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Report

The Role of Recently Derived *FT* Paralogs in Sunflower Domestication

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Summary

Gene duplication provides an important source of genetic raw material for phenotypic diversification [1, 2], but few studies have detailed the mechanisms through which duplications produce evolutionary novelty within species [3–6]. Here, we investigate how a set of recently duplicated homologs of the floral inducer *FLOWERING LOCUS T* (*FT*) has contributed to sunflower domestication. We find that changes in expression of these duplicates are associated with differences in flowering behavior between wild and domesticated sunflower. In addition, we present genetic and functional evidence demonstrating that a frameshift mutation in one paralog, *Helianthus annuus FT 1* (*HaFT1*), underlies a major QTL for flowering time and experienced a selective sweep during early domestication. Notably, this dominant-negative allele delays flowering through interference with action of another paralog, *HaFT4*. Together, these data reveal that changes affecting the expression, sequence, and gene interactions of *HaFT* paralogs have played key roles during sunflower domestication. Our findings also illustrate the important role that evolving interactions between new gene family members may play in fostering phenotypic change.

Results and Discussion

In plants, lineage-specific duplications in the *FLOWERING LOCUS T*-like (*FT*-like) gene family are often found and may be important substrates for evolutionary innovation [7–13]. *FT* plays a crucial and widely conserved role in regulation of flowering time by environmental cues [9, 10, 12, 14–17]. Briefly, genes in the photoperiod pathway integrate cues from the circadian clock and light signaling such that FT is highly expressed in the leaf only under inductive photoperiods [16, 18–20]. FT protein travels from the leaf through the phloem to the shoot apical meristem [12, 21, 22], where, through interactions with additional proteins, it initiates a gene regulatory cascade that promotes reproductive meristem identity [23–25]. Divergence in spatial, developmental or photoperiod-specific gene expression patterns has been observed among lineage-specific *FT*-like paralogs [8, 11, 12, 26, 27], and two rice *FT* paralogs differ in how they promote flowering in different photoperiods [13]. Natural variants in one or more *FT*-like genes have also been linked to variation in flowering in *A. thaliana* and domesticated cereals [11, 17, 28–31]. Here, we have investigated the evolution of *FT*-like genes in the common sunflower, *Helianthus annuus*, and the roles these genes played during domestication.

Recent Expansion and Diverse Fates

Four sunflower FT-like paralogs—*HaFT1*, *HaFT2*, *HaFT3*, and *HaFT4*—were isolated from *H. annuus* by polymerase chain reaction (PCR) and hybridization-based methods. A phylogeny constructed with FT homologs from a diverse set of plants revealed these sunflower paralogs are all more closely related to each other than to homologs from other species (Figure 1), indicating they arose by a series of relatively recent duplications. All paralogs have highly similar sequences and exon-intron structure (Figure 2A; see also Figure S1 and Table S1 available online), though HaFT4 is more divergent and one amino acid shorter than the remaining paralogs. All four duplicates have the conserved FT amino acid at two residues that functionally distinguish FT from TERMINAL FLOWER 1 (TFL1) [32, 33].

The synonymous substitution rate between two sequences, K_s, provides a measure of time since divergence because synonymous sites are expected to evolve neutrally. K_s is ~0.45 for all comparisons between *HaFT4* and the other paralogs (Table S1), a magnitude consistent with duplication during a polyploidy event at the base of the Heliantheae tribe [34]. K_s comparisons of *HaFT1*, *HaFT2*, and *HaFT3* with each other are much lower (~0.04–0.08), indicating these duplications likely occurred within the genus *Helianthus*. *HaFT4* maps to linkage group (LG) 14 of the sunflower genetic map, whereas the other three paralogs all map to the same end of LG6 (Figure 2B), suggesting the recent duplications occurred in tandem. Because gene conversion may homogenize tandem duplicates, further sequencing from additional Heliantheae species will be required to precisely time and order the more recent events.

