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SPATULA, a gene that controls development of carpel margin tissues in *Arabidopsis*, encodes a bHLH protein

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SUMMARY

Studies involving mutants of the gene *SPATULA* indicate that it promotes the growth of carpel margins and of pollen tract tissues derived from them. We show that it encodes a new member of the basic-helix-loop-helix family of transcription factors. *SPATULA* is expressed in marginal and pollen tract tissues throughout their development confirming its role in regulating their growth. It is also expressed in many other tissues where it may act redundantly to control growth, including the peripheral zone of the shoot apical meristem, and specific tissues within leaves, petals, stamens and roots. Expression in the stomium, funiculus and valve dehiscence zone indicates an additional role in abscission. *SPATULA* expression does not require the function of the other carpel development genes *CRABS CLAW* and *AGAMOUS*, although its expression is repressed in first whorl organs by the A function gene *APETALA2*. Further, we have shown that disruptions to gynoecial pattern formation seen in *ettin* mutants can largely be attributed to ectopic SPATULA action. ETTIN's role seems to be to negatively regulate *SPATULA* expression in abaxial regions of the developing gynoecium. *SPATULA* is the first basic-helix-loop-helix gene in plants known to play a role in floral organogenesis.

Key words: *Arabidopsis thaliana*, *SPATULA*, Carpel, Gynoecium, bHLH, Transcription factor, Transmitting tract, *ETTIN*

INTRODUCTION

Angiosperms enclose their ovules in protective leaf-like organs called carpels. To promote fertilization, carpels typically develop specialised tissues that facilitate the passage of the male gametophyte from the exterior to the ovules located within. These tissues usually develop from the carpel margins and include a stigma on which pollen alights, and transmitting tissues within the style and ovary through which the pollen tubes grow. In *Arabidopsis*, two congenitally joined carpels make up a central gynoecium. At the apex, stigmatic tissue develops on top of a short style. The ovary is divided into two locules by a septum that develops by the postgenital fusion of two outgrowths that originate from the regions of carpel fusion.

Recessive mutations in the *SPATULA* (*SPT*) gene specifically disrupt development of the pollen tract tissues including the transmitting tract, style and stigma (Alvarez and Smyth, 1999). These disruptions affect the presumed precursors of these tissues as early as stage 7 when the gynoecial cylinder starts to elongate (J. Alvarez and D. R. S., unpublished). Reduced growth results in the reduction or absence of septum tissue, especially in apical regions. A cleft is often present separating the two carpels at the apex, and stigmatic tissue is also severely reduced. The only tissue absent in strong *spt* mutants is the transmitting tract within the septum and style that generate an extracellular matrix. Despite this, fertilisation usually occurs, although at a reduced frequency. *spt* fruits are shorter than wild type and wider in the medial plane especially towards the apex, resulting in a spatula-like appearance (Alvarez and Smyth, 1999).

Genetic analysis has revealed that *SPT* acts in parallel with two other genes to specify all components of the mature gynoecium (Alvarez and Smyth, 1999). The C-class gene *AGAMOUS* (*AG*) specifically promotes the characteristic cellular morphology of the carpel wall and the development of a stylar apical outgrowth. *CRABS CLAW* (*CRC*), however, promotes the narrow, parallel-sided shape of carpels as opposed to the ovate shape of leaves. Genetic and molecular data suggest that while these genes are probably activated independently, regulatory interactions amongst them may fine-tune their expression (Alvarez and Smyth, 1999; Bowman and Smyth, 1999).

Another gene that effects the pattern of carpel development is *ETTIN* (*ETT*) (Sessions and Zambryski, 1995; Sessions, 1997). The gynoecia of strong *ett* mutants have no valve tissue within the ovary, and instead produce a style-like gynophore topped by a bifurcated and everted stigma. Interestingly, *spt* mutations suppress many aspects of the *ett* phenotype suggesting that abnormalities seen in *ett* mutant gynoecia may result from ectopic SPT activity (Alvarez and Smyth, 1998).

To help understand SPT function at the molecular level, we report the cloning of the *SPT* gene by chromosome walking and analysis of its expression pattern. *SPT* encodes a basic helix-loop-helix (bHLH) transcription factor that is expressed continuously in the margins of developing carpels, presumably supporting their growth. It is also expressed in a range of other tissues where its

redundant functions may include growth promotion and tissue abscission. Whereas *SPT* expression can occur independently of the other carpel genes *CRABS CLAW* and *AGAMOUS*, it is negatively controlled by *ETTIN*. This key interaction is essential for correct tissue patterning within the gynoecium.

MATERIALS AND METHODS

Plant material

The weak *spt-1* and strong *spt-2* mutant alleles have been described previously (Alvarez and Smyth, 1999). Another strong mutant *spt-3* was obtained subsequently. All were isolated in the Landsberg *erecta* background using ethylmethane sulphonate. *ap2-2* and *crc-1* single mutant and *ap2-2 ag-1* double mutant lines were bred previously (Alvarez and Smyth, 1999), as were *ett-3* single and *ett-3 spt-2* double mutants (Alvarez and Smyth, 1998). Plants were grown at 20-25°C in constant light. Floral stages follow Smyth et al. (1990).

Initial mapping and generation of recombination markers

The *SPT* locus is less than one map unit below *APETALA2* (*AP2*) on chromosome 4 (Alvarez and Smyth, 1999). Six marked recombination points between *AP2* and *SPT* were generated by crossing *ap2-2 spt-2* double mutants in the Landsberg *erecta* ecotype to wild-type Columbia plants and selecting F_2 recombinants. Their frequency indicated that *SPT* is approximately 0.3 map units below *AP2*.

Recombination markers distal to *SPT* were generated by crossing *spt-2* to *aintegumenta-9* (*ant-9*) in C24 ecotype background (Elliott et al., 1996) and selecting F_3 families segregating for the double mutant phenotype. Between 13 and 17 recombinant families were identified out of 345 families tested, giving an estimated map distance of between 1.9 and 2.5 map units.

Mapping the *SPT* candidate region

Yeast Artificial Chromosomes (YACs) containing the *AP2* gene were identified by screening the EG (Grill and Somerville, 1991) and EW (Ward and Jen, 1990) YAC libraries with a 7.2 kbp genomic fragment that contains *AP2* (present in plasmid pLE 7.2 (Jofuku et al., 1994)). The left end of YAC EG7G11 was used to isolate a genomic clone, λMH3, from an EMBL3 phage library made using Landsberg *erecta* DNA. This was used to isolate two further phage genomic clones (λMH1 and λMH2) as well as a cosmid genomic clone (cosMH1) from a Columbia library obtained from the Arabidopsis Biological Resource Center (Olszewski et al., 1988).

cDNAs corresponding to florally expressed genes located within the candidate region were isolated by using λMH1, λMH3 and cosMH1 as probes to screen a cDNA library made from L*er* inflorescences containing buds up to stage 12 (Weigel et al., 1992).

Sequencing of mutant alleles

The genomic sequence of ESSA AP2 contig 1 (GenBank accession number Z99707) (Terryn et al., 1999) was used in conjunction with sequence data obtained from the candidate cDNAs to design PCR primers that amplified the candidate genes from L*er* wild-type and *spt* mutant plants. *Pfu* DNA polymerase (Stratagene, La Jolla) was used on at least two independent DNA preparations per genotype. These were sequenced directly.

