Tomato Yield Heterosis Is Triggered by a Dosage Sensitivity of the Florigen Pathway That Fine-Tunes Shoot Architecture

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Abstract
The superiority of hybrids has long been exploited in agriculture, and although many models explaining “heterosis” have been put forth, direct empirical support is limited. Particularly elusive have been cases of heterozygosity for single gene mutations causing heterosis under a genetic model known as overdominance. In tomato (Solanum lycopersicum), plants carrying mutations in SINGLE FLOWER TRUSS (SFT) encoding the flowering hormone florigen are severely delayed in flowering, become extremely large, and produce few flowers and fruits, but when heterozygous, yields are dramatically increased. Curiously, this overdominance is evident only in the background of “determinate” plants, in which the continuous production of side shoots and inflorescences gradually halts due to a defect in the flowering repressor SELF PRUNING (SP). How sp facilitates sft overdominance is unclear, but is thought to relate to the opposing functions these genes have on flowering time and shoot architecture. We show that sft mutant heterozygosity (sft/+)) causes weak semi-dominant delays in flowering of both primary and side shoots. Using transcriptome sequencing of shoot meristems, we demonstrate that this delay begins before seedling meristems become reproductive, followed by delays in subsequent side shoot meristems that, in turn, postpone the arrest of shoot and inflorescence production. Reducing SFT levels in sp plants by artificial microRNAs recapitulates the dose-dependent modification of shoot and inflorescence production of sft/+ heterozygotes, confirming that fine-tuning levels of functional SFT transcripts provides a foundation for higher yields. Finally, we show that although flowering delays by florigen mutant heterozygosity are conserved in Arabidopsis, increased yield is not, likely because cyclical flowering is absent. We suggest sft heterozygosity triggers a yield improvement by optimizing plant architecture via its dosage response in the florigen pathway. Exploiting dosage sensitivity of florigen and its family members therefore provides a path to enhance productivity in other crops, but species-specific tuning will be required.

Introduction

More than a century ago, simple garden studies by Darwin revealed a remarkable phenomenon in which crossing related varieties of plants produced hybrid progeny with superior growth and fecundity compared to their parents [1]. Understanding this hybrid vigor began with population genetics theories postulating that outcrossing facilitates adaptation and improves fitness by shuffling allelic diversity to thwart inbreeding depression [2]. However, it was the agricultural exploitation of hybrid vigor, or “heterosis,” in both crop and animal breeding that propelled rapid advancements in agriculture. Despite the appeal of hybrid vigor, evidence that a model known as overdominance might also contribute to heterosis [5–8]. Overdominance has long been an appealing explanation, because theoretically heterozygosity at only a single gene is needed to cause heterotic effects, presumably from intra-locus allelic interactions functionally superseding any one allelic form. However, the relevance of overdominance for yield and whether allelic interactions are the underlying cause remains controversial, primarily because quantitative trait locus (QTL) mapping studies reporting overdominant QTL have failed to pinpoint responsible genes [12–16]. Importantly, though, there have been scattered reports of single gene overdominance over the years, and among these have been several unexplained examples from yeast, plants, and animals involving heterozygosity for single gene loss-of-function mutations [17–24].

We previously reported a dramatic case of overdominance for tomato yield in multiple environments and planting densities resulting from loss-of-function mutations in the gene SINGLE
Author Summary
For over a century, it has been known that inbreeding harms plant and animal fitness, whereas interbreeding between genetically distinct individuals can lead to more robust offspring in a phenomenon known as hybrid vigor, or heterosis. While heterosis has been harnessed to boost agricultural productivity, its causes are not understood. Especially controversial is a model called “overdominance,” which states in its simplest form that a single gene can drive heterosis, although multiple overdominant genes can also contribute. In tomato, a mutation in just one of two copies of a gene encoding the flowering hormone called florigen causes remarkable increases in yield, but it is not known why. We show that yield increases are triggered by a fine-tuning of florigen levels that cause subtle delays in the time it takes all shoots to produce flowers. The resulting plant architecture maximizes yield in varieties that dominate the processing tomato industry. We show that while similar changes in flowering occur when one copy of florigen is mutated in the model crucifer Arabidopsis, yield is not increased, suggesting that, while manipulating florigen holds potential to improve crop productivity, the tuning of florigen and related genes will have to be tailored according to species.

FLOWER TRUSS (SFT) encoding the generic flowering hormone florigen [25]. Tomato yield, on both a per plant basis and in the context of tons per acre, depends partly on fruit size, but is mainly driven by the production of dozens of multi-flowered inflorescences and resulting fruit clusters that develop according to the “sympodial” growth habit [26]. The defining feature of sympodial plants is the shoot apical meristem (SAM) ends growth by differentiating into a terminal flower after producing a set number of leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, produces just three leaves, and growth then renews from a specialized axillary (i.e. sympodial) meristem (SYM) that, in tomato, products where fruit quality is less relevant, must be managed greenhouse growth and maintain fresh market quality [28].

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The discovery that sft/+ heterozygosity in an sft/+ SP background dramatically increases fruit production while only modestly increasing sympodial shoot regeneration to create a new optimum for fruit yield, we explored tomato sft heterosis from a developmental and molecular perspective. We show that while similar changes in flowering occur when one copy of florigen is mutated in the model crucifer Arabidopsis, yield is not increased, suggesting that, while manipulating florigen holds potential to improve crop productivity, the tuning of florigen and related genes will have to be tailored according to species.