After duplication, gene copies may have several evolutionary fates including nonfunctionalization, retention for additional dosage, neofunctionalization, subfunctionalization, and differential improvement of ancestral functions [2]. We surveyed spatial expression patterns of the *HaFT* genes in wild *H. annuus* to examine what processes have acted on these paralogs (Figure 2C). Because *HaFT1*, *HaFT2*, and *HaFT3* have extremely similar coding sequences, primers that uniquely amplify each copy could not be designed. Instead, *HaFT2* expression was distinguished by restriction digest. All PCRs exhibiting expression were also cloned, and 24 clones per reaction were sequenced to verify the contributing paralog(s). *HaFT4* and *HaFT2* exhibited similar expression patterns; however, *HaFT1* expression diverged in two notable ways. *HaFT1* was not expressed in leaves but, unlike *HaFT2* and *HaFT4*, was expressed in the shoot apex. Sequencing of cloned PCR products confirmed these results. *HaFT1* also exhibited an alternative splice form retaining part

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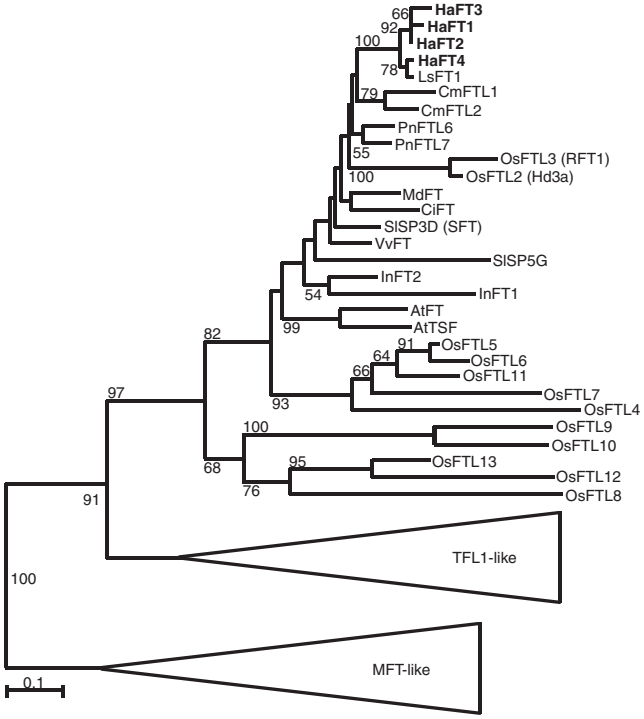


Figure 1. Recent Duplications of Sunflower *FT*-like Genes

Maximum likelihood phylogeny based on amino acid sequences of plant FT proteins. Bootstrap percentages > 50% shown above branches. Species abbreviations: *Antirrhinum majus*, Am; *Arabidopsis thaliana*, At; *Citrus unshiu*, Ci; *Cucurbita maxima*, Cm; *Helianthus annuus*, Ha; *Ipomoea nil*, In; *Lactuca sativa*, Ls; *Malus x domestica*, Md; *Oryza sativa*, Os; *Populus nigra*, Pn; *Solanum lycopersicum*, Sl; and *Vitis vinifera*, Vv. See also Tables S1 and S6.

of the third intron, which, though in frame, contains premature stop codons. Depending on whether the single copy ancestor of these genes was expressed in the shoot apex, either subfunctionalization or neofunctionalization may have preserved *HaFT1*, though gain of a new expression domain is the more parsimonious explanation.

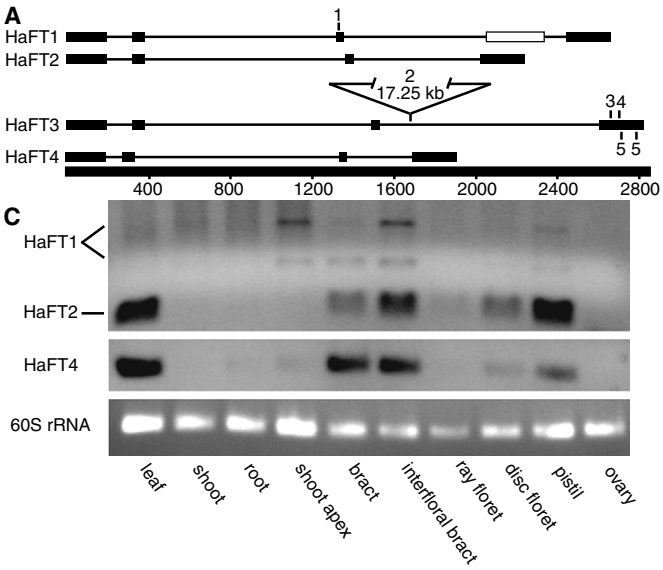
HaFT3 expression was not detected by sequencing reverse-transcriptase (RT)-PCR products from any tissue (0/192 clones sequenced total across 8 tissues), and four mutations likely to disrupt function (Figure 2A) were found in 54 of 60 wild and domesticated accessions of *H. annuus* surveyed and in sister species *H. argophyllus* (Tables S2 and S3). These findings provide strong evidence consistent with nonfunctionalization of *HaFT3*.

