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The *SELF-PRUNING* gene of tomato regulates vegetative to reproductive switching of sympodial meristems and is the ortholog of *CEN* and *TFL1*

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SUMMARY

Vegetative and reproductive phases alternate regularly during sympodial growth in tomato. In wild-type 'indeterminate' plants, inflorescences are separated by three vegetative nodes. In 'determinate' plants homozygous for the recessive allele of the *SELF-PRUNING (SP)* gene, sympodial segments develop progressively fewer nodes until the shoot is terminated by two consecutive inflorescences. We show here that the *SP* gene is the tomato ortholog of *CENTRORADIALIS* and *TERMINAL FLOWER1*, genes which maintain the indeterminate state of inflorescence meristems in *Antirrhinum* and *Arabidopsis* respectively. The *sp* mutation results in a single amino acid change (P76L), and the mutant phenotype is mimicked by overexpressing the *SP* antisense RNA. Ectopic and overexpression of the *SP* and *CEN* transgenes in tomato rescues the 'indeterminate' phenotype, conditions the replacement of flowers by leaves in the inflorescence and suppresses the transition of the vegetative apex to a reproductive shoot. The *SELF-PRUNING* gene is expressed in shoot apices and leaves from very early stages, and later in inflorescence and floral primordia as well. This expression pattern is similar to that displayed by the tomato ortholog *LEAFY* and *FLORICAULA*. Comparison of the sympodial, day-neutral shoot system of tomato and the monopodial, photoperiod-sensitive systems of *Arabidopsis* and *Antirrhinum* suggests that flowering genes that are required for the processing of floral induction signals in *Arabidopsis* and *Antirrhinum* are required in tomato to regulate the alternation between vegetative and reproductive cycles in sympodial meristems.

Key words: Growth habit, Reproductive switching, Sympodial shoot, Determinate meristem, *SELF-PRUNING* (*SP*), *CENTRORADIALIS* (*CEN*)/*TERMINAL FLOWER1* (*TFL1*), Tomato

INTRODUCTION

Shoot development in flowering plants is a continuous process ultimately controlled by the activity of the shoot apical meristem (Sussex, 1989). The growth habit of plants is defined by the pattern of vegetative and reproductive appendages arising along the shoot axes and by the way in which lateral branches arise. It determines, therefore, yield and other agronomic traits of a given crop plant, be it annual or perennial. Shoots of all higher plants feature one or other of two basic growth habits, monopodial or sympodial. Each is found also in families of the most primitive plants, such as liverworts, mosses and cycads (see Bell, 1992 for review).

In *Arabidopsis* and *Antirrhinum*, two photoperiod-sensitive monopodial model plants, the vegetative shoot apical meristem gives rise to leaves until the appropriate photoperiodic cues result in the transition to inflorescence development (Bradley et al., 1996b; Shannon and Meeks-Wagner, 1993; Ma, 1998). During the inflorescence phase, the same shoot apical meristem continues the sequential initiation of inflorescence branches and solitary flowers (with or without subtending bracts), resulting in a single cycle consisting of vegetative, inflorescence and floral phases, with a clear separation of the vegetative and reproductive phases.

By contrast, in the sympodial shoots of the day-neutral tomato plant the vegetative and reproductive phases alternate regularly. The primary (juvenile) shoot is terminated with a cymose inflorescence after the production of 8-12 leaves. Growth then continues from the uppermost lateral (axillary) bud just below the inflorescence (Figs 1, 2A). This shoot then generates three more leaves before terminating in turn with another inflorescence, and so on. The shoot is thus composed of an indefinite number of reiterated sympodial units each consisting of three vegetative nodes and a terminal inflorescence. Each unit arises from the most proximal vegetative node of the preceding unit. The seemingly upright continuity of the tomato stem results from the new sympodial segments displacing each inflorescence, via more vigorous growth, into lateral positions (see Sawhney and Greyson, 1972; Silvy, 1974; Atherton and Harris, 1986; for descriptions of the

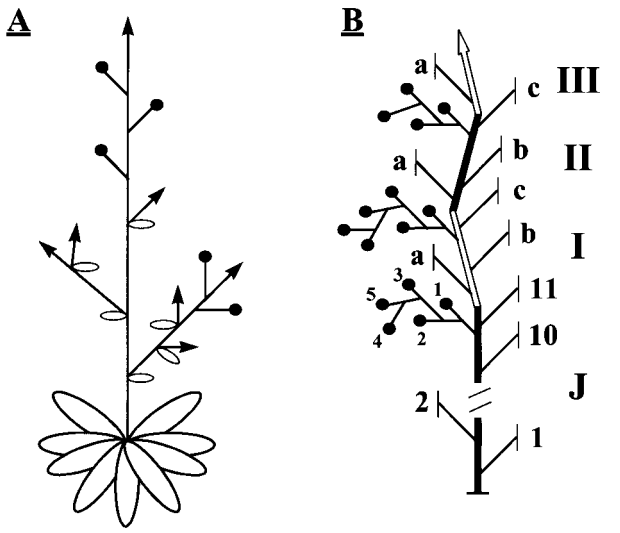


Fig. 1. Shoot architecture of *Arabidopsis* and tomato. (A) Monopodial organization of *Arabidopsis* shoots**.** The indeterminate vegetative apex generates leaves on its flanks before changing to an indeterminate floral apex that extends indefinitely (arrow) as flowers are now generated in succession upon its flanks. Side arrows indicate coflorescences arising in the axils of cauline leaves and black circles represent solitary flowers. (B) Sympodial organization of tomato shoots. The primary vegetative shoot (J, leaves 1-11 in this example) is terminated by a flower. Subsequently, a vegetative shoot arises in the axil of the leaf just below the inflorescence. This first sympodial segment unites with the basal part of the leaf that subtends it thus placing it above the inflorescence and in addition displacing the inflorescence sideways. Reiterated units consisting of three nodal leaves (a, b, c in sympodial sections I and II) and a terminal inflorescence, are then generated indefinitely. New flowers (black circles) arise successively to the side of each earlier arising flower in a zig-zag pattern to generate the scorpioid inflorescence.

tomato system, and Child, 1979; Weberling, 1989 for a general discussion of the sympodial habit). It has been suggested that the more advanced monopodial shoot evolved from the sympodial pattern by reduction of side branches, while the sympodial shoot, in turn, evolved from earlier primitive dichotomous branching models via sequential loss of branching (Stewart, 1964).

In this study we begin to dissect the genetic system that regulates growth habit and alteration of phases in the sympodial shoot of tomato by describing the cloning, expression and some genetic interactions of the *SELF-PRUNING* (*SP*) gene. A recessive allele of the *SP* gene confers accelerated termination of sympodial units by the inflorescence, resulting in a limited growth of the shoot, a bushy, compact constitution and nearly homogeneous fruit setting (Yeager, 1927; MacArthur, 1932; Went, 1944; Calvert, 1965; Picken, 1986; Atherton and Harris, 1986). The recessive *sp* gene was the single most important genetic trait in the development of modern agrotechniques for this crop plant, because the ‘determinate’ growth habit facilitates mechanical harvest.

A principal assumption of our work is that *Arabidopsis*, *Antirrhinum* and tomato employ similar genes to regulate their growth habits, but that these genes operate in different meristematic contexts: monopodial, indeterminate and photoperiod-sensitive in the former two species, and sympodial, determinate and day-neutral in the latter (Hareven et al., 1996; Parnis et al., 1997). The very same gene may, therefore, display altered expression patterns and, when mutated, perhaps a different range of phenotypic alterations. In such cases a better and more comprehensive understanding of gene function would result from the study of complementary plant systems.