Complementation of the *spt* mutant phenotype

A 5,094 bp *Pst*I fragment, containing the putative *SPT* gene and 1.3 kb of upstream DNA, was sub-cloned from cosMH1 into the binary vector pBIN19 and transformed into *spt-1* and *spt-2* mutant plants by infiltration with *Agrobacterium tumefaciens*.

In situ hybridization

Plant tissues were fixed, embedded in paraffin wax and sectioned as previously described (Long et al., 1996). The slide pretreatment, hybridization and washing steps were carried out according to the method of Braissant and Wahli (Braissant and Wahli, 1998). Hybridisation was in 50% formamide, 5× SSC at 59°C, followed by washing in 0.2× SSC at 55°C. Antisense DIG-labelled transcripts were synthesised from cDNA 5 (after linearization with *Bam*HI) using T7 RNA polymerase and DIG-labelled UTP (Roche) according to the manufacturer's instructions. The expression pattern was qualitatively identical to that obtained using a shorter cDNA 3.5 template that lacks the conserved bHLH domain. Control sense probes did not yield detectable signal.

RESULTS

Isolation of the *SPT* gene

The *SPT* locus was located within a 20 kbp region below the AP2 gene (Fig. 1). cDNAs corresponding to seven genes within this region were isolated and five were partially sequenced. At this point, the sequence of the genomic region surrounding *AP2* was released (Terryn et al., 1999). This information enabled the rapid sequencing of candidate genes corresponding to the cDNAs. One gene, represented by cDNA 3.5, contained different mutations in three independently isolated *spt* mutant strains. This proved to correspond to the *SPT* gene because a 5,094 bp genomic *Pst*I fragment containing only the candidate gene fully complemented *spt-1* and *spt-2* mutant phenotypes (Fig. 2). 15 independent transformants were obtained, and the restored phenotype co-segregated with the insert in subsequent generations.

SPT encodes a bHLH protein

cDNA 3.5 corresponds to predicted gene 44 in the AP2 contig of Terryn et al. (1999) (also called AT4g36930 by the Martinsried Institute for Protein Sequences, see http://www.mips.biochem.mpg.de/proj/thal/). Comparison of cDNA 3.5 and the predicted ORF suggested that it is not full length. Two longer cDNAs (5 and 9) were isolated, and the longest, cDNA 9, extends upstream of the putative start codon (Fig. 3A).

The *SPT* gene consists of seven exons encoding a predicted protein of 373 amino acids (Fig. 3A). Database searches revealed that SPT contains a bHLH domain (boxed) homologous to that of transcription factors found in plants, fungi and animals (Atchley and Fitch, 1997; Littlewood and Evan, 1998). bHLH proteins bind DNA via a stretch of approximately 13 amino acids (the 'basic' region) that lies adjacent to a helix-loop-helix region which facilitates homo- or hetero-dimerization (Fig. 3B). SPT also contains a putative bipartite nuclear localisation signal (Daingwall and Laskey, 1991) that overlaps the basic domain between amino acids 194 and 210. Protein structure prediction programs (Rost and Sander, 1994) identified two other α-helical regions in the N-terminal region, one charged and the other amphipathic.

Within the bHLH domain, the known protein most similar to *SPT* is PHYTOCHROME INTERACTING FACTOR3 (PIF3) from *Arabidopsis* (43 out of 49 amino acids identical; Fig. 3B) (Ni et al., 1998). SPT is less closely related to proteins that regulate anthocyanin biosynthesis in plants (B-peru, R-1c, DEL and JAF13; 21-23 amino acids identical), the rd22BP1 protein that regulates response to dehydration and abscisic acid treatment in *Arabidopsis*, and a regulator of phaseolin seed

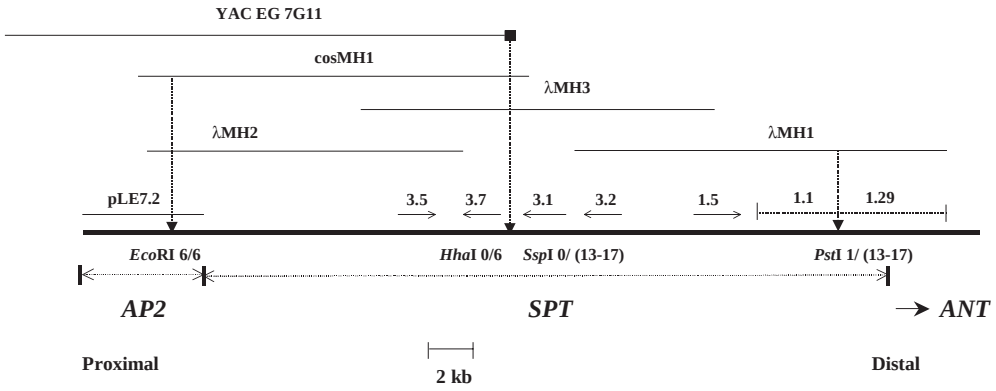


Fig. 1. The chromosome walk to *SPT* from *AP2*. Plasmid pLE7.2 (containing *AP2*) hybridized to YAC EG 7G11. The left end of this (black box) identified two RFLPs (central vertical arrow) that were not separated from the *SPT* locus by recombination breakpoints between *SPT* and the two flanking loci *AP2* (0/6 recombinants) and *ANT* (0/13-17 recombinants). A contig of the candidate region was generated involving phage clones λMH3, λMH1, λMH2 and the cosmid cosMH1. RFLPs detected by marginal subclones from this contig (left and right vertical arrows) indicated that the candidate region had been spanned. Seven cDNAs, 3.5 (encoding a putative bHLH), 3.7 (unknown), 3.1 (unknown), 3.2 (protein kinase), 1.5 (RNA binding), 1.1 (not sequenced) and 1.29 (not sequenced) were isolated and mapped within the contig (1.1 and 1.29 partially mapped). Sequencing of the gene corresponding to cDNA 3.5 revealed three different mutations corresponding to three independently isolated *spt* alleles.

storage proteins (PG1). Searches using SPT sequences outside the bHLH domain revealed no significant similarity to any other proteins.

SPT expression in the wild-type gynoecium

SPT expression was monitored in developing flowers using in situ hybridisation (Fig. 4A). From floral stage 4, *SPT* is expressed in an inverted conical domain at the apex of the floral meristem. Judging from its location and shape, this may reflect the anlage of the gynoecial cylinder. The cone-shaped pattern becomes refined by stage 6 as seen in longitudinal (Fig. 4B) and transverse (Fig. 4C,D) sections. At this stage expression is most intense in medial regions that correspond to the margins of the two co-joined carpel primordia (Fig. 4C).

