Newly Discovered Alleles of the Tomato Antiflorigen Gene SELF PRUNING Provide a Range of Plant Compactness and Yield

Min-Sung Kang 1,†, Yong Jun Kim 1,†, Jung Heo 1, Sujeevan Rajendran 1, Xingang Wang 2, Jong Hyang Bae 3, Zachary Lippman 2,4 and Soon Ju Park 1,*

1 Department of Biological Science and Institute of Basic Science, Wonkwang University, Iksan 54538, Korea; duke0357@naver.com (M.-S.K.); youngjun6381@naver.com (Y.J.K.); dandy3745@wku.ac.kr (J.H.); sujeevr@outlook.com (S.R.)
2 Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA; xinwang@cshl.edu (X.W.); lippman@cshl.edu (Z.L.)
3 Department of Horticulture Industry, Wonkwang University, Iksan 54538, Korea; bae@wku.ac.kr
4 Howard Hughes Medical Institute, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA
* Correspondence: sjpark75@wku.ac.kr
† These authors contributed equally to this work.

Abstract: In tomato cultivation, a rare natural mutation in the flowering repressor antiflorigen gene SELF-PRUNING (sp-classic) induces precocious shoot termination and is the foundation in determinate tomato breeding for open field production. Heterozygous single flower truss (sft) mutants in the florigen SFT gene in the background of sp-classic provide a heterosis-like effect by delaying shoot termination, suggesting the subtle suppression of determinacy by genetic modification of the florigen–antiflorigen balance could improve yield. Here, we isolated three new sp alleles from the tomato germplasm that show modified determinate growth compared to sp-classic, including one allele that mimics the effect of sft heterozygosity. Two deletion alleles eliminated functional transcripts and showed similar shoot termination, determinate growth, and yields as sp-classic. In contrast, amino acid substitution allele sp-5732 showed semi-determinate growth with more leaves and sympodial shoots on all shoots. This translated to greater yield compared to the other stronger alleles by up to 42%. Transcriptome profiling of axillary (sympodial) shoot meristems (SYM) from sp-classic and wild type plants revealed six mis-regulated genes related to the floral transition, which were used as biomarkers to show that the maturation of SYMs in the weaker sp-5732 genotype is delayed compared to sp-classic, consistent with delayed shoot termination and semi-determinate growth. Assessing sp allele frequencies from over 500 accessions indicated that one of the strong sp alleles (sp-2798) arose in early breeding cultivars but was not selected. The newly discovered sp alleles are potentially valuable resources to quantitatively manipulate shoot growth and yield in determinate breeding programs, with sp-5732 providing an opportunity to develop semi-determinate field varieties with higher yields.

Keywords: tomato; sympodial growth; semi-determinate; core collection; yield

1. Introduction

Tomato is a major horticultural crop that continues to be intensively bred to improve cultivation-related traits, such as plant height, shoot determinacy, fruit size, and shape, through the selection of beneficial genetic variants [1,2]. Comparative genomics using large-scale genomic resources have identified many genes and alleles that were selected during tomato domestication and modern breeding [3–5]. Exploration of these genetic variations provides insight into the management of quantitative traits in tomato breeding [6]. For example, fruit size enlargement, a major domestication syndrome, originates in part from natural variations in fasciated (fas) resulting in the downregulation of the stem-cell repressing
genes SICLAVATA3 (SICLV3) and locule number (lc), resulting in an expanded expression domain of the stem-cell promoting gene SIWUSCHEL (SIWUS) [7,8]. The improvement of fruit mass under domestication combined the mutations in these two genes, which function in the conserved CLV-WUS negative feedback circuit [9]. Based on the molecular control of the CLV-WUS circuit, cis-regulatory variants of SICLV3 engineered by CRISPR-Cas9 genome editing recreated the effects of fas and lc, and further provided a continuum of fruit size, suggesting that this major domestication and breeding trait can be fine-tuned by expanding allelic diversity in these and other fruit size genes [10].