Robust, efficient, and universal sunflower transformation protocols are as yet undeveloped; however, because *FT* activity is widely conserved, *HaFT* function is testable by heterologous complementation. To determine whether the three expressed *HaFT* genes were functionally equivalent to *A. thaliana FT*, we overexpressed the coding region of each paralog from the CaMV35S promoter in Columbia-0 plants and *ft-1* mutants raised in long days. Wild alleles of all three sunflower paralogs accelerated flowering in both wild-type and mutant backgrounds (Figure 3, Table S4, Figure S2), indicating these sunflower paralogs all encode functional copies of *FT*.

QTL Characterization

The three *HaFT* paralogs that map to LG6 colocalize with a QTL explaining 7%–36% of flowering time variation, depending on cross and environment, in crosses between wild and domesticated sunflower (Figure 2B) [35–37]. Although *HaFT3* appears nonfunctional, *HaFT1* and *HaFT2* are strong candidates for the gene underlying the QTL. Because *FT* is involved in photoperiodic floral induction, we characterized the photoperiod response of parents of a wild × elite-crop QTL population. Plants from the wild parent's population, Ann1238, exhibited a short-day response, flowering ~10 days earlier in short and intermediate days than under long days (Figure 4A). The domesticated parent line, CMSHA89, had the opposite behavior—long-day response—flowering ~10 days earlier in long days. Comparing flowering time in each group's inductive photoperiod, the domesticated line flowered ~10 days earlier than the wild population.

To determine what aspect of this divergence in flowering behavior the LG6 QTL controls, we developed a near isogenic line (NIL) for this region by genotypic selection during backcrossing for five generations of a descendant of the original



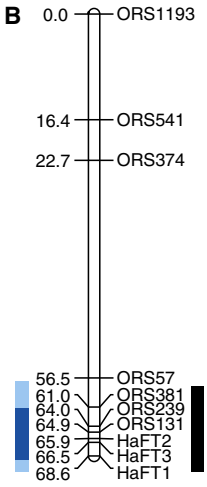


Figure 2. Gene Structure, Map Position, and Spatial Expression of *HaFT* Paralogs

(A) Exon-intron structure of *HaFT* coding sequences shown to scale. The open rectangle in *HaFT1* denotes an alternatively spliced exon. Numbered sites mark the locations of the frameshift in *HaFT1* (1) and putative loss-of-function mutations in *HaFT3* segregating in natural populations (2–5). *HaFT3* mutations include a 17.25 kb insert in the third intron (2), a 7 bp deletion (3), a 1 bp deletion (4), and two cosegregating premature stop mutations (5).

(B) Genetic map of LG6 indicating the map positions of *HaFT1*, *HaFT2*, and *HaFT3* relative to previously mapped QTL region (black) [35], and relative to the wild introgression into a domesticated background in NILs (blue, introgression end points occur within light blue regions).

(C) Spatial expression of *HaFT1*, *HaFT2*, and *HaFT4* in wild sunflower assayed by RT-PCR. See also Figure S1 and Table S3.