As the gynoecial cylinder elongates vertically during stage 7, *SPT* expression becomes fully localised to medial domains. Expression is limited to internal regions (Fig. 4E) except at the apex where it extends to the outer surface (Fig. 4A, left flower). During late stage 8, as adaxial cells within these medial regions undergo periclinal divisions to form the medial ridge, *SPT* expression becomes restricted to a small number of cells at the leading edge of this outgrowth (Fig. 4F). During stages 9-11, expression occurs throughout the developing septum that arises from this ridge (Fig. 4G-I), including the differentiating transmitting tract cells (Fig. 4H,I). Stigmatic papillae also express *SPT* from their inception (Fig. 4I). By stage 12, however, expression dissipates in the septum and the stigma (data not shown).

Within the gynoecium, expression is also detected in initiating ovule primordia (Fig. 4G). Later, signal becomes restricted to the epidermis of the developing funiculus and in the cells that give rise to the integuments (Fig. 4H). As the inner and outer integuments lengthen, *SPT* expression appears strongest in the cells that are elongating at their tip (Fig. 4J). Expression also appears in the megaspore mother cell before it undergoes meiosis. After fertilization, *SPT* expression remains distally in the funiculus at least until stage 15 (Fig. 4K).

At stage 12 (just before anthesis) *SPT* expression becomes detectable throughout the valve regions of the carpel walls (excluding the epidermis and vascular bundles; Fig. 4L). During growth of the silique, this expression gradually becomes restricted to the margins of the valves in cells that will later become the valve dehiscence zone (Fig. 4M).

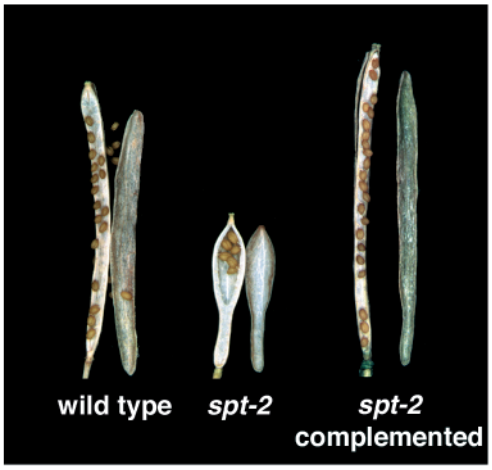


Fig. 2. Complementation of the *spt-2* mutant. In the *spt-2* mutant silique (centre), septum development is restricted to the basal half and seed set is limited to the apex. In the silique of a complemented *spt-2* mutant plant (right) the carpels are fully fused, the internal septum is restored, and the siliques are of similar length to the wild type (left).

To summarise, *SPT* expression within the gynoecium is detected in the initiating and developing medial regions, and then in the developing septum and stigma. *SPT* expression also occurs in sub-regions of developing ovules, and in the wall and dehiscence zone of the maturing fruit.

SPT is widely expressed in other tissues

Although the *spt* mutant phenotype is limited to the gynoecium, *SPT* is also expressed in many other tissues.

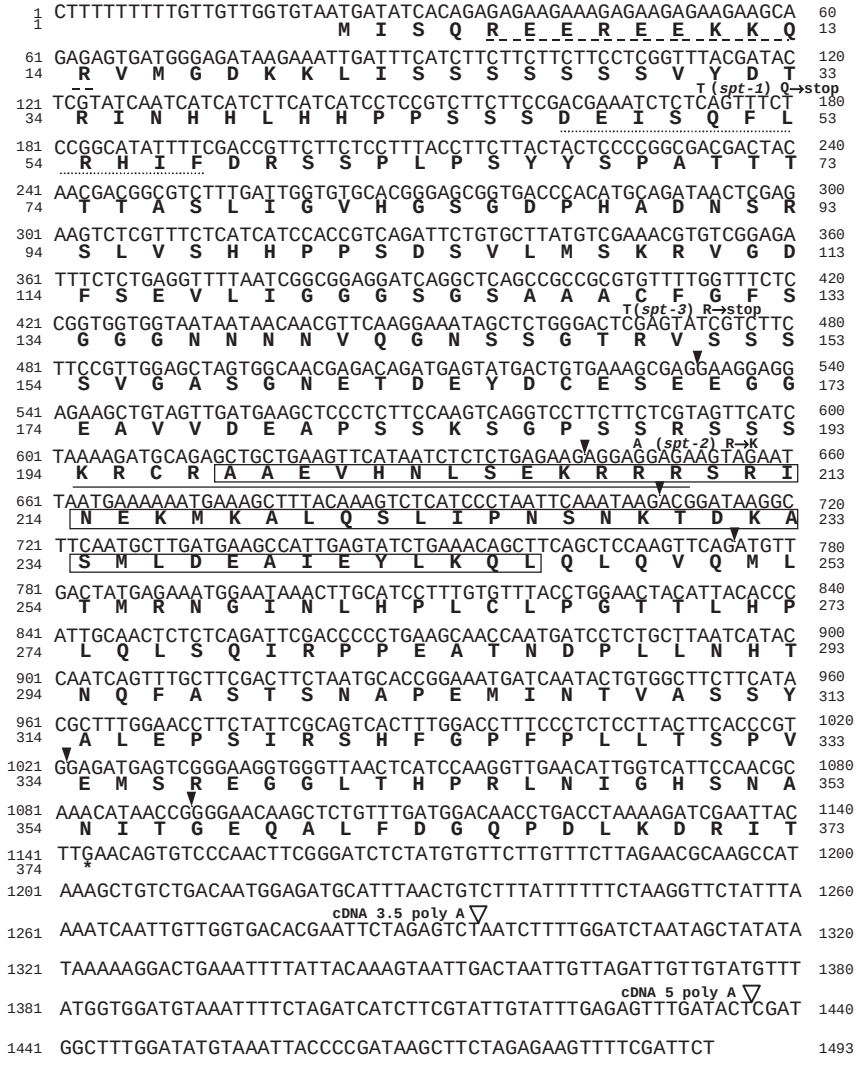
Considering developing floral organs from stage 5, *SPT* transcripts are apparently absent in sepals, but weak expression appears in the initiating petals and stamens. As the petals develop during stages 7-12, this weak expression persists (Fig. 4N) but becomes restricted to the adaxial epidermis (Fig. 4O). Expression also persists early within the developing stamens (Fig. 4D) but then quickly fades. Expression reappears in the

vicinity of the archesporial cells by stage 7 (Fig. 4P) and then intensifies in the cells undergoing divisions to produce the sporogenous and secondary parietal cells of the anther locules during stage 8 (Fig. 4Q). Expression continues in the cells of the tapetum and microspore mother cells but then fades as meiosis is initiated. By stage 12, weak expression is also detectable in the stomium regions (Fig. 4R) and in the filaments.

SPT expression was also detected within the shoot apical meristem, during both vegetative and an inflorescence stages (Fig. 4A). This was investigated further by examining serial transverse sections of the inflorescence apex (Fig. 5). *SPT* expression is strong in the peripheral zone of the meristem, occurring continuously from the epidermis down to the upper pith cells. Within this region, expression seems to be particularly strong in locations that correspond to anlagen of three successive flower buds yet to arise (P –3, P –2 and P –1 in Fig. 5C). In contrast, expression is weak in the anlage of the next floral primordium that will develop (P0), and is excluded in the three latest floral primordia that have already arisen (P1, P2 and P3).