We report here that the tomato *SELF-PRUNING* gene is the functional ortholog of the *CENTRORADIALIS (CEN*) gene in *Antirrhinum* and the *TERMINAL FLOWER1 (TFL1)* gene of *Arabidopsis* that have recently been cloned (Bradley et al., 1996a; 1997). Mutations of either *TFL1* or *CEN* cause production of an inflorescence with fewer flowers and a terminal aberrant flower, whereas wild-type plants normally develop an indeterminate inflorescence lacking any terminal differentiation (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1996a). Mutations in *TFL1* (*Arabidopsis)* but not in *CEN* (*Antirrhinum*) also impart early flowering (Shannon and Meeks-Wagner, 1991). The *CEN* and *TFL1* genes are thought to be negative regulators of the *FLORICAULA (FLO)* and *LEAFY (LFY)* genes, respectively (see Ma, 1998 for review). Mutations in *FLO* and *LFY* condition the conversion of early floral meristems into leafy shoots, while over-expression of *LFY* results in early flowering in *Arabidopsis* and other species (Weigel and Nilsson, 1995).

It has been suggested that a modified expression of the *CEN/TFL1* gene may be responsible for the diversity of inflorescence structures among plant species (Alvarez et al., 1992; Bradley et al., 1996a). The present analysis of the *SELF-PRUNING* gene demonstrates that the same gene system that decides the fate of inflorescence meristems in *Arabidopsis* and *Antirrhinum* controls, in tomato, the determinacy of sympodial meristems and thus the processes in which vegetative and reproductive shoots alternate.

MATERIALS AND METHODS

Plant material

The following tomato (*Lycopersicon esculentum*) lines were provided by Dr C. M. Rick, University of California Davis: *an/+* (LA536), *tmf* (LA2462), *blind* (LA59) *sft* (LA2460), VFNT-Cherry *SP* (LA2756), VFNT-Cherry sp^2 (LA2705). M82 sp^1 and wild-type 93-137 are ‘determinate’ and ‘indeterminate’ lines respectively, grown in our laboratory. Confirmation of double mutant genotypes was conducted by regular test crosses. Plants were grown in air-conditioned glass houses at 20°C and 25°C night and day temperatures respectively. Light conditions were not strictly controlled.

Transgenic plants

The *SP* and *CEN* cDNA clones were inserted in both sense and antisense orientations into pCGN1548 to be expressed under the 35S CaMV promoter (Benfey and Chua, 1990). RK9/8 *sp* (Pnueli et al., 1994; Hareven et al., 1996), *an/an:sp/sp* and *an/+:sp/+* plants for transformation were maintained in culture vessels using cuttings. The latter two lines were derived from selected readily transformable F_2 lines derived from a cross of RK9/8 *sp/sp:*+/+ × +/+*:an/an*. Leaf disc transformation was conducted essentially according to Horsch et al. (1985) and McCormick (1991).

Cytological procedures

For scanning electron microscopy we followed the procedure of Alvarez et al. (1992). In situ hybridizations with digoxigenin-labelled RNA probes were done according to the manufacturer's procedure (Boehringer Mannheim) as referred to in Pnueli et al. (1994) and Hareven et al. (1996). Antisense and sense cRNA probes were generated with the T3 and T7 polymerases from the opposing promoters of the BlueScript (SK+) vector (Stratagene). No specific signals were observed when sense RNA probes were employed. As a positive control, the tomato gene encoding the small subunit of ribonucleotide reductase (RNR; Lifschitz and Egea-Cortines unpublished) was subcloned in the same vector, and its anti-sense cRNA used for hybridization. RNR is regulated by the cell cycle, marking cells in S phase, and the expected scattered signal confirmed the technical success of the procedure (results not shown).

Molecular techniques and material

A genomic library of tomato was prepared from M82-*sp*^{1} DNA in the λ FIX vector (Stratagene) using partial *Sau*3A digest. The genomic library represented 1.4×10^{6} independent clones. cDNA libraries were prepared in the λ ZAPII vector (Stratagene) from mRNA of wild-type apices, about 0.5 cm long, containing the second and third sympodial segments, and also from *anantha* inflorescences. Preparation of RNA, DNA blotting, PCR techniques and sequencing were carried out according to established procedures (Ausubel et al., 1988).

RESULTS

Development of the sympodial shoot and inflorescence in wild-type and *sp* mutant plants

The sympodial unit of the tomato shoot is by definition determinate (i.e. its growth is terminated when it differentiates into a flower), yet the wild-type growth habit is classified as 'indeterminate' in reference to the continuous production of an unrestricted number of sympodial units (Figs 1B and 2A), rather than the developmental status of the apical meristem proper. 'Determinate' *self-pruning* (*sp)* mutant plants are so-called because a limited number of sympodial shoots arise before further extension of the main apex ceases. This does not occur straight away, but the number of vegetative nodes arising on successive sympodial shoots is gradually reduced from three to two to one, until the vegetative phase is by-passed completely with the production of two successive inflorescences (Fig. 2B; Yeager, 1927; MacArthur, 1932). We have also found that the 'determinate' habit is enhanced in lateral branches which develop from the more proximal axillary buds of the sympodial segments. Thus the *sp* mutant does not alter the overall sympodial architecture of the plant, but it disrupts the regularity with which vegetative and reproductive phases alternate.

As first reported by MacArthur (1932), *sp* does not accelerate the appearance of the first inflorescence that terminates the juvenile primary shoot. The number of leaves to the first inflorescence varies in different genetic backgrounds and under different physiological conditions but it is always similar in sibling *SP* and *sp/sp* plants. Observations of 40 plants from each of the two isogenic derivatives of VFNT Cherry used in our work have confirmed that in both *SP/SP* and *sp*^{2}*/sp*^{2} lines the first inflorescences appear after 10-12 leaves are produced. In the same isogenic lines we have found, however, that the nodes between leaves of the 'determinate' isogenic line are invariably 10-15% shorter than their 'indeterminate' counterparts throughout development.

The wild-type primary vegetative apex after eight leaves is shown in Fig. 3A, and the progressive enlargement of the apical dome that is fated to form an inflorescence, along with the first sympodial meristem is shown in Fig. 3B. The progressive production of flowers from the first inflorescence is enumerated in Fig. 3C,D. Comparison of Fig. 3D with 3E illustrates the difference between 'indeterminate' wild-type and 'determinate' *sp* mutant apices. In Fig. 3E the first sympodial segment that was formed at the axil of the last leaf (leaf 11) is composed of an inflorescence and only one leaf (leaf L1). A more advanced *sp* mutant 'determinate' apex is shown in Fig. 3F.

