Development 126, 2387-2396 (1999)
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DEV0226



# *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains

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*Accepted 12 March; published on WWW 4 May 1999*

## SUMMARY

**Studies of plants with mutations in the *CRABS CLAW* gene indicate that it is involved in suppressing early radial growth of the gynoecium and in promoting its later elongation. It is also required for the initiation of nectary development. To gain further insight, the gene was cloned by chromosome walking. *CRABS CLAW* encodes a putative transcription factor containing a zinc finger and a helix-loop-helix domain. The latter resembles the first two helices of the HMG box, known to bind DNA. At least five other genes of *Arabidopsis* carry the same combination of domains, and we have named them the yabby family. The new helix-loop-helix domain itself we call the yabby domain. Consistent with the mutant phenotype, *CRABS CLAW* expression is mostly limited to carpels and nectaries. It is expressed in gynoecial primordia from their inception, firstly in lateral sectors where it may inhibit radial growth, and later in the epidermis and in four internal strips. The internal expression may be sufficient to support longitudinal growth, as carpels are longer in a *crabs claw* promoter mutant where expression is now confined to these regions. The patterns of expression of *CRABS CLAW* in ectopic carpels of floral homeotic mutants suggest that it is negatively regulated by the A and B organ identity functions, but largely independent of C function. *CRABS CLAW* expression occurs in nectaries throughout their growth and maturation. It is also expressed in their presumptive anlagen so it may specify cells that will later develop as nectaries. Nectaries arise from the floral receptacle at normal positions in all A, B and C organ identity mutants examined, and *CRABS CLAW* is always expressed within them. Thus *CRABS CLAW* expression is regulated independently in carpels and nectaries.**

Key words: *Arabidopsist thaliana*, Flower development, *CRABS CLAW*, Carpel, Nectary, HMG box, Yabby domain, Zinc finger

## INTRODUCTION

Analysis of homeotic flower mutants has revealed that the identity of floral organs is specified by a limited set of key regulatory genes, acting alone and in combination. A model, now known widely as the ABC model, has been shown to apply to a range of plant species (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). A key regulatory gene specifying carpel identity in *Arabidopsis* flowers is *AGAMOUS* (*AG*) (Bowman et al., 1989, 1991). This C function gene encodes a transcription factor of the MADS family (Yanofsky et al., 1990). A combination of genetic and molecular experiments, including ectopic expression of *AG* in transgenic plants, has shown that *AG* is sufficient for the specification of carpels within the wild-type flower (Bowman et al., 1989, 1991; Mizukami and Ma, 1992).

Even so, some carpel properties, such as stigmatic tissue, fusion of floral organs along their margins, and the production of ovules, can arise even in the absence of AG activity (Bowman et al., 1991). Other genes must therefore exist that specify aspects of carpel development in the absence of *AGAMOUS* gene function. In a screen for mutations that specifically disrupt carpel morphogenesis, two genes, *CRABS CLAW* (*CRC*) and *SPATULA* (*SPT*), that act in this capacity have been identified (Alvarez and Smyth, 1999). *CRC* apparently controls the width of the gynoecium and its elongation, whereas *SPT* promotes development of carpel margins and the tissues that arise from them. In addition, *CRC* is required for the development of nectaries that arise at the base of the stamens in *Arabidopsis* flowers (Davis, 1994a; Smyth et al., 1990).

In this paper we describe the structure and expression of the *CRC* gene to further understand its functions. *CRC* is a member of a family of genes that encode proteins with a zinc finger-like domain and a putative helix-loop-helix with sequence similarity to part of the HMG box. Thus CRC is likely to act as a transcriptional regulator. *CRC* mRNA is mostly limited to developing carpels and nectaries. Its expression pattern in wild-

type and ABC homeotic mutant flowers suggests that its expression is negatively controlled by A and B functions but can occur independently of C function. Furthermore, *CRC* directs nectary development independently from both its carpel functions and the influence of the ABC genes.

## MATERIALS AND METHODS

### Plant material

Two mutant alleles, *crc-1* and *crc-2*, have been described (Alvarez and Smyth, 1999) and a further five are reported here. All were isolated in the Landsberg *erecta* background using ethyl methane sulphonate, except for *crc-2* which was induced by gamma ray irradiation. Single and multiple mutant lines of floral organ identity genes used for in situ hybridisation were bred previously (Bowman et al., 1991; Alvarez and Smyth, 1999). The *leunig* (*lug*) mutant *lug-3* was obtained from Zhongchi Liu (Liu and Meyerowitz, 1995). The *clavata1-1* (*clv1-1*) and *clv2-1* markers used for chromosome walking were originally provided by Maarten Koornneef. All mutant lines were in the Landsberg *erecta* background. Plants were grown in constant light at 20-25°C.

### RFLP mapping

Initial mapping experiments using RFLP markers lbat453 and lbat315 localized *CRC* to the lower arm of chromosome 1 near *AP1*. Plants homozygous for both *crc* and one or other of the flanking morphological markers, *clv1* or *clv2*, were crossed to wild-type Niederzenz (Nd-0) plants. The resulting $F_2$ was screened for plants that were either *crc* or *clv* single mutants, indicating recombination had occurred between *crc* and the respective *clv* locus. 70 recombinants were isolated between *crc* and *clv1* over a distance of approximately 9 cM, and 74 recombinants were isolated between *crc* and *clv2* over a distance of approximately 8 cM. Of the latter, a single recombinant was detected between the molecular marker *AP1* (pYK65; Mandel et al., 1992) and *crc*, placing *CRC* 0.2 cM distal to *AP1*. The proximal to distal order of markers is thus *CLV2 AP1 CRC CLV1*.