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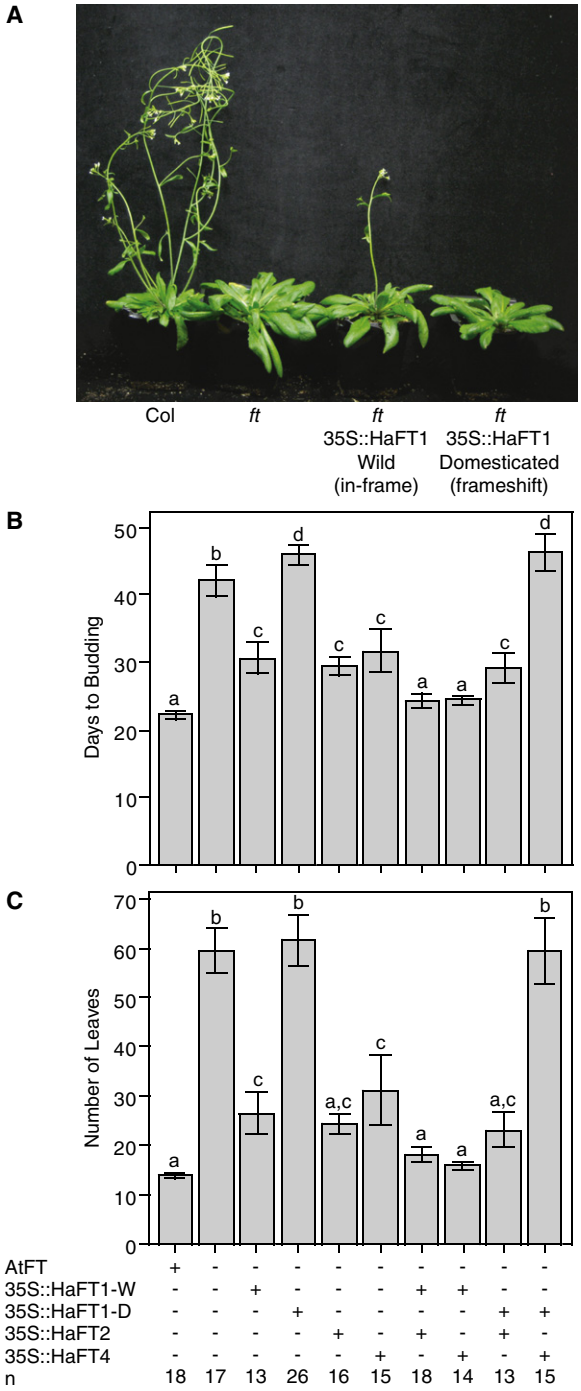

Figure 3. Frameshift-Carrying Domesticated *HaFT1* Allele Has Dominant-Negative Effect

(A) Overexpression of in-frame wild allele of *HaFT1* complements the *A. thaliana ft* mutant whereas overexpression of the frameshift-carrying domesticated allele does not. Plants photographed 36 days after germination.

(B and C) Days to budding (B) and rosette leaf number (C) of plants overexpressing 0, 1, or 2 HaFT paralogs in an *ft* background. 35S::HaFT1-W plants overexpressed the wild, in-frame *HaFT1* allele; 35S::HaFT1-D plants overexpressed the domesticated, frameshift-carrying *HaFT1* allele. Means for each genotype were compared with a general linear model, and pairwise comparisons were performed with Tukey's multiple comparison test. Different letters above the 95% confidence intervals denote significantly different phenotype distributions. See also Figure S2 and Table S4.

recombinant panel to CMSHA89 followed by selfing (Figure 2B). Photoperiod response was then characterized for individuals homozygous for the domesticated allele in the LG6 QTL region (DD), heterozygous (DW), and homozygous for the wild allele (WW).

Like the domesticated parent, all genotypes showed a long-day response (Figure 4B). Thus, this QTL does not mediate the photoperiod response difference between the parents. A photoperiod-specific difference in flowering time was observed between genotypes however. WW plants budded ~4 days earlier than DD plants or the domesticated parent only in the inductive long-day photoperiod (Figure 4B). These results were similar in direction and magnitude to the QTL effect observed previously [35]. Similar results were obtained on replication (Table S5), and a larger sample size provided sufficient statistical power to show the DW phenotype was intermediate. Additional phenotyping revealed photoperiod-specific differences for 9 of 13 traits including a 7-day difference in anthesis date and 1 cm difference in disc diameter between DD and WW plants in long days. Because it is unlikely that many genes in a small genomic region would independently affect diverse traits in a photoperiod-specific manner, we expect these additional differences could be direct pleiotropic effects or indirect effects of the change in developmental timing caused by allelic variation in a single gene.

Evaluation of Candidate Paralogs

The NIL phenotypes suggest a model for the action of the gene underlying the QTL. First, the photoperiod-specific effect of the QTL on flowering suggests a gene with photoperiod-specific expression or action. Second, because DD plants flower later than WW plants, the domesticated allele likely contains a loss-of-function or dominant-negative mutation.

