributions of Bayesian phylogenetic histories for each trait are overlaid to estimate the total duration of the histories that is spent in each of the potential character state combinations for the two traits. If transitions between states for one trait coincide with transitions between states for the other trait, then character states will more often co-occur along histories, producing a signal of association between the traits.

***F_3 mapping panel growout*—**To evaluate the potential complexity of the genetic architecture underlying an intraspecific photoperiod response transition, we characterized flowering time segregation patterns in an F_3 mapping population raised under multiple day length conditions. An individual grown from seed collected in Manitoba, Canada (MB; Whitewater Lake Wildlife Refuge; 49.23°N, 100.24°W) was reciprocally crossed to an individual from Kansas, United States (KS; I-70 Exit 53 near Colby; 38.85°N, 99.42°W) populations to generate F_1's (Fig. 2). These two populations were previously demonstrated to be day-neutral (MB) and facultative short-day (KS) flowering (Blackman et al., 2011a). F_2's were generated by reciprocally intercrossing an F_1 individual from each of the reciprocal parent crosses. Finally, 37 families of F_3's were generated by randomly pairing and mating F_2's within each of the two reciprocal F_2 populations (Fig. 2). Seeds from the MBxKS F_3 families as well as MB and KS seeds derived from intrapopulation crosses not involving the F_3 parent individuals were germinated as described above and sown in 4″ pots. Seedlings were divided between two growing conditions: long days (16 h light) and short days (11 h light), both kept at constant temperature of 25.5°C (long days: $N_{F3} = 258$, 7.0 ± 0.7 individuals per F_3 family, $N_{MB} = 14$, $N_{KS} = 6$; short days: $N_{F3} = 259$, 7.0 ± 0.8 individuals per F_3 family, $N_{MB} = 9$, $N_{KS} = 7$). These photoperiods span the maximum photoperiod at MB and the minimum photoperiod at KS during the natural growing seasons of these populations. The positions of flats containing eight plants each were randomized weekly within growth rooms (Environmental Growth Chambers, Chagrin Falls, Ohio, USA). Light intensity was adjusted such that plants in each treatment received equivalent quantities of photosynthetically active radiation per day in both growth rooms (163 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in long days, 237 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in short days). The number of days from germination to budding (R1 stage; Schneiter and Miller, 1981) was recorded as a measure of flowering time for every plant. Statistical analyses were conducted with the program JMP Pro 10.0.2 (SAS Institute, Cary, North Carolina, USA). Due to variation in germination success among families, some families were only sown into a single flat. Consequently, we first used a reduced data set including only families sown in multiple flats to model the impacts of photoperiod as a fixed factor and family, photoperiod × family interaction, and flat nested within photoperiod as random factors on days to budding in a series of nested restricted maximum likelihood linear mixed models. Because flat nested within photoperiod explained <0.1% of the residual variance in the full model, we report results on the full data set not including flat as a factor. The Castle–Wright estimator for number of effective factors, which provides a rough estimate for the number of contributing loci, was calculated as $(\text{Mean(MB)} - \text{Mean(KS)})^2 / \{8 \cdot [\text{Variance}(F_3) - (\text{Variance(MB)} + \text{Variance(KS)})/2]\}$ (Castle, 1921; Lynch and Walsh, 1998).

***Genotyping*—**To determine whether sequence variants in *HaFT4* cosegregated with flowering in our mapping population, we first screened for polymorphism

in the *HaFT4* gene by amplifying and sequencing a 556-bp fragment from a set of eight F_3 individuals by a nested PCR procedure (common forward primer: 5′-ATATTCCCGCGACCACTGGAGCACGTTTTGG-3′; external reverse primer: 5′-TATAGCCTCCGTTGCCACAGACTA-3′; internal reverse primer: 5′-GGTGCAATATTTGCATGCCAGGGA-3′). Primary and secondary PCR reactions were run following a touchdown protocol (95°C for 2 min; 15 cycles of 95°C for 30 s, 60–45°C for 30 s reducing the annealing temperature by 1°C per cycle, and 72°C for 1 min; 20 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 1 min; 72°C final extension for 10 min). Because *HaFT4* shares sequence similarity with other *HaFT* paralogs, multiple bands amplified for several samples. Consequently, PCR products were run out on a 1% agarose gel stained with ethidium bromide, and excised bands were purified (Wizard SV Gel Kit; Promega, Madison, Wisconsin, USA). Each amplified product was then sequenced by Sanger sequencing using an Applied Biosystems 3130 capillary sequencer to specifically isolate the *HaFT4* sequence using the common forward and internal reverse primer above.