### Establishment of YAC, cosmid, and cDNA maps

Yeast artificial chromosome (YAC) clones of genomic fragments covering *AP1* were obtained from Usha Vijayraghavan (Vijayraghavan et al., 1995). A single recombinant was detected between the left end of one of these, YAC EW5H4 (p5H4-L), and *crc* in the *crc clv1* mapping population. As *AP1* crossed a recombinant on the other side of *CRC*, the *CRC* gene was deduced to be present within EW5H4.

Sub-clones of the region encompassed by YAC EW5H4 were isolated from two cosmid libraries. One was made using Columbia genomic DNA and obtained from the Arabidopsis Biological Resource Center (Olzewski et al., 1988) (clone N3-5), and a second was constructed from EW5H4 itself using the pOCA18 vector (Olzewski et al., 1988) (J series clones).

Florally expressed cDNAs were identified using labelled, gel-isolated EW5H4 DNA as a probe on a cDNA library constructed from mRNA isolated from flowers of stage 12 and younger (Weigel et al., 1992). These were grouped and ordered within the YAC sequence.

### In situ hybridization

In situ hybridizations were performed with nine classes of cDNA. In all cases except cDNA 38 and 73, the polyA tail was removed by sub-cloning the 5′ portion. In cDNA 56 (*CRC*), a 470 base pair subclone containing the 5′ end of the cDNA to the *Hin*dIII site in the fifth exon was used to synthesize anti-sense RNA probes. The in situ hybridization procedure was performed using $^{35}$S-labelled antisense probes as previously described (Drews et al., 1991).

### Complementation of the *crc-1* mutant

An 11 kb *Eco*RI fragment of cosmid J12 containing the *CRC* gene was initially sub-cloned into Bluescript (p12RI). A 7 kb *Xba*I-*Eco*RI fragment from this was the sub-cloned into pBIN19. The resulting plasmid was introduced into *Agrobacterium* strain AGL1, and *crc-1* mutant roots transformed and regenerated by standard methods.

## RESULTS

### Phenotypic defects in *crc* mutants

#### *crc* gynoecia exhibit growth defects

Mutations in *CRC* result in several phenotypic alterations in the gynoecium compared to wild type (Alvarez and Smyth, 1999). It is wider and shorter throughout development, and contains fewer ovules. It fails to fuse at the apex and has a reduced amount of style tissue. It occasionally consists of three carpels, suggesting that the floral meristem exhibits a slight loss of determinacy. We have examined seven recessive mutant


**Fig. 1.** Phenotypic characterization of *crc* mutants. (A) Siliques of Landsberg *erecta* wild-type (wt) and of four *crc* mutant alleles. The siliques of strong *crc* mutants *crc-1* and *crc-3* are broader laterally and about half the length of wild type. Style tissue is reduced in *crc-1*, *crc-3*, and *crc-4* siliques. The weaker *crc-2* allele is closer to the wild-type in fruit length and exhibits nearly normal style growth. (B-C) Lateral SEM views of a wild-type (B) and a *crc-1* (C) mutant flower, showing the absence of nectary (n) development in the *crc-1* flower. In each case, a lateral sepal and two petals have been removed. (Note that the lateral stamen has failed to develop in each flower.) (D) Siliques of *crc-1* mutant plants (left), and of complemented *crc-1* plants carrying a 7 kb fragment of wild-type DNA spanning the *CRC* locus (right). The two carpels now fuse at their apex, generate a normal number of ovules, and nectaries develop in the appropriate position in the third whorl (arrow). However, the plants are not fully complemented as they retain some style defects (arrowhead).

alleles of *crc*, two of which (*crc-1* and *crc-2*), have already been described (Alvarez and Smyth, 1999). The phenotype of *crc-3* is closely similar to that of the strong mutant *crc-1* (Fig. 1A), as are *crc-5* and *crc-6*. In *crc-4* the gynoecium is also similar to that of *crc-1* with respect to the loss of fusion and determinacy, but its length and the number of ovules produced is slightly increased (Fig. 1A). The phenotype of the *crc-7* mutant is weaker again in all aspects, while the *crc-2* mutant allele has the weakest phenotype (Alvarez and Smyth, 1999). In particular, its gynoecium is longer, and it produces significantly more ovules. Carpel separation and the reduction in style tissue are also less severe (Fig. 1A).

### *crc* flowers lack nectaries

In wild-type flowers, nectaries arise at stage 9, well after all other floral organs (Davis, 1994a; Smyth et al., 1990). They first appear as relatively undifferentiated outgrowths from the floral receptacle at positions that vary between flowers. One or two of these outgrowths are always seen at the base of each lateral stamen. If these stamens are absent (as occurs in one quarter of Landsberg *erecta* flowers; Smyth et al., 1990), a nectary arises in the space the stamen normally occupies (Fig. 1B,C). Nectary outgrowths are also frequent at the base of medial stamens. By stage 12 (just prior to bud opening), secretory stomata are visible at the apex of the outgrowths. In cross sections of flowers at this stage, the nectary tissue is seen to occupy a continuous ring that encircles the floral receptacle between the perianth organs and the stamens (Davis, 1994a). It interconnects the prominent outgrowths, and also encircles the lateral stamens. Nectar secretion is visible in wild-type flowers at stage 13 when the bud opens. Strikingly, in *crc-1* mutant flowers there is no evidence of nectary development at any stage (Fig. 1C), and the same applies to all other *crc* mutants examined, including the weakest allele *crc-2*.