Results
sft/+ heterozygosity suppresses sympodial shoot termination in determinate tomatoes

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Delauges in flowering time and sympodial termination were also observed on side shoots (Figure S1A–C), indicating a whole plant effect from sft/+ heterozygosity that explains the increase in total inflorescence number (Figure S1D) [25]. Thus, postponement of sympodial termination in sp mutants from sft/+ heterozygosity is based on recurring weak delays of all main and side shoot sympodial flowering transitions.

sft/+ heterozygosity weakly delays the primary flowering transition

Initiation and perpetuation of tomato sympodial growth depends on a gradual flowering transition culminating in PSM termination in a process mediated in part by accumulating florigen product from SFT counterbalancing repressive signals from SP. Regardless of whether SP is mutated, mutations in SFT cause late

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flowering and produce vegetative inflorescences, and strong alleles fail to initiate sympodial growth (Figure 2A) [27]. Our observation that precocious sympodial termination was delayed in sft/+ sp plants beginning with the first sympodial shoot (Figure 1C) led us to ask whether the flowering delay might commence in the PSM where sft homozygous mutant phenotypes first manifest. Surprisingly, whereas flowering time of sft/+ heterozygotes alone was not significantly different from sp mutants and WT, sft/+ sp plants were slightly later flowering (Figure 2A). We pinpointed this weak semi-dominant effect more precisely by evaluating developmental progression (ontogeny) of meristems. Like vegetative shoots, multi-flowered inflorescences of tomato are based on sympodial growth [26]. Just before the PSM transitions to a terminal floral meristem (FM), a sympodial inflorescence meristem (SIM) initiates perpendicularly, and this process reiterates several times to produce the characteristic zigzag inflorescence [30]. At 20 days after germination (DAG), we quantified SIM production in the primary inflorescence and found that sft/+ sp plants were on average one SIM behind sp mutants (Figure 2B–D). At this same point, while the first SYM of sp plants had already given rise to the first or second FM-SIM pair of the second inflorescence, most sft/+ sp SYMs were still in the reproductive transition (no FM evident morphologically) or starting the development of the first SIM-FM pair (Figure 2E–G). Thus, having only one fully functional allele of SFT delays the flowering transitions of both primary and sympodial shoots in sp mutants.

sft/+ heterozygosity delays seedling development and primary shoot meristem maturation

Our developmental findings suggested that sft/+ overdominance and yield increases might commence with a semi-dominant delay of the primary flowering event. The flowering transition is paralleled by a maturation of seedlings marked by changes in morphological complexity and molecular states (e.g. transcriptomes) of leaves [27,31]. As leaves of sft/+ sp plants are indistinguishable from those of WT and sp, we captured global gene expression patterns of the 6th expanding (3 cm) leaf, which is when differences in meristem ontogeny first appear (Figure 2B–G, Figure 3A and Dataset S1). In comparing sp single and sft/+ double mutant leaf mRNA-Seq generated transcriptomes with those of sft/+ sp plants, we found 839 differentially expressed genes among all genotypes. Previous studies comparing gene expression between hybrids and parents involved whole genome heterozygosity and reported thousands of differentially expressed genes representing all modes of gene action (e.g. dominant, recessive, additive, overdominant, etc.) [6,8,32]. Surprisingly, despite having heterozygosity at only a single gene in an otherwise homozygous background, we observed expression changes in all directions (Dataset S2). One possible explanation among many for this complexity is that SFT is involved in multiple feedback loops and regulates major signaling cascades [33]. However, our primary interest was not to classify and compare these expression differences to whole genome heterozygotes or to dissect transcriptional regulatory networks controlled by SFT or SFT, but rather to use the RNA-Seq data as a quantitative molecular phenotyping tool to determine if there are changes in seedling maturation caused by sft/+ heterozygosity before gross morphological differences in shoot architecture become apparent.

The Digital Differentiation Index (DDI) algorithm identifies transcriptional marker genes whose expressions peak at chosen reference stages to identify stage-enriched marker genes and then queries these marker genes from transcriptomes of “unknown” tissues to predict their maturation states relative to the references
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transcripts. We tested this by over-expressing artificial microRNAs against SFT (35S:amiR-SFT) in the sp background [35,36]. In addition to SFT, the artificial microRNAs were designed to target the Arabidopsis thaliana SFT ortholog, FLOWERING LOCUS T (FT), to assess their broad efficacy, and were incorporated into two different Arabidopsis pre-microRNA templates, At pre-mir-16b and At pre-mir-319a, to guard against differential amiR backbone efficiencies (Figure 5A). In Arabidopsis, 35S:amiR-SFT/FTb1669d and 35S:amiR-SFT/FTb319a transformants exhibited late flowering phenotypes equivalent to β mutants (Figure S2B–C). In tomato, six of eight first generation (T1) transformants showed sp suppression phenotypes, and we selected three lines representing the range of observed suppression for further analysis. SFT transcript abundance was evaluated in these lines by quantitative RT-PCR, revealing a range of knockdown levels by the artificial microRNAs (Figure 5B). We evaluated progenies from two 35S:amiR-SFT/FTb1669d (referred to as amiR-SFTa and amiR-SF Tb) and one 35S:amiR-SFT/FTb319a (referred to as amiR-SFTc) transformants, and found that the amiR-SFTa produced an average of one additional sympodial unit and inflorescence compared to non-transformed sp mutants, closely resembling the dosage effects of sp+ heterozygosity (Figure 5C). amiR-SFTc showed greater suppression, terminating sympodial growth after producing often more than two additional units, while amiR-SF Tb fully suppressed sp to indeterminacy like WT plants (Figure 5C). Notably, the level of suppression of sp determinacy corresponded with the level of knockdown of SFT; e.g. the indeterminate line, amiR-SFTb, showed the greatest reduction of SFT transcripts (Figure 5B–C). In all six lines, we failed to find strong sp sp double mutant phenotypes of reverted inflorescences or loss of sympodial growth, suggesting only weak alleles of SFT were created with the 35S:amiR-SFT gene – an effect that is also consistent with often observed weak target knockdown by artificial microRNAs [35,36]. Importantly, we found delayed flowering time in successive sympodial units like in sp sp heterozygotes, and all three amiR-SFT progeny populations exhibited delayed primary shoot flowering time (Figure 5D). Thus, tuning SFT dosage transgenically mimics the effects of sp+ heterozygosity, further illustrating that a classical epistasis relationship between the sp and SFT mutants is ultimately responsible for the overdominant effect on yield.