For the three single nucleotide polymorphisms (SNPs) that were segregating in the F_3's, we designed primers for genotyping following the derived cleaved amplified polymorphic sequences (dCAPS) method (Neff et al., 1998, 2002). PCR products were again amplified with a nested procedure, substituting the SNP-specific primer for the forward primer (SNP +1472: 5′-TATTATAAGATATAAAGAAATCATG-3′; SNP +1624: 5′-AATATCTTAGCAAATAGCTAACCTA-3′) or reverse primer (SNP +1615: 5′-AAGTGACCAATGCCACACTATGGTA-3′) in the secondary reaction. Digestions containing 5 μL of the secondary PCR product were completed following the manufacturer's instructions with enzymes BfaI (SNP +1472 and SNP +1615; New England Biolabs, Ipswich, Massachusetts, USA) or KpnI (SNP +1624; Thermo Fisher Scientific, Asheville, North Carolina, USA). Digested PCR products were separated on 2% agarose gels stained with ethidium bromide and scored for genotyping. Subsets of F_3 individuals that flowered earliest (N = 16) and latest (N = 19) under long days were genotyped, and the haplotype frequency distributions of these groups were compared with a log-likelihood ratio test (Sokal and Rohlf, 1995).

RESULTS

Transitions in the short-day requirement for flowering are common and correlated with geography—Many perennial and several annual sunflower species, particularly those with ranges restricted to the southern United States, did not flower over a 6-mo period under long-day conditions in the greenhouse (Table 1; Fig. 1). Ancestral state reconstruction by stochastic mapping (Huelsenbeck et al., 2003) revealed substantial lability in this aspect of photoperiodic flowering within the *Helianthus* clade. In general, transitions to an obligate short-day requirement for flowering appear to evolve readily, while losses of an obligate short-day requirement are less likely by comparison (Fig. 1; Appendix S3). For instance, analyses conducted with the full *Helianthus* phylogeny and our observed data yielded posterior expectations (mean [SD]) for the numbers of transitions in photoperiod response as follows: gain (7.71 [1.90]), loss (1.45 [1.62]). When we included observations for several additional species made by other investigators (Appendix S4), thus further constraining possible simulated outcomes, we observed a similar pattern, though with fewer predicted transitions to an obligate short-day requirement: gain (5.72 [1.42]), loss (1.37 [1.67]). Analyses conducted with the exclusively diploid phylogeny yielded higher posterior expectations for loss of the obligate short-day requirement, but also increased variability among simulated character histories (Appendix S3).

As expected based on previous work (Heiser et al., 1969; Timme et al., 2007), a perennial life history was predicted to be ancestral, with few transitions to annual life histories (means ~2 in all analyses, corresponding to *H. porteri* and sections *Helianthus* and *Agrestes* sensu Schilling and Heiser, 1981). A transition back from an annual to a perennial life history in the lineage of *H. carnosus* is suggested, though this may be an artifact of uncertain phylogenetic placement relative to *H. agrestis* and *H. porteri* (Timme et al., 2007). Photoperiod response and life history (perennial vs. annual) were significantly associated: D = 0.19, $P < 0.001$ for the observed data set, D = 0.10, $P < 0.001$ for extended data set. Presence of an obligate short day requirement co-occurred significantly more often along histories with perenniality than with annuality, while absence of an obligate short day requirement co-occurred significantly more often along histories with annuality than with perenniality. Likewise, photoperiod response was also significantly correlated with geographic range (restricted to southern United States vs. widespread or endemic elsewhere): D = 0.16, $P < 0.001$ for the observed data set, D = 0.24, $P < 0.001$ for the extended data set. Presence of an obligate short day requirement co-occurred significantly more often along histories with restriction to the southern United States than with an alternate range type, while absence of an obligate short day requirement co-occurred significantly more often along histories with an alternate range type than with restriction to the southern United States.

TABLE 1. *Helianthus* species accessions surveyed for photoperiod response diversity.

Species	Accession	Location	Obligate short-day flowering
H. agrestis	Ames 30848	Florida	Y
	Ames 30849	Florida	Y
H. annuus	KS	Kansas	N
H. argophyllus	PI 435627	Texas	Y
	PI 494569	Texas	Y
H. atrorubens	PI 664694	Georgia	Y
H. carnosus	PI 649956	Florida	Y
H. debilis subsp. *silvestris*	PI 468686	Texas	N
H. debilis subsp. *vestitus*	PI 468694	Florida	N
H. decapetalus	PI 649972	Virginia	N
H. divaricatus	PI 503209	Virginia	N
H. exilis	PI 649895	California	N
H. maximilliani	PI 592333	Manitoba	N
H. mollis	PI 664610	Oklahoma	N
H. nuttallii	PI 531045	Oregon	N
	PI 592346	Saskatchewan	N
H. occidentalis subsp. *plantagineus*	PI 494595	Texas	Y
H. petiolaris	PI 586922	Colorado	N
	PI 613762	North Dakota	N
H. porteri	PI 649914	Georgia	Y
H. praecox subsp. *hirtus*	PI 468847	Texas	N
H. praecox subsp. *praecox*	PI 468851	Texas	N
H. radula	Ames 30853	Florida	Y

Notes: Accession = USDA Germplasm Resources Information Network ID (http://www.ars-grin.gov) except KS, which was reported by Blackman et al. (2011a). Location = state or province where accession was originally collected. Y = yes, N = no.