The same serial sections (Fig. 5A-D) allow the pattern of expression in developing flower primordia to be mapped up to stage 4. *SPT* expression first appears in stage 2 primordia, in two domains that may correspond to the presumptive abaxial and adaxial sepal anlagen (bud P5 in Fig. 5B). During stage 3, expression now appears to be absent in the initiating sepals but it is present throughout the region interior to them (buds P7

A.



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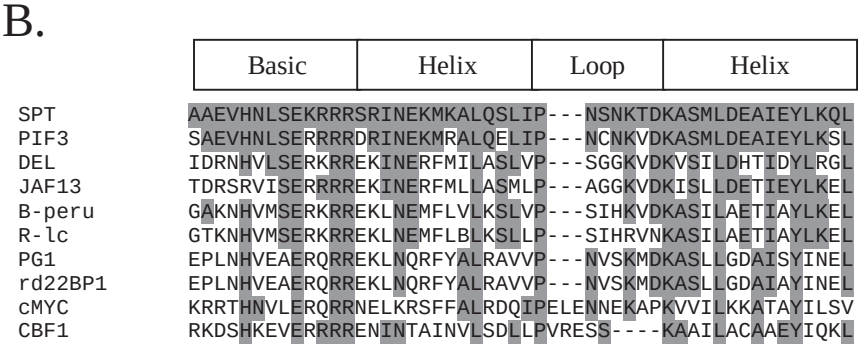

Fig. 3. Sequence of the *SPT* coding region and analysis of the predicted protein product. (A) Nucleotide sequence of cDNA 9 and the deduced amino acid sequence (GenBank accession number: AF319540). Intron positions are denoted by solid triangles and the polyadenylation sites for cDNA 3.5 and cDNA 5 by open triangles. The location of the bHLH domain (boxed) and putative bipartite NLS (solid underline) are shown as well as a predicted charged helix (dashed underline) and an amphipathic helix (dotted underlined). The nucleotide and predicted amino acid changes corresponding to the three *spt* mutations are shown above the nucleotide sequence. (For each allele, genomic DNA from positions –179 to +1862 was fully sequenced, and no other changes were detected.) (B) Alignment of the bHLH domain from SPT and a number of characterized proteins from plants, animals and yeast. The proteins include PHYTOCHROME INTERACTING FACTOR3 (PIF3) from *Arabidopsis* (Ni et al., 1998); four genes that regulate anthocyanin synthesis (DELILA (DEL) from *Antirrhinum* (Goodrich et al., 1992), JAF13 from *Petunia* (Quattrocchio et al., 1998), B-peru from maize (Radicella et al., 1991), R-lc from maize (Perrot and Cone, 1989)); phaseolin G-box binding protein (PG1) from bean (Kawagoe and Murai, 1996); rd22 binding protein (rd22BP1) from *Arabidopsis* (Abe et al., 1997); cMYC from humans (Bernard et al., 1983); and Centromere Binding Factor (CBF1) from yeast (Cai and Davis, 1990). Amino acids conserved between SPT and other proteins are shaded.

and P8 in Fig. 5A). Within this region, expression now falls away in the central zone, leading to the inverted cone of expression seen in stage 5 buds (discussed above, Fig. 4B).

SPT expression was also detected in young leaves, stipules, maturing pith cells of the stem, in differentiating vascular cells, and in the lateral root cap (results not shown).

SPT expression is not directly controlled by SPT or CRC

To determine if SPT positively regulates its own transcription, its expression was examined in developing gynoecia of *spt-2* mutants. Before any mutant disruptions to growth are observed, expression appears to match that in the wild type (Fig. 6A). After stage 7, expression is reduced but this is directly correlated with the reduced growth or absence of tissues in *spt* mutants (Fig. 6B). By stage 11, expression was detected only occasionally in the epidermal cells of the unfused *spt-2* septum (Fig. 6C). Also, no changes in expression were seen in tissues unaffected by loss of SPT function. Thus it seems likely that *SPT* expression is not autoregulated.

The *CRABS CLAW* gene product apparently acts to suppress the radial growth of carpels while promoting their longitudinal growth (Alvarez and Smyth, 1999). *crc* mutant carpels are unfused towards the apex, and pollen tract tissue development is reduced somewhat. Strikingly, *crc spt* double mutants exhibit a much more dramatic loss of carpel fusion and pollen tract tissue than either single mutant, suggesting that the function of these genes overlap somewhat (Alvarez and Smyth 1999). Genetic evidence indicates that this occurs in part by the promotion of SPT activity by CRC function. To test this, *SPT* expression was examined in *crc-1* mutants. The level of expression during stage 6-8 appeared similar to wild type (Fig. 6D). The observed reduction in *SPT* expression at later stages seems to be attributable to reduction in septum and transmitting tract development (Fig. 6E, F). Thus it seems that CRC does not directly regulate *SPT* transcription.

SPT expression is negatively regulated by AP2 in first whorl organs

Genetic evidence suggests that AP2 negatively regulates the expression of *SPT* in the first whorl (Alvarez and Smyth, 1999). In *ap2-2* mutants, the first whorl medial organs contain all cell types present in wild-type carpels, including septum, transmitting tract and stigmatic cells that are controlled by SPT. However, unlike normal carpels, unfused carpels develop a flange of ectopic stigma tissue from their lateral margins as well as on top.

In unfused medial carpels of *ap2-2* mutants, *SPT* transcripts were detected in all those tissues, and their precursors, that normally express *SPT* in the wild-type gynoecium (Fig. 6G). Further, the ectopic development of stigmatic papillae is associated with ectopic expression of *SPT* along the outer edge (Fig. 6H). These results confirm that AP2 normally prevents expression of *SPT* in the first whorl.

SPT is expressed in the absence of AG activity

The role of *AG* in carpel development has been inferred in part by assessing its role in controlling development of the ectopic carpels that develop in the first whorl of *ap2* mutants (Bowman et al., 1991). Interestingly, when *AG* is in mutant form in addition to *AP2*, the outer whorl organs frequently retain many carpel features including stigmatic and septal tissue. However, when *SPT* is mutant in addition to *AG*, as in *ap2-2 ag-1 spt-2* triple mutants, all the marginal pollen tract tissues such as stigma and septum are lost and the organs closely resemble leaves (Alvarez and Smyth, 1999). These data indicate that despite the loss of *AG* activity, *SPT* remains active and is necessary for the development of most of the remaining carpel features.

This proposal was confirmed, as the pattern of *SPT* expression in *ap2-2 ag-1* carpelloid organs (Fig. 6I) was similar to that in *ap2-2* single mutants (Fig. 6G,H). Even so, *SPT* expression was less intense in the *ap2-2 ag-1* organs, and this reduction is associated with the somewhat reduced degree of carpelloidy seen in the doubly mutant organs. In addition, *SPT* did not accumulate at the apex where the stylar prominence is lacking as a consequence of the loss of AG activity (Alvarez and Smyth, 1999). Taken together, these results show that SPT can act independently of AG, but that its expression is supported to some degree by coincident *AG* expression.