A dosage effect from florigen mutant heterozygosity is conserved in Arabidopsis, but does not cause heterosis

As florigen is a universal inductive signal for flowering that several flowering pathways converge upon [37,38], we wondered if and how florigen mutant heterozygosity in a different system might affect growth, and specifically whether heterosis would result. We tested this by creating orthogonal mutant combinations in Arabidopsis thaliana, which is a monopodial plant in which a single flowering event converts the SAM into a continuously growing inflorescence meristem (IM) that produces flowers laterally, in contrast to the tomato sympodial growth habit in which multiple flowering transitions occur. Despite this difference, Arabidopsis ft (ft) mutants are likewise late flowering [39] and completely epistatic over the early flowering and precocious termination of inflorescence meristems of ft (sp) mutants [40]. To evaluate potential dosage effects of ft+ heterozygosity, we phenotyped progeny from ft-2/+ ft-1-2 plants, in which the ft-2 mutation, a strong allele, segregates in the ft-1 background (Figure 6A). We measured flowering time by counting rosette leaves and found a clear dosage effect in ft-2/+ ft-1 plants compared to ft-1 single and ft-2 ft-1 double mutants (Figure S3A). We next tested for heterosis by quantifying yield related traits, including plant height, number of axillary shoots, and, as a parallel

[31]. DDI revealed that ft/+ sp 6th leaf maturity was in between ft sp and sp, indicating that ft/+ heterozygosity delays maturation of sp plants already as young seedlings (Figure 3B, Dataset S3). We next asked whether the change in SFT dosage might be sensed in the PSM before it transitioned to flowering. We previously captured and quantified transcriptomes of five developmental stages of PSM maturation, which revealed a meristem maturation clock underlies a gradual transition of the PSM to a reproductive state [34]. The transition meristem (TM) stage of this clock is marked by increasing expression of flowering transition genes [34], and we therefore chose this stage for molecular phenotyping and comparison (Dataset S2 and S3). Importantly, TMs can be collected at precisely matched ontogenetic points, defined by initiation of the last leaf and indistinguishable meristem morphologies of tall round domes (Figure 4A–C) [34]. Assuming based on the primary inflorescence of ft mutants reverting into a vegetative shoot, and consistent with ft epistatic over sp, DDI revealed that the TM of ft sp double mutants exhibited a severely delayed maturation, most closely matching a vegetative meristem state (Figure 4D). In contrast, whereas sp TM maturity was indistinguishable from WT, the ft/+ sp TM was delayed relative to sp and therefore intermediate between sp single and ft sp double mutants (Figure 4D). Importantly, we also profiled the first SYM using the DDI algorithm [31]. Given that seedling development of sp is delayed compared to sp based on extreme late flowering, the sft 6th expanding leaf was designated an early leaf calibration point. Dark and light green curves indicate sft sp and sp maturation score distributions based on 124 DDI-defined marker genes. The black curve for the sft/+ sp 6th leaf indicates an intermediate maturation state. Numbers above indicate average maturation scores.

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Suppression of SFT by artificial microRNA phenocopies the dosage effects of sp+ heterozygosity

Our findings that single gene overdominance traced back to cumulative delays on recurring flowering transitions led us to reason that the dosage effects of sp+ heterozygosity might be recapitulated by simply partially reducing levels of functional SFT
to tomato yield, the number of siliques, flowers, and flower buds (Figure 6B–C and Figure S3B–D). Surprisingly, ft/+ tfl plants showed semi-dominance for plant height and total yield (Figure 6B and C), and similar effects were observed for a moderate second allele of ft (Figure S3E). Thus, whereas the dosage effect on flowering time from florigen mutant heterozygosity is conserved in the monopodial growth habit of Arabidopsis, it does not translate to heterosis.