The transition from facultative short-day to day-neutral flowering is genetically simple—For examining the genetic architecture of photoperiod response divergence between *H. annuus* populations, populations of F_3 individuals were derived from reciprocal crosses of a day-neutral MB parent to a facultative short-day KS parent. Plants from 37 MB × KS F_3 families (Fig. 2) were raised in growth rooms under short days and long days. F_3 plants flowered earlier on average under short days (N = 258, mean ± SE = 39.5 ± 0.3 d) than under long days (N = 259, mean =

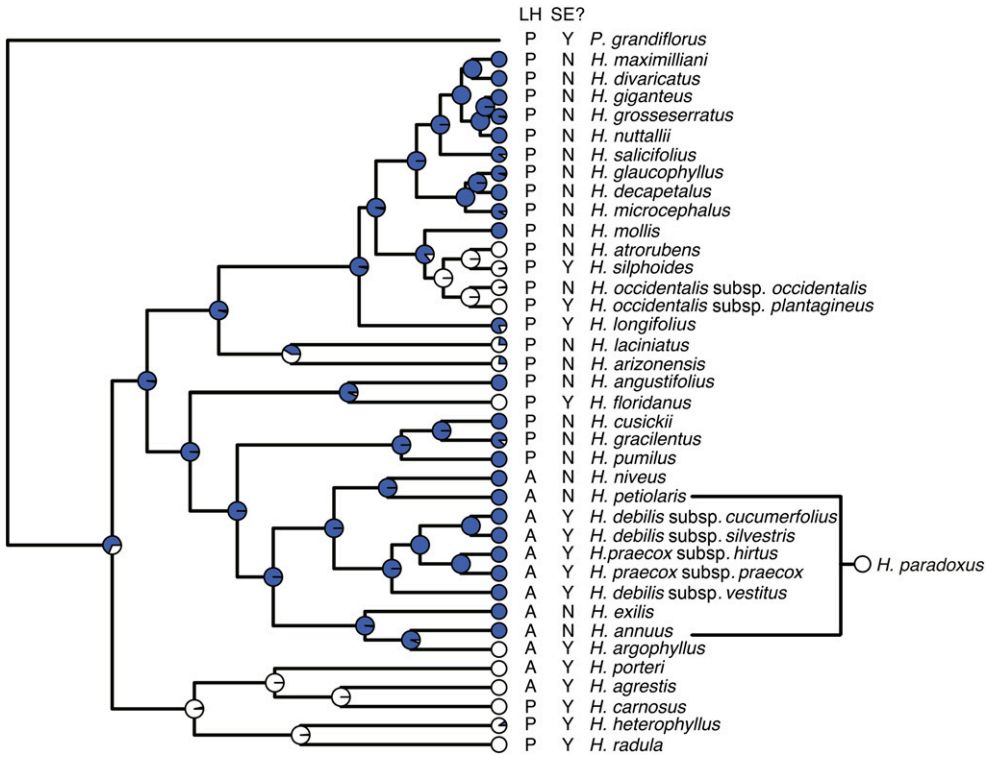

Fig. 1. Ancestral character state reconstruction for photoperiod response in *Helianthus* indicates multiple transitions from absence to presence of an obligate short-day requirement for flowering. Posterior probabilities of each character state are shown as fractions of the pie charts at the nodes and tips of the tree. Blue = no obligate short-day requirement, white = obligate short-day requirement. Outgroup taxon *P. grandiflorus* was excluded from stochastic mapping. Outcome depicted based on default priors, extended character matrix (Appendix S4), and pruned polyploid-inclusive maximum-likelihood phylogeny (Appendix S2). A reticulation event is depicted for *H. paradoxus*, a homoploid hybrid species derived from *H. annuus* and *H. petiolaris*. Tree was plotted with the phytools R package (Revell, 2012). Character states used in correlation analysis are listed by species for life history (LH: A = annual, P = perennial) and endemism to the southeastern US and Texas (SE?: Y = yes, N = no).

