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CRABS CLAW and *SPATULA*, two *Arabidopsis* genes that control carpel development in parallel with *AGAMOUS*

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SUMMARY

To help understand the process of carpel morphogenesis, the roles of three carpel development genes have been partitioned genetically. Mutants of *CRABS CLAW* cause the gynoecium to develop into a wider but shorter structure, and the two carpels are unfused at the apex. Mutants of a second gene, *SPATULA*, show reduced growth of the style, stigma, and septum, and the transmitting tract is absent. Double mutants of *crabs claw* and *spatula* with homeotic mutants that develop ectopic carpels demonstrate that CRABS CLAW and SPATULA are necessary for, and inseparable from, carpel development, and that their action is negatively regulated by A and B organ identity genes. The third carpel gene studied, *AGAMOUS*, encodes C function that has been proposed to fully specify carpel identity. When AGAMOUS function is removed together with the A class gene *APETALA2*, however, the organs retain many carpelloid properties, suggesting that other genes are also involved. We show here that further mutant disruption of both CRABS CLAW and SPATULA function removes remaining carpelloid properties, revealing that the three genes together are necessary to generate the mature gynoecium. In particular, *AGAMOUS* is required to specify the identity of the carpel wall and to promote the stylar outgrowth at the apex, *CRABS CLAW* suppresses radial growth of the developing gynoecium but promotes its longitudinal growth, and *SPATULA* supports development of the carpel margins and tissues derived from them. The three genes mostly act independently, although there is genetic evidence that CRABS CLAW enhances AGAMOUS and SPATULA function.

Key words: Flower development, Carpel, Gynoecium, *AGAMOUS*, *CRABS CLAW*, *SPATULA*, *Arabidopsis thaliana*

INTRODUCTION

Carpels are the female reproductive unit of flowers. They enclose the ovules that bear the female gametophyte, and they provide a screen that is penetrated only by appropriate male gametes. At maturity, they generate the fruit that harbours the developing seeds. The identity of carpels and other floral organs is specified by three classes of homeotic genes (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). These encode three identity functions, named A, B and C, that are active within the developing flower in concentric overlapping fields. When A function acts alone, sepal identity results, A and B together specify petals, B and C in combination result in stamen development, and C function alone specifies carpels. A and C functions have additional roles in mutually preventing each others action, with A blocking C function in the perianth (sepals and petals), and C preventing A function in the reproductive organs (stamens and carpels).

Only one C function gene is known. In *Arabidopsis* this is *AGAMOUS* (*AG*). In *ag* mutant flowers (Bowman et al., 1989, 1991), A function is no longer blocked from the central whorls. Petals now develop in place of stamens, and, instead of carpels, four sepals arise that constitute the outer whorl of another inner flower. The role of AG in specifying carpel identity can also be seen indirectly in mutants of A function genes including *APETALA2* (*AP2*) (Bowman et al., 1989, 1991; Kunst et al., 1989). In these, *AG* is no longer under negative control in the perianth and it is ectopically expressed there (Drews et al., 1991). Rather than developing into sepals, the first whorl organs now display the properties of normal fourth whorl carpels. Similar sepal-to-carpel transformations are observed when AG is constitutively expressed (Mizukami and Ma, 1992). (Continued proliferation in the center of the floral meristem in *ag* mutants is the result of disruption of a different function of AG, imposition of determinacy; Mizukami and Ma, 1995; Sieburth et al., 1995.)

Even so, AG function is not absolutely required to generate all carpel properties. When AG activity is in turn removed from *ap2* A function mutants, floral organs nevertheless retain many carpel properties including style, stigma and ovule production (Bowman et al., 1991). Other carpel genes must be involved.

To identify further genes involved in carpel development, we undertook a screening program for carpel-specific mutants (Alvarez and Smyth, 1998). We argued that such genes are likely to include primary targets of AG regulation, as well as genes acting independently of AG. Mutants of two genes,

CRABS CLAW (*CRC*) and *SPATULA* (*SPT*), were isolated which have different developmental defects largely exclusive to the gynoecium. In this study, the relationships between the two genes, *CRC* and *SPT*, and with *AG* and other organ identity genes are defined. Both CRC and SPT function were found to be inextricably associated with the carpel development program. Analysis of the interactions of CRC and SPT with AG has further revealed that, rather than being direct downstream targets of *AG*, *CRC* and *SPT* play relatively independent roles in controlling different aspects of carpel development in parallel with *AG*.

MATERIALS AND METHODS

The Landsberg *erecta* ecotype was used throughout. Plants were grown under daylight supplemented with continuous cool white fluorescent light at 20-25°C. Seeds were mutagenised with 25 mM ethyl methane sulphonate (EMS) for 12 hours, or 25 krad of γ irradiation from a ^{60}Co source, and mutants identified by screening second generation plants. The loci of mutants were mapped by crossing them to a tester line carrying ten mutant markers, and linkage was detected among F_2 segregants.

Multiple mutant plants were generated by intercrossing homozygous mutants (or heterozygotes if plants were both male and female sterile), and the desired mutant combinations recognised among the phenotypic categories seen in the F_2 segregants. Their genotypes were confirmed by checking that they arose in the expected Mendelian proportions, and, if possible, by progeny testing. The *agamous* mutant used was *ag-1*, a null allele (Yanofsky et al., 1990). The *apetala2* and *pistillata* mutant alleles used, *ap2-2* and *pi-1*, are also null alleles (Jofuku et al., 1994; Goto and Meyerowitz, 1994). For phenotypic analysis, the first 15-20 flowers on the primary inflorescence shoot were examined unless otherwise specified.

For light microscopy and scanning electron microscopy (SEM), samples were immersed in 2% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 6.8) and vacuum infiltrated for up to one hour. For sections, specimens were then washed, dehydrated in an ethanol series, and infiltrated and embedded in LR White resin. Sections of 2 μm were cut, dried onto slides and stained with 0.3% toluidine blue (w/v) in 1% sodium tetraborate (w/v, pH 9) for 30 seconds. For SEM, glutaraldehyde-fixed tissues were further fixed in 1% OsO_4 before dehydration through a graded ethanol series and critical point drying using liquid CO_2. Specimens were coated with platinum in an Eiko 1B.5 sputter coater, dissected if necessary, and viewed using a Hitachi s570 scanning electron microscope. To view the vasculature, cleared whole gynoecia were prepared. They were fixed in 3:1 ethanol:glacial acetic acid (v/v) for 24 hours, rinsed in 0.1 M potassium phosphate buffer (pH 7.5) for 2 hours, cleared in 1 M NaOH at room temperature for 12 hours, and washed in the same buffer. They were then mounted whole in buffer on a slide, and observed using dark-field optics.