**Discussion**

Crop yields derive from a complex integration of fitness-related traits founded on developmental and physiological mechanisms for organ production and biomass accumulation. Thus, studying heterosis inevitably involves a broad analysis of the myriad mechanisms controlling plant growth. It is therefore perhaps not surprising that recent gathering of vast genetic, phenotypic, and molecular data on cases of heterosis from diverse systems has suggested that multiple non-mutually exclusive system-specific mechanisms are likely at work [8–10,41]. Looking at heterosis from the developmental perspective, it would be reasonable to assume a priori that flowering would have a major role given that selection of allelic variation for flowering time regulators has been a major contributor to adaptation, domestication, and maximizing crop yields through classical and modern breeding [42]. In rice, for example, alleles of strong effect from various flowering regulators, many showing epistatic interactions, were selected to enable growth at different climates and day lengths [43–45].
same was achieved in maize, but, instead, dozens of loci of small additive effect were found to be involved [46]. In both rice and maize, and as occurred during the domestication and breeding of many crops, this selection enabled a shift from an extended period of flowering in wild populations to uniform flowering, which provided sudden bursts of yield that facilitated agronomic practices, particularly harvesting [42]. Interestingly, the genetic path leading to high yielding tomatoes has differed from other major crops in that domestication has mostly acted on fruit size to increase yield with little evidence for selection on flowering [47–50]. Indeed, while there is certainly flowering time and architectural variation among distantly related wild tomato species [51], cultivated tomatoes and their wild progenitor, *S. pimpinellifolium*, share nearly identical flowering times and indeterminate growth habits, suggesting there was little or no standing genetic variation for artificial selection to act upon [52]. Only with the relatively recent discovery of *sp* did a change in flowering provide a major agronomic shift in how tomato was grown in the field, enabling a burst of flower production and yield on compact plants grown at high density, which gave rise to the processing tomato industry [26]. In this regard, in contrast to maize where altered flowering times are frequently observed in hybrids [10,53], cultivated tomato hybrids do not differ substantially from their parental inbreds for flowering time, inflorescence production, or overall plant architectures. Only upon introgressing quantitative trait loci (QTL) from distantly related wild species are heterotic effects on yield observed, a subset of which have been tied to changes in flowering and plant architecture, but the causative genes have not been identified [54]. Thus, our dissection of *sft* heterosis is the first to expose a direct link to flowering and resolve the underlying mechanism.

Figure 5. Reducing *SFT* transcripts with artificial microRNAs mimics the dosage effects of sft/+ heterozygosity. (A) Artificial microRNAs targeting tomato *SFT* and *Arabidopsis FT*. Shown are alignments of amiR-SFT/FTAT164b and amiR-SFT/FTAT319a with the complementary region of *SFT* and *FT*. G–U wobbles and mismatches between the two amiR-SFT/FTs and the target are highlighted in the target sequence with bold blue and red, respectively. (B) Quantitative RT-PCR measurements of tomato *SFT* transcript levels in amiR*SFT* plants showing knock down. Results shown are from using primers targeting *SFT* transcripts 5’ to the amiRNA binding site, consistent with reports of primer-dependent transitivity occurring at the 3’ to 5’ direction upon the initial target cleavage, resulting in degradation of the 5’ cleaved product of the target but not the 3’ product [80,81] (Figure S2). Bars indicate relative expression level and error bars indicate standard deviation among replicates. (C) Depending on the strength of suppression, amiR*SFT* plants produce at least one additional sympodial unit and inflorescence compared to *sp* alone, indicating that reducing *SFT* transcript levels by artificial microRNA partially suppresses *sp* sympodial termination, mimicking the dosage effect of sft/+ heterozygosity. Note that some amiR*SFTc* progeny plants showed indeterminacy, whereas amiR*SFTb* progeny plants were always indeterminate, indicating that a stronger suppression of *SFT* completely suppresses the *sp* phenotype and reverts the plants to normal sympodial cycling. Differences in sympodial unit and inflorescence numbers between amiR*SFT* and *sp* plants were tested by Wilcoxon rank sum test and significance levels are marked by asterisks (* P<0.05, ** P<0.01, *** P<0.001). (D) amiR*SFT* plants have delayed primary shoot flowering time compared to *sp* and WT controls, similar to sft/+ heterozygosity. Bars indicate average leaf numbers with standard deviations. Genotypes and sample sizes are shown below. Differences in leaf numbers between amiR*SFT* and *sp* plants were tested by Wilcoxon rank sum test and significance levels are marked by asterisks (* P<0.05, ** P<0.01, *** P<0.001).

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Our combined developmental and molecular phenotyping of sft/+ overdominance has exposed a novel principle for how tomato plant architecture and yields might be further optimized by taking advantage of the surprising and remarkable level of dosage sensitivity within florigen and the florigen pathway. The genetically induced reduction in dosage of florigen from sft/+ heterozygosity causes a slight delay in the transition to reproductive growth that, in the context of recurring flowering events of the sympodial habit and the sp mutant background, translates to cumulative overdominance. Indeed, this heterosis example, like many others [20–24,55], is conditional. Yet, it is this genetic and developmental conditionality that suggests sft/+ heterosis could be considered less about heterozygosity and heterosis per se and more about the potential to genetically fine-tune SFT expression levels to manipulate yield in a way that domestication and breeding efforts have not yet capitalized on, perhaps because standing allelic diversity for florigen and members of its pathway is limited. In this respect, we propose that additional directed quantitative manipulation of the relative doses of SFT to SP might enable further fine-tuning of flowering, sympodial cycling, and inflorescence production. For example, as yet undiscovered, or artificially created [56,57], transcriptional or loss-of-function alleles of SFT and SP of various strengths could be combined in different genetic constitutions to pinpoint an even higher optimum of plant architecture to maximize yield. In an even simpler scenario, homozygosity for very weak mutant alleles of SFT in a strong sp background, or homozygosity for weaker mutant alleles of SP alone, could potentially match or exceed fruit production of the sft+/sp genotype. Finally, beyond tweaking SP and SFT, partial suppression of sp determinacy by generating mutations in other pathway genes, especially those encoding components of the florigen activating complex [58], could provide novel alleles and breeding germplasm that natural variation might not be able to provide.