In terms of inflorescence structure, the first apical dome of the reproductive meristem is fated to form the first flower (Fig. 3B). Reiteration of this process occurs, with each new meristem appearing at a right angle and in an alternating orientation to its predecessor (Fig. 3C,E,F). This results in the characteristic scorpioid architecture of the tomato

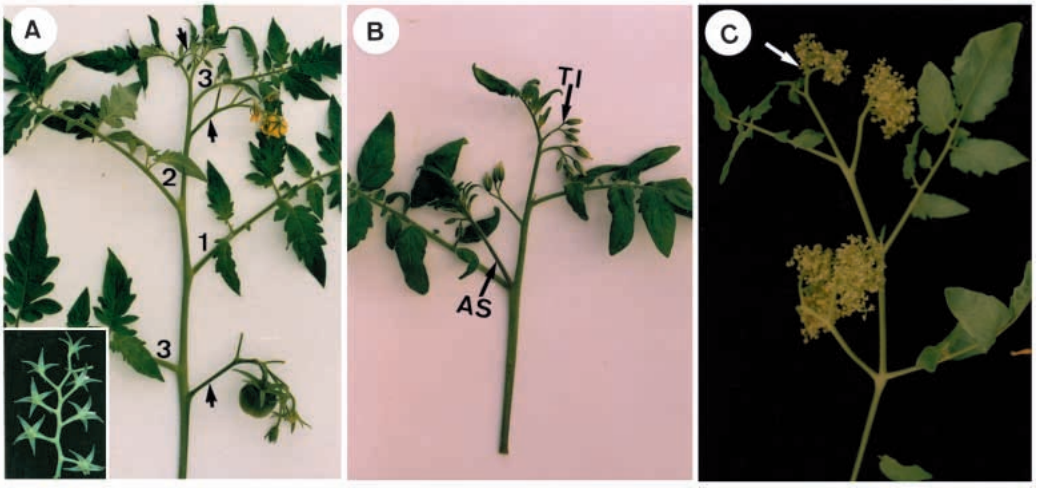


Fig. 2. 'Indeterminate' and 'determinate' shoots of tomato. (A) Indeterminate (*SP*) shoot: One full-size sympodial segment is shown. It consists of three leaves and a terminal inflorescence. The third leaf of such a unit (No. 3) appears above the inflorescence because it is united with the new, fast growing sympodial unit. Arrows indicate three consecutive inflorescences. The insert features a scorpioid (zig-zag) tomato inflorescence. (B) 'Determinate' (*sp/sp*) shoot. Only one nodal leaf separates the first two inflorescences. Arrows mark the terminal inflorescence (TI) and an axillary shoot (AS) developing below the older inflorescence. (C) Shoot of *an sp* double mutant. Note the distance between inflorescences and the termination of the shoot just as in the 'determinate', (*sp/sp)* plants, in B.

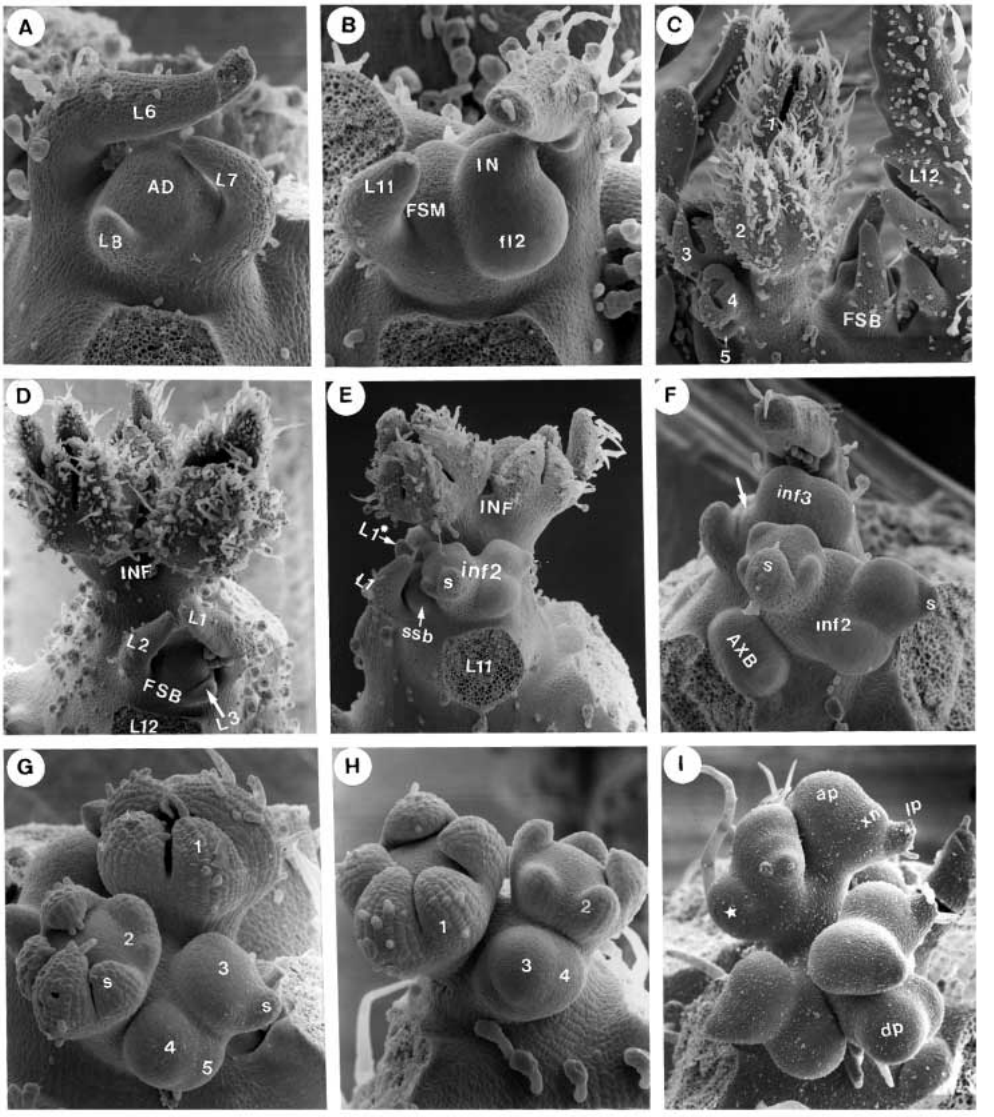


Fig. 3. The development of 'indeterminate' and 'determinate' sympodial apices; scanning electron micrographs. (A-D) The primary apical meristem of 'indeterminate' shoots. (A) Vegetative apex with eight leaves. The apical meristem (AD) will generate 2-3 more leaves before termination. (B) Primary shoot after eleven leaves. The shoot consists of an apical inflorescence meristem (IN) that will differentiate into the first flower, a second floral meristem (fl2), and the axillary meristem that will give rise to the first three-nodal sympodial segment (FSM). (C) First sympodial bud (FSB) at the axil of leaf No. 12. Flowers of the first terminal inflorescence, to the left, are numbered sequentially. (D) Front view of the first sympodial bud at a similar stage as in C showing its three nodal leaves (L1-L3). (E,F) Primary apices and first sympodial segments of 'determinate' *sp* mutant plants. (E) In the axil of L11 has emerged the first sympodial shoot consisting of one leaf (L1) and an inflorescence (inf 2). A second one-nodal segment with inflorescence meristem (ssb, arrow) and one leaf (arrow, L1*) has arisen in the axil of leaf L1. (F) Advanced state of a 'determinate' apex. The second sympodial bud has by now developed as the third inflorescence. The next sympodial bud (arrow) may form an inflorescence only and the shoot is then terminated as in Fig. 2B. An axillary bud (AXB) is developing at the axil of the second leaf below the dissected first terminal inflorescence. (G,H) Scorpioid (zig-zag) development of inflorescence shoots. Near top view of two inflorescences of two indeterminate wild-type plants illustrating the terminal position of the first flowers, the origin and position of younger floral primordia and the alternative directions of lateral floral appendages. (I) A branch of the *anantha* compound inflorescence. One tripartite unit consists of the apical dome (ap) which is formed first in each unit, a presumptive leaf primordium (lp) and an axillary meristem (xm). A bipartite unit (dp) consists usually of two regular domes. A newly emerged meristem is marked with a star.