RESULTS

Functions of CRC and SPT deduced from their single and double mutant phenotypes

Structure of the wild-type gynoecium

The mature wild-type gynoecium of *Arabidopsis* has been described in detail elsewhere (Hill and Lord, 1989; Smyth et al., 1990; Sessions and Zambryski, 1995). In brief, it consists of a stigma, style and ovary joined to the floral receptacle by a very short stem (Fig. 1A,B). The ovary is divided medially into two locules. Each of the lateral walls is occupied largely by valve tissue. These are joined to two vertical strips of tissue, the repla, that run medially. In the mature fruit, the valve separates from the replum exposing the seeds. Internal to each replum, a septum grows across the ovary and fuses post-genitally with septal tissue growing from the other side. A row of ovules arises from placentae on each side of the elongating septum. The upper region of the gynoecium narrows into a short style that is capped by a dry stigma. Inside the style and septum, a core of transmitting tract cells develops. These secrete an extra-cellular matrix that supports pollen tube growth. Studies of mutants have revealed that the gynoecium consists of two congenitally fused carpels joined along their edges where replum, septum and placental tissues arise (Okada et al., 1989).

crabs claw mutations affect growth of the gynoecium

Two alleles of the *CRABS CLAW* (*CRC*) gene were isolated, a stronger EMS allele *crc-1* and a weaker γ-induced allele *crc-2*. The *CRC* locus was mapped to chromosome 1, just below *APETALA1*. The stronger mutation *crc-1* results in a gynoecium that is shorter overall than wild type but wider both laterally and medially (Fig. 1C,D). The two carpels are characteristically unfused in their upper third. Above the point of fusion, each carpel extends outward in the lateral plane, before bending inwards such that each half of the greatly abbreviated style is now nearly horizontal, and the two clusters of stigmatic papillae intermingle. All cell types of the wild type are present in appropriate locations. The only ectopic structure seen is an occasional ovule that arises from the replum outside the ovary. Overall the total number of ovules per gynoecium is reduced relative to wild type. One further characteristic is the occasional presence of more than two carpels, especially in the first formed flowers (Table 1). These are inserted medially. The only other mutant defect observed was the abolition of nectary development (see Bowman and Smyth, 1999). The weaker allele, *crc-2*, shares all these defects but they are less severe, especially the reduction in gynoecial length.

spatula mutations affect carpel fusion and abolish transmitting tract production

Two EMS alleles of the *SPATULA* (*SPT*) gene were obtained, a weaker mutant allele *spt-1* and a stronger allele *spt-2*. The *SPT* locus is on chromosome 4, less than 1 map unit below *APETALA2*. *spatula* mutants have a spectrum of changes different from that seen in *crc* mutants (Fig. 1E,F). The *spt-2* gynoecium at anthesis is narrower in the stylar region, and there is reduced development of stigmatic papillae at the tip. It is also somewhat flatter, being expanded laterally. In some cases the two carpels are unfused on one or both sides in the upper-most regions. The style is hollow, and the septum is under-developed and unfused, especially toward the top. There is no transmitting tract, as cells that secrete extra-cellular matrix are absent from the style and septum. The length of the gynoecium is not significantly different from wild type, although it carries about 20% fewer ovules. However only a quarter of these go on to develop as seeds. This appears to be the result of inefficient pollen tube growth associated with loss of the transmitting tract. Again, the weaker allele, *spt-1*, shares all the disruptions but mostly to a lesser degree. Defects in other floral or vegetative organs were not detected.

Table 1. Additional carpels and internal organs in *crc* single and multiple mutant flowers

	Number of carpels/gynoecium			Whorls of internal organs	
Genotype	2	3	4	1-10	Indeterminate
crc-1	76	2	2	0	0
crc-2	77	1	2	0	0
crc-1 spt-2	74	2	4	2	0
crc-1 spt-2/+	74	4	2	0	0
crc-1 ag-1/+	39	31	10	31	7

Entries show the number of flowers (total of 80 scored for each genotype).

Interactions between *crabs claw* and *spatula* mutant phenotypes

To test if CRC and SPT share redundant functions, the double mutant was created. Some redundancy seems likely, as the mature *crc-1 spt-2* gynoecial organs are markedly different from either single mutant (Fig. 1G,H). They are now boat-shaped, elongated at the tips and typically unfused except at the base. While the tissue morphology of the carpel wall closely resembles that of wild type, there is a marked reduction in other carpel tissues including stigmatic papillae, style, septum, transmitting tissue and ovules. In addition, there tends to be a greater loss of flower meristem determinacy than in *crc-1* single mutants, with stamens occasionally being observed interior to the fourth whorl carpels (Table 1). Plants heterozygous for *spt-2* and homozygous for *crc-1* show slightly increased loss of carpel properties compared with *crc-1* (results not shown), suggesting that the level of SPT function is important in *crc* mutant background. This effect is not allele specific.

Tests of the involvement of CRC and SPT in ectopic carpel development

Double mutants of *crc-1* and *spt-2* with the B class mutant *pistillata-1*

Mutations of either B class gene *PISTILLATA* (*PI*) or *APETALA3* (*AP3*) cause carpelloid organs to arise in what would normally be third whorl stamen positions (Bowman et al., 1989, 1991). To test whether *CRC* and *SPT* are ectopically active in these third whorl organs, the phenotype of flowers doubly mutant for each of *crc-1* and *spt-2* with the null allele *pi-1* (Bowman et al., 1989; Goto et al., 1994; Hill and Lord, 1989) were investigated.

In *pi-1* mutant flowers the third whorl is highly variable in structure, consisting of carpels and carpelloid filaments that demonstrate various degrees of connate fusion with each other and with fourth whorl carpels (Bowman et al., 1989; Hill and Lord, 1989; Fig. 2A). In *crc-1 pi-1* double mutants, the third whorl organs are less fused allowing a significant reduction in carpelloid properties to be deduced (Fig. 2B). Compared with unfused carpels in other genotypes (*ap2-2* for example, see below), they have little style and stigmatic tissue, and relatively few ovules arise from their edges. In flowers of plants homozygous for *spt-2* and *pi-1*, an even greater diminution in carpelloid development is observed (Fig. 2C). These reductions suggest that both CRC and SPT are now active in the third whorl of *pi* mutants and promote their feminisation. That is, B function normally acts to prevent the inappropriate action of CRC and SPT in the third whorl of the *Arabidopsis* flower.

Double mutants of *crc-1* and *spt-2* with the A class mutant *ap2-2*

Mutations in the A class gene *AP2* cause carpelloid organs to develop in the outermost whorl (Bowman et al., 1989; 1991; Kunst et al., 1989). To determine whether CRC and SPT contribute to this ectopic carpellody, double mutants were made between each of *crc-1* and *spt-2* and the null mutant *ap2-2* (Bowman et al., 1991; Jofuku et al., 1994). Before this, however, a detailed analysis of the carpelloid elements within the first whorl medial organs of *ap2-2* single mutants was carried out for later comparisons (Fig. 3A-D).

Firstly, the shape of the unfused carpels is linear with parallel edges. The edges, especially around the apex, are involuted, and the whole organ bends inwards so that it appears hooded (Fig. 3A). Secondly, the wall of the valve region is closely similar in structure to the ovary wall of the central gynoecium (Fig. 3A,B). Even so, tissue with leaf-like properties (including occasional stellate trichomes) and sepal characteristics can occur, especially in the first produced flowers. Thirdly, there is a remarkable change in the distribution of stylar and stigmatic cells. Instead of being confined to the tip of the gynoecial tube, these now arise along the edges of the unfused organ as well (Fig. 3A,B). They are intercalated between replum-like cells and a flange of septum cells (Fig. 3C), and appear as a lobe of tissue projecting from the ovary wall (Fig. 3B). The stigmatic cells typically decrease in extent toward the base of the carpel (Fig. 3D). These changes occur in all unfused carpels, including those often present in the centre of the *ap2-2* flower. It seems that, when a carpel is unfused, the valve and replum differentiate relatively normally, as do the septum and the ovules internally, but that the lateral edges now enter the style and stigma differentiation program that is normally confined to the apex.