Genetic pathways that provided inflection points in cultivation-related traits have been well characterized in tomato, thus offering insight into the genetic and molecular changes that drove large-scale field cultivation [11,12]. For more than a century, SELF PRUNING (sp) mutants have precociously terminated tomato shoot growth naturally, and determinate tomato cultivars have been bred to have a shoot architecture suitable for mechanical fruit harvesting and fresh-market field production [13]. SELF-PRUNING (SP), a phosphatidylethanolamine binding protein (PEBP) family gene, was characterized as a positive controller of sympodial growth, which is defined by continuous cycling of vegetative-reproductive shoot units along primary and axillary shoots. This cycling is based on a balance of opposing flowering signals from SP and SINGLE FLOWER TRUSS (SFT) in axillary meristems [14]. Sympodial growth is completely inhibited in strong sft loss-of-function alleles, resulting in reversion of inflorescences to vegetative shoots with sparse production of flowers [15]. SP and SFT are homologs in the florigen gene family. Both of their encoded proteins bind to 14-3-3 adapter proteins in the cytoplasm of SAM cells and translocate into the nucleus, forming florigen activation complexes (FAC) through binding with the bZIP transcription factor SSP (ortholog of Arabidopsis FD) [16,17]. SFT induces the expression of floral transition genes such as homologs of FRUITFUL1 (SIFUL1/TFUL1 and SIFUL2/TFUL2 [18,19]. Conversely, SP was suggested to function in a similar system as FAC, but with the opposite function in tomato [17,20]. Therefore, the balance between SFT and SP expression is a crucial factor in determining the flowering time and growth pattern of tomato sympodial shoots. This balance model suggests that an advanced tomato shoot structure could be improved by finding a new balance between SP and SFT expression [12,15]. Indeed, hybrid studies of sft mutants showed that the partial suppression of shoot termination could increase yield by exploiting new balances of florigen signals in the sp background [21,22]. Moreover, the dosage sensitivity model of florigen was further supported using induced (EMS and genome-edited) mutations in SP, SFT, and SSP in various single-mutant higher-order mutant homozygous and heterozygous combinations [10,17]. For example, engineered promoter alleles of SP induced by the CRISPR/Cas9 system represented a continuum of quantitative variations for sympodial growth depending on the levels of SP expression, which is a new genetic resource to fine-tune and optimize plant determinacy and productivity in distinct breeding programs and agronomic conditions [10].

Since sp-classic has been known dominantly by marker-assisted selection breeding, only sp-classic was used for studying the interaction with modifiers from other genes/alleles that suppress or enhance sympodial growth. This means that we may have missed different sp alleles in the germplasm, which could be functionally stronger or weaker allele than sp-classic.

In this study, we hypothesized that germplasm resources provide an opportunity to find new sp alleles, potentially of different allelic strength, that would allow to find additional ways to tune architecture and yield/productivity. We then screened for and identified previously unknown genetic variants of SP from a collection of determinate genotypes in large tomato core collection (CC). By grouping the classic sp mutant and potential new sp alleles from 242 accessions phenotypes as determinate, we collected as determinate growth lines by genotyping classic sp mutants to isolate new sp alleles. The sympodial shoot growth and tomato fruit harvest of new sp alleles were carefully quantified compared to sp-classic. The molecular state of the new sp mutant was quantified using
molecular markers to show that quantitative differences in determinacy are based on altered meristem maturation, which translates to differences in overall plant architecture and yield between four sp genotypes.

2. Results
2.1. Isolation of New sp Alleles from Core Collections

Recent reports have revealed that the suppression of sp can increase yields in determinate tomato cultivars [17,21]. We hypothesized that weak sp alleles could be isolated from sp allelic variations naturally collected or genetically modified (Figure 1), as a semi-determinate sp allele was selected among the Si^{CR-pro} alleles [10].

![Figure 1](image-url)

**Figure 1.** Tomato plant architecture of sp-classic and hypothetical weak sp allele. (A) Diagrams depicting tomato plant architecture of sp-classic and hypothetical weak sp allele. The dark green bars and ovals represent the primary shoot and associated leaves in the main shoot. Alternating white green and dark green bars and ovals indicate successive sympodial shoots. Arrows indicate axillary shoots and black lines indicate inflorescences. Red- and orange-colored circles represent maturing fruits. (B) Representative main shoots from sp-classic and hypothetical weak sp allele. Three-month-old plants are shown. Arrowheads indicate inflorescences. L, leaf. Scale bars, 5 cm.

To screen for possible new alleles of SP, we selected 242 core collections (CCs) pre-categorized as determinate or semi-determinate by Zamir’s lab. We genotyped them using the sp-classic CAPS marker. Genotyping this subset using PCR with the sp-classic marker revealed 52 CC lines that did not carry the sp-classic mutation (Table S1). In contrast, 47 CC lines showed the same pattern as the SP wild-type genotype, 5 lines showed one band that was smaller than that of the SP genotype, suggesting a deletion event at the SP locus (Figure 2A). Furthermore, phenotyping of sympodial shoot growth validated that 14 out of 52 CCs were phenotypically determinate growth tomatoes (Table S1). The remaining lines showed indeterminate growth, which was either due to the mis-categorization of determinacy or outcrossing with indeterminate genotypes in the open field. To test whether the 14 determinate CC lines were new sp alleles, PCR and Sanger sequencing of the SP promoter and coding region was performed. Five lines were shown to amplify a short PCR product 1 kb upstream and the first exon of SP, and also a 750 bp deletion in the SP promoter region from the middle of the first exon was identified (Figure 2B). Of the remaining lines, five lines amplified a short PCR product on the SP coding region including the 1st and 2nd exon and the 1st intron. This reflected that CCs have an identical 175 bp deletion in the 1st intron region (Figure 2C,D). Surprisingly, the last four CC did not show any difference between the PCR products in the SP promoter and SP coding region, but sequencing identified two synonymous substitutions on the second and third exons, converting lysine to glutamine (D99N) and alanine to aspartic acid (Q128K), respectively.
were allelic for Ubiquitin (sp-2446 valine to leucine (V99L); transcripts were mis-spliced, suggesting that the intronic deletion caused a strong loss-of-function (Figure 2D,G). In contrast, while SP accumulated two small transcripts, indicating a knockout allele (Figure 2F). The 1st intron deletion that showed the promoter deletion allele. From these,