SPT expression is negatively regulated by the ETTIN gene product

ettin (*ett*) mutant gynoecia exhibit developmental defects including the ectopic development of stigmatic and transmitting tract tissue at the expense of carpel wall tissue (Sessions and Zambryski, 1995; Sessions, 1997; Fig. 7A,C). Interestingly, *spt* is largely epistatic to *ett* in this regard (Alvarez and Smyth, 1998; Fig. 7B-D). To test if this abnormal tissue development results from ectopic *SPT* expression, its expression pattern was examined in the developing gynoecia of *ett-3* mutant flowers.

Ectopic transcripts of *SPT* were apparent throughout gynoecium development. As early as stage 6, *SPT* expression appeared more intense in the lateral regions of the primordium than in wild type (Fig. 7E, compare Fig. 4C). Ectopic expression became more obvious during stage 7, when it was detected in abaxial cells (Fig. 7F) which, by stage 8, appeared to be undergoing periclinal divisions (Fig. 7G). As ectopic outgrowths of style and stigmatic tissue develop towards the apex during stage 10, *SPT* expression remains restricted to the outer, periclinally dividing cell layers (Fig. 7H). The layers underlying these appear to be stylar cells rather than valve tissue. Together, these observations are simply explained if ETT normally prevents *SPT* expression in sub-regions of the wild-type gynoecium.

In addition to ectopic septum and stigma cells, *ett* mutant gynoecia develop a stalk or gynophore in the basal region at the expense of ovary tissue (Sessions and Zambryski, 1995; Sessions, 1997; Fig. 7C). Longitudinal sections reveal that although *SPT* expression does not occur in the extending gynophore from stage 7 onwards (Fig. 7K,L), it does extend to the base of the gynoecial primordium at stage 6 (Fig. 7I,J).

The epistasis of *spt* over *ett* (Fig. 7A-D) provides an opportunity to define the early domain of ectopic SPT expression in the *ett spt* double mutant gynoecial primordium without the confounding morphological aberrations seen in the *ett* single mutant. During stage 6, *SPT* expression occurs in its normal medial domain, and also ectopically in a ring that encircles the primordium (Fig. 7M). Significantly, this ring corresponds to the domain of *ETT* expression at this stage (Sessions et al., 1997). Later, ectopic expression is maintained in abaxial regions, although it becomes more patchy (Figs 7N-P). This expression

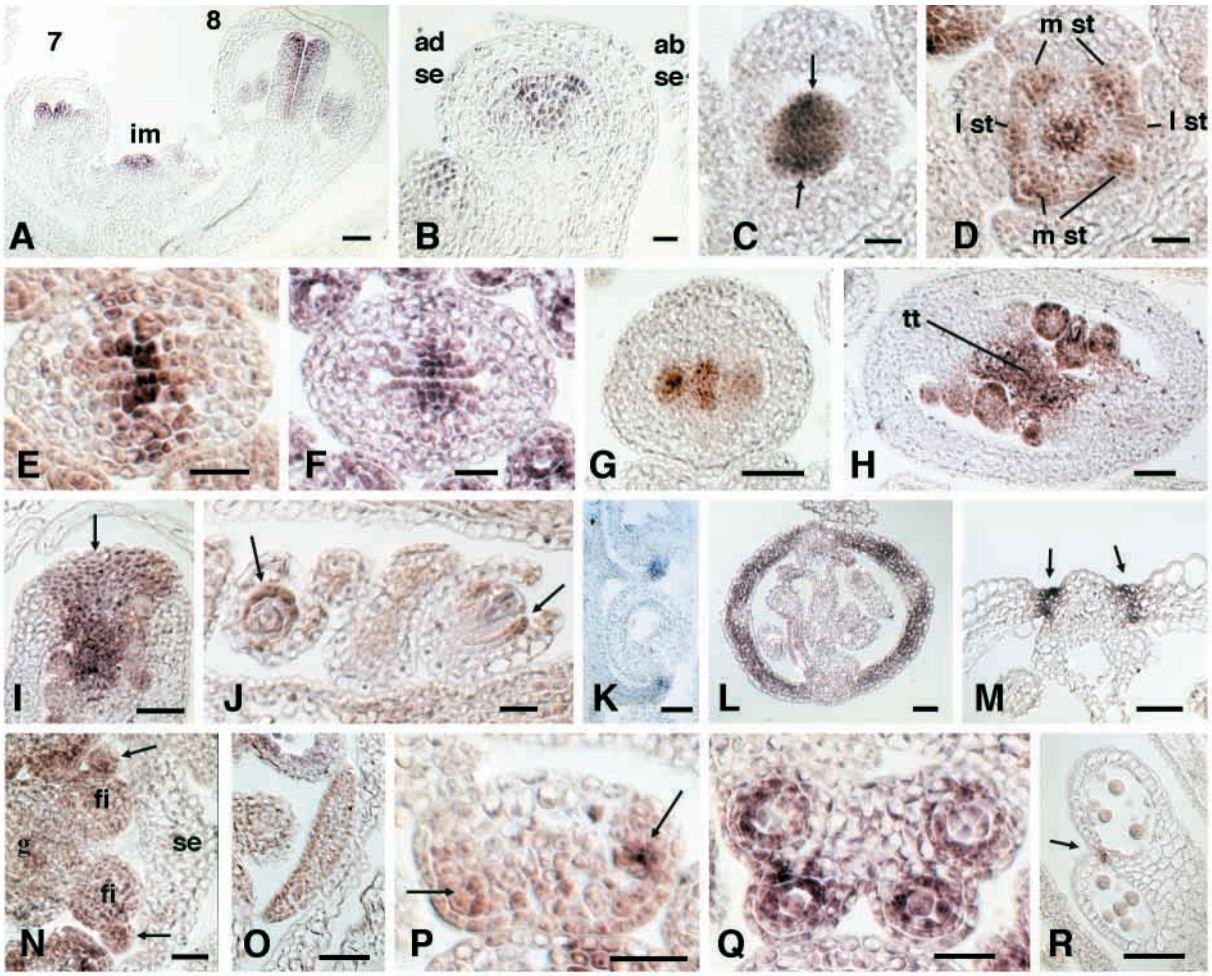

Fig. 4. *SPT* expression patterns in wild-type floral tissues. (A) Longitudinal section of an inflorescence, showing the inflorescence meristem (im) and medial views of stage 7 and 8 flowers. (B) Medial longitudinal section of a stage 6 bud (ad se, adaxial sepal; ab se, abaxial sepal). (C,D) Transverse sections of a stage 6 bud (m st, medial stamens; l st, lateral stamens). Signal at the apex of the gynoecial primordium (C) is strongest in the medial regions (arrows). A transverse section 24 μm below that shown in C shows expression in the stamen primordia and in a domain at the centre of the bud (D). (E-H) Transverse sections of gynoecia at stage 7 (E), stage 8 (F), stage 10 (G) and stage 11 (H; tt, transmitting tract). (I) Glancing longitudinal section of the apex of a stage 11 gynoecium showing expression within the developing stigma (arrow). (J) Ovules of a stage 12 gynoecium showing expression in the cell at the tips of the inner integuments (arrows). (K) Longitudinal sections of developing seeds at stage 15. (L) Transverse section of a stage 13 gynoecium. (M) Transverse section of the medial region of a stage 17 silique showing expression in the dehiscence zone (arrows). (N) Transverse section of a stage 8 flower showing expression in the petal primordia (arrows; fi, filament; g, gynoecium; se, sepal). (O) Transverse section of a stage 11 petal. (P) Transverse section of a stage 7 anther showing expression in the parietal and sporogenous cell layers (arrows). (Q) Transverse section of a stage 8 anther. (R) Transverse section of a stage 12 anther showing expression in the stomium (arrow). Bars, 25 μm (A,C-F,J,N,P,Q); 10 μm (B); 50 μm (G-I, K-M,O); 100 μm (R).

occurs in regions where ectopic outgrowth occurs in *ett-3* single mutants, but such outgrowths are absent in the *spt-2 ett-3* double mutant. Thus at this stage of development, ectopic *SPT* expression is apparently required to promote these ectopic outgrowths rather than being simply associated with them.