Importantly, although there is tremendous diversity among angiosperms in when and where inflorescences and flowers form, the SFT/SP system is highly conserved [38,59,60], suggesting the aforementioned concepts could be applicable to other plants. Yet, our findings in Arabidopsis imply that while dosage effects on flowering time from florigen mutant heterozygosity will be broadly conserved, yield benefits might not be, and species-specific outcomes will likely trace back to differences in growth habits. The lack of meristem termination and recurring flowering events in the monopodial growth habit of Arabidopsis means that florigen mutant heterozygosity is sensed only once during development, and that no compounding of the semi-dominant dosage effect is possible. Indeed, increasing yield in Arabidopsis simply requires a larger plant, which can be achieved by delaying and prolonging flowering either environmentally through short day growth conditions or genetically through mutations in flowering regulators like FT. Consistent with this, we found that homozygous ft mutants were the highest yielding of all genotypes (Figure 6). At first glance, this would suggest limited possibilities for exploiting our findings beyond tomato; however, for some breeding goals, such as improving biomass, delaying flowering quantitatively and predictably through an allelic series of florigen mutants in either the homozygous or heterozygous condition could prove valuable to customize plant architecture and size for particular agronomic needs. Remarkably, yield benefits from heterozygous mutations in florigen orthologs have been found in at least one plant that lacks sympodial growth. In a strikingly similar example to tomato, a major domestication QTL for flowering time in sunflower traces back to a deletion in a duplicated paralogous FT gene that causes heterosis for both seed size and weight when heterozygous under short day conditions [61]. In another example, a classical report of overdominance for sorghum yield involves heterozygosity for an as yet uncharacterized late flowering mutant that has all the hallmarks of being defective in florigen or a florigen pathway component [62]. Thus, heterozygosity for florigen mutants holds potential for broadly improving crop yields, which, in hindsight, is perhaps not surprising given that selection for beneficial alleles of various strengths in florigen family genes, especially orthologs of SFT and SP, was key for the domestication of barley [63], beets [64], beans [65,66], grape [67], potatoes [68], roses [69], soybeans [70,71], sunflower [61], tobacco [72], and likely many other plants. With these examples in mind, and considering our findings in Arabidopsis, we suggest that sft/+ heterozygosity in a dose-dependent epistatic relationship with sp may represent only one of several ways to genetically tailor florigen levels, and that hunting for new alleles in existing germplasm or engineering custom alleles could allow an optimal fine-tuning of florigen and its pathway to...
maximize flowering, inflorescence production, and other yield components in these and other crops. The potential to broadly manipulate agronomic traits by florigen and its family members in diverse plant species stems not only from roles in flowering time, but also as general coordinators of diverse physiological processes affecting multiple aspects of plant growth and fertility [30]. Thus, parallel to how mutations in biosynthesis genes for the hormone gibberellin created the dwarf mutants that propelled the Green Revolution [73], our findings provide compelling evidence that manipulating florigen family genes can provide a new path to meet current breeding challenges associated with a rapidly changing climate.

Materials and Methods

Tomato plant growth conditions, genotyping, and phenotyping

The sp mutant was first reported more than 80 years ago and arose spontaneously, and the strong sp mutant allele used in this study, sft-7187, was isolated from a fast neutron mutagenesis screen performed in tomato cultivar M82, and has a two nucleotide deletion that truncates the C-terminal portion of the protein [26,27,74]. All mutants were backcrossed to M82 at least four times to eliminate background mutations prior to the original yield trials [25]. For all experiments in this study, plants were grown in controlled greenhouse conditions at Cold Spring Harbor Laboratory. Greenhouses were supplemented with artificial light from high-pressure sodium bulbs (50 μmol/m2/sec; 16 h/8 h) and daytime temperature was 78°F and nighttime temperature was 65°F, with a relative humidity of 40–60%. Tomato F2 generation seeds derived from self fertilization of an sft-7187 mutant and three bands for the mutant was first reported more than 80 years ago and sft mutant allele used in this study. sft-7187 mic fragments of the cetrimonium bromide (CTAB) DNA extraction protocol. Geno-

genotyping. Total genomic DNA was extracted using a standard CTAB DNA extraction protocol. The sft-7187 mutant was first reported more than 80 years ago and sp plant growth conditions, genotyping, and daytime temperature was 78°F.