56.4 ± 0.7 d). Mean flowering time did not differ between F_3 populations derived from reciprocal F_1 crosses under both photoperiod conditions, either when photoperiod is treated as a single fixed factor (long days: $t = 1.11$, df = 256; $P = 0.27$; short days: $t = 1.32$, df = 257; $P = 0.19$) or when included in a restricted maximum likelihood linear mixed model with family and flat specified as random factors (long days: $F_{1,232} = 0.045$, $P = 0.83$; short days: $F_{1,235} = 0.004$, $P = 0.95$). Thus, we found no evidence for cytoplasmic or other parent-of-origin effects on phenology.

We expected to observe specific effects of photoperiod response loci under long-day conditions, which are inductive for day-neutral MB but not facultative short-day KS. Individuals reconstituting the extremes of the parental phenotype distributions were recovered in significant proportions in our F_3 panel (Fig. 3A), providing a strong qualitative indication that the divergence between populations for this trait is genetically simple. Notably, five or more F_3 families within the panel recovered each of the parental phenotype distributions as well (Fig. 3B), indicating that individuals with fully parental genotypes at flowering time loci also segregated out in high proportions in the F_2's such that they were randomly paired in multiple matings. Parental value F_3's are also recovered under short days, although no individuals recovered the extremes shown by some KS plants, which flowered aberrantly late under short days (online Appendix S5). The unexpected behavior of the KS plants may be explained by inbreeding depression. KS was the only set of plants generated by crossing individuals derived from the same maternal plant, and the seeds also exhibited exceptionally poor germination and seedling establishment frequencies. Little evidence of transgressive segregation was observed in either condition (Fig. 3B, Appendix S5).

Several additional analyses suggest the divergence in photoperiod response between MB and KS is likely to be genetically simple and largely controlled by few loci of major effect. A Castle–Wright estimator for the minimum number of effective factors, n_e (Castle, 1921), responsible for flowering time variation

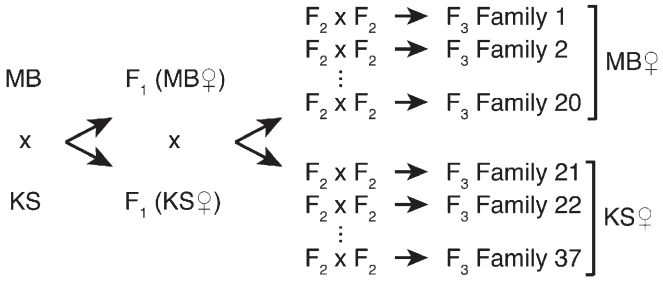

Fig. 2. Cross design of an intraspecific, wild *Helianthus annuus* F_3 mapping population. Cytoplasmic inheritance from MB or KS through reciprocal crosses of the parental and F_1 generations is denoted by MB♀ and KS♀, respectively. The F_2's were randomly paired within each maternal lineage.

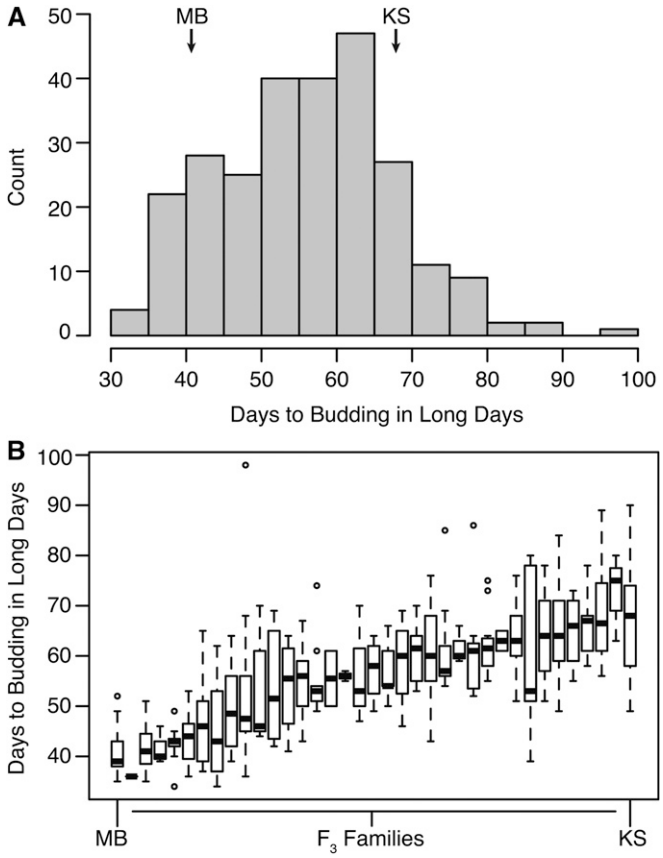

Fig. 3. *Helianthus annuus* F_3s derived from a day-neutral MB × facultative short-day KS cross recover parental flowering time values under long day conditions. (A) Distribution of individual F_3 flowering times. Arrows depict means of parental genotypes. (B) Flowering time distributions by parental or F_3 family shown as boxplots with outliers. F_3 families shown from left to right in order from smallest to largest mean flowering time.

under long days was 3.95. Because the Castle–Wright estimator is known to underestimate the actual number of loci when a strict model of additive gene action is violated (Lynch and Walsh, 1998), we present it here only as a ballpark quantitative estimate consistent with our qualitative observations suggestive of a simple genetic architecture.