scripts were examined using semi-quantitative RT-PCR from the SAM of 17 DAG seedlings molecular differences in the three newly discovered sp alleles, two deletional sp alleles and amino acid substitutional sp allele. To examine molecular differences in the three newly discovered sp alleles, SP expression and transcripts were examined using semi-quantitative RT-PCR from the SAM of 17 DAG seedlings that showed the promoter deletion allele. From these, sp-2798 failed to produce SP transcripts, indicating a knockout allele (Figure 2F). The 1st intron deletion sp allele, sp-2857, accumulated two small SP transcripts in SYMs. Sanger sequencing revealed that all mutant alleles and wild type (sp-classic) in the shoot apex of SYM stage. Ubiquitin (Ub) transcripts were used as PCR control. M, size marker.

Figure 2. Identification of new sp alleles from core collections: (A) Genotyping by sp-classic CAPS marker. PCR products were digested by ScrFI cutting. SP and sp-classic are controls. Red asterisks, amino acid substitution sp mutants; green asterisks, new deletion sp mutant in SP locus. (B,C) Gel shift of PCR product amplifying 1kb SP promoter including the first exon (B) and amplifying the first exon and intron in SP locus (C). (D) Diagrams showing the SP gene structure and the locations of sp mutations. Dashed black lines indicate deletion regions of sp mutant. Dashed green and gray lines indicate mis-splicing of SP transcripts. red fonts, nucleotide substitution; white boxes, UTR; gray boxes, exon. (E) Partial alignment of SP homologs showing the external loop domain (yellow line box), residues binding to a 14-3-3 protein (red line boxes), and cue residues for florigen/antilflorigen function (blue line boxes). red arrows, sites of mutation substituted amino acid. (F,G) Semi-quantitative RT-PCR analysis of SP expression (F) and PCR amplification of full-length SP transcript including ORF (G) in sp mutant alleles and wild type (SP) at the shoot apex of SYM stage. Ubiquitin (Ub) transcripts were used as PCR control. M, size marker.

In summary, along with complementation tests that confirmed all determinate lines were allelic for sp-classic, all determinate CCs were due to mutations in the SP locus: sp-classic, two deletional sp alleles and amino acid substitutional sp allele. To examine molecular differences in the three newly discovered sp alleles, SP expression and transcripts were examined using semi-quantitative RT-PCR from the SAM of 17 DAG seedlings that showed the promoter deletion allele. From these, sp-2798 failed to produce SP transcripts, indicating a knockout allele (Figure 2F). The 1st intron deletion sp allele, sp-2857, accumulated two small SP transcripts in SYMs. Sanger sequencing revealed that all sp-2857 transcripts were mis-spliced, suggesting that the intronic deletion caused a strong loss-of-function (Figure 2D,G). In contrast, while sp-5732 showed relatively unchanged
SP expression compared to WT plants, the two amino acid changes were in conserved positions among all SP orthologs (Figure 2E,F). In conclusion, based on the combined analysis of SP expression, transcript sequencing, and protein modification, the two deletion mutants sp-2798 and sp-2857 are predicted to knock out SP, whereas the amino acid changes in sp-5732 are predicted to compromise SP protein function.

The discovery of three new sp alleles was surprising, especially given that all known determinate breeding lines and associated hybrid varieties are based on sp-classic. To determine when sp alleles arose and were largely used in breeding, we used resequencing data from 588 diverse tomato genomes to analyze the sp allele frequency. sp-classic was not detected in distantly related wild Solanum species or the progenitor species of domesticated tomatoes (S. pimpinellifolium), consistent with sp-classic first being documented nearly 100 years ago [13]. Indeed, sp-classic is only found in early domesticated genotypes (S. lycopersicum var. cerasiforme) and the ‘vintage’ accessions, which comprise cultivars that diverged approximately 75 years ago [23], reaching near-fixation in the processing cultivars. This suggested that sp-classic emerged during the early stages of modern breeding and were selected in modern processing cultivars (Figure 3, Table S2). Interestingly, we found that new deletion sp allele was detected at low frequencies (0.1-0.2%) in accessions from wild relatives to modern tomato cultivars, indicating that sp-2798 was not selected, and may remain in a state of drift as cryptic variants during tomato breeding (Figure 3, Table S2), perhaps because its strong alleles result in too severe of a determinate growth habit. We also detected sp-5732 with low frequencies (0.01-0.06%) in the first domesticated types and the ‘vintage’ accessions of the early domesticates and cultivars. Notably, this allele is completely absent in modern tomato cultivars, suggesting that marker-assisted selection may have eliminated sp-5732 due to preference for a moderate effect on determinacy from sp-classic allele that breeders use for breeding fresh market and processing cultivars (Figure 3, Table S2).