Florigen Optimizes Plant Architecture in Heterosis

Arabidopsis plant growth conditions, genotyping, and phenotyping

Arabidopsis thaliana plants were grown in the greenhouse under long day (16 h light, 8 hr dark) conditions in 32-cell flats with two plants per cell. Individual seeds were delivered to the corner of each cell to avoid growth competition during germination. The seeds were stratified at 4°C for 4 days before transferring to a long day greenhouse maintained at 21°C. All mutant lines were acquired from the Arabidopsis Biological Resource Center (ABRC) and originated from EMS mutagenesis in the Landsberg erecta (Ler) background. Homozygous tfl1-2 mutant plants were crossed to a moderate (ft-1) and strong (ft-2) allele of ft. Individual F1 plants from each cross were self-fertilized to generate F2 populations segregating for both tfl1-2 and ft mutants. Plants homozygous for the tfl1-2 mutation and heterozygous for the ft-2 mutation were self-fertilized to generate F3 populations fixed for the ft-2 mutation and segregating for the ft-2 mutation. Tissue was harvested from young rosette leaves and DNA was extracted using a standard CTAB DNA extraction protocol. The sft-2 and ft-2 mutations were detected using derivative CAPS (dCAPS) assays. A fragment of TFL1 was amplified by PCR using the primers “tfl1-2 dCAPS-F” 5’-AAACGTCTCAGTCTTGTTTTCCTC-3’ and “tfl1-2 dCAPS-R2” 5’-AAATGAAAAGAAAGAATAAATATAAGGTAAG-3’ and a fragment of FT was amplified using “ft-2 dCAPS-F2” 5’-CCTCTGTA-CACATGGAAAAACCTTTTGTTG-3’ and “ft-2 dCAPS-R2” 5’-AAATCCTGGATGTTAAATGTTGAG-3’. Both TFL1 and FT fragments were amplified using a touchdown PCR program: initial denaturation at 95°C for 3 minutes, then 10 cycles at 95°C for 20 seconds, 65°C for 30 seconds (decreased by −0.5°C/cycle), 72°C for 30 seconds followed by an additional 30 cycles at 95°C for 20 seconds, 52°C for 30 seconds, 72°C for 30 seconds and ending with a final extension at 72°C for 10 minutes. Underlined nucleotides in the above mentioned sequences introduce a new restriction site in the wild type PCR amplicons. TFL1 PCR amplicons were digested using KpnI for 3 hours at 37°C, which cuts wild type but not mutant sequences. FT PCR amplicons were digested using HaellII for 3 hours at 37°C, which cuts wild type but not the ft-2 mutant sequences. Wild type versus mutant banding patterns was resolved on a 3% half MetaPhor agarose-half regular agarose gel. Phenotyping was completed in the F3 generation, and we compared sft-2 ft-2 double, sft-2 ft-2/+ and sft-2 single mutants. Homozygous single mutants and wild type Ler-0 were grown at the same time for comparison. Phenotyping and imaging was performed when the plants completed flowering and inflorescence meristems stopped growing (6–8 weeks after germination). The height of each plant was measured along the main shoot of the plant from where the base emerged from the rosette to top of the shoot. The number of rosette leaves, axillary shoots, siliques, open flowers, and floral buds were also recorded as measures of flowering time and yield. For each measured trait, the mean and standard deviation was calculated for each genotype. The means were compared using a
Global gene expression profiling (mRNA-Seq) of tomato leaves and meristems

Tomato homozygous sp mutants, sfl sp double mutants and F1 single gene heterozygotes of sfl/+ sp plants were used for leaf and meristem expression profiling experiments. All sfl/+ sp plants originated from F1 seeds of direct crosses between the sp and sfl sp parents, and a subset of F1 plants were confirmed by PCR genotyping to ensure 100% sp/heterozygosity. Seeds were germinated in petri plates on water-soaked Whatman paper at 28°C for 72 hours until the root radicles emerged. The germinated seeds were then transplanted to 72-cell insert flats with pre-wet soil and placed in the greenhouse. The plants used for leaf expression profiling were transplanted to two-gallon pots (three plants per pot), and tissue from the 6th young expanding leaf from each plant was collected and immediately frozen in liquid Nitrogen when the leaves reached 3 cm in length. Total RNA was extracted using a Qiagen RNeasy mini total RNA extraction kit according to the manufacturer’s protocol. Growth of seedlings for meristem expression profiling was monitored daily under a dissecting microscope using the meristem morphological cues marking previously defined maturation stages [34]. At the transition maturation (TM) stage, the cotyledons and leaves were removed from seedlings and the shoot apices with 3 cm hypocotyl attached were collected and stored in 100% acetone followed by vacuum infiltration for 30 minutes. Meristem tissue was dissected from the fixed stems using a surgical blade following the lines shown in Figure 4A-C and E-F under a dissecting microscope after confirming the morphology that marks the TM stage. Total RNA was extracted from the dissected meristem tissues with an Arcturus PicoPure total RNA extraction kit (Life Technologies). Except for the sp SYM, which is difficult to capture in high numbers because of a rapid termination, for all genotypes, tissue was harvested and prepared for mRNA-Seq construction for two biological replicates, and sp SYM was subjected to two technical replicates. As reported previously [34], two replicates were sufficient to quantify meristem maturation stages using the DDI algorithm, which was our primary goal in the expression analysis.

RNA-Seq library preparation

For all tissues, poly-A containing mRNA was purified from total RNA using Invitrogen oligo-dT DynaBeads for mRNA-Seq library construction using the ScriptSeq v2 RNA library preparation kit (Epicentre). The maximum amount of mRNA input (50 ng) was used when possible to maximize the library output. The final PCR enrichment step was carried out following the standard protocol with 15 cycles and primers with barcode indices supplied by Epicentre to create barcoded mRNA-Seq libraries. The quantity and size distribution of each individual barcoded mRNA-Seq library was detected with a High Sensitivity DNA Chip on a Bioanalyzer 2100 machine (Agilent). The final concentration of each library was verified by qPCR using a KAPA library quantification kit and based on these results, four to six barcoded libraries were pooled together with equal concentration for one lane of Illumina paired-end (PE) 100 bp sequencing on an Illumina HiSeq sequencing machine (Dataset S1). All reads files were deposited to SGN (ftp://ftp.solgenomics.net/transcript_sequences/by_species/Solanum_lycopersicum/libraries/illumina/LippmanZ/) and the mean RPKM values of meristems are visualized on an eFP browser (http://tomatolab.cshl.edu/efp/cgi-bin/efpWeb.cgi, SFT heterosis panel).