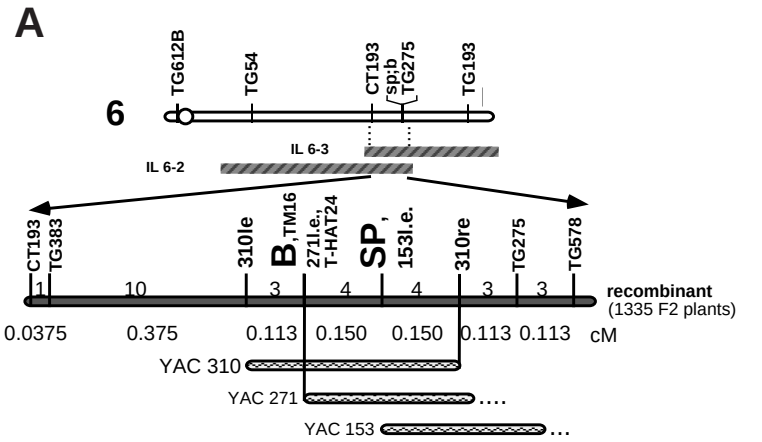
inflorescence (insert in Fig. 2A, see Sawhney and Greyson, 1972 and Silvy, 1974 for details). The second flower may arise on either the left or right hand side of the first flower's pedicel (Fig. 3G,H). Which side it adopts is determined by the direction of the spiral phylotaxy shown by the leaves. It is significant that inflorescence structure in wild-type and *sp* mutant plants is indistinguishable.

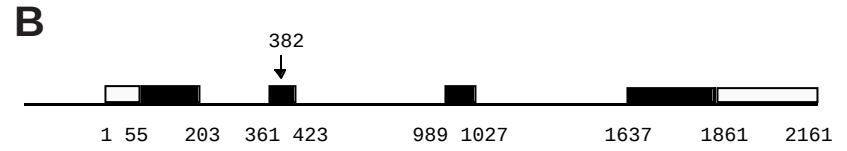
Absence of flower differentiation in *anantha* mutants does not affect the progressive reduction in the sympodial vegetative phase of *sp* mutants

To assess whether the eventual determinacy of the sympodial process in *sp/sp* double mutants is dependent upon flower production, we examined the phenotype of sp^1/sp^1 *an/an* double mutant plants. The ever-proliferating meristematic units of *anantha* inflorescences (Helm, 1951; Paddock and Alexander, 1952) are cauliflower-like and never produce flowers, yet they emerge every three internodes as normal in the 'indeterminate' genetic background. As shown in Fig. 2C, the shoot of the sp^1/sp^1:*an/an* double mutant is 'determinate', indicating that floral differentiation is not essential for the increased determinacy of *sp/sp* sympodial shoots. In Fig. 3I, meristematic units of the *anantha* inflorescence are shown. Note that di- or tri-partite units can be formed on the flanks of a former meristematic dome or from the axil of units within the inflorescence that also carry a leaf primordium.

Fine mapping and cloning of the *SP* gene

To clone the *SP* gene we used a map-based procedure. The *SP* gene has been localized to position 106 on the genetic map of





C

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                                                                    AGTAGATTTTGTTTTGTTCTTATAATTAATTAATAATTAACTCTAAATATATAT 54
ATG GCT TCC AAA ATG TGT GAA CCC CTT GTG ATT GGT AGA GTG ATT GGT GAA GTT GTT GAT 114
 M   A   S   K   M   C   E   P   L   V   I   G   R   V   I   G   E   V   V   D      20
TAT TTC TGT CCA AGT GTT AAG ATG TCT GTT GTT TAT AAC AAC AAC AAA CAT GTC TAT AAT 174
 Y   F   C   P   S   V   K   M   S   V   V   Y   N   N   N   K   H   V   Y   N      40
GGA CAT GAA TTC TTT CCT TCC TCA GTA ACT TCT AAA CCT AGG GTT GAA GTT CAT GGT GGT 234
 G   H   E   F   F   P   S   S   V   T   S   K   P   R   V   E   V   H   G   G      60
GAT CTC AGA TCC TTC TTC ACA CTG ATC ATG ATA GAT CCA GAT GTT CCT GGT CCT AGT GAT 294
 D   L   R   S   F   F   T   L   I   M   I   D   P   D   V   P   G   P   S   D      80
CCA TAT CTC AGG GAA CAT CTA CAC TGG ATT GTC ACA GAC ATT CCA GGC ACT ACA GAT TGC 354
 P   Y   L   R   E   H   L   H   W   I   V   T   D   I   P   G   T   T   D   C     100
TCT TTT GGA AGA GAA GTG GTT GGG TAT GAA ATG CCA AGG CCA AAT ATT GGA ATC CAC AGG 414
 S   F   G   R   E   V   V   G   Y   E   M   P   R   P   N   I   G   I   H   R     120
TTT GTA TTT TTG CTG TTT AAG CAG AAG AAA AGG CAA ACA ATA TCG AGT GCA CCA GTG TCC 474
 F   V   F   L   L   F   K   Q   K   K   R   Q   T   I   S   S   A   P   V   S     160
AGA GAT CAA TTT AGT AGT AGA AAA TTT TCA GAA GAA AAT GAA CTT GGC TCA CCA GTT GCT 534
 R   D   Q   F   S   S   R   K   F   S   E   E   N   E   L   G   S   P   V   A     180
GCT GTT TTC TTC AAT TGT CAG AGG GAA ACT GCC GCT AGA AGG CGT TGA                 578
 A   V   F   F   N   C   Q   R   E   T   A   A   R   R   R   *                 195
TATATCGACAAATTAAAAGCATCTACAATTATATAATAATTAGTGCTGGACGGACTACTACTACTATATGCTTTTTAAT 657
ATTATATTAATTTAATAAGACATGCAGACTTAAATTTTATATTATGTATGTATATGGGGGTTAATGGTTGTTCACCCTC 736
ATGACTTAATGCCAATGGCTTAATTATAAGCACAATGTAATGTAATATCATCAATGTTTCACTTAATTAATATAATTCT 815
ATGTGTGTTTTCTC POLY A                                                          829
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D

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Sp  MASKMC.EPLVIGRVIGEVVDYFCPSVKMSVVYN.NN..KHVYNGHEFFPSSVTSKPRVEVHGGDLRSFFTLIMIDPDV
Cen **A*VSSD*********D***H*TST*****I**S**SI********L***A***T*********M********T****
Tfl *GTRVI.***IM***V*D*L*F*T*TT**N*S**.KK...Q*S****L*****S******I**********V******

Sp  PGPSDPYLREHLHWIVTDIPGTTDCSFGREVVGYEMPRPNIGIHRFVFLLFKQKKRQTIS.SAPV.SRDQFSSRKFSEE
Cen ***********************S***K***S**********************GQAML*P**VC**G*NT***TQ*
Tfl ******F*K********N******AT**K***S**L***S********V**R**Q*RV*FPNI*..***H*NT***AV*

Sp  NELGSPVAAVFFNCQRETAARRR
Cen ****L******************
Tfl YD**L********A*******K*
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Fig. 4. Chromosomal mapping, genetic organization and amino acid sequence of the *SELF-PRUNING* gene. (A) RFLP mapping of *SP*. Schematic map locations of the major RFLP markers of chromosome 6 are indicated above the open bar at the top. The hatched bars below represent the genetic size of the two polymorphic chromosomal regions, IL6-2 and TL6-3, that overlap in the *SP* region. Fine mapping of *SP* and linked flanking markers along with the number of recombinants and calculated distances are shown respectively above and below the expanded shaded bar. The most adjacent polymorphic markers that were used to isolate the three YAC clones (wavy lines) are TG275 and the tomato MADS box gene TM16. The B (β carotene) gene is tightly linked to TM16 and the cDNA clone corresponding to the tomato homolog of HAT24 marks the left end of YAC271. The map position of left (l.e.) and right (r.e.) ends of all three YAC clones are also indicated. (B) Physical map of the *SP* genomic clone. Exons are represented by boxes. Open and filled boxes indicate untranslated and translated sequences respectively. Arrow marks the site of the *sp* mutation. (C) Nucleotide and deduced amino acid sequence of the *SP* cDNA clone. Arrows indicate the position of introns. The proline in position 76 (boxed) is replaced by leucine as a result of a CCT to CTT change. (D) Comparison of amino acid sequences of *SP*, *CEN* (Bradley et al., 1996) and *TFL1* (Bradley et al., 1997). Dots indicate missing residues and compatible replacements are underlined. Proline in position 76 is marked by an arrowhead.