## Positional cloning of *CRC*

Mapping placed *CRC* between *CLV2* and *CLV1* on chromosome 1, very close to and distal to *AP1* (see Materials and Methods). YAC EW5H4 was shown to span the *CRC* locus (Fig. 2). To identify *CRC*, a strategy of determining expression patterns of candidate cDNAs was adopted. Using the YAC as a probe, 80 florally expressed cDNAs were isolated from approximately $2\times10^5$ inflorescence clones. Fifty one of these corresponded to *AP1*, while the other 29 comprised 14 non-cross-hybridizing classes. Four could be eliminated as *CRC* candidates from their map position (Fig. 2). The expression patterns of 9 of the remaining 10 were examined by in situ hybridization to mRNA of developing flowers. Four exhibited distinct spatial and temporal expression patterns (Fig. 2), and one, cDNA56, displayed a carpel and nectary specific expression pattern strongly implicating it as a transcript of the *CRC* gene.

Cosmid sub-clone J12 spans the candidate cDNA (Fig. 2), and a 7 kb fragment of this was introduced into *crc-1* mutant plants. This complemented the mutant phenotype, although the wild phenotype was not fully restored in that the two carpels occasionally failed to fuse properly and style development was often reduced (Fig. 1D). This 7 kb clone contains approximately 3.5 kb 5′ to the putative *CRC* transcription initiation site. A larger genomic sub-clone including approximately 6 kb of sequence 5′ to the coding region has subsequently been shown to fully complement the mutant phenotype (Y. Eshed and J. L. B., unpublished). Based on these data, and sequence analysis of seven *crc* mutant alleles (see below), we conclude that cDNA 56 represents a transcript of *CRC*.

## *CRC* encodes a protein with zinc finger and helix-loop-helix motifs

The *CRC* gene has seven exons and encodes a protein of 181 amino acids (Fig. 3). The putative protein contains a $C_2C_2$ zinc finger-like domain near its amino terminus (Klug and Schwabe, 1995). There is a serine rich and proline rich domain (17 out of 20 amino acids) in its central portion, characteristic of activation domains of transcription factors (Mitchell and Tjian, 1989). In the carboxyl third of the predicted protein a potential helix-loop-helix domain occurs (Rost, 1996) with similarities to the first two helices of the three found in HMG boxes (Baxevanis and Landsman, 1995) (see Discussion). Near the

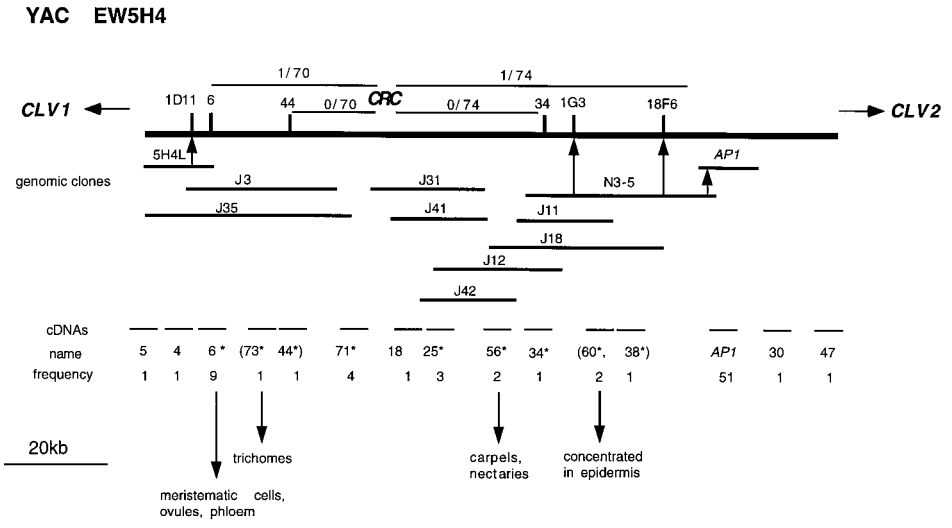


**Fig. 2.** Cloning of *CRC* by chromosome walking. *CRC* was placed within YAC EW5H4, whose map is shown. One out of 70 recombinants between *CRC* and *CLV1* was crossed by a sub-clone made from the left end of YAC EW5H4 (5H4-L), and 1 out of 74 recombinants between *CRC* and *CLV2* was crossed by *AP1*, close to right end. Other markers listed within YAC EW5H4 were located as RFLPs using ends of other YACs (YUP1D11, YUP1G3, EW18F6, Vijayraghavan et al., 1995) or cDNAs (6, 44 and 34) as probes. To obtain smaller genomic clones, YAC EW5H4 was sub-cloned in cosmids (N3-5 and J series). To identify candidate genes, cDNA clones were identified using the YAC EW5H4 as a probe. Their names and frequencies are shown, and, except for those shown in parentheses, they were ordered unambiguously. The expression patterns of nine cDNAs from the relevant region (asterisks) were examined by in situ hybridization, and four that showed a tissue-specific expression pattern are indicated. Candidate cDNA 56 revealed a pattern expected of *CRC*.