Interactions between ETT and *SPT* within the flower seem to be limited to the gynoecium as we observed no differences in *SPT* expression in other floral organs of *ett-3* mutants.

DISCUSSION

SPT is a novel bHLH transcription factor

SPT encodes a transcription factor of the well-known bHLH family that includes c-Myc. This family is relatively ancient, dating back at least to the common ancestor of plants, animals and fungi. In animals, many family members are known to act in regulatory networks that control cell proliferation and the generation of specific cell types, including components of myogenesis, neurogenesis, sex determination and haematopoiesis (Littlewood and Evan, 1998). By contrast, *SPT* is one of the first bHLH transcription factors to be identified in plants that controls morphogenetic processes.

Of plant bHLH proteins whose function has been established, many regulate anthocyanin biosynthesis (DEL, JAF13, B-peru and R-1c; Fig. 3B). Other characterised functions include regulation of response to abscisic acid and dehydration (rd22BP1), and regulation of the expression of seed storage genes

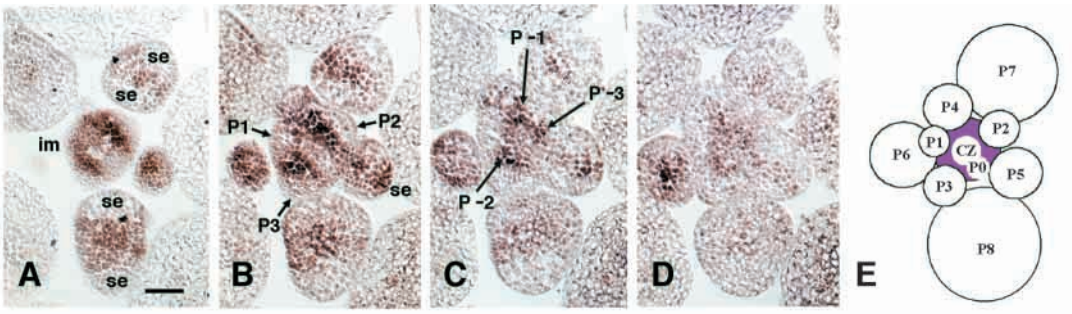

Fig. 5. *SPT* expression pattern in serial transverse sections of an inflorescence meristem. Bud primordia are numbered according to their relative age from P1 to P8. Bud anlagen are numbered P –3 to P0. Expression is high in the anlagen of flowers and sepals, but falls away as they initiate. (A) Section at the apex showing expression in the peripheral zone of the inflorescence meristem (im). Expression is excluded from the developing medial sepals (se) of two stage 3 buds corresponding to P7 and P8. (B) Section 8 μm below A. Expression is weaker in the central zone of the inflorescence meristem, and is excluded from the youngest floral primordia (P1, P2 and P3) on its flanks. Expression in the stage 2 bud (P5) occurs in two domains corresponding to anlagen of the abaxial sepal (se) and the inner adaxial sepal. (C) Next serial section showing expression concentrated in regions destined to form flower primordia (P –3, P –2 and P –1). (D) Last serial section showing attenuation of expression deeper in the inflorescence meristem. (E) Diagram indicating the relative ages of the floral primordia shown in A-D (P0 to P8) and the expression of *SPT* in the peripheral zone (pink). Bar, 50 μm.

(PG1; Fig. 3B). All these proteins share a region of homology amino terminal to their bHLH domains. In B-peru, this region interacts with the Myb transcription factor C1, at least in yeast cells (Goff et al., 1992). rd22BP1 is also thought to interact with the myb protein AtMYB2 to activate a putative target gene *rd22* (Abe et al., 1997). SPT does not contain this N-terminal domain and so is unlikely to interact with a MYB partner in this way.

The bHLH protein most closely related to SPT, PHYTOCHROME INTERACTING FACTOR3 (PIF3) from *Arabidopsis*, also lacks such conserved amino-terminal sequences. Instead, PIF3 contains an N-terminal PAS domain that is thought to mediate a direct interaction between PIF3 and phyA and phyB (Ni et al., 1998). Again, SPT lacks such a PAS domain and there seem to be few if, any parallels, in SPT and PIF3 functions.

Mutant *spt* phenotypes and associated mutational changes

Despite having the weakest known phenotype, the *spt-1* mutant allele is predicted to generate a truncated protein of just 50 amino acids (Fig. 3A). Perhaps *spt-1* translation re-initiates at codon 107, similar to the re-initiation event seen in translation of the bHLH protein R-lc of maize (Damiani and Wessler, 1993). The stop codon associated with the strong *spt-3* allele occurs further downstream at codon 149 (Fig. 3A), so re-initiation may not occur in this case. The other strong mutant allele, *spt-2*, results in an arginine to lysine substitution in the basic region (Fig. 3A). Significantly, although this is a conservative substitution, it abolishes DNA binding for a number of other bHLH proteins, including E12 (Sieber and Allemann, 1998), E47 (Voronova and Baltimore, 1990) and TFEB (Fisher et al., 1993).

SPATULA functions directly to promote proliferation of specific tissues within the gynoecium

By matching *SPT* expression patterns with *spt* mutational disruptions in the developing gynoecium (Alvarez and Smyth, 1999), we conclude that SPT function directly promotes the growth of medial regions where the two carpels are congenitally fused. Growth is retarded specifically in these