chromosome VI (Rick and Butler, 1956; Stevens and Rick, 1986). Fine mapping of *SP* was achieved through the analysis of 1335 plants derived from F_1 hybrid plants between the 'determinate' (*sp*/*sp*) *L. esculentum* line M82 and the introgression line IL6-3 that carries the wild-type allele of *SP* and the dominant marker *B* from *L. pennellii*. A comprehensive description of the mapping procedure using the *L. pennellii* introgression lines (IL) is given in Eshed and Zamir (1994, 1995). Two RFLP markers, TG275 (Tanksley et al., 1992) and TM16 (Pnueli and Lifschitz, unpublished), were found to flank the *SP* gene, based on the analysis of 31 recombinant individuals (Fig. 4A). Oligoprimers for the two markers were then used to isolate three overlapping YAC clones from the wild-type library constructed by Martin et al. (1992). The mapping of the three YAC clones with respect to the position of *SP* and the flanking recombinant markers is depicted in Fig. 4A.

By the time subclones of YAC153 were examined, Bradley et al. (1996a) reported the successful cloning of the *CENTRORADIALIS* gene (*CEN*) from *Antirrhinum* and its possible identity with *TERMINAL FLOWER1* (*TFL1*) from *Arabidopsis*. Using the *CEN* probe (courtesy of Dr E. Coen) we found that it recognised a 4 kb *Eco*RI fragment present in YAC153 as well as in the tomato genome. The *Antirrhinum CEN* cDNA clone was then used to isolate, from a genomic library constructed from M82 *sp/sp* DNA, the genomic clone shown in Fig. 4B. RFLP mapping of the 5′ non-coding region of the tomato clone as well as of the *CEN* clone verified that they are inseparable from *SP* and further evidence for *SP* being the *CEN/TFL1* ortholog is presented below. Both the *CEN* probe and the *Arabidopsis* EST probe, representing the *Arabidopsis TFL1* gene (Bradley et al., 1996a, 1997), hybridized to additional genomic bands indicating the presence of a small family of 4-5 *CEN*-like genes in tomato. These polymorphic markers were mapped to loci outside chromosome 6.

A cDNA clone corresponding to the putative *SP* gene was isolated from mRNA of the wild-type 'indeterminate' line 93-137 following amplification by RT-PCR using 5′ and 3′ flanking primers derived from the genomic sequence. The sequence of the wild-type cDNA clone was identical to that of the *sp* mutant genomic clone (where they overlapped), except that the genomic clone encoded leucine instead of proline in position 76 (i.e. P76L, CCT→CTT, Fig. 4C). The genomic library was prepared from M82 (*sp/sp*) DNA and we have verified that a PCR product generated from M82 mRNA also contains the P76L alteration. The C→T transition in the proline codon results also in the abolition of a rare *Scr*F1 restriction site. Southern blot analysis established that this site is missing in genomic DNA of ten 'determinate' cultivars but is found in DNA from plants of six 'indeterminate' lines (results not shown).

Only two recessive mutant alleles of the *SP* gene have been reported to date. We found that the RT-PCR product of the second allele, that in VFNT Cherry sp^2/sp^2, also contains leucine rather than proline in position 76 whereas a parallel PCR product from the VFNT Cherry *SP*/*SP* isogenic line carries proline in this position. Almost all commercial 'determinate' varieties most probably carry the original sp^1 allele reported by Yeager (1927) and MacArthur (1932). The sp^2 allele was discovered by Dr C. M. Rick as a single plant in a VFNT Cherry *SP*/*SP*, background. It was surprising, therefore, that the same amino-acid alteration was found in the VFNT Cherry sp^2 allele. In order to examine the possibility that sp^2 has not arisen independently of sp^1 but rather is the result of a rare cross-fertilization, the DNA fingerprints of the two lines were compared using a satellite GATA probe and several RFLP markers. They were found to be almost identical. If sp^2 is an sp^1 'contaminant', its background must have been similar to the VFNT Cherry background. Alternatively, sp^2 could be a newly arising mutant allele within the VFNT Cherry background. One possibility is that further mutations in the *SP* gene have not been identified because they do not result in a 'determinate' phenotype. However, this is unlikely given that multiple mutant alleles have been found in the orthologous *CEN* and *TFL1* genes (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1996a, 1997).

Wild-type tomato plants of the sibling species *L. pennellii*

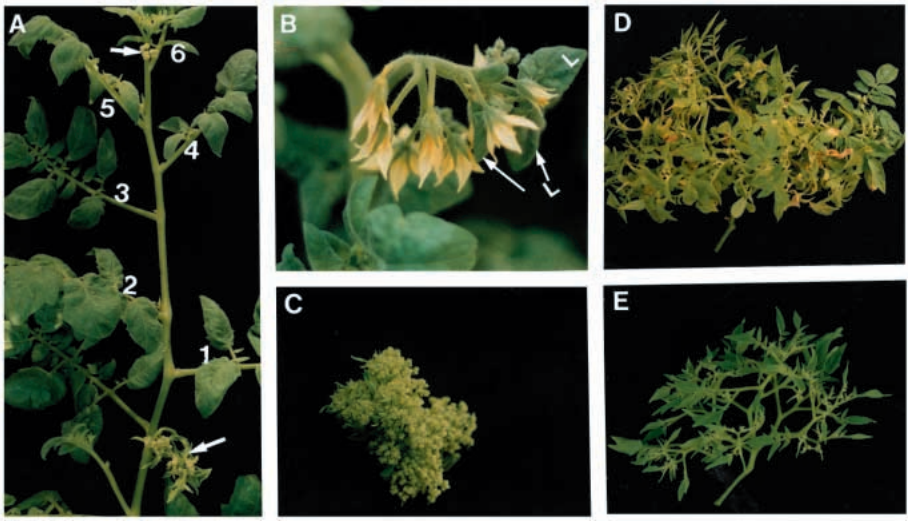

Fig. 5. Over-expression of *SP* in transgenic tomato plants. (A) Increased nodal spacing in *an:sp* plants overexpressing the *35S::SP* transgene. The six leaves separating the two inflorescences are numbered. The inflorescence at the bottom of the picture has already initiated the formation of leaves typical of such transgenes (see D and E). (B) Inflorescence of a determinate plant expressing the *35S::SP* sense transgene. In addition to reverting to the indeterminate habit the transgenic plants also form inflorescences in which flowers are replaced by leaves (arrows). (C) Inflorescence of transgenic *an/an* plants expressing *SP* antisense RNA. No difference from the progenitor inflorescence is observed. (D) Inflorescence of *an/an* plant expressing the *35S::SP* (sense) RNA. An intermediate phenotype with respect to the 'leafiness' is shown. (E) More extreme form of leafy inflorescence in a transgenic *anantha* plant. The structure in 5D closely resembles the inflorescence of *jointless anantha* double mutant plants. The extremely leafy inflorescence in E is most similar to those formed by *falsiflora* mutant plants.

are also 'indeterminate' but with two, rather then three, nodal leaves between successive inflorescences. Regular 'indeterminate' pattern is thus not synonymous with a three nodal spacing. The two nodal pattern is recessive to the three nodal pattern of cultivated tomato (*L. esculentum*) in F_1 hybrids of the inter-species cross. A genomic PCR product of the *SP* gene from *L. pennellii* has proline, not leucine, in position 76 and the remainder of the deduced amino acid sequence is also identical to that of *L. esculetum*.