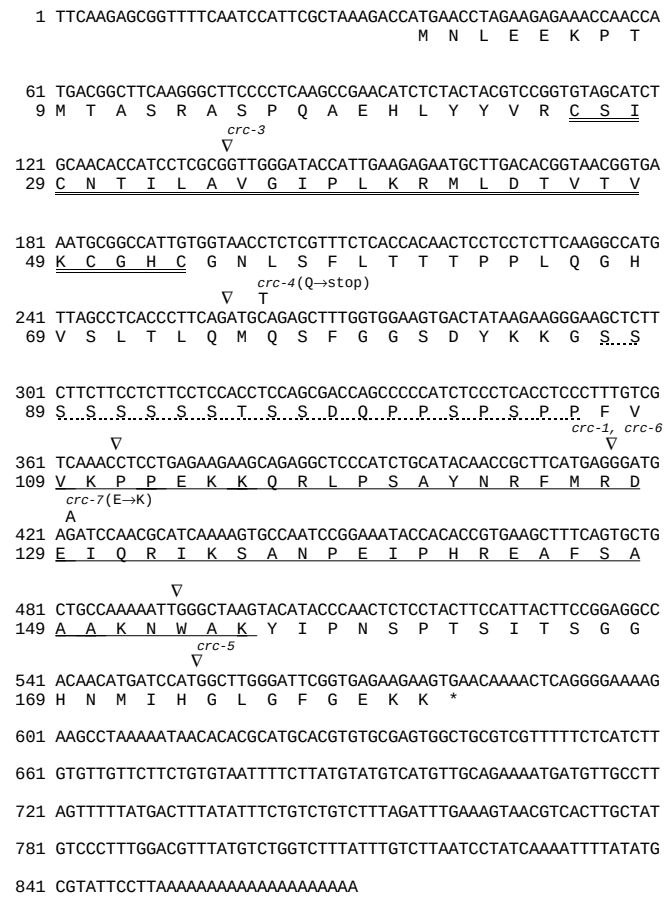


**Fig. 3.** Sequence of cDNA clone 56, and the deduced amino acid sequence of the CRC protein. Intron positions are indicated by inverted triangles. The zinc finger-like domain (double underline), a serine/proline rich domain (dotted underline), and the yabby (helix-loop-helix) domain (single underline) are indicated. The locations of the *crc-1, 3, 5* and *6* mutations are shown above the intron where they result in a G to A change in the splice site acceptor in each case. The *CRC* cDNA sequence has been submitted to GenBank (Accession number: AF132606).

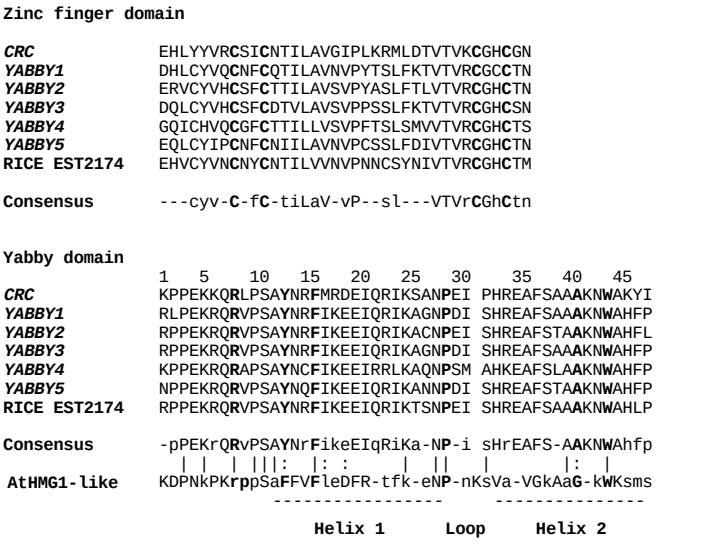


**Fig. 4.** Sequence alignments of yabby family proteins, showing the zinc finger-like domain (above), and the yabby helix-loop-helix domain (below). In the consensus sequences, capital letters designate identical residues, and lower case denote positions conserved in all save one or two of the proteins. In the zinc finger, cysteine residues proposed to chelate zinc are shown in bold. The consensus yabby domain has been aligned with the consensus sequence of five HMG1-like proteins from *Arabidopsis thaliana* (AtHMG; Stemmer et al., 1997). Only the first two helices of the three found in HMG boxes are involved (vertical line, identical amino acids; colon, related amino acids). To maximise the alignment, a gap has been inserted in the loop of the yabby domain. Residues strongly conserved in all HMG boxes (Baxevanis and Landsman, 1995) are indicated in bold.

amino end of this helix-loop-helix domain there is a cluster of basic amino acids that could potentially serve as a nuclear localization signal (Raikhel, 1992). Thus, sequence analysis strongly suggests that CRC is part of the transcriptional apparatus.

Database searches revealed sequence similarity between *CRC* and four genes sequenced by the *Arabidopsis* genome project and an expressed sequence tag of rice (Fig. 4). All show close similarity in both the zinc finger and helix-loop-helix domains and thus define a new gene family. We have named this the 'yabby' family after the Australian fresh water crayfish as a link to the founding member of the family, *CRABS CLAW*. We have recently identified an additional gene (*YABBY2*), and estimate that the family comprises 6-7 members in *Arabidopsis* (Kellee R. Siegfried and J. L. B., unpublished).