![Figure 3](image-url)

**Figure 3.** Allele frequencies of sp alleles in accessions classified as wild Solanum species (distant relatives and S. pimpinellifolium, the wild progenitor of domesticated tomato), early domesticates and cultivars (S. lycopersicum var. cerasiforme and S. lycopersicum vintage), and modern cultivars (fresh-market and processing). Number of accessions is indicated in parentheses.

### 2.2. Comparisons of Shoot Structure and Yield Harvest among sp Mutants

To test the idea that the three sp alleles provide a range of determinacy, we performed comparative studies of sympodial shoot termination and yields. To directly compare shoot growth and termination among sp variants, the new sp alleles were introgressed into the processing cultivar M82 by backcrossing at least three times (BC2F2 or BC3F2) to establish near isogenic lines (NILs). We then quantified leaf numbers from the reference sp-classic and the new sp alleles on the primary shoot meristem (PSM) and successive SYMs on the main and axillary shoots. Shoot termination at the shoot apices of the three-month-old plants was
also recorded (Figure 4A). Primary shoots accounted for seven to nine leaves in all sp alleles, indicating a similar flowering time in PSM among sp alleles (Figure 4B). sp-2587 and sp-2798 did not show much difference in the leaf number produced by the main shoot compared to the sp-classic, and no significant differences were observed for the leaf number produced in the axillary shoot compared to the sp-classic (Figure 4C,D). Although all shoots eventually terminated in the mature plants of all the sp genotypes, the leaf numbers of the main shoot and axillary shoot were higher in sp-5732 compared to sp-classic (Figure 4C,D). These results indicate that overall flowering time and sympodial shoot cycling and termination is weaker in both primary shoot and axillary shoot systems compared to sp-classic, similar to the effects of sft and ssp heterozygosity (sft-4537/+ ssp2129/+, respectively) [17].

**Figure 4.** Comparison of flowering time with leaf numbers on the primary and sympodial shoot among sp alleles. (A) Diagrams depicting sympodial shoot growth on the main and axillary shoot in determinate tomato. Alternating dark green and light green bars represent the primary and successive sympodial shoots in main shoot. Alternating black and gray bars indicate the successive sympodial shoots in an axillary shoot. Ovals on the colored bars indicate leaves produced in each shoot. Red- and orange-colored circles represent maturing fruits. (B) Quantification and comparison of flowering time in the main shoot in three sp mutant alleles. sp-classic segregated from BC2 or BC3F2 generation was used as the control. (C,D). Quantification and comparison of leaves produced by five successive sympodial-shoot initially produced by primary shoot (C) and by axillary shoot (D). Mean values (± s.d) of leaves produced by each sympodial shoot (alternative grey and white color bar) were compared to those for sp-classic and sp allele segregated. The sum of the leaves from five sympodial shoots was used to test statistical significance in (C,D). D, terminated shoot; p values determined via two-tailed, two-sample t-test; * p value < 0.05.
We next compared fruit yield among three sp variants; homozygous mutants from BC4F3 generations of sp-2798 and sp-5732 were compared with the control sp-classic. All three genotypes were grown under field conditions with controlled watering and nutrients. Shoot growth and termination of the genotypes grown under field conditions were identical to previous results, with more lateral organs and delayed flowering time and sympodial cycling and termination in sp-5732 (Figure S1). Notably, the semi-determinacy of sp-5732 translated to higher overall yield, with significantly increased plant mass, red fruits, and total fruit harvests, compared to other sp genotypes (Figure 5A–E). The Brix value, representing sugar content, was also increased in sp-5732 (Figure 5F). The Brix yield of sp-5732 increased by more than 50% with better fruit quality than that of sp-classic (Figure 5G). We validated these findings in a second yield trial, which showed a consistent yield increase of more 40% in sp-4537 compared to the sp-classic in the open field (Figure S2).

Figure 5. Variations in crop productivity induced by sp alleles at field. (A) Representative plant size and yield from sp-classic as control, sp-2798, and sp-5732. (B–G) Statistical comparisons of mean values (±S.E.M.) for plant weight (B), red fruit weight (C), green fruit weight (D), total yield (E), Brix (F), and Brix yield (G) from sp-classic (black bars), sp-2798, and sp-5732 (white bars). Asterisks indicate significantly different yields. Different letters indicate significant differences between samples according to a one-way ANOVA followed by Tukey’s HSD post-hoc test (p < 0.05). Asterisks indicate significant differences with sp-classic by Tukey–Kramer test; * p value < 0.05, ** p value < 0.01. n, number of replicates.