Nonetheless, this estimate may be conservative because only a subset of the genetic factors contributing to segregating variation in flowering under long days are expected to be directly responsible for photoperiod response divergence; loci contributing to later flowering of KS relative to MB regardless of photoperiod are also captured in that estimate. Consistent with this expectation, F_3 family means under short days and long days are partially but not fully correlated (Fig. 4A; N = 32 of 37 families with plants raised under both photoperiod conditions; $r = 0.53$, $P = 0.002$). Testing this expectation more rigorously with nested linear mixed models, we find significant effects of photoperiod, family (likelihood ratio test: G = 122.07, $P <$ 0.001) and family × photoperiod interaction (likelihood ratio test: $G = 37.05$, $P < 0.001$) on flowering time in the F_3's ($R^2 = 0.71$, $F_{\text{photoperiod (1,505)}} = 211.3$, $P < 0.0001$ for full model). These separate effects are evident when family means are plotted as reactions norms (Fig. 4B). Genetic variation affecting mean time to flowering across environments segregates independently from genetic variation affecting photoperiod response. For example,

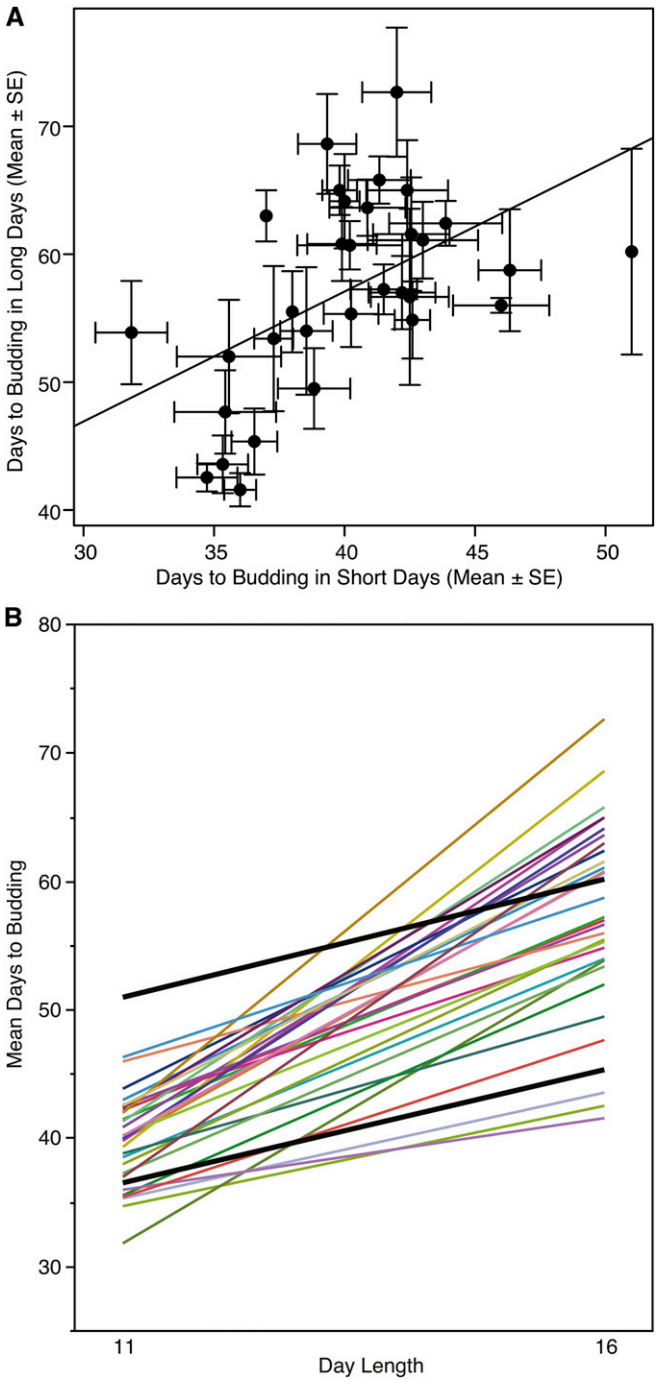

Fig. 4. Cross-environment expression of flowering time in MBxKS F_3 families of *Helianthus annuus* indicates divergence both at loci with effects under all conditions and at photoperiod response loci. (A) Bivariate plot showing the means ± SE for each F_3 family grown under short- and long-day conditions. Absence of error bars from a family indicates either (1) that only one individual in that family was grown in that condition or (2) that all individuals from that family flowered on the same day in that condition. (B) Mean flowering times for each F_3 family shown as reaction norms with respect to day length. Two families with equivalent photoperiod responses but different mean flowering times across conditions are highlighted by the wider, black reaction norms. Photoperiod, family, and their interaction all had significant effects on flowering time ($R^2 = 0.71$ for full model, $P < 0.001$ for all terms by likelihood ratio test).

two families with equivalent photoperiod responses (i.e., mean in long days – mean in short days) differ in their mean flowering time across environments [i.e., (mean in long days + mean in short days)/2] by ~15 d (Fig. 4B, wider black reaction norms).