The *crc-1*, *crc-3*, *crc-5* and *crc-6* mutations involve single base pair changes at the 3′ splice acceptor sites of the fourth, first, sixth and fourth introns of the *CRC* gene, respectively (Fig. 3). In all cases, the change is from AGG to AAG, and presumably results in the splice site being frame-shifted one nucleotide further downstream. In the case of *crc-3* this would yield a truncated protein four amino acids into the second exon that contains only the first half of the zinc finger domain, likely resulting in a non-functional protein. The other three splice site alleles all have phenotypes indistinguishable from *crc-3* suggesting that they too are null alleles. The *crc-4* mutation is associated with a single base pair change that results in a stop at codon 76 (Fig. 3). How this could result in a phenotype slightly weaker than null is not clear. In *crc-7*, an intermediate mutant, there is a single nucleotide change that results in lysine replacing a conserved glutamate within the first helix of the helix-loop-helix domain. Finally, in the weak allele *crc-2* the sequence of the coding regions and the splice site junctions did not reveal any changes. However, a DNA rearrangement involving at least 5 kb was detected approximately 1.5 kb 5′ from the transcription start site.

## *CRC* expression pattern in wild type

In situ hybridization of mRNA (Fig. 5) and mRNA gel blots (not shown) indicate that the expression of *CRC* is restricted to flowers. Within flowers, strong expression is detected in carpels and nectaries.

In carpels, expression commences during stage 6 (Smyth et al., 1990) when the gynoecial primordium first becomes distinct (Fig. 5A,B). Expression occurs in the lateral region of each carpel, as two half cylinders separated by an unlabelled zone where the carpels join (Fig. 5B). By stages 7-8, carpel expression resolves into two distinct domains, epidermal and internal (Fig.

5C-H). Epidermal expression occurs mostly on the outer surface but there is also some on the inner surface. On the outside, expression at first extends from the base of the gynoecium to the tip, extending fully around its circumference (Fig. 5E-H). By about stage 10, this expression declines in the presumptive replum, while persisting in the valve regions (Fig. 5I). By mid stage 12 expression has disappeared in the latter as well. Inside the gynoecium, epidermal expression occurs in valve regions at a low level during stages 7-9 (Fig. 5F). Turning to the internal expression, labelling is first observed in discrete zones within the walls of the gynoecium in stage 7-8 flowers (Fig. 5C,D). In cross sections these are seen as four striking patches of signal in groups of cells adjacent to where placental tissue will develop (e.g. Fig. 5G, arrows). These continuous strands occupy the full length of the elongating cylinder, declining first in apical regions in stage 8-9 flowers (Fig. 5E), and disappearing early in stage 10, soon after the ovule primordia arise (Fig. 5I). No expression is detectable in the placental regions themselves, nor in the septum, stigma and ovules.

Nectaries arise from the receptacle at the base of the stamens at stage 9. From stage 6, *CRC* expression occupies an almost continuous ring of receptacle cells between the stamen and sepal primordia, including regions where nectaries will later develop (Fig. 5B-D). Expression is strong in nectaries from their initiation at stage 9, and continues in all nectary outgrowths throughout floral development (Fig. 5L) until at least stage 14 (post-pollination), although it is stronger in surface layers than in the core. Expression also continues in most of the ring of intervening cells.

Weak *CRC* expression can also be seen in buds from late stage 4. It occupies the core of growing sepal primordia (Fig. 5K), often as twin, finger-like fields. It may also be seen at stage 5 in several small clusters of cells within the receptacle, internal to the sepals (Fig. 5J). The origin of these has not been resolved. Finally, weak expression can also be seen in developing petals during stages 9 and 10 when they are rapidly enlarging (not shown). As in sepals, this expression frequently occupies twin cores of tissue.

### *CRC* expression in *crc* mutants

In carpels of the presumed null mutants *crc-1* and *crc-3 CRC* expression appears to be largely unaltered (Fig. 6A). This suggests that *CRC* mRNA is relatively stable in *crc* mutants, and that functional CRC protein is not required for the maintenance of *CRC* expression in carpels. Even so, expression does not persist as late as in wild type. Outside the gynoecium, a ring of *CRC* expression is clearly seen in the stage 6 receptacle as in wild type. Even though nectary development does not occur, a low signal persists in this region at least through to stage 12 (not shown).

The spatial pattern of *CRC* expression in the weaker *crc-2* mutant that retains just 1.5 kb of its promoter is different. In gynoecia the initial expression at stage 6 is missing. The later internal expression domains within the carpels are largely intact, although epidermal expression is also almost completely abolished (Fig. 6B). In addition, *CRC* mRNA is not detectable outside the gynoecium, including those cells that normally give rise to nectaries.

### Control of *CRC* expression by *SPATULA*

In *spt* mutant plants, the carpels show reduced growth of the style, stigma and septum, and lack transmitting tissue in both the style and the septum. There is some gene dosage evidence that *CRC* supports SPT function, although not vice versa (Alvarez and Smyth, 1999). Unexpectedly, however, *CRC* mRNA expression is apparently boosted in *spt-2* mutant carpels compared to wild-type (compare Fig. 6C with Fig. 5F). In contrast, levels of *CRC* expression in *spt-2* nectary regions appear unchanged. Thus *SPT* appears to down-regulate *CRC* expression in carpels but not in nectaries.

### Control of *CRC* expression by the ABC genes

#### Expression in A class mutants

Two A class genes, *APETALA2* (*AP2*) and *LEUNIG* (*LUG*), have been shown to repress C class activity, represented by *AGAMOUS* (*AG*), at the transcriptional level in the outer two whorls of the *Arabidopsis* flower (Drews et al., 1991; Liu and Meyerowitz, 1995). To determine if they also regulate *CRC*, we examined *CRC* expression in *ap2* and *lug* mutant flowers (Fig. 6D-H).