Read mapping and analysis

All mRNA-Seq reads were trimmed to 50 bp to remove the bases with low qualities and mapped using Bowtie [75] to the tomato reference CDS [76] with paired-end relationships maintained. Trimming the reads to 50 bp also made the libraries comparable to our previous mRNA-Seq libraries [34] for combined DDI analyses. The lack of size selection step in the Epicentre ScriptSeq v2 mRNA-Seq library preparation protocol allowed lower initial mRNA input but produced a larger insert size range (150 bp~1000 bp), which lowered the successful mapping with proper distance between paired-end reads. Mapping to predicted CDS also reduced the mapping rate due to failed mapping of reads coming from 5’ and 3’ UTR regions. However, the higher total read number from Illumina HiSeq compensated for the relatively lower mapping rates, yielding comparable mapped read numbers and sequencing depth to previous mRNA-Seq libraries that allowed for differential expression analysis and molecular phenotyping by DDI [34]. The resulting bam alignments were sorted and indexed by SAMtools [77], and the number of reads mapped to each CDS was counted to calculate the raw counts for all libraries. The raw counts from leaf and TM tissues across three genotypes were normalized using the TPM method. The distribution of gene expression levels were modeled following a negative-binomial distribution and tag-wise dispersion were estimated based on two replicates. Finally, exact tests for differential expression were conducted based on the replicates in pairwise comparisons. All normalization and differential expression tests were conducted using the edgeR package [78,79]. Although only two replicates were performed, we classified gene expression patterns from comparing sfl/+ sp heterozygotes and homozygous parents into 12 categories belonging to five major classes: additive, recessive, dominant, overdominant and underdominant (Dataset S2) using a threshold of two-fold change and P-value<0.01. Numbers of genes in each category were counted and their proportions in each category relative to all differential expressed genes were calculated for the 6th young leaf and TM, respectively, revealing all categories of gene expression changes were detected (Dataset S2).

Digital Differentiation Index analyses

Raw counts for the leaf expression profiles (including sp, sfl/+sp and sfl sp 6th young leaves) were incorporated into a master leaf data set. Raw counts for the meristem expression profiles (including sp and sfl/+sp TM and SYM) were incorporated into a master meristem data set that includes all raw counts from our previous meristems profiling experiments [34]. For both master data sets, all raw counts were then summarized over replicates and normalized against number of mapped reads and CDS lengths to calculate RPKM values for DDI analyses [31]. DDI selects samples with known or pre-determined maturation states in the whole data set as calibration points, and then identifies marker genes that show maximum expression at each calibration point. These genes characterize the calibration points molecularly. DDI checks the marker gene expressions in the samples that are submitted to query (the ‘unknown’ samples) and quantifies the ‘unknown’ samples’ maturation states relative to the calibration points. For each marker gene, DDI compares expression levels between ‘unknown’ samples and each calibration point and calculates a ‘maturation score’. Collectively, all marker genes generate a distribution of maturation scores for the ‘unknown’ sample [31]. Importantly, curves showing multiple ‘peaks’ reflect a mixed molecular maturation state for the queried tissue, as different marker genes give different maturation estimates. This is most evident in sfl sp double mutants that still transition to...
flowering, but at a much slower rate compared to wild type and with vegetative reversion of the inflorescence, indicative of a mixed vegetative-reproductive state. At the same time, a Student’s t-test of average maturation score difference between calibration and unknown samples was conducted for each unknown meristem sample, yielding a P-value for the significance of the maturation state difference. For each prediction, this P-value was obtained for comparisons between the unknown sample and temporarily successive calibration points, in order to generate a ‘gradient’ of meristem similarity plotted in heat-maps in the form of scaled 1/(-log10P). For example, to predict the maturation state of sft/+ sp SYM using the first replicate of WT EVM, MVM, LVM, TM and FM [the Early, Middle, and Late Vegetative Meristems (EVM: 5th leaf initiated; MVM: 6th leaf initiated; LVM: 7th leaf initiated), the Transition Meristem (TM: 8th leaf initiated), and the Flower Meristem (FM)] as calibration points, P-values were calculated for maturation state comparisons SYM vs. EVM, SYM vs. MVM, SYM vs. LVM, SYM vs. TM and SYM vs. FM, respectively. The P-values were then transformed into 1/(-log10P) and scaled across five values into a zero to one range (scaling was done for each prediction independently). Because smaller P-values indicate larger differences in maturation scores, the scaled 1/(-log10P) values quantify the relative similarity of the sft/+ sp SYM to each of the five calibration points. With the master leaf data set, DDI analyses were conducted using sft sp and sft 6th young leaves as two calibration points to predict maturation stages of sft/+ sp leaf maturation. With the master meristem data set, DDI analyses were conducted using five WT primary shoot meristem (PSM) stages as calibration points to predict maturation stages of sft, sft/+ sp and sft sp meristems. As in [34], one replicate of calibration samples was used for marker gene identification (Dataset S8), a second replicate of calibration samples treated as unknowns was predicted and plotted to set the boundaries of maturation stages (colored curves and boxes in Figure 4D and Figure 4G), and averaged RPKM values of predicting leaves and meristems were used to generate and plot the predicted distribution of maturation scores. All parameters for DDI analyses were as previously described [34]. All DDI analyses were carried out using modified R scripts as described previously [34].

Artificial microRNA construction and transformation

Artificial microRNAs were designed to repress both tomato SFT and Arabidopsis FT with two different backbones (Figure S1) [55]. The artificial microRNA amir-SFT/FT (mSFT) and amirR-SFT/FT (mSFT) were synthesized by DNA2.0 and Bio S&T, respectively, and transformed into both tomato and Arabidopsis plants and phenotyped for repression of SFT and FT, respectively (Figure 5B, Figure S2). Tomato plants carrying mSFT transgenes were measured for sympodial unit and inflorescence number, and phenotyping stopped after counting five or more sympodial units with two or more leaves in each unit and classified as indeterminate. The means of phenotypes were compared using a Student’s t-test (Wilcoxon rank sum test when phenotype distribution is not a normal distribution).