***Factors acting in* cis- *to* HaFT4 *are not responsible for the change in photoperiod response*—**Because previous work indicated that upregulation of *HaFT4* expression under long days likely contributes to the day neutrality of MB (Blackman et al., 2011a), we tested whether sequence variation in *HaFT4* was associated with flowering under long days by bulked segregant analysis (Michelmore et al., 1991). Sequencing of a genomic fragment containing the third intron and fourth exon of *HaFT4* from eight F_3's revealed three polymorphic sites: SNP +1472 (A/G), SNP +1615 (C/G), and SNP +1624 (A/G). SNPs are named based on position relative to the first base pair of the *HaFT4* start codon (GenBank GQ884984). We then designed dCAPS markers to genotype the SNPs for the MB and KS parent individuals of the cross, the two F_1s used to generate the F_2's, and the subsets of MBxKS F_3's that flowered earliest and latest under long days. Four segregating haplotypes were recovered, as expected based on the patterns of inheritance of the four parental alleles by the outbred F_1's.

We reliably scored genotypes at all three SNPs for 16 of the 18 earliest flowering F_3's (~6.2% of the total F_3 population in long days) and 19 of the 20 latest flowering F_3s (~7.3% of the total F_3 population in long days). Ratios of haplotype frequencies in both bulks differed significantly from a 1 : 1 : 1 : 1 expectation (Table 2; $X^2 = 13.89$, df = 3, $P = 0.003$), which may have resulted from segregation distortion or genetic drift between the F_2 and F_3 generations. However, no difference in haplotype frequency distribution was observed between the early and late flowering bulks (Table 2; log-likelihood ratio test, $G = 0.752$, df = 3, $P = 0.86$). Consolidating the haplotypes by parent of origin yields similar results (log-likelihood ratio test, $G = 0.617$, df = 1, $P = 0.43$). Given the expected level of recombination present in the F_3 population (~2.5 Mb/cM in another sunflower F_3 population; Burke et al., 2002), the extent of local linkage disequilibrium should provide sufficient power to detect cosegregation with a major effect *cis*-regulatory polymorphism using markers in the *HaFT4* open reading frame. Thus, we conclude that the changes responsible for the difference in photoperiod response between MB and KS map elsewhere in the genome and likely encode *trans*-acting regulators of *HaFT4*.

DISCUSSION

The seasonal timing of flowering is a critical determinant of plant fitness and crop yield, and genetic variation for photoperiod response in the wild relatives of crop plants may provide an important resource for tailoring crops to thrive in local or future climates. Our results indicate that photoperiod response varies widely among wild sunflower species and that transitions in photoperiod response may be characterized by a simple genetic architecture. Thus, marker-assisted manipulation of photoperiod response during introgression of traits from wild relatives may prove an effective means for increasing yield and the efficiency of crop improvement in sunflower.

TABLE 2. *HaFT4* haplotype frequencies in groups of earliest and latest flowering F_3 individuals.