To evaluate whether *HaFT1* or *HaFT2* fit these criteria, we compared their expression and cDNA sequence in the parental genotypes and NIL plants. In the wild parent, expression of both candidates was upregulated by the inductive photoperiod, short days (Figures 4C and 4E). In the domesticated line, the inductive photoperiod (long days) also upregulated *HaFT2* but *HaFT1* was expressed in both photoperiods, and similar patterns were observed for DD, DW, and WW plants (Figures 4C–4F, Figure S3C). Thus, regulatory changes responsible for expression differences of these genes between the parents mostly act in *trans*. There was one notable difference between the genotypes: *HaFT2* peak abundance was 3–4-fold higher in DD plants than in DW or WW plants, consistent with a 4–5-fold increase in the domesticated parent relative wild parent (Figures 4E and 4F) and indicative of a *cis*-regulatory difference affecting *HaFT2* peak abundance. This change is unlikely to underlie the QTL effect, however. Increased *HaFT2* expression in DD plants should accelerate flowering, but these plants flowered later than WW plants. DD, DW, and WW plants differed in relative abundance of the longer *HaFT1* splice form; however, these differences were not photoperiod specific and thus also unlikely to cause the observed phenotypic differences.

We did find a coding sequence difference that could explain the LG6 QTL effect. Although the wild and domesticated HaFT2 amino acid sequences were identical, the domesticated allele of *HaFT1* differed from the wild allele by a frameshift mutation (TG→C) in the third exon (Figure 2A). The frameshift does not create a premature stop codon, but rather leads to a protein 17 amino acids longer than wild-type that is half novel sequence (Figure S1). No premature stop mutations

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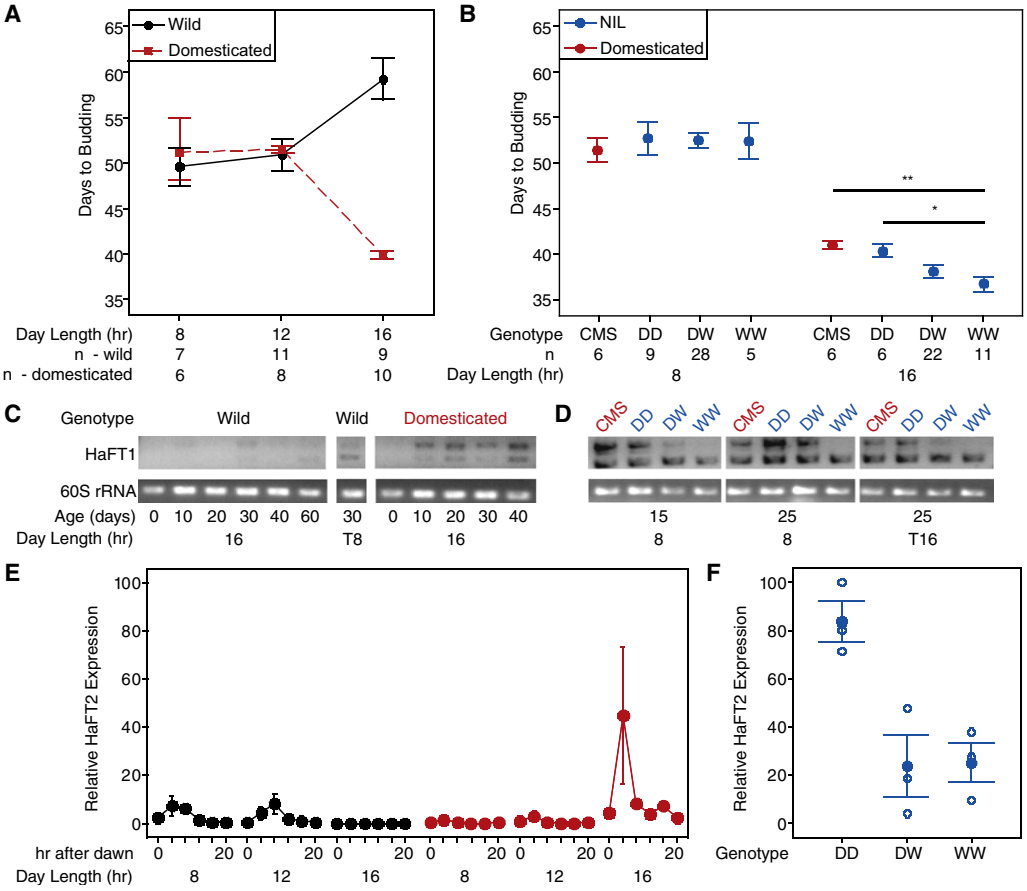

Figure 4. Flowering Time, *HaFT1* Expression, and *HaFT2* Expression in Parental and NIL Plants

(A) Days to budding of wild (black) and domesticated (orange) parents in three photoperiods. Mean ± SE is shown.