The effect of disruption of CRC function in these ectopic carpels was then examined in *crc-1 ap2-2* double mutant flowers (Fig. 3E,F). The medial outer whorl organs develop ostensibly as unfused carpels but tend to be slightly broader and more leaf-like than in *ap2-2* single mutants (Fig. 3A). There is a greater abundance of phylloid epidermal characteristics on their abaxial surface, such as irregularly shaped cells, intervening stomata and stellate trichomes. Stylar epidermal cells with their prominent cuticular ridges are less abundant, and stigmatic papillae are also reduced and restricted to the upper regions (Fig. 3E,F). The number of marginal ovules is greatly reduced, but a significant flange of septal tissue typically remains.

When first whorl organs of the *spt-2 ap2-2* double mutant are examined, there is a more striking loss of stigmatic papillae, and septal tissue is also mostly lost (Fig. 3G,H). Involution of the margins is also reduced compared with *ap2-2* single mutants (Fig. 3A), and there is a blunt-ended prominence at the apex associated with stylar cells. Similar disruptions are seen in the fourth whorl. (In passing, in both *crc-1 ap2-2* and *spt-2 ap2-2* there is a reduced degree of connate and adnate fusion of first whorl organs, and an increased number of free standing stamens interior to the first whorl.)

The phenotypes of the *crc-1 ap2-2* and *spt-2 ap2-2* double mutants demonstrate that CRC and SPT are both active in the outer whorl of *ap2-2* mutant flowers, and that they promote the same carpel attributes as they do in the fourth whorl of wild-type flowers. Thus A function normally plays a role in

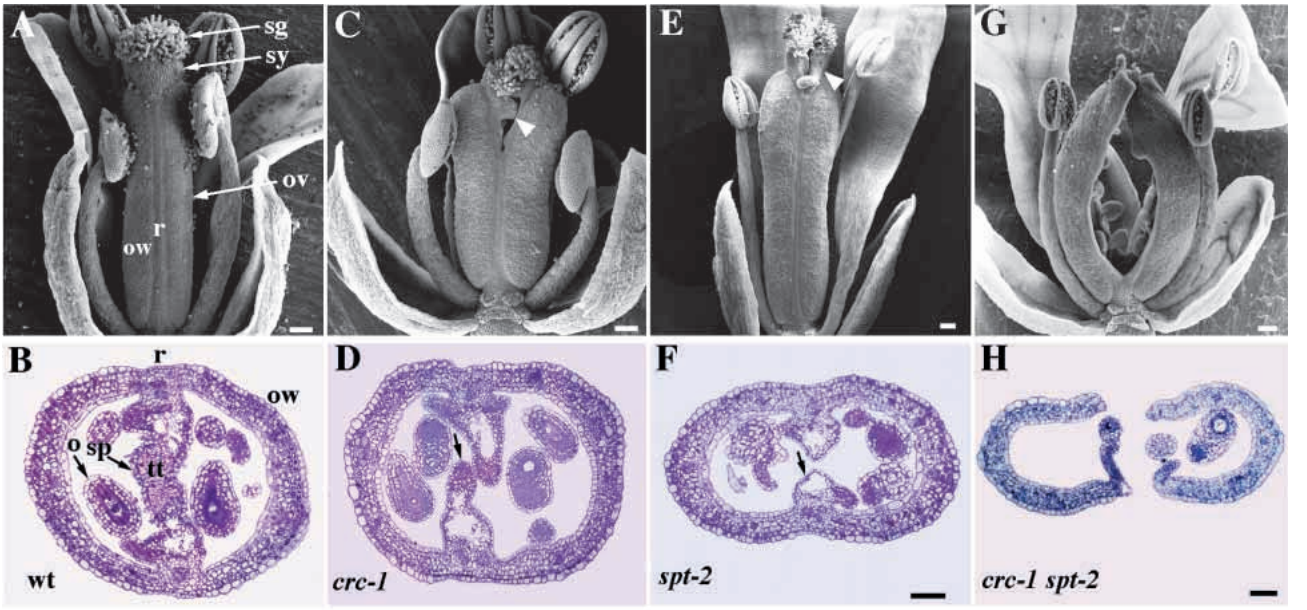

Fig. 1. Structure of the mature gynoecium of wild type, *crc-1* and *spt-2* single mutants, and the *crc-1 spt-2* double mutant. SEMs (above) are medial views of flowers at anthesis (stage 13), with some sepals, petals and stamens removed. Transverse sections (below) are ovaries of the same stage and genotype shown in the same orientation. (A,B) Wild type, showing the ovary (ov), style (sy) and stigma (sg) of the gynoecium. The two carpels are fused medially. The ovary walls (ow) are joined by a narrow replum (r). Inside the replum, a false septum (sp) has grown internally to divide the ovary into two locules. Extra-cellular matrix is secreted by cells within the septum and acts as a pollen tube transmitting tract (tt). Ovules (o) arise from placental regions close to, or within, each side of the septal outgrowths. (C,D) *crc-1*, showing the wider but shorter gynoecium that is unfused in the upper third (arrowhead). The septum is also unfused in the section shown, although transmitting tract cells are present (arrow). (E,F) *spt-2*, showing the medially flattened gynoecium with the two carpels unfused in the stylar region (arrowhead). Transmitting tract cells are absent (arrow). (G,H) *crc-1 spt-2*, showing greatly reduced fusion of the carpels, and reduced development of the style, stigma, septum and ovules. Scale bars: A,C,E,G 100 μm; F,H 50 μm; B,D same magnification as F.

suppressing their carpel promoting activity in the outer whorl. Together with the *pi-1* double mutant analysis, the results reinforce the proposal that wherever normal carpels arise in the *Arabidopsis* flower, *CRC* and *SPT* function necessarily occur.

Interactions between *CRC* and *SPT* and the carpel identity gene *AGAMOUS*

The flowers of *crc-1 ag-1* and *spt-1 ag-1* double mutants are indistinguishable from the *ag-1* single mutant in each case (except that the *crc-1 ag-1* double mutant lacks nectaries). This is consistent with the *ag-1*-induced disruption to the fourth whorl (where another flower arises) occurring before any disruptions resulting from *crc-1* and *spt-2* mutation.