2.3. Comparisons of Molecular States Using DEG Markers

Gene expression biomarkers are promising tools for better monitoring plant states for crop yield. To isolate the biomarkers for quantification of the molecular state of sympodial shoot meristems (SYM) between indeterminate and determinate growth, we compared the transitional meristems (TM) and SYM transcriptomes of SP and sp-classic profiled in our previous studies (Figure 6A,B) [17,24]. We previously defined TM as the stage of primary shoot meristem (PSM) switching to the reproductive phase and shows a broader and taller dome shape than vegetative stage PSM with a smaller last formed leaf (Figure 6A). SYM is a specialized axillary meristem in sympodial growth plant, which develops in the axil of the last leaf on the PSM and terminates after producing only three leaves under SP but producing zero to two leaves in sp-classic (Figure 6B). Notably, TM and SYMs were
collected and profiled at precisely matched morphogenic points in our previous studies (Figure 6A,B) [17,24]. Based on the normalized read counts of cDNA sequences (ITAG3.0), we identified 811 ‘TM DEGs’ between TMs of the genotypes, and 520 ‘SYM DEGs’ between SYMs of the genotypes; and a total of 984 ‘total DEGs’ between TM and SYM paired with genotypes using DESeq2 with cut-off criteria: log$_2$ fold-change $\geq$ 2, false discovery rate (FDR) < 0.05, and fragments per kilobase million (FPKM)/sample $\geq$ 3 (Tables S3–S5) [24].

To explore the DEGs more deeply according to gene expression pattern, the DEGs were clustered into seven (I–VII) according to hierarchical expression in two tissues of the genotypes using hclust (Figure 6C; see Methods). Clusters I–IV (520 genes) were grouped as ‘Single DEGs’ containing genes differentially expressed in either TM or SYM. Cluster V–VII (464 genes) were grouped as ‘Co DEGs’ containing genes differentially expressed in both TM and SYM (Figure 6C, Table S5). Therefore, these five groups of DEGs all indicate the patterns of DEG expression.

To biologically categorize each DEG into the five groups, we performed gene ontology (GO) enrichment analysis using the PANTHER classification system [25]. Single DEGs showed high enrichment for protein folding, developmental processes, single-multicellular organism processes, and multicellular organism processes GO terms in biological processes (Figure 6D). Specifically, the protein folding term was highly enriched in TM DEGs, but the remaining terms were enriched in SYM DEGs, reflecting tissue-specific functions. Notably, TM DEGs and Co DEGs were highly enriched in terms of biosynthetic processes, ribosome formation, and nuclear transport, reflecting overall differences in the development of SP and sp-classic (Figure 6D, Table S6). Regarding molecular function, the terms for DNA binding, transcription, and DNA binding RNA polymerase were highly enriched in single DEGs and SYM DEGs, which is consistent with the enrichment of the nuclear term of the cellular component. This reflects a high difference in the regulation of transcription in SYMs between SP and sp-classic (Figure 6D, Table S6). Altogether, SYM DEGs were functionally enriched in the developmental processes and transcription regulation, indicating SYM-specific functions.

To monitor the molecular state of sp alleles, we first isolated 55 SYM DEGs categorized under the development process and regulation of nucleic acid-templated transcription, which are major enriched GO terms, and then selected six biomarkers according to molecular functions related to the potential downstream genes of FAC, sympodial growth, and strong expression difference in sp-classic (Table S7). The 55 DEGs were comprised of the potential targets of FAC, such as SIFUL1/TFUL1, SIFUL2/TFUL2, MADS-BOX PROTEIN 20 (MBP20), the transcription factors (TF) controlling sympodial shoot and inflorescence structure at the post-transition stage such as BLIND [26], LONG INFLORESCENCE (LIN), JOINTLESS 2 (J2), and ENHANCE OF JOINTLESS 2 (EJ2) [27], and the genes physically interacting with FAC targeting MADS TFs such as RIPENING INHIBITOR (RIN), J2, and EJ2 (Table S7) [18]. Functionally unknown TFs, such as MYB, NT-FA, AP2, AT-hook motif, homeobox, and cold-shock-domain TFs were also characterized as highly downregulated or upregulated genes in the SYM of sp-classic. Therefore, two FAC downstream genes (SIFUL2 and MBP20), two genes related to sympodial growth at the post-transition stage (J2 and EJ2), and two functionally unknown and downregulated TFs (Solyc12g009050 and Solyc01g006930) in sp-classic were selected as biomarkers for comparison of the molecular stages of SP alleles (Table S7).
Figure 6. Hierarchical clustering analysis and GO term enrichment assay using DEGs between SP and sp-classic. (A,B) Microdissection of the TM stage (A) and SYM stage (B) was used for RNA extraction [24]. Dashed lines indicate dissected tissue lines. (C) Hierarchical clustering of DEGs at the TM and SYM stages between SP and sp-classic. Clustering is visualized by heatmap, and seven clusters (I–VII) are grouped based on the dendrogram. The FPKM expressions are normalized to row-wise Z-Scores. (D) Enrichment of gene ontology functional analysis of DEGs. -Scaled -log_{10}(p values) are shown in the heat map (Table S7). ‘Single’ indicate DEGs showing differential expression in only one meristem stage. ‘Co’ indicate DEGs on both meristem stage. Red fonts indicate the major terms in functional analysis of ‘Single’ DEGs group.