(B) Days to budding of domesticated (CMS, orange) and NIL plants (blue) homozygous for the domesticated LG6 QTL region (DD), heterozygous (DW), and homozygous wild (WW) in short and long days. Mean ± SE is shown. Differences among genotypes tested by general linear model corrected with Tukey's multiple comparison test. **$p < 0.02$; *$p = 0.058$.

(C) *HaFT1* shoot apex expression in developing wild and domesticated parents in long days. Age measured as days after sowing. T8 plants experienced 20 long days followed by 10 short days.

(D) HaFT1 shoot apex expression in CMS, DD, DW, and WW plants 15 and 25 days after sowing in short days. T16 plants experienced 15 short days followed by 10 long days.

(E) HaFT2 leaf expression in wild (black) and domesticated (orange) parents every 4 hr on the 30th day after sowing. Mean ± SE for three biological replicates is shown.

(F) HaFT2 leaf expression in long-day grown DD, DW, and WW plants 4 hr after dawn, 30 days after sowing. Mean of three technical replicates per biological replicate (open circles) and mean ± SE for three biological replicates (filled circles) shown. Relative expression expressed as delta-delta-Ct normalized to 60S rRNA and scaled to the highest individual measurement. See also Figure S3 and Table S5.

are present in the long splice form of this allele, meaning differential nonsense-mediated decay could explain expression variation of this form in the NILs.

The frameshift mutation in the domesticated allele is consistent with the observation that DD plants flowered later than WW plants, but it is more difficult to explain the QTL's photoperiod-specific effect on phenotype because *HaFT1* was expressed in both photoperiods in all NIL genotypes. If *HaFT1* affects flowering whenever expressed and the frameshift is a simple loss of function, the effect of the QTL on flowering would be expected in both photoperiods. Alternatively, if the frameshift mutation has a dominant-negative effect, then it could have photoperiod-specific action through interference with the function of other *FT* paralogs. Because *HaFT2* and *HaFT4* were expressed only in long days in the CMSHA89 background (Figure S3), dominant-negative action of *HaFT1* would be photoperiod specific.

To determine whether the domesticated and wild *HaFT1* alleles are functionally distinct, we took a heterologous complementation approach. Full-length cDNAs of the in-frame wild allele and frameshift-carrying domesticated allele were overexpressed in Columbia-0 and *ft* mutant *A. thaliana* backgrounds. The two constructs differed only by the frameshift mutation.

Although overexpression of the in-frame wild allele accelerated flowering in both backgrounds, overexpression of the frameshift-carrying domesticated allele did not (Figure 3A, Table S4). Indeed, *ft* transformants carrying the frameshift allele were slightly delayed in flowering relative to untransformed *ft* mutants (Figure 3B), though a similar delay was not observed in the wild-type background nor evident from leaf counts (Table S4). These results indicate that the frameshift mutation functionally alters the HaFT1 protein and provided us a preliminary indication that it may not be a simple loss of function.