Transgenic copies of *SP* and *CEN* convert the determinate phenotype of *sp/sp* plants

In order to verify the functional identity of the cloned *SP* gene, tomato plants over-expressing 'sense' and 'antisense' transgenes of *SP*, as well as the 'sense' transgene of *CEN*, were generated. All genes were fused with the ubiquitously acting 35S promoter. Three lines were transformed, two 'determinate' and one 'indeterminate'. Line 1, TK9/8 - sp^1, is an extreme 'determinate' (mutant) line, line 2, *sp/sp:an/an*, is a 'determinate' line with *anantha* mutant inflorescences (see Figs 2C and 3I), and line 3, *sp/+:an/+*, is an 'indeterminate' line with a wild-type phenotype but with a reduced dosage of *SP* and *AN*.

In the two 'determinate' mutant lines, lines 1 and 2, the 35S::*SP*-sense gene resulted in a restoration of the 'indeterminate' phenotype in 80% of the Kan^R plants. In addition, however, overexpression of *SP* in T_1 *anantha* plants (line 2) but not in the other two lines, confers a variable increase of nodal spacing with between three and eight leaves now arising before the next inflorescence (Fig. 5A). The increase in the number of leaves between inflorescences is more conspicuous in late sympodial segments of both main shoots and side branches. In contrast, over-expression of the sense version of 35S::*sp* (P76L) (the mutant gene) failed to rescue the 'determinate' phenotype of 14 Kan^R plants of lines 1 and 2. This supports the proposal that the P76L alteration represents the mutational change in the *sp* allele.

Turning to the effect of expression of the 35S::*SP*-antisense transgene, this successfully converted eight of eleven 'indeterminate' plants from line 3 into regular fertile 'determinate' plants. By contrast, its over-expression in 'determinate' plants of lines 1 and 2 resulted in no further phenotypic alterations, suggesting that the *sp* allele may represent the most extreme mutant phenotype.

In addition to its effect on the sympodial shoot in lines 1 and 2, over-expression of the 35S::*SP*-sense gene resulted in morphogenetic changes to the inflorescence in all lines. In lines 1 and 3, frequent replacement of flowers by leaves was observed (Fig. 5B). Over-expression in *anantha* mutant plants (line 2) resulting in an inflorescence where hundreds of *anantha* meristems developed as regular leaves or even shoots (Fig. 5C-E). Replacement of flowers by leaves mimics the

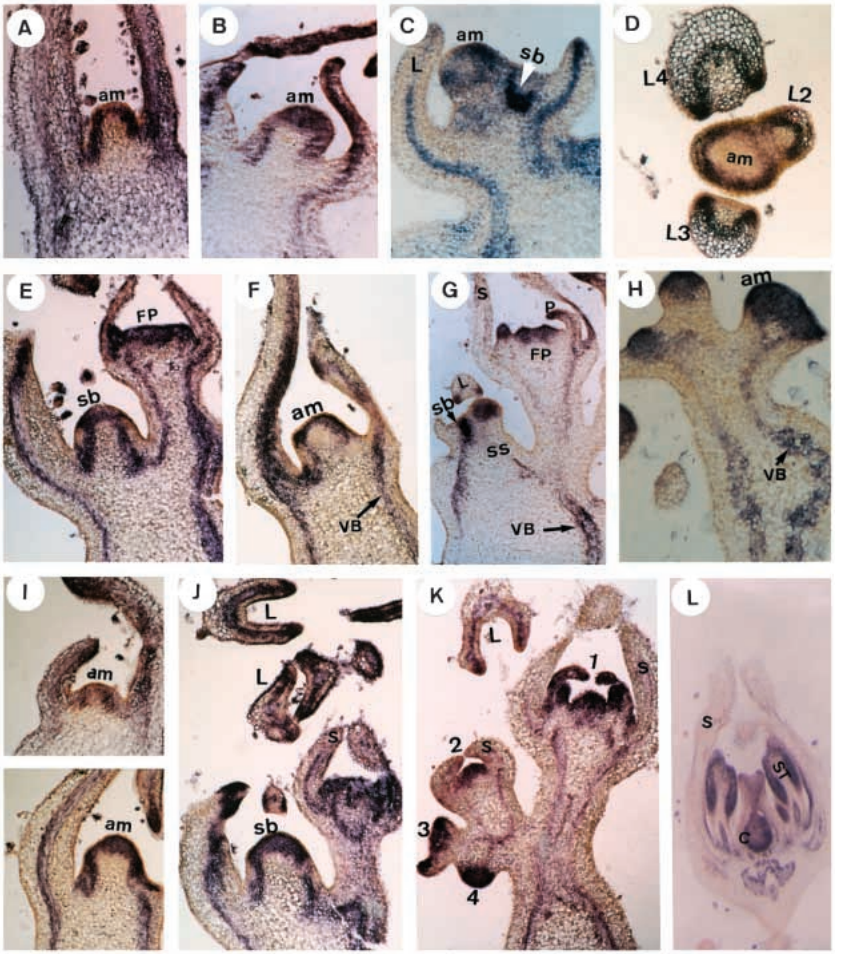

Fig. 6. *SP and T-FLO* are expressed in identical domains. (A-G) In situ localization of the *SP* transcripts in wild-type plants. (A) Longitudinal section in apex of a seedling with three true leaves. (B,C) Near median section of primary shoots with 10 leaves just before or during transition to flowering. The strong signal (arrowhead in C), marks the emergence of the first presumptive sympodial shoot. (D) Cross section from apex at a stage similar to that in B and C. (E) Emerging sympodial bud (sb) in the axil of a leaf. The floral primordium (FP) represents the first flower of the inflorescence that terminated the preceding shoot. (F) Advanced apex of an axillary bud with three leaves, two of which are seen. (G) Expression of *SP* in floral primordium (FP). Organs of the new sympodial shoot which is at a stage comparable to the one shown in C and D are at the lower left side. (H) Expression of *SP* in primordia of an *anantha* inflorescence. (I-L) Localization of *T-FLO* transcripts in organs of a wild-type plant. (I) Top: apex from a seedling with 3-4 leaves. Bottom: longitudinal section of the same apex shown in F but probed with *T-FLO*. (J) Section of the same apex as in E. Note also the localization of T-LFY transcripts in cross-section of leaves (L). (K) Wild-type inflorescence. The section cuts through four floral primordia, number 4 is the youngest. (L) Young flower, longitudinal section. Digoxigenin labelled antisense cRNA was use as a probe. Sense RNA was used as a negative control probe and antisense RNA of the RNR gene (see Materials and Methods) as a positive control probe. am, apical meristem; C, carpel; FB, floral primordium; L, leaf; P, petal; sb-sympodial bud; S, sepals; ss, sympodial shoot; ST, stamen; VB, vascular bundles.

phenotype of *jointless1* homozygous plants (Rick and Butler, 1956), while ramified inflorescences of the type shown in Fig. 5D and E are typical of *falsiflora* mutant plants (Stubbe, 1963).