To quantify the molecular states in the SYM of sp-5732 between indeterminate and determinate sympodial growth, we dissected the SYMs of SP, sp-classic, and sp-5732 using a stereoscope (Figure 7A). Notably, meristem morphology of SYM was nearly indistinguishable among three SP alleles, with vegetative stages appearing as small dome structures producing the two leaf primordia (Figure 7A). The expression of biomarkers in the SYMs of SP and sp-classic provided a calibration stage between indeterminate and determinate
symodonal shoot growth, thus enabling direct comparisons of the SYM states in sp-5732 using real time quantitative RT-PCR (qRT-PCR) (Figure 7A–C). The expression patterns of all biomarkers were identical to the differences seen in the FPKM between SP and sp-classic.

In the SYM of sp-5732, qRT-PCR results indicated that the expression of all biomarkers, including SP, was intermediate between the values of SP and sp-classic. MBD20 and SlFUL2 expression was upregulated to approximately half of the sp-classic value in the SYM of sp-5732 (Figure 7D,E). J2 and EJ2 were weakly expressed and slightly upregulated in the SYM of sp-5732 compared to that in SP, reflecting that the SYM of sp-5732 is in the pre-transitional stage before sympodial growth and termination, like the wild type (Figure 7F,G). Solyc12g009050 and Solyc01g006930 were downregulated to nearly average values between SP and sp-classic in SYM of sp-5732, indicating that the biomarkers may play a role in the phase transition to the floral stage in SYM and confirming that the selected biomarkers successfully evaluated the maturation states of other types of SYM (Figure 7H).

![Figure 7](image-url)
3. Discussion

3.1. Allelic Variations in SP and Delay of Meristem Maturation and Sympodial Shoot Termination

An important goal of crop genetics and genomics is to identify all allelic variation in key productivity genes and establish quantitative genotype-to-phenotype relationships on growth and development to assess their value in breeding programs [28]. Natural variation in tomato SP and its homologs in many crops have been central in agronomic adaptions and yield enhancements in many crops, serving as inflection points that transformed indeterminate growth to determinate compact architectures. In tomato, this major change occurred within the last century, as is based on reduced SP activity mitigating the inhibition of florigen-based SFT signals in the sympodial shoot system. That all-determinate breeding in tomato appears to be based on the single allele of sp-classic suggests that breeders selected for a specific modified balance of florigen–antiflorigen (SFT-SP) signals to provide a level of determinacy that optimizes productivity in open field processing and fresh market production systems. Alternatively, sp-classic dominance in breeding may simply be serendipitous, and breeders have been fine-tuning determinacy with second site modifiers to adjust growth habit and yield over the last 100 years. In this regard, the prolonged SYM maturation—and thus extended sympodial cycling—of sp-5732, unlike other sp alleles, could be an as yet unrealized variant to improve yield by providing predictable breeding of semi-determinate growth.

Screening for sp alleles using determinate CC lines revealed three new sp alleles. Two deleterional alleles were knock-out mutants showing no expression of SP in sp-2798, and expressing shortened and truncated transcripts due to mis-splicing of SP transcripts in sp-2587. Amino acids substituted in sp-5732 and sp-classic were highly conserved in CETS proteins but were not key amino acids binding with 14-3-3 and were not distinct FLOWERING LOCUS T (FT) and TFL1-like homologs, indicating that structural variations of SP could indirectly and negatively affect the functions of antiflorigen (Figure 2E). Although we could not clearly explain the differences in functional defects among sp alleles, including between sp-5732 and sp-classic, allelic variations in sympodial structure showed that sp-5732 produced more leaves in each SYM and could produce more sympodial shoots in the plants than other sp alleles, reflecting that sp-5732 delayed maturation in SYMs and termination of sympodial cycling is due to a weak allele effect relative to the other three alleles. Thus, sp-5732 is similar to CRISPR-Cas9 engineered SP promoter alleles that provided a continuum of sympodial shoot termination, from modified indeterminate growth to semi-determinate and determinate growth [10]. Specifically, one sp promoter allele resulted in semi-determinate growth, also producing more leaves than the sp-classic and occasionally re-initiated sympodial shoots on primary and side shoots [10]. In support, comparisons of sympodial leaf production and biomarker expression among all natural sp alleles indicate that sp-5732 has a prolonged vegetative phase during sympodial shoot maturation compared with that of sp-classic (Figures 4 and 5).