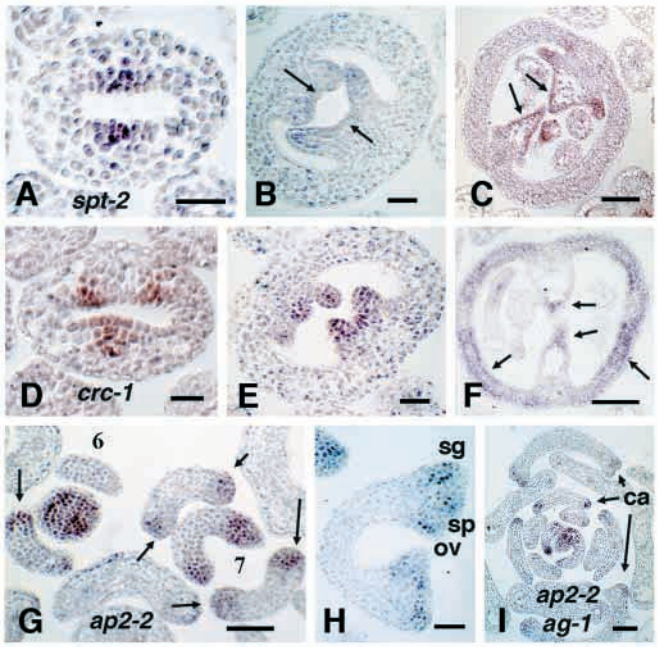

Fig. 6. *SPT* expression in *spt*, *crc*, *ap2* and *ap2 ag* gynoecia. (A-C) Transverse sections of *spt-2* gynoecia at stage 8 (A), stage 10 (B) and stage 12 (C). Note absence of expression between the ovule primordia in B (arrows), and faint expression in the epidermis of the unfused septum in C (arrows). (D-F) Transverse sections of *crc-1* gynoecia at stage 8 (D), stage 10 (E) and stage 12 (F). Note *SPT* expression in the septal cells and carpel walls in F (arrows). (G) Transverse section through two *ap2-2* flowers at stage 6 and stage 7 showing expression in the first whorl carpel margins (arrows). (H) Transverse section of an *ap2-2* first whorl carpel at the stage when the ovules are initiated. Note expression corresponding to the position of the developing ovules (ov), septum (sp) and stigmatic tissues (sg). (I) Transverse section of an *ap2-2 ag-1* double mutant flower. Weak expression is detected in the margins of carpelloid leaves (ca). Bars, 25 μm (A,B,D,E,H); 50 μm (I,G); 100 μm (C,F).

regions as the newly arising gynoecium begins to elongate. Later, development of the internal gynoecial ridge and septum is also compromised, and stylar and stigmatic cells are less abundant. *SPT* is expressed specifically in these regions throughout their development, suggesting that SPT acts directly and cell autonomously to promote their growth, and that SPT function is required continuously.

There is only one cell type that absolutely requires SPT function. Transmitting tract cells that produce extra-cellular matrix within the style and septum do not arise in strong *spt* mutants (Alvarez and Smyth, 1999). *SPT* is expressed within these cells continuously as they grow and as they mature in the wild type, suggesting that SPT is absolutely required for their differentiation as well as for the cell proliferation that generates them.

As the gynoecium develops into the mature silique, *SPT* is expressed in the ovary wall. In *spt* mutants, the silique is shorter than in the wild type, suggesting that here, too, SPT function normally promotes growth. From stage 12, *SPT* expression in the ovary wall parallels that of the MADS box gene *FRUITFULL* (*FUL*; Mandel and Yanofsky, 1995; Gu et al., 1998), and these two genes may share regulatory functions.

SPATULA may redundantly control growth and dehiscence in other tissues

SPT is expressed in many other tissues of the plant, although the lack of mutant disruption in these regions shows that SPT function is not necessary. That SPT is likely to have redundant functions is not surprising given that it belongs to a gene family estimated to contain 100 members in *Arabidopsis* (Reichmann and Ratcliffe, 2000). Furthermore, the ability of these proteins to function as heterodimers suggests that SPT function may be differentially specified in different tissues through dimerization with a range of distinct partners, as is commonly the case for animals bHLH proteins (Littlewood and Evan, 1998).

Many of the tissues in which *SPT* is expressed are actively growing. Within the flower, *SPT* expression is present in proliferating cells within ovule primordia, in the lengthening funiculus and in elongating cells of the integuments. It is present in developing petals throughout their growth. Stamen primordia, too, express *SPT*, as do growing sub-regions of the maturing anther.

Within the inflorescence meristem, *SPT* is expressed in the peripheral zone, but not in newly arising primordia. This pattern shows parallels with that of the homeodomain gene *SHOOT MERISTEMLESS* (*STM*; Long et al., 1996). STM has been proposed to maintain a population of undifferentiated cells within the meristem (Endrizzi et al., 1996), a function no longer required when primordia arise. Unlike *STM*, however, particularly strong *SPT* expression seems to be specifically associated with locations deeper within the meristem where several successive flower primordia are destined to arise. SPT might play a role in defining these positions and promoting their growth. Interestingly, the pattern of *SPT* expression in young flower primordia as sepals arise, and as carpels arise, shows parallels with the inflorescence expression pattern.

Another common theme associated with *SPT* expression is cell separation. We localised *SPT* expression to the stomium of the anther, the abscission zone within the funiculus, and the dehiscence zone of the silique. In the last case, *SPT* expression becomes restricted to the edges of the valves of the maturing silique in a pattern that resembles that of the *SHATTERPROOF* MADS genes (*SHP1* and *SHP2*; Savidge et al., 1995; Flanagan et al., 1996; Férrandiz et al., 2000). In s*hp1 shp2* double mutants, cells at the borders of the carpels fail to differentiate appropriately and do not become lignified, which in turn prevents pod shatter (Liljegren et al., 2000). Valve dehiscence is not affected in *spt* mutants, and SPT may play a redundant role downstream of the SHP proteins.

The regulation of *SPT* expression by CRC, AP2 and AG

Our results indicate that CRC does not regulate *SPT* expression directly. The level of *SPT* transcription seems unaffected in tissues that arise normally in *crc* mutant plants. The *CRC* gene encodes a YABBY family transcription factor that is expressed in lateral regions of the initiating gynoecium, and later in its walls and epidermis (Bowman and Smyth, 1999). Thus its expression domain does not overlap with that of *SPT*, consistent with the lack of a direct effect. A corollary of this is that the boost to CRC transcription seen in *spt* mutant gynoecia (Bowman and Smyth, 1999) is also likely to be indirect. Such indirect downstream effects could account for the more severe phenotypic disruptions seen in *spt crc* double mutants, and in *crc* mutants carrying only one copy of the active *SPT* gene (Alvarez and Smyth, 1999).

Likewise, *SPT* expression is not absolutely dependent upon AG function even though their expression domains are coincident early in gynoecium development (Liu et al., 2000). However, we have shown that the negative regulation of *SPT* expression in first whorl floral organs is dependent upon the function of the *AP2* gene. This negative control also applies to *AG* (Drews et al., 1991) and *CRC* (Bowman and Smyth, 1999).

Consequences of SPT activity in *ett* mutants

In *ett* mutants, *SPT* is ectopically expressed on the outer surface of the developing gynoecium as well as internally. Later this abaxial expression is specifically associated with the proliferation and differentiation of transmitting tract and stigmatic cells that appear on this surface in *ett* mutants (Sessions and Zambryski, 1995; Sessions, 1997). Thus, our results suggest that ETT patterns gynoecium tissues in part by partitioning *SPT* expression, which in turn specifies distinct cell fates.

The situation in more basal regions of the *ett* gynoecium is less clear. Instead of valve tissue, a stalk or gynophore develops. Interestingly, this aspect of the *ett* phenotype is also substantially suppressed by the loss of SPT function. Because *SPT* expression is not detected during the development of the gynophore after stage 6, either SPT activity promotes its growth before stage 7, or SPT controls its development non-autonomously.