For quantitative RT-PCR of SFT transcript abundance in the amirRNA lines, cotyledon tissue was collected from two-week old seedlings for total RNA extraction with Qiagen RNeasy mini total RNA extraction kit including DNase treatment with RNase-free DNase (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was then synthesized using the SuperScript III First-Strand Synthesis System with oligo dT (Invitrogen). Ubiquitin mRNA (Solyc03g063100) from the amirSFT binding site were quantified with 1 ul of cDNA using Phusion High-fidelity DNA polymerase (NEB), qTm SYBR Green Supermix (Bio-Rad). A loss of transcripts was detected 5’ to the amirRNA binding site, consistent with reports of primer-dependent transcriptivity occurring at the 3’ to 5’ direction upon the initial target cleavage, resulting in degradation of the 5’ cleaved product of the target but not the 3’ product [80,81]. Primers pairs used were: 5’-CGTG- GGTGCTGCTAGAAGAG-3’ and 5’-ACGAAGGCTCTT- GACCTTTTC-3’ for Ubiquitin (UB); 5’-CCTTGAGGCCCTCCG- CAAGTTA-3’ and 5’-GGGTGGACGATAACAAAAGT-3’ for sft/+ sp (upstream); 5’-GACAACTTAGGGTCCGAAAAAACA-3’ and 5’-AGCGGACACAGGTAAAACCAA- 3’ for 3’sp SFT (downstream). Two biological replicates of qRT-PCR were performed on the CFX96TM Real-time PCR System (Bio-Rad). qRT-PCR data were calculated from the number of PCR cycles needed to reach the linear phase for each SFT transcript from amirSFT lines and normalized against Ubiquitin using the qbase PLUS Data-Analysis Software.

Supporting Information

Dataset S1 Design of the mRNA-Seq expression profiling experiments, including genotypes, tissues, replicates, total read numbers and mapping rates.

Dataset S2 Global gene expression profiling from two tissue types, 6th young expanding leaf and TM, grouped as percentages of differentially expressed genes in 12 possible gene action categories when comparing sft, sft/+ sp and sft sp. There are five major classes of gene action: additive (semi-dominant), dominant, recessive, overdominant and underdominant. Subcategories for each major class of gene action are represented by cartoon bar graphs. The first sheet shows the summary statistics of classification in two tissues and results of Fisher’s exact tests for significant differences between the percentages in each gene action category. The following sheets show detailed information of the genes, including gene IDs, mean RPKM values, log fold changes for three pairwise comparisons, and P-values from differential expression tests. The 12 gene expression categories are classified based on a threshold of two-fold change and P-value< 0.01 between genotypes. All possible modes of gene action were observed in both tissues.

Dataset S3 Marker genes selected by DDI and used in maturation score estimations all meristem DDI analyses involving the 6th expanding leaf, TM stage, and SYM stage. Included are gene IDs and functional annotations from tomato gene annotation iTAG version 2.3 [76].

Figure S1 sft/+ mutant heterozygosity delays precocious axillary shoot termination in determinate tomato. (A) Compared to sp mutants, sft/+ sp plants show delayed primary flowering time on both basal and proximal axillary shoots similar to the main shoot (Figure 2A). Although no statistically significant (P = 0.11), there is a trend towards a delay on the proximal lateral shoots of sft/+ sp plants (B) sft/+ sp plants produce more sympodial units before sympodial cycling terminates on both basal and proximal axillary shoots, similar to the main shoot (Figure 1B). (C) On both axillary shoots, sft/+ sp plants produce more leaves in the first three sympodial units, indicating a delay in precocious termination similar to the main shoot (Figure 1C). (D) Compared to sp mutants, sft/+ sp plants produce more inflorescences on each plant.
Genotypes and sample sizes are shown below, and error bars indicate standard deviations of averages. Statistical significance was tested by Wilcoxon rank sum test, and significance levels are indicated by asterisks (*P<0.05; **P<0.01; ***P<0.001).

Figure S2 Artificial microRNAs (amiRNA) targeting the SFT and FT genes. (A) Quantitative RT-PCR measurements of tomato SFT transcript levels using primers targeting 3′ to the amiRNA binding site. Note that transcript levels show little or no reduction compared to 5′ of the amiRNA binding site (Figure 5B), consistent with reports of primer-dependent transitivity occurring at the 3′ to 5′ direction upon the initial target cleavage, resulting in degradation of the 5′ cleaved product of the target but not the 3′ product [80,81]. Bars indicate relative expression level and error bars indicate standard deviation among replicates. (B) The At pre-amiR-SFT/FT-4614b and pre-amiR-SFT/FT-4611a sequences that were introduced into the plants along with theoretical representations of the RNA secondary structure. The fold-back structure in each of the sequences is emboldened and the miRNA representations of the RNA secondary structure. The fold-back structure in each of the sequences is emboldened and the miRNA representations of the RNA secondary structure.

Figure S3 Dose-dependent suppression of Arabidopsis thaliana siliques; (D) Total number of floral buds; Note that number of siliques; (B) Total number of axillary shoots; (C) Total number of leaves; (A) Total number of rosette leaves; (B) Total number of axillary shoots; (C) Total number of siliques; (D) Total number of floral buds; Note that number of rosette leaves and siliques showed semi-dominance caused by /+/ heterozygosity. (E) Representative plants from left to right of wild type Ler-0 (WT), ft-2/1 single mutants, ft-1/+/ft-2, and ft-1 single mutants. Like for ft-2, ft-1 mutants are completely epistatic over /ft-2 mutants, and therefore ft /ft-2 double mutants (not shown) are not significantly different from ft single mutants (Figure 6).

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Author Contributions
Conceived and designed the experiments: KJ KLL SJP JPA ZBL. Performed the experiments: KJ KLL SJP JPA. Analyzed the data: KJ KLL JPA. Wrote the paper: KJ KLL ZBL.

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