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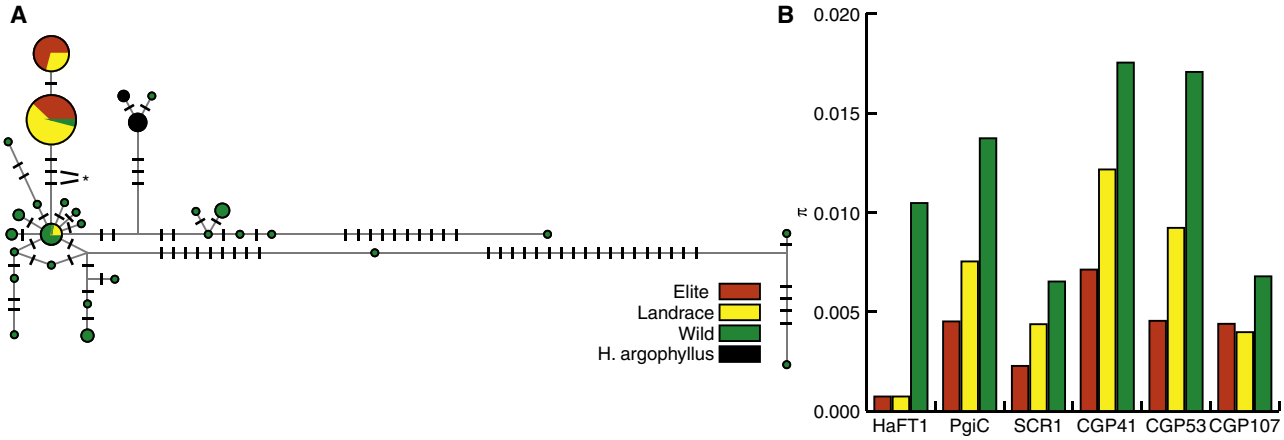

Figure 5. HaFT1 Frameshift Distinguishes Wild from Domesticated Accessions and Experienced a Selective Sweep during Domestication

(A) Median joining haplotype network constructed from 711 bp region of *HaFT1* sequenced from elite-bred (red), landrace (yellow), and wild (green) *H. annuus* and wild *H. argophyllus* (black). TG→C frameshift mutation (*) and a noncoding SNP define a branch separating nearly all domesticated lines from nearly all wild accessions. The number of hatchmarks on a branch indicates the number of substitutions.

(B) Average pairwise nucleotide diversity (π) for *HaFT1* and five putative neutral loci in samples of elite-bred, Native American landrace, and wild *H. annuus*. See also Table S2.

To test whether the *HaFT1* frameshift could cause a dominant-negative effect with photoperiod-specific action by interfering with other *HaFT* paralogs, we crossed *ft* plants overexpressing the *HaFT1* frameshift allele to *ft* plants overexpressing either *HaFT2* or *HaFT4*. We predicted that if the frameshift allele interferes with *HaFT2* or *HaFT4* function, then it should suppress the complementation of late flowering in *ft* mutants by these paralogs. Transgenic *ft* mutants overexpressing the in-frame allele of *HaFT1* were also crossed to plants overexpressing *HaFT2* or *HaFT4* for comparison. The genotypes of cross progeny and transgene overexpression were confirmed by RT-PCR (Figure S2D).

When overexpressed in either the *ft HaFT2* or *ft HaFT4* background, the wild allele further accelerated flowering and reduced leaf number relative to plants without this transgene (Figures 3B and 3C, Table S4). This is consistent with its expected effects and demonstrates that transgenics carrying multiple *HaFT* transgenes do not exhibit any generalized cosuppression. In the *ft HaFT2* background, overexpressing the *HaFT1* frameshift allele did not alter flowering time. In contrast, overexpressing this allele in the *ft HaFT4* background significantly delayed flowering, fully suppressing complementation of *ft* by *HaFT4* (Figures 3B and 3C, Table S4).

Thus, the photoperiod-specific effect of the LG6 QTL region on NIL phenotypes may be mechanistically explained by dominant-negative action of the *HaFT1* frameshift mutation. Although *HaFT1* expression is not photoperiod specific, *HaFT4* is only expressed under long-day conditions (Figure S3). Therefore, dominant-negative interference of the domesticated allele of *HaFT1* with *HaFT4* and a consequent delay in flowering only occurs in the inductive photoperiod. Whether the long form of the domesticated allele shares this functionality is an uninvestigated though intriguing possibility. Several aspects of *HaFT* paralog expression, transport, and redundancy in sunflower were not recapitulated in our experiments, and the eventual development of sunflower transformants will be required confirm our findings. One potential mechanism for the dominant-negative interaction between the domesticated allele of *HaFT1* and *HaFT4* could be interference with binding of *HaFT4* to shoot apex proteins required for floral induction. Similar interference mechanisms have been proposed to explain the effects of chimeric *FT/TFL1* constructs or *TFL1* on flowering also based on transgenic studies [33, 38].