ETTIN has been proposed to control apical-basal patterning of the gynoecium by controlling the position of two radial boundaries (Sessions, 1997; Sessions et al., 1997). These boundaries are proposed to shift as a consequence of loss of ETTIN function. However, boundaries between tissue types are apparently close to normal in *ett* mutants, providing *spt* is also mutant (Alvarez and Smyth, 1998). Hence our results suggest that the distortions to pattern formation seen in *ett* single mutants are largely a secondary consequence of ectopic *SPT* expression.

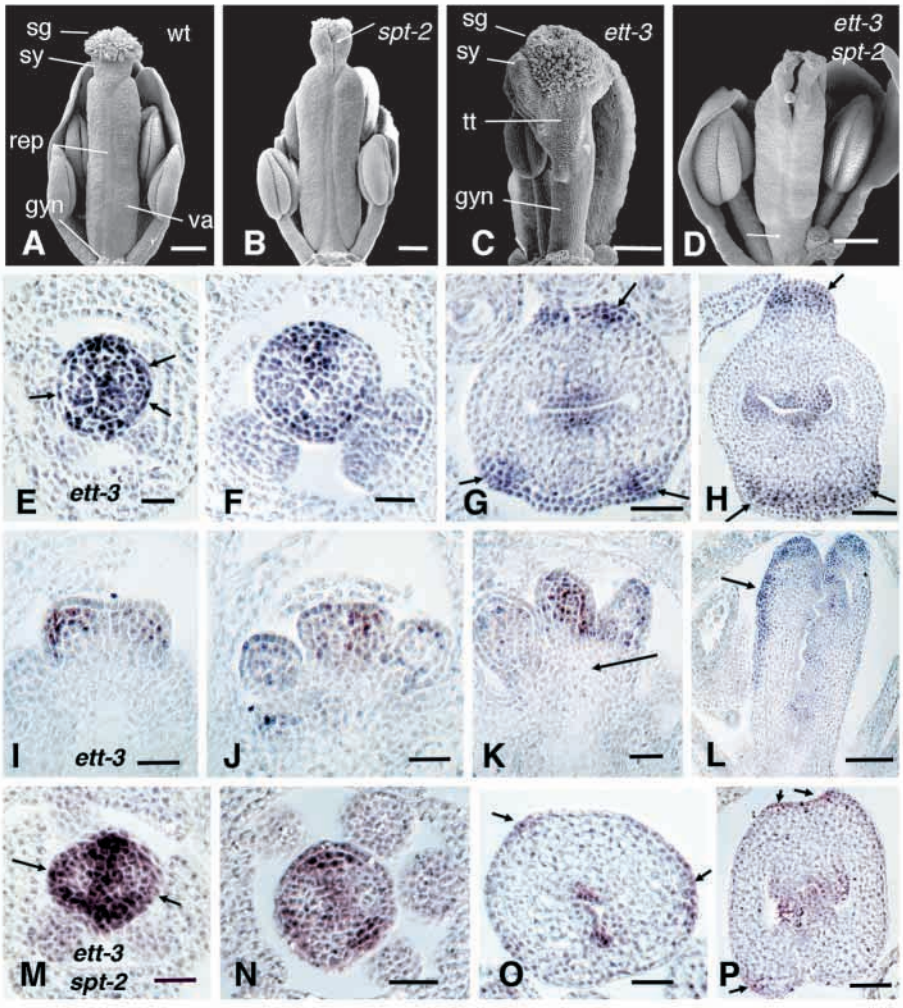

Fig. 7. Interactions between *spt-2* and *ett-3*. (A-D) Scanning electron micrographs of dissected stage 12 flowers of the wild type (A), *spt-2* (B), *ett-3* (C) and *ett-3 spt-2* (D). Note unfused apex of *spt-2* gynoecium (arrow in B). In *ett-3* single mutants, note the increase in stigma (sg), style (sy),transmitting tract (tt), and gynophore (gyn) tissues, and the reduction in valve (va) and replum (rep) tissues, compared with the wild type (A). Significantly, these *ett-3* disruptions are mostly ameliorated in the *ett-3 spt-2* double mutant (arrow indicates remaining small gynophore in D). (E-H) *SPT* expression in transverse sections of developing *ett-3* gynoecia at stage 6 (E), stage 7 (F), stage 8 (G), and stage 10 (H). *SPT* is ectopically expressed laterally from stage 6 (arrows in E) and later in abaxial proliferating cells (arrows in G and H). (I-L) *SPT* expression in longitudinal sections of developing *ett-3* gynoecia at stage 6 (I,J), stage 7 (K), and stage 10 (L). I and J are representative sections from a series through a stage 6 gynoecium in the medial and lateral planes, respectively. *SPT* expression is not detected within the developing gynophore (arrow in K), but it increases towards the apex (arrow in L). (M-P) *SPT* expression in transverse sections of developing *ett-3 spt-2* double mutant gynoecia at stage 6 (M), stage 7 (N), stage 8 (O), and stage 10 (P). *SPT* is ectopically expressed in an abaxial ring (arrows in M). After stage 8, *SPT* expression is mostly restricted to the epidermis (arrows in O and P). Bars, 25 μm (E,F,I-K,M-O); 50 μm (G,P); 100 μm (L); 200 μm (A-D).

ETTIN, auxin and *SPT* regulation

Is this control of *SPT* expression by ETT direct or indirect? There is some evidence that it is direct. Firstly, during stages 6-8 *ETT* is expressed in an abaxial cylinder within the wild-type gynoecial primordium (Sessions et al., 1997). This pattern coincides closely with the pattern of ectopic *SPT* expression in *ettin* mutants. Secondly, ETT is a member of the Auxin Response Factor (ARF) family (Sessions et al., 1997). Characteristically such proteins bind TGTCTC motifs that occur within Auxin Response Elements (AuxREs; Ulmasov et al., 1997; Ulmasov et al., 1999). Significantly several AuxRE-like elements occur within the *SPT* promoter. We propose that ETT may bind to such sequences and block SPT transcription in the developing gynoecium. (In other parts of the plant, however, including the petals and stamens, this relationship does not hold as the *SPT* and *ETT* expression patterns overlap; Sessions et al., 1997; this study.)

AuxREs are known to confer auxin responsiveness (Guilfoyle et al., 1998), suggesting that auxin may also regulate *SPT* transcript levels. Rather than ETT being involved here, it seems more likely that other ARFs act to trigger SPT expression. This proposal is supported by the recent finding that treating *spt* mutant gynoecia with inhibitors of polar auxin transport suppresses the *spt* mutant phenotype, especially in apical regions (Nemhauser et al., 2000). Such treatment may cause a build up of auxin in apical regions, and an auxin signalling pathway promoted by SPT may be strengthened (Nemhauser et al., 2000). In contrast, treatment of gynoecia of an intermediate *ett* mutant strengthens the *ett* mutant phenotype (Nemhauser et al., 2000). Given our results, this enhancement is likely to be caused in part by increased ectopic *SPT* expression, perhaps in response to increased auxin concentrations towards the apex.

It is now important to test whether or not *SPT* expression is induced by auxin, not only in developing gynoecia but also in other growing tissues where *SPT* is expressed.

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