3.2. Molecular Changes in Sympodial Shoot Termination

The molecular basis of local SFT/SP balance in sympodial shoot termination is the target competition system in which SP competes with SFT to bind with 14-3-3 to assemble FAC [20]. Our transcriptome analysis isolated SYM-specific upregulated genes containing SIFUL1/TFL1, SIFUL2/TFL1, and MBD20 in sp-classic, which are known as the potential direct targets of FAC, reflecting anti-FAC (SP:14-3-3:SSP) suppressed FAC target genes and phase transition (Figure 7D,E). Significant upregulation of J2, EJ2, LIN, and RIN in the sp-classic indicates that the SYM stage takes place during or after transition as J2, EJ2, and LIN are markers of sympodial inflorescence development, and RIN functions in fruit ripening after the transition to the floral stage (Table S7). Conversely, two novel NF-YAs were significantly downregulated in sp-classic, indicating that NF-YAs play a role in the suppression of floral phase transition. These results suggest that the overexpression of NF-Y subunits such as NF-YA, NF-YB, and NT-YC alters flowering time and that NF-Y complexes regulate floral transition by highly redundant and complicated mechanisms.
in Arabidopsis [29]. In this respect, the state of SYM-producing second leaf primordium was molecularly evaluated as a state during or after floral transition in *sp-classic*, reflecting the precocious termination of the sympodial shoot, whereas wild-type SYM is still in the vegetative stage, indicating indeterminate reiterations of sympodial shoots.

### 3.3. Crop Performance through Modulation of the Antiflorigen/Florigen Signals

Late-flowering Micro-Tom variants rearranged with a continuum of flowering time indicated the scope for genetic resources with high biomass and fruit harvest under *sp* background [30]. Our yield trials using the newly discovered *sp* alleles and *sp-classic* indicated that moderate suppression of *sp* determinate growth enhanced the harvest of biomass and fruits. *sft* mutations as homozygotes genetically suppress sympodial shoot development in the main and axillary shoots of *sp* mutants, indicating the complete epistasis of *sft* over *sp* [15,31]. This epistasis is dosage dependent, as *sft/+* heterozygotes partially suppress *sp-classic* determinacy. Homozygous *ssp* mutants that disrupt FAC activity also suppress *sp* determination into indeterminate growth of the main and axillary shoots, as do heterozygotes in a similar dosage-dependent manner as *sft* alleles [17]. Across all these single and double mutant homozygous and heterozygous genotypic combinations, the flowering time of sympodial shoots was delayed, as reflected, for example, in the delayed expression of downstream MADS TFs expression, which are targeted by FACs [18]. Furthermore, double hybrids genetically combined with FAC components showed a progressive increase in overdominant fruit harvest due to an even greater quantitative suppression of sympodial shoot cycling and termination, suggesting that mutations in the florigen pathway could provide a broad toolkit to boost crop productivity [17]. As elaborated above, engineered *SP* promoter alleles mimic *sft/+* and *ssp/+* effects on determinacy, providing another route to achieving semi-determinate growth and potentially higher-yielding cultivars [10]. Here, we have added to this toolkit by identifying the new amino acid substitution *sp-5732* allele, which showed greater biomass and fruit harvest under field growth conditions.

Finally, our *sp* allele frequency assay indicated that the *sp-classic* was mainly used for breeding modern tomato cultivars. However, other alleles have cryptic variations that are hidden resources of natural variations. We reported that *sp-4537* improves fruit harvest under determinate growth. We strongly suggest breeders take advantage of *sp-4537* and other alleles to cultivate high-yielding modern processing tomatoes with optimizing plant determinacy.

### 4. Materials and Methods

#### 4.1. Plant Materials and Genotyping

Tomato seeds of 242 CC inbred lines were obtained from Zamir’s lab at the Hebrew University of Jerusalem. All plants were grown using a water irrigation system at the farm of the Cold Spring Harbor Laboratory and Wonkwang University from April to July. *sp-classic* genotyping markers were used to screen for wild-type *SP* among the CC lines (Table S8). *SP* CDS were amplified and sequenced to isolate the other *sp* alleles. To transfer new *sp* alleles into the tomato cultivar M82, the CC lines were backcrossed more than three times with cultivar M82. The new *sp* alleles were genotyped using their genotyping markers (Table S8). The progeny of BC3F2 or BC2F2 were used for phenotyping, and the progeny of BC3F3 and BC3F4 were used for all yield trials in this study.

#### 4.2. Tissue Collection, RNA Extraction, and RT-PCR

To extract total RNA from *sp-classic*, *sp-2798*, and *sp-5732*, shoot apices were collected between 13 and 17 days after germination (DAG) from plants grown in a greenhouse. As defined in a previous report [24], transitional meristems (TM) and sympodial shoot meristems (SYM) were imaged using a stereoscope. More than 30 meristems were dissected and collected from shoot apices fixed by acetone fixation for RNA stabilization, as previously reported [24]. Total RNA was extracted using the PicoPure RNA Extraction kit (Arcturus) and treated with the RNase-Free DNase Set (Qiagen, Valencia, CA, USA),
according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis using ReverTra Ace-α® (TOYOBO, Osaka, Japan). RT-PCR was performed using i-Taq™ DNA Polymerase (Intron) and a T100™ Thermal Cycler system (Bio-Rad, Hercules, CA, USA). Real-time quantitative RT-PCR (qRT-PCR) was used to verify the expression of biomarkers in the sp alleles. Two biological replicates of TM and SYM were used for qRT-PCR, and the expression values were analyzed using the CFX96TM Real-time PCR System (Bio-Rad, Hercules, CA, USA). The threshold cycle (Ct) values were calculated and normalized against Ubiquitin using the StepOne™ software v2.3 (Applied Biosystems, Ltd., Waltham, MA, USA). Primer information is shown in Table S8.