Indirect genetic evidence for some interplay between CRC and AG functions was seen, however, in that in a *crc-1* homozygous mutant background, *ag-1* heterozygotes now have a distinguishable phenotype. In particular, there is a marked decrease in flower meristem determinacy. This is reflected by an increased number of carpels in the fourth whorl (more than half of flowers now have three or more carpels) (Fig. 4A), and proliferation of further organs occurs within the carpels of nearly half of all flowers (Fig. 4B; Table 1). These organs occur as alternating groups of stamens and carpels separated by an elongating pedicel. The mature gynoecium may also be elevated on a small gynophore (Fig. 4A), and there are reductions in style and stigma development and in ovule numbers. This effect is not allele specific. Thus *AG* is semi-dominant in a *crc* mutant background, and it may be that CRC function quantitatively promotes the AG determinacy function to some degree.

Significant carpel properties remain in *ap2-2 ag-1* double mutants

The carpel identity role of AG has been inferred in part from the observation that ectopic first whorl carpels arise when AG function is released from AP2 inhibition, as in *ap2-2* mutants. Significantly, however, carpelloid features have been reported to develop in these organs even when AG function is also removed in *ap2-2 ag-1* double mutants (Bowman et al., 1991). We have therefore further characterised these AG-independent carpelloid properties in these flowers before investigating which, if any, are controlled by CRC and SPT.

In *ap2-2 ag-1* double mutants the linear, hooded shape of the outer medial organs and internal carpelloid organs is retained (Fig. 5A). Also, the lobes of style cells and stigmatic papillae, the septal flange, and the ovules that line the sides of the *ap2-2* single mutant organs (Fig. 3A-D) are also frequently present, especially in earlier flowers (Fig. 5A,B). The first major change seen associated with loss of AG function involves the specialised cells of the ovary wall. The outer epidermis now shows variable vegetative properties, including leaf-like cells in combination with the very long cells characteristic of sepals (Fig. 5B). Internally, too, there is a striking change. The ovary wall of wild-type carpels (Fig. 5C) (and of *crc-1 spt-2* double mutants; Fig. 5D) is made up of six specialised cell layers. The first whorl carpels of *ap2-2* single

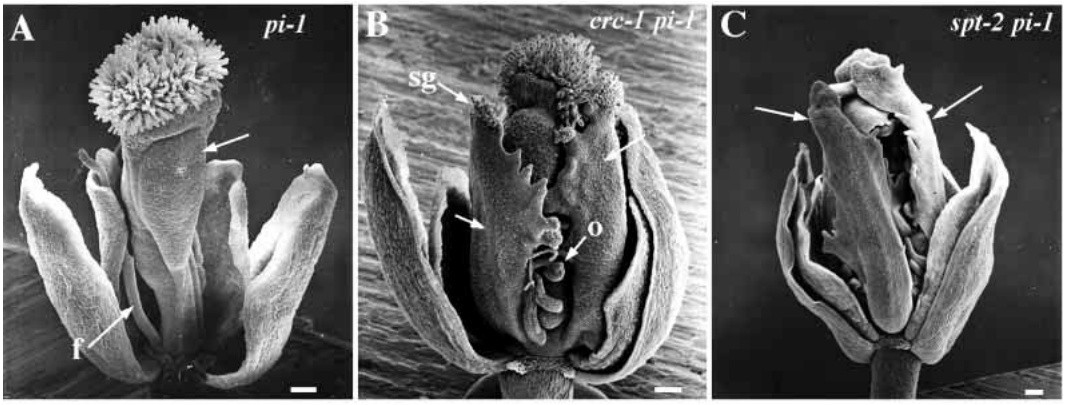

Fig. 2. SEMs of flowers of the B function mutant *pistillata-1* (*pi-1*) alone or in combination with *crc-1* or *spt-2*. (A) *pi-1*. The third floral whorl is variably carpelloid, and the organs are fused to each other and the fourth whorl (arrow). Some are filamentous (f). (B) *crc-1 pi-1*. Unfused third whorl organs (arrows) are still carpel-shaped but have reduced stigmatic papillae (sg) and ovules (o) generated from their edges. (C) *spt-2 pi-1*. Unfused third whorl organs (arrows) reveal stronger reductions in carpellody. Stigmatic outgrowths are lacking, and ovules are mostly vestigial. Scale bars: 100 μm.

mutants have a very similar structure (Fig. 5E). In *ap2-2 ag-1* first whorl organs, however, the adaxial layer normally made up of many thin cells that will ultimately be lignified in the mature fruit (Spence et al., 1996), is frequently replaced by a row of palisade mesophyll cells (Fig. 5F), just as is seen in the equivalent adaxial region of leaves.

A second major effect of removing AG function is a reduction in development of a stylar prominence. When the carpelloid organs are unfused, the tip now has a tapered, leaf-like point that is typically devoid of stigmatic papillae (Fig. 5G, compare Fig. 3A). The same conclusion can be reached from fused organs (Fig. 5H), where examination of the vasculature confirms severe stylar reduction. In wild-type gynoecia (Fig. 5I), the medial vascular bundles extend into the style and branch extensively producing a number of blunt-ended xylem elements (Fig. 5J; Sessions and Zambryski, 1995). In *ap2-2 ag-1* flowers, apical extension of the medial vasculature occurs only sporadically (Fig. 5K). Instead, it extends to the shoulders of the organ before converging with the central vascular bundle.