Null mutations in *AP2*, such as *ap2-2*, result in the homeotic conversion of the medial first whorl into carpel-like organs (Bowman et al., 1991). *CRC* is expressed in these ectopic carpels (Fig. 6D), and the pattern is similar to that seen in normal fourth whorl carpels (Fig. 5D). Expression occurs as soon as the organs are initiated (stage 2). In addition, the level of *CRC* expression is consistently higher than in control wild-type flowers.

The partial loss-of-function allele, *ap2-1*, has leaf-like organs rather than carpels developing in the outer whorl positions (Bowman et al., 1989). In this case, the AP2 function that represses AG activity is not disrupted, at least up to stage 6 of development (Drews et al., 1991). Surprisingly, however, *CRC* is ectopically expressed in these outer whorl organs almost from their inception (stage 3, Fig. 6F). *CRC* mRNA is still present in a variable pattern at stage 8-9, with the signal occurring primarily at the margins of the organs and in the abaxial epidermis (Fig. 6E).

Mutations in *LUG* result in similar but less conspicuous carpelloid transformations in the outer whorl (Liu and Meyerowitz, 1995). Gynoecium development is also affected in that the carpels fail to fuse properly, and they have horn-like projections at the top of the valve in place of lateral regions of the style. Additionally, septal fusion and ovule development is disrupted. *CRC* is ectopically expressed in the first whorl organs of *lug-3* mutants (Fig. 6G,H). As in *ap2-2* flowers, it is expressed in late stage 2 flowers at, or slightly prior to, the inception of the first whorl (Fig. 6G). By stage 8-9, expression is restricted to the margins and the abaxial epidermis (Fig. 6G,H), although some weak signal can be detected in interior cells. In *lug-3* fourth whorl carpels, the internal expression domains are largely absent (Fig. 6H). Thus, *LUG* influences that pattern of *CRC* expression in two ways, firstly by repressing *CRC* in the outer whorl of the flower, and secondly, by strongly promoting the internal expression domains in the carpel.

The conclusion is that A function genes are negative regulators of *CRC* expression in the outer whorl of *Arabidopsis* flowers, as they are of *AG*.

#### Expression in B class mutants

The two B class genes of *Arabidopsis*, *PISTILLATA* (*PI*) and *APETALA3* (*AP3*), encode MADS box genes (Goto and