Selection During Early Domestication

We examined the historical importance of the HaFT1 frameshift mutation during domestication by sequencing a 711 bp region containing the third exon and surrounding intron sequence from a diversity panel of elite-bred, Native American landrace, and wild *H. annuus* (Figure 5, Table S2). Nearly all elite (36/36) and landrace (36/38) alleles contained the frameshift mutation whereas nearly all wild alleles (44/46) were in-frame. This dramatic allele frequency change between wild populations (q = 0.05) and early domesticates (q = 0.95) strongly suggests selection acting during early domestication. Of the four exceptions, the two landraces heterozygous for the in-frame allele may result from recent admixture between domesticated and wild *H. annuus* or an incomplete selective sweep. Both wild lines segregating for the frameshift allele were from Oklahoma. These may indicate recent admixture, but could also represent the allele's area of origin.

Other aspects of *HaFT1* sequence diversity support these conclusions (Figure 5A). A substitution in the second intron was in complete linkage disequilibrium with the frameshift. A single nucleotide polymorphism unique to domesticated lines was also found, suggesting the sweep occurred sufficiently long ago and the allele remained sufficiently isolated from wild germplasm that new variation has accumulated without passing into wild plants by gene flow.

We tested whether *HaFT1* shows a signature of a selective sweep by using a maximum-likelihood adaptation of the Hudson-Kreitman-Aguadé (MLHKA) test [39]. The test compares the likelihood of a strictly neutral model to a model where a candidate gene is under selection. We sequenced portions of five putatively neutral control loci on the same diversity panel. A separate multilocus HKA test verified that these genes did not depart from neutrality. MLHKA tests were then conducted for elite, landrace, and wild datasets separately to determine the timing of selection. These tests found that *HaFT1* was evolving neutrally in wild *H. annuus* populations

($p = 0.281$) but under selection in landrace ($p = 0.048$) and elite lines ($p = 0.015$). Reduction of *HaFT1* average pairwise nucleotide diversity (π) with domestication was much greater than reductions in neutral gene nucleotide diversity with domestication, consistent with this result (Figure 5B).

Linkage disequilibrium decays rapidly in domesticated sunflower ($r^2 = 0.32$ at 5 kb, $r^2 = 0.1$ at ~100 kb [40]). Because neither *HaFT2* nor *HaFT3* is present in BAC sequence 60 kb upstream or 51 kb downstream of *HaFT1,* it is unlikely this sweep affected all three paralogs. Indeed, MLHKA analysis of *HaFT3* sequences we obtained revealed no signature of selection during early domestication ($p = 0.095$).

Conclusions

Our results demonstrate that multiple recently evolved *FT*-like duplicates have met a variety of fates and have played diverse roles in flowering time divergence between wild and domesticated sunflower. Expression divergence in one paralog, *HaFT2*, caused by both *cis*- and *trans*-regulatory effects, is associated with a shift to earlier, long-day responsive flowering. A frameshift mutation in a second paralog, *HaFT1*, maps to a major flowering QTL, and affects developmental timing through interference with the function of a third paralog, *HaFT4*. Furthermore, comparison of sequence diversity to neutrally evolving loci revealed a signature of selection on *HaFT1* during early domestication.

Together, to our knowledge, our findings provide the first functional and population genetic evidence identifying an early domestication gene in sunflower. Like most previously identified early domestication genes, *HaFT1* is involved in transcriptional regulation and the domesticated allele is not a simple null [41]. Retention of the frameshift allele through modern breeding despite selection for earlier, more synchronous flowering likely occurred due to absence of genetic variation in modern crop progenitors and possibly also favorable pleiotropic effects. Selection on flowering time during modern breeding must have acted at other loci, perhaps including *HaFT2* where we located *cis*-regulatory differences predicted to promote early flowering.

Our findings also illustrate how gene duplication may foster evolutionary change by creating an opportunity for new gene-gene interactions within gene families to evolve and produce natural variation. As many transcriptional regulators and signaling molecules participate in gene complexes, often as homo- or heterodimers, we speculate that origin and modulation of within-gene family interactions made possible by gene family expansion is an important contributor to biological diversity [42].

Accession Numbers

GenBank accession numbers of deposited sequences (GQ884199–GQ885119) are listed in Table S6.

Supplemental Information

Supplemental Information includes three figures, seven tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.01.059.

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