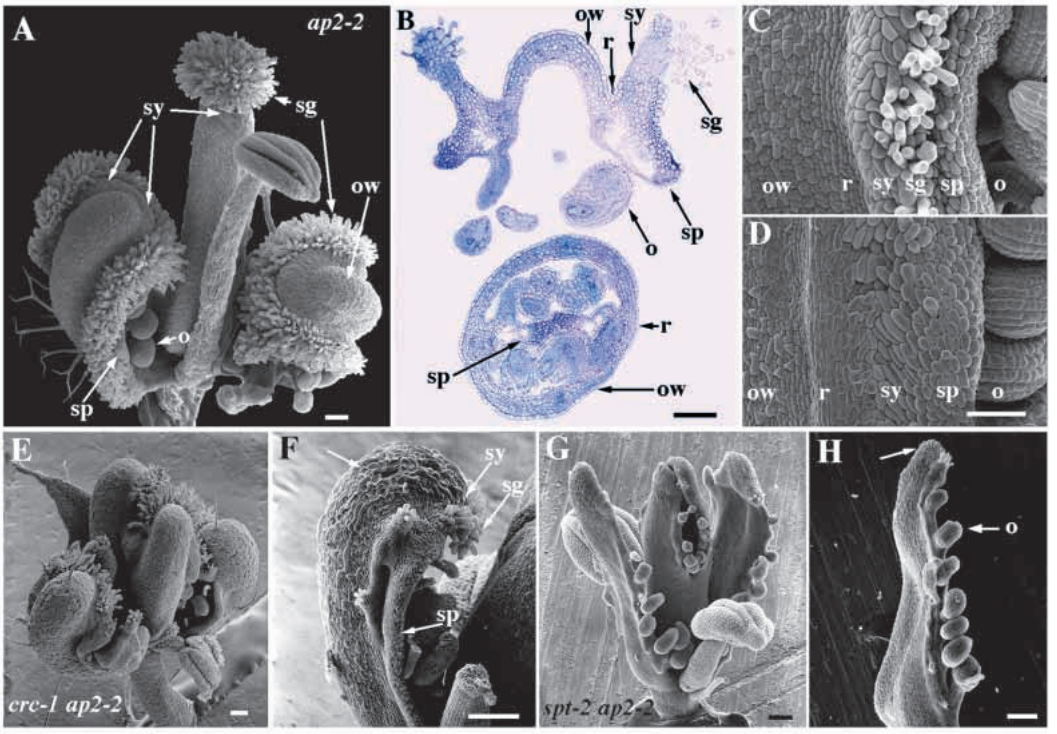

Fig. 3. The A function mutant *apetala2-2* (*ap2-2*), alone and in combination with *crc-1* and *spt-2*, showing reductions in carpelloid properties of first whorl organs. (A,B) SEM (A) and transverse section (B) of *ap2-2* flowers at anthesis. The medial first whorl organs (above in B) resemble unfused carpels with a hood-shaped ovary wall (ow) and flanking replum (r). However, stylar tissue (sy) and stigmatic papillae (sg) now arise as a continuous lobe along the unfused lateral edges as well as at the apex. A flange of septal cells (sp) arises internal to this lobe, and ovules (o) arise internal to this. The central fused gynoecium (below in B) is normal. (C,D) Higher magnification of the epidermis at the unfused edge of *ap2-2* medial first whorl organs. A progressive transition in cell types occurs: ovary wall (ow), replum (r), style (sy), stigma (sg), septum (sp), ovules (o). Stigmatic development is pronounced near the apex of the organ (C), but is progressively reduced toward the base (D). (E,F) *crc-1 ap2-2* flower (E), and medial first whorl organ (F). Incorporation of *crc-1* has reduced carpelloid properties. First whorl organs are now somewhat wider but remain hooded, and the lobe of style (sy) and stigma (sg) tissue around their edges is much reduced. The septal flange (sp) remains. Epidermal cells of the carpel wall are more sepal- and leaf-like (arrow in F). (G,H) *spt-2 ap2-2* flower (G) and medial first whorl organ (H). These are even less carpelloid. The first whorl organs are flatter, although a stylar outgrowth ('prominence') is apparent at their tip (arrow in H). Both the stigmatic lobe and the flange of septal tissue are greatly reduced around the edges, although ovules (o) still arise. Scale bars: A,B,E,F,G,H, 100 μm; C,D, 50 μm.

Table 2. Summary of phenotypic effects of *crabs claw, spatula* and *agamous* mutants on medial first whorl organs

Genotype*	Shape	Valve tissue	Replum, style, stigma and septum	Ovules
(*AG*) *CRC SPT*	linear, hooded	present	present	present
(*ag-1*) *CRC SPT*	linear, hooded	absent	variably present	variably present
(*ag-1*) *crc-1 SPT*	ovate, hooded	absent	reduced, patchy	variably present
(*ag-1*) *CRC spt-2*	linear, flat	absent	absent	sporadic, vestigial
(*ag-1*) *crc-1 spt-2*	ovate, flat	absent	absent	very sporadic,vestigial

*Mutant for *ap2-2*, so ectopic AG expression occurs (*AG*) unless *ag* is also mutant (*ag-1*).

Thus AG function, at least when no longer repressed by A function, does not specify the full carpel development program, but determines the identity of the ovary wall and promotes growth of the style.

Most carpelloid properties are lost when *crc* and *spt* are combined with *ap2* and *ag*

To test the role of CRC and SPT in specifying the remaining AG-independent carpelloid properties, a line generating *ap2-2 pi-1 ag-1* triple mutant plants was grown and the *crc-1* and *spt-2* mutants incorporated into it separately and together. The ABC triple mutant was used instead of the AC double to avoid the lateral patches of stamen tissue that are sometimes seen in first whorl medial organs of *ap2-2 ag-1* double mutants (Bowman et al., 1991).

All organs of the *ap2-2 pi-1 ag-1* triple mutant genotype are carpelloid except for the lateral first whorl organs (Fig. 6A). Adjacent organs frequently fuse, even though they may not strictly occupy the same whorl but rather lie in alternate positions. Where they fuse, the development of stigmatic papillae no longer occurs from the lateral margins, but is typically restricted to the unfused commissural position at the apex (Fig. 6A). A replum also develops between the fused organs, and a septum and ovules usually arise internally. The septum houses transmitting tissue that is capable of supporting pollen tube growth (not shown).

When CRC function is compromised in addition to AG, leaving SPT function solely present (in the *crc-1 ap2-2 pi-1 ag-1* quadruple mutant), the organs are still hood-shaped but are now somewhat shorter and broader and tend to be unfused (Fig. 6B). They continue to show some irregular development of stigma, style, septum and ovule development along their edges, although the extent is significantly reduced. On the other hand, when SPT function is mutant (in the *spt-2 ap2-2 pi-1 ag-1* quadruple) and only CRC function presumably remains, all organs are longer with parallel edges (Fig. 6C), and they are always unfused. There is a marked loss of marginal carpelloid tissues, with no sign of stigma, style, septum or transmitting tract development. The only structures seen are short stalked outgrowths on the margins that occasionally show some ovule-like differentiation of integuments, or a small cap of stigma-like cells.

Finally, when CRC and SPT functions are both compromised (in *crc-1 spt-2 ap2-2 pi-1 ag-1* pentuple mutants), the organs are now more ovate, and marginal outgrowths are further reduced (Fig. 6D). Trichomes may occur on the abaxial surface, especially in the first-formed flowers. The organs are not fully leaf-like, however, in that the epidermal morphology on both outer (abaxial) and inner (adaxial) surfaces retains some sepal-like properties, especially in later formed flowers.

These observations are summarised in Table 2. The conclusions are that, in the absence of AG function, CRC strongly promotes the longitudinal growth of floral organs, whereas SPT promotes both involution of the edges and development of the carpel-specific tissues that arise from them.

DISCUSSION

In this study, we make deductions about the role of wild-type genes from their mutant phenotypes. It is necessary to spell out several caveats involved in this approach. First, the changes seen in individual mutants may reflect the primary disruption, and/or they may be secondary downstream changes. For instance, CRC appears to enhance *AG* and *SPT* activity as evidenced by the partial dominance of *ag* and *spt* in *crc* homozygotes. Thus, in *crc* single and multiple mutant combinations, some elements of the phenotype may arise due to reductions in the activity of other non-mutant genes.

Second, epistasis can have a range of interpretations. If one mutant is epistatic to another, the first gene may normally activate the action of the second, or the second gene may negatively regulate the first. Alternatively, the epistatic gene may normally provide the conditions necessary for the second to function. We argue that the latter is the trivial explanation for *ag* being epistatic to *crc* and *spt*, in that the *ag* single mutant results in the inappropriate activity of A class genes such as *AP2* that promote perianth organs and suppresses CRC and SPT function in carpel morphogenesis.

A third situation where qualification is necessary occurs when the phenotype of a double mutant includes properties not seen in either single mutant. This could mean that the genes have partially overlapping functions, and it is only when both are mutant that the function is revealed. The potential complication here is that the further properties lost in the double mutant may be far downstream or an indirect consequence of the initial disruption point of one or both genes. For instance, to uncover the effects of CRC and SPT in the *ag* mutant background required us to introduce the *ap2-2* mutant. Our analysis then requires us to assume that the loss of AP2 does not significantly modify carpel morphogenesis. We further assume that in the *ap2-2 ag-1* background any other genes that may become active in the absence of AG activity do not antagonise carpel morphogenesis. In this regard we concede that additional A class genes may be involved, as evidenced by the sepalloid characteristics remaining when CRC, SPT and A, B and C functions are all disrupted.