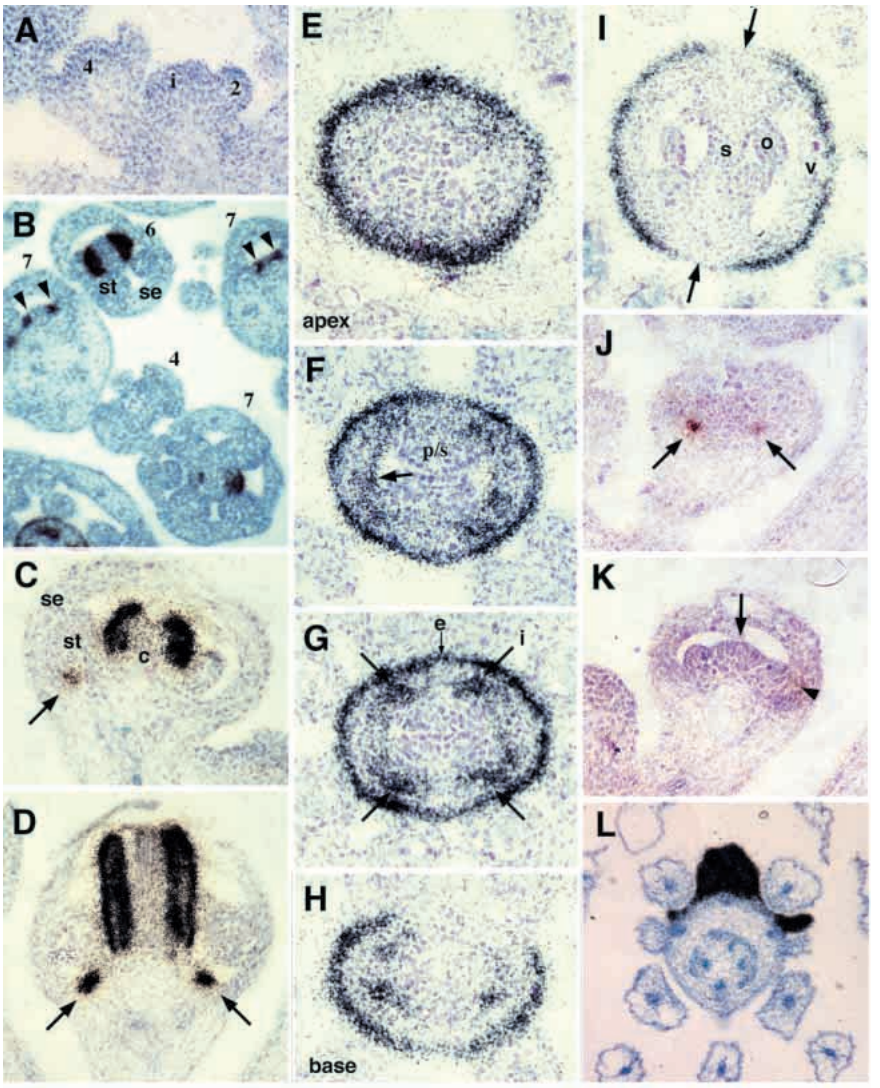


**Fig. 5.** Expression of *CRC* in wild-type flowers shown by in situ hybridization. (A) Longitudinal section of an inflorescence meristem (i), and a stage 2 (2) and a stage 4 (4) bud. *CRC* expression is not detectable. (B) Transverse section of an inflorescence above the apical meristem. In the gynoecium, strong *CRC* expression commences at stage 6 in the lateral regions of each carpel. Expression in two of the stage 7 buds are in regions of the floral receptacle that will give rise to nectaries (arrowheads). Numbers refer to floral stages. se, sepal; st, stamen. (C,D) Longitudinal sections of stage 7 (C) and stage 9 (D) flowers in the lateral plane. *CRC* expression is detected in the lateral regions of the developing gynoecial cylinders. In the stage 9 flower, two distinct domains can be seen, epidermal and internal. Expression can also be seen at the base of stamens (arrows) where nectary primordia arise at stage 9. se, sepal; st, stamen; c, carpel. (E-H) Series of transverse sections of a stage 8-9 gynoecium. Two distinct domains of expression can be detected, epidermal (e) and internal (i) (G). The outer epidermal domain extends from the base of the carpel to the tip, and fully around its circumference. In contrast, expression in the inner epidermis (arrow in F), and the four internal patches, cannot be detected in the most apical region (H). No expression is observed in cells that will give rise to the placenta and septum (p/s in F). (I) Transverse section of a stage 11 gynoecium. The outer epidermal expression has declined in the presumptive replum (arrows), while persisting in the valve regions. The internal expression domain is no longer detected. s, septum; o, ovule; v, valve. (J,K) Longitudinal sections of a stage 5 flower. K is through the center of the floral meristem, J is 16 μm lateral to this. *CRC* mRNA occurs in small groups of the receptacle cells, lying in the groove internal to the developing sepals (arrows in J). Weak expression also occurs in the core of sepals (arrowhead in K), but no expression is observed in the cells that will give rise to the carpels (arrow in K). (Silver grains appeared brown in this series.) (L) Expression in nectary tissue. In an oblique transverse section near the base of the stamens of a stage 13 flower, CRC expression occurs in the nectarial outgrowths, and in a continuous ring of cells around the receptacle (only partly present in this section). One of the lateral stamens is absent, and a large nectary occupies the space.

Meyerowitz, 1994; Jack et al., 1992), and mutations in them result in homeotic transformations of stamens to carpels (Bowman et al., 1989, 1991; Hill and Lord, 1989). These carpels are fused congenitally with each other and any internal carpels, resulting in an enlarged multi-carpellate gynoecium. In both *pi-1* (Fig. 6L) and *ap3-3* (not shown) mutant flowers, we observed ectopic *CRC* expression associated with these third whorl carpels. Expression commences at about late stage 4 or early stage 5, with the subsequent cellular expression patterns paralleling those seen in wild-type fourth whorl carpels. Thus B function normally negatively regulates CRC expression in the third whorl.

## Expression in C class mutants

Carpels do not develop in mutants of the C class gene *AGAMOUS* (*AG*). In their place another flower arises (Bowman et al., 1989, 1991). As expected, no carpel-like pattern of CRC expression occurs in *ag-1* single mutants (Fig. 6I). On the other hand, nectaries usually develop outside the third whorl of *ag* flowers despite their homeotic transformation from stamens to petals (Davis, 1994b; S. F. Baum and J. L. B., unpublished), and *CRC* mRNA is detected in these regions (Fig. 6I).

## Expression in multiple mutants

Because carpels are absent in *ag* mutants, they are not useful for testing if *CRC* carpel expression is regulated by AG, or if it is involved in specifying carpel properties in the absence of AG. These possibilities can be tested, however, by comparing *CRC* expression patterns in first whorl organs of *ap2-2* flowers and *ap2-2 ag-1* flowers (Alvarez and Smyth, 1999). In the former, the organs are carpelloid through the ectopic activity of AG. In the latter, AG activity is removed. Even so, the *ap2-2 ag-1* organs retain a carpelloid shape, with reduced but significant marginal outgrowths of style, stigma, septum and