Haplotype	SNP +1472	SNP +1615	SNP +1624	Early bulk frequency (*N*)	Late bulk frequency (*N*)
MB 1	A	G	A	0.09 (3)	0.10 (4)
MB 2	A	G	G	0.31 (10)	0.39 (15)
KS 1	G	G	A	0.41 (13)	0.32 (12)
KS 2	G	C	A	0.19 (6)	0.18 (7)
				(Total $N = 32$)	(Total $N = 38$)

***Extensive evolutionary lability in photoperiodic flowering in* Helianthus—**Here, we have documented multiple transitions between the absence and presence of an obligate short-day flowering requirement across wild sunflower species. Assuming absence of a short-day requirement as the ancestral state, at least four independent gains are apparent (Fig. 1). Our Bayesian stochastic mapping estimates for gains of a short-day requirement during the evolutionary history of diploid *Helianthus* species predicted additional gains as well as losses, although it is possible these estimates are elevated because observations were not made for all the included taxa. For instance, no observations were recorded for the clade containing *H. laciniatus* and *H. arizonensis*, and the posterior expectations for the alternative states are intermediate. Extending the data set with previous observations also reduced the number of predicted transitions (Appendix S3). Even so, our estimates are conservative in another respect. We did not include homoploid hybrid or polyploid taxa in our analyses, and additional independent changes in short-day requirement may have occurred in the history of these lineages. Indeed, *H. paradoxus*, a homoploid hybrid species, is an obligate short-day plant derived from *H. annuus* and *H. petiolaris* parents that lack a short-day requirement for flowering (Fig. 1; B. Blackman, unpublished data).

The overall variability of photoperiod response as a trait across the genus is likely to be even greater than our results suggest for two major reasons. First, in scoring only one or two accessions per species, our greenhouse survey did not capture the full extent of intraspecific variation in the short-day requirement or its threshold value. Second, only one specific aspect of photoperiod response—whether there is a threshold day length above which plants will not flower—was scored. Whether the photoperiod responses of plants that will flower in long days are day-neutral, obligate or facultative long-day, facultative short-day, or another type clearly varies within and between species as well (Allard and Garner, 1940; Kays and Nottingham, 2008; Blackman et al., 2011a). As mentioned above, photoperiod response transitions are a major component of clinal differentiation for flowering time among wild populations of *H. annuus*, and similar transitions likely contribute to ecotypic or clinal differentiation for flowering observed in *H. argophyllus* or *H. maximilliani* (Kawakami et al., 2011; B. Moyers, University of British Columbia, personal communication). Given that the shifts in photoperiod response observed in *Helianthus* to date are more frequent and diverse than those described in many other wild taxa (Thomas and Vince-Prue, 1997), future investigations with greater taxonomic and environmental sampling are certain to further burnish this group's value for exploring the genetic mechanisms and ecological pressures driving diversification in the seasonal timing of flowering.

An initial insight in this vein is the signal of correlated evolution that we detected between photoperiod response and geographic range. *Helianthus* species with an obligate short-day requirement for flowering are more likely to be found in the southeastern United States and Texas than elsewhere. Several

developmental and ecophysiological hypotheses may explain this finding. This coastal region, bordered by the Atlantic Ocean and Gulf of Mexico, is characterized by long, hot, and humid summers that may be unfavorable for growth or reproduction. Although populations of *H. annuus* in Texas likely escape these harsh conditions by flowering in the long days of late spring (Blackman et al., 2011a), most perennials and some annuals in this region appear to have repeatedly favored an alternate strategy of germinating in the spring but postponing flowering until the arrival of cooler, less humid falls.

We expect delayed flowering will increase plant size at maturity and, consequently, plant fecundity. However, other environmental agents may act to favor the specific seasonal timing afforded by a short-day requirement over allelic variation that delays flowering irrespective of seasonal conditions. For instance, pollen fertility decreases at temperatures above 30°C in *H. annuus* (Seiler, 1997), and such high temperatures are regularly experienced during summers in this region. Elevated temperatures during seed maturation also reduce yield in *H. annuus* (Ploschuk and Hall, 1995). These constraints may explain the correlated shifts in range and photoperiod response to the extent that they are important in other *Helianthus* species. Biotic interactions may also be relevant. For example, postponing flowering until humid summer conditions have passed may discourage establishment of fungal pathogens on heads or dispersed seed (Gulya et al., 1997).

The signal of correlated evolution for photoperiod response and life history may indicate that an obligate dependence of flowering on environmental signals is of greater importance in perennials. For instance, flowering in multiple years or enhancing vegetative growth through delaying flowering until the second year (or later) may constrain the flexibility to maintain genetic variation in these requirements. This in turn may promote habitat specialization but come at a cost in terms of the capacity for establishment in novel or marginal environments.

Photoperiod response and wild relatives as tractable sources of genetic variation to enhance crop productivity—Our results indicate that the genetic basis of diversity in photoperiod response among wild sunflowers may be characterized by changes at a few loci of moderate to major effect, a pattern similar to findings from crosses between sunflower cultivars or between wild and cultivated accessions. Parental phenotypes were recovered in early generation hybrids derived from crossing a day-neutral parent from Manitoba to a short-day parent from Kansas (Fig. 3). In addition, the pattern of reaction norm variation among F_3 generation families suggests that the divergence between the parents is due to loci that have photoperiod-independent effects as well as loci with photoperiod-specific effects (Fig. 4).