Bearing in mind these caveats, we make the following deductions.

The carpel-promoting activities of CRC and SPT are restricted to the gynoecium

A number of genetic tests have demonstrated that *CRC* and *SPT* are active wherever carpelloid organs arise in the flower, even in the absence of the carpel gene *AG*. They appear to perform the same function in ectopic carpels as they do in the wild-type gynoecium. There is no genetic evidence that they are active in other floral organs.

The C function gene *AG* encodes a transcription factor of the MADS family (Yanofsky et al., 1990), but it seems unlikely that AG directly activates *CRC* and *SPT* expression. The presence of many carpel characteristics in the organs of *ap2-2 pi-1 ag-1* flowers that are ultimately lost when either *CRC* or *SPT* are also mutant (see Table 2) demonstrates that neither CRC nor SPT lie directly downstream of AG function.

The fact that *CRC* and *SPT* activity promote carpel development in the outer whorl organs of *ap2-2* flowers suggests that their action in this region of wild-type flowers is prevented by A class genes. This is similar to the cadastral function that AP2, and the product of another A function gene *LEUNIG* (Liu and Meyerowitz, 1995), have on the C function gene *AG* (Bowman et al., 1989; 1991; Drews et al., 1991). However, unlike AG, which reciprocally antagonises A function, the absence of carpel features attributable to CRC and SPT activity in *ag-1* mutant flowers suggests that CRC and SPT do not have an equivalent cadastral function with respect to A function. Together, these results suggest that the activities of *CRC* and *SPT* in promoting carpel development will be restricted to wherever AG alone is active. In this regard, although CRC and SPT are not formally downstream of AG activity, AG appears essential for their action, acting as a buffer against potentially negative AP2 action. For this reason CRC and SPT activities in promoting carpel morphogenesis are inseparable from AG activity when AP2 activity is not compromised.

Double mutants between *crc-1* and *spt-2* and the B function gene *pi* reveal that CRC and SPT are active in the third whorl carpels of *pi* mutants. It is only here where B class activity is absent (and the potentially inhibiting A function is still blocked by C function) that the carpelloid promoting activities of *CRC* and *SPT* become active. This implies that B class activity is antagonistic to the activity of *CRC* and *SPT* in promoting carpellody.

AG controls the identity of the carpel valve and growth of the stylar prominence

When AP2 function is compromised, most carpel features may arise independent of AG function. The only carpel tissue types not seen in first whorl medial organs of *ap2-2 ag-1* double mutants are those of the ovary wall or valve (Table 2). These are now almost fully vegetative. Their outer carpel-like epidermis is absent, and cells resembling the epidermis of leaves and sepals are present. Trichomes may also be abundant, especially in the first-formed flowers (see also Bowman et al., 1991). The thin longitudinal cells underlying the inner epidermis that will become lignified in the fruit (Spence et al., 1996) are also absent, and the mesophyll cells that replace them often include an incipient palisade layer as seen in leaves.

Even though the valve is not carpel-like, the organs retain their carpel-like shape. They are hooded, and their margins are parallel (Table 2). The only difference in shape is the absence of a flattened extension at the organ's incurved tip. This appears to represent reduced growth of the 'stylar prominence' (although style cells themselves can still differentiate on the lateral margins). Examination of the vasculature of carpelloid organs in *ap2-2 ag-1* flowers has revealed that they lack the specialised xylem cells normally present in styles (Sessions and Zambryski, 1995). It may be that AG promotes development of the stylar prominence by activating growth-promoting genes such as *KNAT1*. This shoot meristem gene is expressed in the style, and when expressed ectopically in leaves it induces lobing in which the vascular strands are ramified in a style-like fashion (Chuck et al., 1996).

CRC suppresses radial growth but enhances longitudinal growth of the developing gynoecium

The main role of CRC seems to be to control aspects of carpel growth. Differentiation is not affected in that all carpel tissue types arise in *crc* mutants. The mature gynoecium is shorter and wider than in wild-type, the increased width being evident from its inception (J. A., unpublished). This suggests that CRC suppresses excess lateral expansion but promotes longitudinal growth of the developing carpel. This proposal is reinforced by observations in the *ap2-2* mutant background when *crc* is also mutant (Table 2). The unfused first whorl organs consistently become ovate and more leaf-like in shape. By contrast, when CRC function is not disrupted, the organs are linear with parallel edges. The mechanism by which CRC may produce such proposed growth effects is unclear (see Bowman and Smyth, 1999, for discussion). A detailed analysis of cell division patterns within wild-type and mutant carpels would help resolve this question. The role of CRC in promoting ovule initiation may also be direct or indirect, although the former is supported by the observation that ectopic external ovules occasionally arise from the replum in *crc* mutant gynoecia.

A distinctive feature of the *crc-1* mutant phenotype is that the carpels are unfused in the upper third of the gynoecium. From growth analysis, we postulate that this may be a secondary consequence of the increase in width of the mutant gynoecium (J. A., unpublished). Results from multiple mutant combinations support this, in that whenever *crc* is present in mutant form, unfused carpelloid organs are ovate (Table 2). Thus the upper region of *crc* single mutant carpels may be narrower and less likely to show congenital fusion than lower regions.

SPT is required for transmitting tract development, and promotes differentiation of tissues at carpel margins

Unlike CRC, disruption to SPT function results in the complete loss of a specific tissue type. The transmitting tract within the style and septum is absent in any plant homozygous for *spt*. There is also a marked reduction in the extent of stigma production and some reduction of the style. Internally, too, core tissues of the style and septum are reduced, especially in the apical region. The number of ovules is also reduced. These reductions are more apparent in the unfused first whorl carpels of *ap2-2 pi-1 ag-1* mutants. When SPT function remains, all these tissue types may be present, although variable in extent

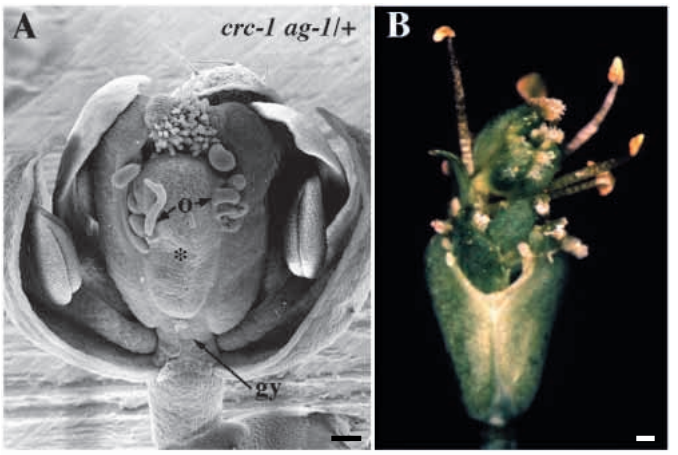

Fig. 4. Reduction in floral meristem determinacy in *crc-1* mutants when AG function is reduced. (A) Medial view of a dissected *crc-1/crc-1 ag-1/+* flower, showing further enlargement of the gynoecium, and a third carpel arising medially between the two lateral carpels (asterisk). The gynoecium has a short stalk (gynophore, gy), and several ovule primordia (o) have arisen from outside the unfused region. (B) Mature *crc-1/crc-1 ag-1/+* flower, showing the eruption of several alternating whorls of stamens and carpels within the original gynoecium. Scale bars: 100 μm.