Over-expression of the heterologous 35S::*CEN* sense gene of *Antirrhinum* (Bradley et al., 1996a) in 'determinate' plants completely rescued the 'indeterminate' phenotype in six out of eight from line 1 and eight out of eleven of line 2. Moreover, *CEN* conferred retarded termination and leafiness of *anantha* inflorescences in line 2 (*sp/sp:an/an*) in the same manner as *SP* does, consistent with the functional identity of the two genes.

SP is expressed throughout development in all organ primordia

We performed in situ hybridization experiments in order to understand the relationship between the expression pattern of *SP* and the recurrent transition of tomato shoot apices from vegetative to the reproductive mode. Apices of primary shoots were examined from the time of germination to the production of the first sympodial shoot. As shown in Fig. 6A-F, *SP* is expressed in vegetative apices of young seedlings with only two or three leaves (Fig. 6A), in floral apices terminating the primary shoot (Fig. 6B-D), in sympodial apices (Fig. 6E), and in apices of axillary buds (Fig. 6F). *SP* is also expressed from the outset in leaf primordia of all stages where its RNA is found predominantly in a well defined and narrow domain around the provascular bundles and in the growing tips (see, for example, Fig. 6C,D). Proximal provascular strands of shoots and all lateral organs, also show the signal (Fig. 6E,F). In the vegetative apices, the gene is expressed in the L2 and L3 cell layers in a ring-like pattern resembling a hollow cone (Fig. 6A-F), but is excluded from the central sub-apical zone and the L1 layer of the apical meristem.

SP is also expressed in the inflorescence and floral meristems and in the primordia of all floral organs. In these mersitems, however, *SP* is expressed in the central subapical domains similar to what is observed for *TFL1* and *CEN* (Fig. 6E,G,H) In developing flowers, *SP* RNA is found in the sporogenic tissues of the anthers and carpels but appears to be excluded later from developing sepals and petals (not shown but see Fig. 6L for identical results). As shown in Fig. 6H, *SP* is expressed in the meristems of *anantha* inflorescences in basically the same pattern as in regular apical reproductive meristems.

In the wild-type, we have not detected any significant changes in the expression level of *SP* along the three nodal units of each sympodial shoot. Also, *SP* continues to be expressed in essentially the same pattern in apices and axillary buds of both earlier and later arising sympodial segments. We have also followed the expression of the gene in later stages of 'determinate' (*sp/sp*) plants but no deviations from the wild-type could be discerned. It is interesting that the *SP* gene is expressed at a particularly high level in all axillary buds along the shoot even though only some of these buds ultimately give rise to side branches.

The tomato *LFY/FLO* ortholog is co-expressed with *SP*

The *TFL1* and *CEN* genes have been proposed to play a role as negative regulators of *LFY* and *FLO*, respectively, in their meristem identity functions (Ma, 1998). We have shown here that over-expression of *SP* alters the fate of floral meristems in tomato inflorescences resulting in them exhibiting more vegetative characteristics, consistent with *SP* potentially functioning to negatively regulate the activity of the tomato *LFY/FLO*-like gene. Thus, the expression of *SP* may be correlated with the absence of expression of the tomato ortholog of the *LFY*/*FLO* gene. We have obtained a cDNA clone of the presumed tomato ortholog (named *T-FLO*) through its homology with the tobacco *NFL* gene (Ron et al., unpublished data). The amino acid sequence of the unique *T-FLO* gene is more than 95% homologous with that of tobacco (Kelly et al., 1995). We find that *T-FLO* is co-expressed with *SP*, temporally as well as spatially (Fig. 6I-L) in all organs at all stages of development. It is particularly notable that *T-FLO* expression is not confined to an identifiably floral phase of tomato development but it is expressed in the vegetative apex and leaf primordia from the early vegetative stage (Fig. 6I). In this regard, its expression matches that of the *NFL* gene in tobacco (Kelly et al., 1995). Significantly, expression of *T-FLO* just as that of *SP* is not detected in the central domain of the shoot apices. *T-FLO* is also co-expressed with *SP* in all floral primordia (Fig. 6 J, K) and later in the sporogenic tissues of anthers and carpels (Fig. 6L), and in *anantha* meristematic units as well (results not shown).

From these data there is no evidence of an antagonistic relationship between *SP* and *T-FLO* at the level of their transcription. *T-FLO* expression is not confined to floral stages of development but is expressed in all developing primordia.

DISCUSSION

It has been shown here that the *SELF-PRUNING* gene of tomato is the ortholog of *CEN* and *TFL1*, genes that maintain the indeterminate state of inflorescence apices in *Antirrhinum* and *Arabidopsis*. *SP*, *CEN* and *TFL1* are each members of a small gene family and are related to a gene known to encode a phosphatidylethanolamine binding protein that may be a component of membrane complexes involved in signal transduction (Bradley et al., 1996a, 1997).

A mutation in *SP* or the suppression of gene activity by antisense RNA results in the premature conversion of the sympodial vegetative apex into a terminal determinate inflorescence shoot but has no effect on the architecture of the inflorescence itself or on the morphology of the flowers. Overexpression of *SP* or *CEN* results in an extended vegetative phase of sympodial shoots and in the replacement of flowers by leaves in the inflorescence. The role of the *SP* gene in tomato thus revolves around the regulation of the cycle of vegetative and reproductive growth inherent in the sympodial system.

The vegetative and reproductive sympodial meristems of tomato

The *sp* mutant phenotype indicates that *SP* clearly acts as part of the system which prevents early flowering in each of the de novo developing sympodial shoot meristems. Since the termination of the vegetative apex in *sp* mutant plants is accelerated with age we infer that the system represented by *SP*, must also be progressively up regulated as the plant ages. At the same time this system must also be proportionally down-regulated in a step-like manner with each internode of the new

sympodial segment to permit transition to flowering after three internodes. By the same token, the regulation and activity of the flowering signals must be tightly controlled in tomato. Their nature is not known (Bernier, 1988), but they must peak after three internodes have been generated and yet they must be sequestered from the new sympodial apex that will arise just ten cells away.

The restrictions placed by the sympodial system notwithstanding, the role of the *SP* gene parallels that of *TFL1/CEN* in an important aspect. Similar to the role of *TFL1/CEN* in the monopodial inflorescence apex, *SP* prevents the premature conversion of a potentially indeterminate shoot meristem into a determinate flower meristem. Considered from a different angle, like *TFL1* in the vegetative monopodial shoot of *Arabidopsis* (Shannon and Meeks-Wagner, 1991), *SP* functions to prevent precocious flowering during vegetative growth of the sympodial segments of the tomato shoot. Even so, it is clear that in tomato, an invariant juvenile phase of vegetative growth occurs before the apex becomes sensitive to the loss of *SP* function and that no mutation in *TFL1* or *CEN* confers the termination of the vegetative shoot with a solitary flower in *Arabidopsis* or *Antirrhinum*. Several mutant lines with solitary flowers in their inflorescence are known in tomato (see Stevens and Rick, 1986 for a list of mutants).

Unlike its effect on the sympodial pattern, a loss of function of the *SP* gene has no consequences for any of the architectural aspects of the tomato inflorescence. This is in contrast to *TFL1* and *cen* mutants that result ultimately in the inflorescence apex being 'taken over' by a terminal flower. However, rather than reflecting a difference in function between *SP* and its orthologs, this is likely to be the consequence of a difference in shoot models between tomato and the other species; vegetative and reproductive monopodial shoots with indeterminate apical meristems in *Arabidopsis* and *Antirrhinum* but vegetative and reproductive sympodial shoots with determinate apical meristems in tomato.