To the extent that these findings are true for other types of photoperiod response transition or at other taxonomic scales in *Helianthus*, they have two key implications. First, if most transitions in photoperiod response in *Helianthus* have similarly simple genetic architectures, then identifying the causal variants or closely linked SNPs will greatly facilitate rapid crop improvement by marker-assisted selection. These benefits could be direct (i.e., introgression of locally adaptive genetic variation to directly modify photoperiod response) or accrue indirectly (i.e., by reducing cycle times when selecting for other desirable traits). Second, the flexibility afforded to breeders by the independent segregation of loci with photoperiod-dependent or photoperiod-independent effects should foster the development of region-specific cultivars.

For instance, breeders in the northern hemisphere have typically selected for early, day-neutral flowering to shorten the growing period and expand the region suitable for cultivation (Putt, 1997). However, in Argentina, a major center of sunflower agriculture, delaying reproductive development until the late-season period of high rainfall results in higher crop productivity (Gonzalez et al., 2011). Introducing flowering time alleles from wild relatives that specifically reduce the threshold day length below which flowering is accelerated or permitted may be a productive means to continue to improve crop yields in this region. Indeed, altering flowering behavior through changes at loci that specifically impact photoperiod response may be particularly valuable, as their effects are likely to be robust to increasing temperatures or temperature variability. Further efforts to characterize the genetic architecture through QTL mapping in this and other intra- and interspecific crosses segregating for variation in photoperiod response will be necessary to confirm whether our findings are sufficiently generalizable to support broader investment of resources into these applied efforts.

The molecular basis of photoperiod response diversity—Although a gain of *HaFT4* expression in long-day conditions is associated with the shift from short-day to day-neutral flowering by MB (Blackman et al., 2011a), we did not detect an association between *HaFT4* genotype and flowering time under long days in our F_3 population (Table 2), likely excluding *cis*-regulatory changes in *HaFT4* as the causal sequence differences. Of the potential alterations in *trans*-acting factors that could be responsible, our previous gene expression studies discount roles for changes affecting several genes upstream in the canonical photoperiod pathway including multiple homologs of *GI*, *CO*, and *FLAVIN-BINDING KELCH-REPEAT F-BOX 1*, as their expression patterns show no clear differences in level or diurnal phase between MB and KS or other short-day populations (Blackman et al., 2011a). Based on the molecular interactions described for the flowering time network in other systems, several alternative possibilities that act downstream or in parallel to these genes in the regulation of *HaFT4* expression are apparent (Andres and Coupland, 2012; Song et al., 2013). First, changes may have evolved that alter the composition of protein complexes that influence the posttranscriptional regulation of CO (e.g., homologs in the *SUPPRESSOR OF PHYA-105* family; Laubinger et al., 2006; Jang et al., 2008) or its binding to the *HaFT4* promoter (e.g., the *HEME ACTIVATOR PROTEIN* complex; Ben-Naim et al., 2006; Wenkel et al., 2006). Such changes may also be mediated by alterations to the function of cryptochromes in regulating the impacts of these CO-interacting complexes on *HaFT4* expression (Zuo et al., 2011) or in influencing other transcriptional regulators that bind the *HaFT4* promoter in parallel (e.g., homologs of *CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1*; Liu et al., 2008). A final possibility is that the variants which contribute to the shift to day-neutrality compromise the function of *trans*-acting repressors that bind to the *HaFT4* promoter and thus discourage flowering in noninductive photoperiods (e.g., homologs of *TEMPRANILLO* and *SCHLAFMÜTZE*; Castillejo and Pelaz, 2008; Mathieu et al., 2009).

Sunflower genetic and genomic resources that are newly available or are in active development promise to accelerate the localization and identification of these and other variants affecting photoperiod response diversity within and between *Helianthus* species. For instance, a draft genome integrated with dense genetic and physical mapping information was recently assembled

(Kane et al., 2011; Bowers et al., 2012). This template will facilitate QTL mapping with genotyping-by-sequencing methods (Andolfatto et al., 2011; Elshire et al., 2011) in our MBxKS F_3 mapping panel. These approaches can be extended to other crosses between *H. annuus* wild populations (e.g., short-day KS to long-day Texan populations) or to crosses between *Helianthus* species that are now being conducted in conjunction with the Global Crop Diversity Trust (L. Rieseberg, University of British Columbia, personal communication). In addition to aiding introgression-based breeding efforts, identifying the causal variants and examining their evolutionary history will provide novel insights into the mechanistic factors and ecological agents that have promoted the emergence of the great diversity and lability in photoperiod response observed in wild sunflowers.

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