(Table 2), but if the *spt-2* mutant is incorporated as well, they almost all disappear. The sole carpelloid structure remaining is the short filiform outgrowths at the margins that occasionally show vestigial ovule-like differentiation. This indicates that SPT normally promotes the development of all these marginal carpel tissues.

Organ fusion is mostly eliminated when the *spt-2* mutant is introduced into the *ap2-2 ag-1 pi-1* mutant background. It may be that SPT normally promotes congenital fusion of the carpels from an early stage, and that precursors of the septum at the margin play a direct role in promoting fusion through positive adhesion. Alternatively, SPT may promote growth at the carpel margins, and changes to fusion and marginal tissue development may be the joint consequences of disruption to this lateral growth. If the latter proposal is correct, the loss of marginal tissue types may be a secondary, downstream consequence of reductions in the substrate required for their initiation.

Do AG, CRC and SPT share any functions?

Notwithstanding the inferences made to date, multiple mutant phenotypes suggest that the functions of the three carpel genes overlap to some degree.

While the phenotype of *spt-2 ag-1 ap2-2 pi-1* mutants highlights the role of SPT in marginal carpel tissue development hinted at by the *spt* single mutant phenotype, it also suggests a redundant role for AG in this process. The reduction in septum, stigmatic papillae and ovule development is clearly greater in *spt-2 ag-1 ap2-2 pi-1* organs than is seen in *spt-2* single mutants.

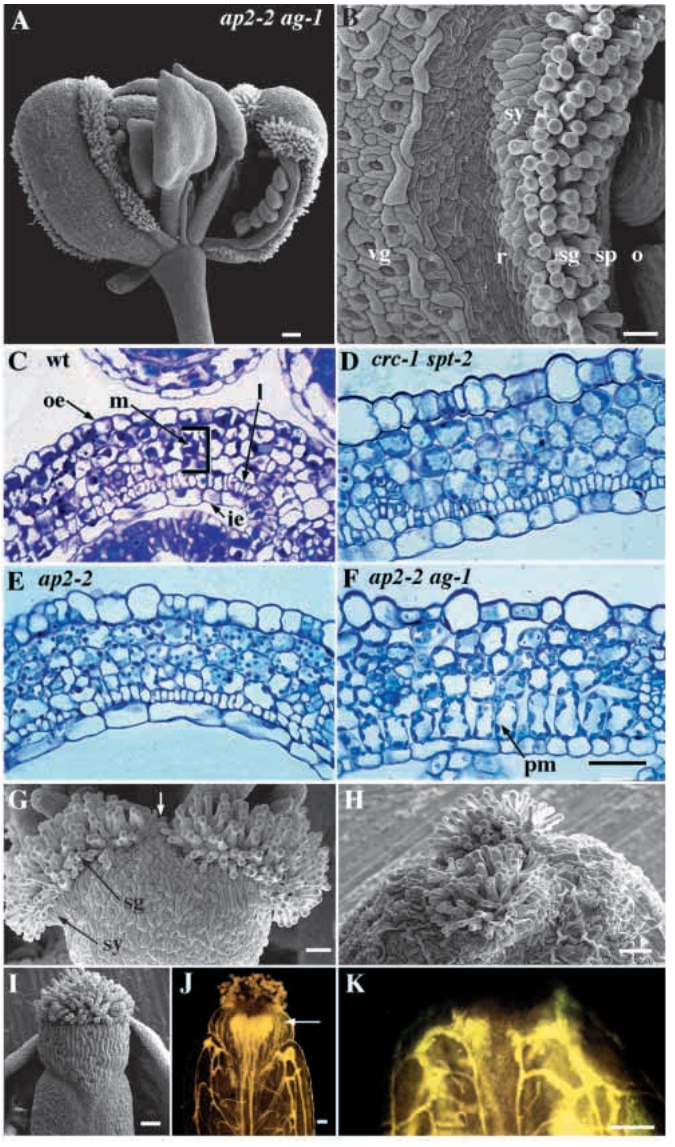

Fig. 5. Effect of loss of AG function on carpelloid properties of first whorl medial organs of *ap2-2*. (A,B) *ap2-2 ag-1* flower in lateral view (A), and the edge of a first whorl organ at higher magnification (B). Many carpelloid properties may remain, including the linear, hooded shape, a lobe of stylar (sy) and stigmatic (sg) cells, a septal flange (sp), and ovule primordia (o). However, the epidermis of the wall is now variably vegetative (vg) with some sepalloid properties (long cells). The replum (r), characterised by rectangular cells, remains in reduced form. (C-F) Transverse sections of carpel walls. The adaxial surface is oriented towards the bottom in each case. The six cell layers of the wild type (C) include an outer (abaxial) epidermis (oe), three layers of mesophyll cells (m), a layer of thin, elongated cells that will be lignified in the mature fruit (l), and an inner (adaxial) epidermis (ie). These are retained in the *crc-1 spt-2* double mutant (D), and in the outer whorl of the *ap2-2* single mutant (E), but when *ag-1* is combined with *ap2-2* (F), the epidermal layers are modified, and the fifth thin cell layer is apparently replaced by a layer of palisade mesophyll (pm). (G-K) Reduced stylar development in *ap2-2 ag-1* double mutants. First whorl organs (G, vertical view), develop both style (sy) and stigma (sg) along the lateral edges, but not at their apex (arrow). When carpelloid organs are fused (H, medial view of fourth whorl organs), the style is almost absent, and the stigma arises in two localised commisural patches (compare with wild type, I). Internally, the core of xylem elements that develops in the wild-type style (arrow in J), is absent in *ap2-2 ag-1* organs, even if fused (K). Scale bars: A,K, 100 μm; B,D, 25 μm; G,H,I,J, 50 μm; C,E,F, same magnification as D.