4.3. Yield Trials under Agricultural Field and Greenhouse

Yield trials were conducted in the Department of Horticulture Industry and in the tomato field at Wonkwang University in 2017 and 2019, as previously described [17]. Seedlings were grown in a greenhouse for 35–40 days and transplanted to the field and greenhouse at the beginning of April. Yield experiments were conducted under wide (1 plant per 0.75 m²) between plants using one irrigation and fertilizer regimes. Each genotype of the sp allele was represented by at least 16 biological replicates in the first trial and 11 replicates in the second trial. All the plants were transplanted in a completely randomized design. Damaged or diseased plants were excluded from the analyses.

4.4. Statistical Analyses of Yield Related Traits and Flowering Time

Harvesting was conducted in the middle of August in 2017 and 2019, when the majority of plants in a trial had over 80% red fruit. Phenotypic measurements of total fruit yield per plant, total number of inflorescences, and plant weight were taken after plants were manually removed from the root. Red and green fruits were collected as mature and immature fruits, and the total fruit yield was the sum of each plant’s red and green fruits. Ten fruits were randomly selected to estimate the average fruit weight and total soluble solids content (mainly sugars). The latter was referred to as the Brix value and was measured using a digital Brix refractometer (ATAGO). Brix yield was the total Brix content in the total yield per plant. Mean values for each measured yield parameter were analyzed using the “Fit Y by X” function and statistically compared using a Tukey-Kramer multiple comparison test, Dunnett’s ‘compare with control’ test, or t-test, whenever appropriate. At each time point, individual replicate plants were dissected for selected component traits of yield (plant weight, total fruit yield, Brix value, fruit weight, inflorescence number, and flowers per inflorescence). Flowering time was indirectly measured using the leaf number produced by the primary and sympodial shoots. Shoot termination was decided using the main shoot 30 day after transplantation. Shoot termination and flowering time were analyzed using data from a minimum of 12 biological replicates for each genotype.

4.5. RNA-seq Analysis

The RNA-seq data published in previous studies [17,24] were used for the analysis of differentially expressed genes between TM of sp-classic and wild-type plants, and between SYM sp-classic and wild-type plants. The paired-end reads of 2 replicates in SP_TM, sp_TM, sp_SYM, and SP_SYM were downloaded from the following address (sp_TM, sp_SYM, and SP_SYM, https://solgenomics.net/ftp/transcript_sequences/by_species/Solanum_lycopersicum/libraries/illumina/LippmanZ/; SP_TM, SRP090200 (SRA) (accessed on 27 June 2022). Low-quality reads were filtered using Trimmomatic_v0.36 software [32] for primer, adapter, and low-quality sequences. The following parameters were used: ILLUMINACLIP:path/Trimmomatic-0.36/adapters/TruSeq2-PE.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, MINLEN:36. Finally, the filtered reads were confirmed to be of high quality using FastQC v0.101.1 software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, accessed on 27 June 2022). All filtered reads were aligned against annotated cDNAs from tomato ITAG3.0; http://solgenomics.net/organism/solanum_lycopersicum/genome, accessed on 27 June 2022) using the short-
read mapping software Bowtie v2.26 [33], and the abundance of each transcript was estimated with FPKM values using RSEM v1.2.31 [34] with default parameters. The reads of the sp_SYM were split and aligned. The genes in our samples and the raw read counts of the genes served as the foundation for further analysis.

4.6. Differential Expression Gene and Gene Ontology Analysis

Statistical tests of differential gene expression based on raw read counts between each pair of samples involving two stages of M82 tomato meristems (TM and SYM) were conducted using R. Replicates were used in a modified exact test implemented by DESeq2 v1.26.0 [35] to test differential expression in two sample comparisons based on raw read counts. The p-values attained by the Wald test were corrected for multiple testing using the Benjamini and Hochberg’s procedure. As a result, differentially expressed genes achieved greater than log2 two-fold change with FDR < 0.05, and then genes showing less than average 3 FPKM/sample (summed over all stages) were removed. The filtered DEGs with FPKM values were hierarchically clustered using ‘hclust’ from the stats R package (R Core Team, 2019). To biologically categorize each DEGs of the five groups, GO enrichment analysis was performed using the PANTHER classification system (http://geneontology.org, accessed on 27 June 2022) with DEGs showing the expressions of log2 over four-fold change in the filtered DEGs, and enriched GO terms were selected with p-value < 0.01.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23137149/s1.

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