Cronquist (1988) considered the racemose, indeterminate inflorescence to be an "ordinary vegetative axis that had been modified in just two aspects: the leaves are reduced to bracts and every axillary bud develops into a short lateral branch with a single terminal flower". Evolutionary homology between vegetative and reproductive shoots may be applicable in tomato as well. If every sympodial segment of three internodes was reduced to one and terminated by a solitary flower and, in addition, bracts were eliminated, a cymose scorpioid inflorescence architecture would result. This model is consistent with the absence of any inflorescence phenotype in *sp* mutant plants since one would not anticipate that the *sp* mutation would cause a more extreme floral termination on what is already a one-unit floral structure terminating the shoot.

Recently, the tomato inflorescence has been interpreted as being closer to the racemose, indeterminate type, based on the architecture of *anantha* and *falsiflora* mutant inflorescences (Allen and Sussex, 1996). However, our observation that the *sp* mutant has no effect on the inflorescence architecture alone, or in combination with *anantha*, calls this interpretation into question. If the tomato inflorescence is a modified sympodial shoot, as suggested above, evolutionary relations between dichotomous and sympodial models (Stewart, 1964), as mentioned in the introduction, can be applied to make some predictions about the *anantha* (*ap1/cal*-like) and *falsiflora* (*lfy/flo*-like) mutations. These genes, in addition to controlling floral meristem identity, are also required to maintain the cymose configuration of the sympodial inflorescence shoot. When mutated, the ancient dichotomous pattern ramifies.

Transition to flowering and expression patterns of *SP* and *T-FLO*

The expression of *SP* in all apices (Fig. 6) predicts that multiple pleiotropic phenotypes would occur upon its malfunction. However, the *sp* mutation has no effect on the architecture of the primary shoot or the timing (in terms of node number) of its initial termination. Likewise, loss of *SP* does not result in any discernible phenotype in the flowers or the inflorescence. In addition, *SP* is expressed in leaf primordia, but leaf development is not affected in *sp* plants. Finally, over-expression of *SP* results in only subtle changes to phenotype in wild-type plants.

It is improbable that the lack of pleiotropic effects in *sp* mutant plants is due to an idiosyncrasy of the one known *sp* allele. Determinacy of the sympodial shoot is also the main phenotypic consequence seen in a range of transgenic plants expressing the 35S::*SP* anti-sense RNA. The only other effect is some leafiness of the inflorescence.

It may be that the product of the *SP* gene is sequestered or otherwise dispensed with in tissues where it is without mutant phenotype, including reproductive organs and leaves. Alternatively, the role of *SP* may be limited to the regulation of the sympodial shoot apex, and its expression in leaves and other locations may reflect a non-autonomous or systemic action. This speculation is made attractive since leaves generate promotive and inhibitory signals of flowering (see Bernier, 1988 for review, and De Zeeuw, 1956; Leopold and Laur, 1960 for specific examples in tomato). It is notable that in leaves *SP* is expressed predominantly around the vascular bundles. If flowering signals are generated in mesophyll cells, they would have to cross the *SP* domain on their way to the shoot, a scenario rich with attractive speculations. Expression of *SP* in specific locations in an *sp* mutant background would help clarify this point.

A priori, it may be expected that *SP* acts as a negative or antagonistic regulator of the *T-FLO* gene. In *Arabidopsis* and *Antirrhinum*, the *TFL1/CEN* and *LFY/FLO* genes play antagonistic roles in inflorescence and floral meristem identity (Bowman et al., 1993; Shannon and Meeks-Wagner, 1993; Schultz and Haughn, 1993; Bradley et al., 1996a; Simon et al., 1996) and the genes display spatial and temporal differences in their expression patterns (Coen et al., 1990; Weigel et al., 1992; Bradley et al., 1996a, 1997).

The expression of *T-FLO* in vegetative tissues reported here is similar to that recently observed for *LFY/FLO* in *Arabidopsis* (Blazquez et al., 1997; Bradley et al., 1997; Hempel et al., 1997) tobacco (Kelly et al., 1995), pea (Hofer et al., 1997) and *Impatiens* (Pouteau et al., 1997). The vegetative and reproductive expression of the tomato *T-FLO* thus provides a further example of transcription of *LFY/FLO* homologues that does not appear to be absolutely correlated with floral induction. The deduced role of the *SP* gene is to modulate the pattern of growth of the apical meristem in response to these signals. It appears likely that *T-FLO* has a similar but complementary role. Because the two genes seem likely to have opposing roles and yet are expressed coincidentally, their

products may control alternative outcomes of the signalling pathway within individual cells. This may occur within the meristem itself, although the widespread expression of *SP* and *T-FLO* in tomato indicates that their products could intercept the signal some distance away and pass it systemically to the target shoot meristem.

Genetic interactions controlling meristem identity in the tomato inflorescence

The observation that the sympodial shoots and the inflorescence meristems of *anantha* are particularly sensitive to over-expression of *SP* (Fig. 5) indicates that *AN* is also involved in phase switching in the tomato sympodial shoot. Intriguingly, an increased number of internodes between inflorescences is also associated with the *falsiflora* (*fa*) mutation (Stubbe, 1965). The phenotype of *fa* inflorescence is initially similar to that of *anantha* (Allen and Sussex, 1996) but subsequently becomes indistinguishable from that of the *anantha* plant overexpressing *SP* that is shown in Fig. 5D,E. This suggests that *FA* normally functions even more strongly than *AN* to promote the transition to a determinate floral state in the sympodial shoot, a role complementary to that of *SP*. In this regard the absence of any sympodial vegetative phase prolongation in T1 35S::*SP* wild-type plants (line 3) implies that in this background *AN* and *FA* (and possibly other factors) are at a sufficiently high level to fully support the floral transition.

In addition to *SP*, *AN* and *FA*, other genes are known to modify the dynamic balance of the two shoot systems in tomato. Such alterations by *BLIND, TERMINATING FLOWER SINGLE FLOWER TRUSS* and others call for the involvement of additional regulatory functions. The mutant gene *JOINTLESS1* illustrates this point and further illuminates the role of the *SP* gene. A high level of *SP* favors the formation of leaves rather than flowers by the prospective floral meristems (Fig. 5B). This phenotype is identical to that imparted by the *jointless1* mutation and moreover, it has been known for years that *sp* suppresses the formation of leaves in the j^1/j^1*:sp/sp* double mutant inflorescence (Rick and Butler, 1956). Presumably, every meristem with a floral fate undergoes several intermediary developmental shifts and a high level of *SP* or suppression of *JOINTLESS1* arrests the meristems in its early ‘leafy’ stage. Since *JOINTLESS1* is a factor that antagonises the function of *SP* it was expected that the *jointless1* mutation would, like *SP*, confer a leafy (*falsiflora*-like) phenotype in a double mutant with *anantha*.. Consistent with this, the phenotype of *an/an:*j^1/j^1 double mutant inflorescence (unpublished observations) is indistinguishable from that of the *an/an*:35::*SP* over-expressing plants shown in Fig. 5D,E). No genetic factor equivalent to *JOINTLESS1* is known in *Arabidopsis* or *Antirrhinum*.

The coexpression of *SP* and *T-FLO* suggest that regulation of their functions in tomato cannot be satisfactorily explained by simply assigning a negative or a positive role to one gene or another. Our testable working hypothesis is that in the expression domains they both share represent functional complexes that exchange signals by means of transient overlapping components, to regulate the balance between vegetative and reproductive pathways. Our working hypothesis also includes the possibility that the genes *CONSTANS* (Putterill et al., 1995), *ELF3* (Zagotta et al., 1996) and others, which are required for the interpretation of light signals in the monopodial, photoperiod-sensitive species, regulate the recurrent alterations of the vegetative/reproductive cycles in tomato.

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