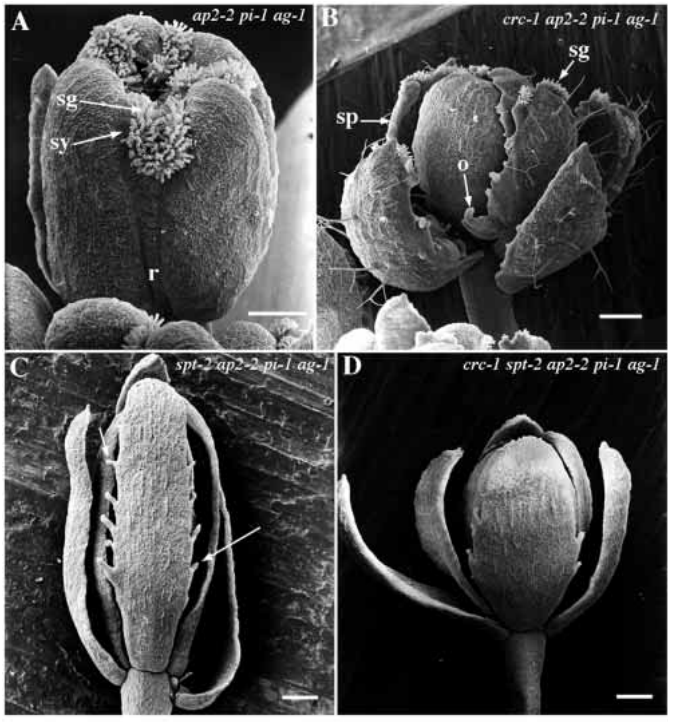

Fig. 6. Loss of carpelloid properties in *ap2-2 pi-1 ag-1* (ABC) triple mutant flowers consequent upon progressively introducing the *crc-1* and *spt-2* mutations. (A) *ap2-2 pi-1 ag-1* triple mutant. Organs in this flower are fused along their edges where replum (r) cells arise, and style (sy) and stigma (sg) development is confined to the unfused commisural positions. Internally, both the septum, including a functional transmitting tract, and ovules may arise. (B) *crc-1 ap2-2 pi-1 ag-1* quadruple mutant. Organs are now more ovate although still hood-shaped, particularly at their apex. Sporadic development of stigma (sg), septal tissue (sp) and ovule-like primordia (o) may occur along their edges. (C) *spt-2 ap2-2 pi-1 ag-1* quadruple mutant. Organs are linear with parallel edges, and have lost any hood-like shape. There is no sign of style, stigma or septal development. Marginal outgrowths may arise (arrows), and these occasionally develop some ovule-like properties. (D) *crc-1 spt-2 ap2-2 pi-1 ag-1* pentuple mutant. The organs are now almost completely vegetative in appearance. They are ovate and flat, with very few marginal outgrowths. Scale bars: 250 μm.

For CRC and SPT, the non-additive phenotype of the *crc-1 spt-2* double mutant gynoecium also implies some overlap in the developmental processes lost in each. However, we know that AG shows a dosage response when CRC function is compromised, so the severity of the *crc spt* defects may be a consequence of AG activity also being marginally reduced. It is insufficient, however, to impair the identity of the ovary wall.

A feature of the *Arabidopsis* carpel is an infolding at the margins which gives the carpel its hooded shape. AG and SPT appear to play the primary role in promoting this process, although their functions are redundant. In *crc-1 spt-2* double mutants, where *AG* is alone active, the carpelloid organs continue to demonstrate infolding at the lateral margins. Also when *AG* activity is alone reduced in the carpel, leaving *SPT* and *CRC* activity (in *ap2-2 pi-1 ag-1* mutants), the carpelloid organs continue to exhibit strongly involuted carpel margins (Table 2). It is only where both AG and SPT activity are absent (in *spt-2 ap2-2 pi-1 ag-1* mutant flowers) that organs exhibit little evidence of marginal involution. A role for AG in promoting marginal involution is consistent with the leaf curling due to ectopic *AG* activity in the *curly leaf* (*clf*) mutation (Goodrich et al., 1997) as well as through over expression of *AG* in 35S-AG plants (Mizukami and Ma, 1992).

Control of floral meristem determinacy

One function of AG, separable from its organ identity role, is to suppress growth at the centre of the flower meristem (Mizukami and Ma, 1995; Sieburth et al., 1995). The minor and variable reduction in the process of flower meristem determinacy seen in *crc-1* single mutants, and the more extensive reduction in *crc-1 spt-2* double mutants (Table 1), most likely occur as a consequence of CRC and SPT normally acting to enhance AG activity in this function. Alternatively, these genes may act separately from AG, but their role in suppressing growth at the centre of the flower can only be detected when AG activity is present. Both possibilities are consistent with AG regulation of flower meristem determinacy being dose dependent in *crc* mutant background (Table 1). In passing, it is worth noting that the increase in organ numbers seen when *crc* and *spt* are each combined with *pi-1* or *ap2-2* may also be a consequence of reduced flower meristem determinacy. This requires more detailed investigation.

Positional information and CRC, SPT and AG function

If the functions of *CRC*, *SPT* and *AG* are simultaneously disrupted, the carpels are converted almost completely to leaf-like organs. This implies that these genes are necessary to specify the normal tissue types and growth characteristics of carpels. But how is positional information established that specifies where and when the genes will act?

Several *Arabidopsis* genes are already known where the mutant gynoecium reveals major changes in pattern elements, including *ETTIN*, *MONOPTEROS*, *PINOID* and *PIN-FORMED* (Nemhauser et al., 1998), and these genes may be involved in the spatial regulation of one or more of *AG*, *CRC* and *SPT*. This seems particularly likely for *ETTIN* with regard to SPT activity, in that the stigmatic lobe and septal tissues that SPT promotes arise ectopically in *ettin* mutants (Sessions and Zambryski, 1995; Sessions et al., 1997). It will be interesting to examine genetic interactions between mutants of *ettin* and *spt* in detail (Alvarez and Smyth, 1998).

The *ap2* null mutant phenotype (Bowman et al, 1991; Kunst et al., 1989; present study) also provides evidence for spatial regulation of carpel tissue development by uncovering a striking 'edge effect' not previously highlighted. When carpels are unfused, as in first whorl medial positions of *ap2-2* mutants, and less frequently toward the centre of the flower, stylar and stigmatic papillae arise inappropriately as a flange of tissue inserted between the normally positioned replum and septum. On the other hand, whenever *ap2-2* carpels are fused, even in the first whorl, ectopic cell types do not arise from the fusion zone. It seems that an unfused carpel edge is somehow sensed during development, and that style and stigmatic cells arise from it. The edge is normally restricted to the apex of the gynoecial cylinder, but when unfused carpels arise, the edge now extends continuously around the periphery. These generalisations extend to *ap2-2 ag-1* and *ap2-2 pi-1 ag-1* genotypes as well. SPT function may somehow be involved, as

development of stigmatic papillae is lost from the margins of unfused *spt-2 ap2-2* carpels. Future cloning of *SPT* and analysis of its expression will shed light on this proposal.

Conclusion

In this study we have shown that simultaneous disruption of AG, CRC and SPT functions (in the absence of AP2 function) results in the loss of almost all carpel features within floral organs. In their place, leaf-like organs arise. From our analysis, we propose that AG does not control the full carpel program but defines only the identity of the carpel wall and growth of the stylar prominence. On the other hand, SPT promotes growth of carpel margins and the differentiation of specialised tissues from them, and CRC promotes carpel elongation. The three genes carry out these functions relatively independently, and all are required simultaneously for normal carpel ontogeny. An understanding of how they bring this about requires further study of their products and mechanisms of action. To this end, the cloning of *SPT* is in progress, and the nature of the *CRC* gene and its product are described in the following paper (Bowman and Smyth, 1999). *CRC* encodes a regulatory protein, and its pattern of expression in normal and mutant plants throws light on some of the interpretations made here.

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