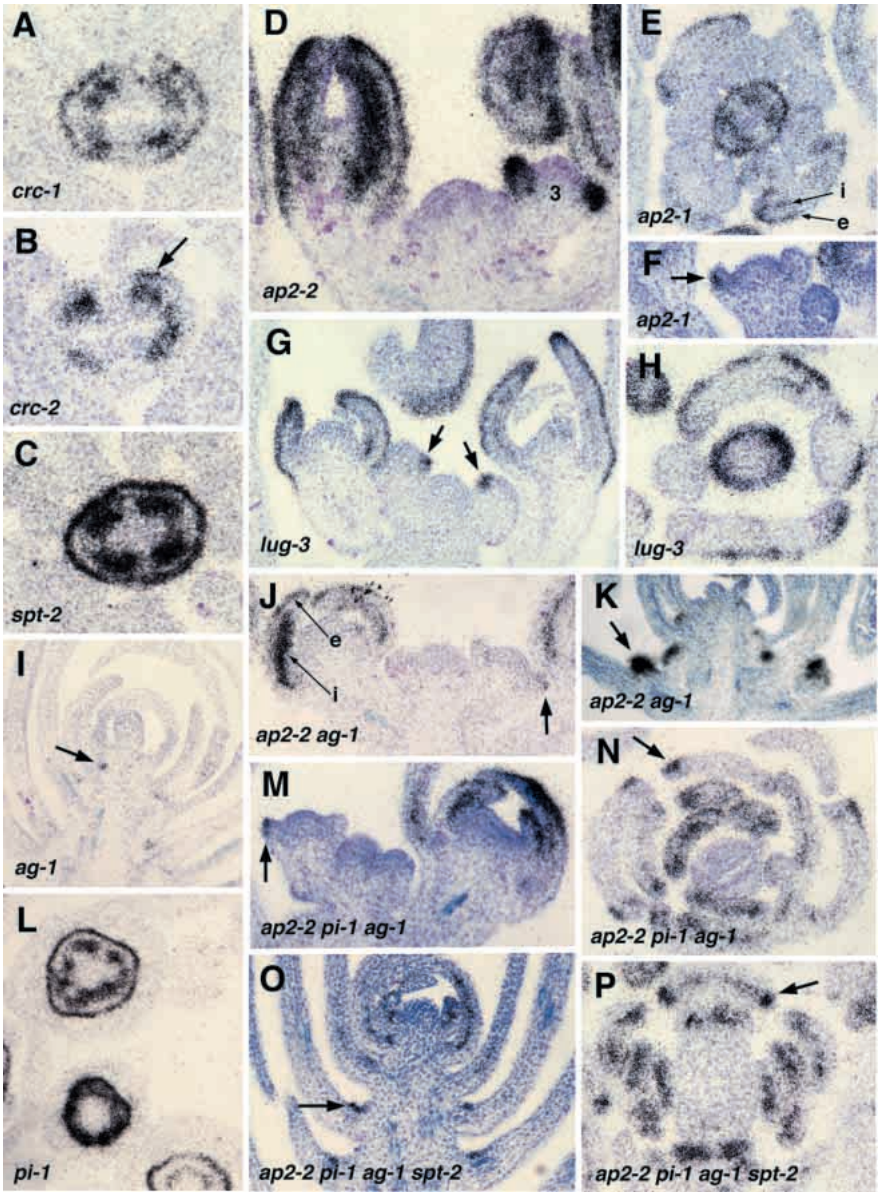


**Fig. 6.** Expression of *CRC* in mutant flowers shown by in situ hybridization. (A) Transverse section of stage 9-10 gynoecium of the strong mutant *crc-1*. Both epidermal and internal expression domains are detectable. (B) Transverse section of stage 9-10 gynoecium of the weaker mutant *crc-2*. The internal expression domain is largely intact, but the epidermal domain is mostly absent. Occasionally a small region of epidermal expression occurs just abaxial to one of the internal patches (arrow). (C) Transverse section of stage 9-10 *spt-2* gynoecium. *CRC* expression is consistently higher than in wild-type controls (see Fig. 5F, from the same experiment), although the spatial pattern is not altered. (D) Longitudinal section of an *ap2-2* inflorescence apex. Expression can be seen in the first whorl carpelloid organs of a stage 3 flower (3). Later, epidermal and internal expression occurs in the developing first whorl carpels (left), parallelling that seen in wild-type carpels (Fig. 5D). (E,F) Expression in the weaker mutant *ap2-1*. Expression is seen first on the margins of the first whorl vegetative organs at stage 3 (arrow in F). At later stages (e.g. transverse section of a stage 9-10 flower in E), expression in the medial first whorl organs again occurs in two domains, epidermal (e) and internal (i), even though these organs develop few carpel-like properties. (G,H) Expression in *lug-3* mutant flowers. Expression is initially detected in the first whorl carpelloid organs in late stage 2 (arrows in G). Subsequently, expression is confined primarily to the epidermis (G,H). In the fourth whorl carpels, only the epidermal expression domain is evident (H). (I) Transverse section of an *ag-1* flower. Expression is mostly limited to the presumptive nectaries at the base of third whorl petals (arrow). (J,K) Longitudinal sections of *ap2-2 ag-1* double mutants, showing an inflorescence apex (J), and a mature flower (K). Signal is first detected in stage 3 flowers (arrow in J). Epidermal (e) and internal (i) expression domains are seen in the carpelloid leaf-like organs of the first whorl (J). Expression levels in the nectaries (arrow in K) are higher than in *ag-1* single mutants (I). (L) Transverse sections of *pi-1* mutant flowers at stage 7-8 (below) and stage 8-9 (above). Expression patterns in the amalgamated third and fourth whorl carpels parallel those in the wild-type carpels. (M,N) Expression in the *ap2-2 pi-1 ag-1* triple mutant, showing a longitudinal section of an inflorescence apex (M), and a transverse section of a mature flower (N). Signal is first detected in stage 3 flowers (arrow in M). Marginal and internal expression domains are observed in the carpelloid leaf-like organs found in all whorls. As they mature, expression becomes restricted to the margins, often on the abaxial side alone (arrow in N). (O,P) Expression in mature flowers of the *ap2-2 pi-1 ag-1 spt-2* quadruple mutant, in longitudinal (O) and transverse (P) section. Expression patterns are similar to those observed in *ap2-2 pi-2 ag-1* flowers (M,N), although it is relatively intense (arrow in P indicates marginal domain). Signal is also evident in the presumptive nectaries (arrow in O).

ovules. It has been shown genetically that the role of AG in these organs is limited to specifying the identity of the valve and promoting growth of the stylar prominence (Alvarez and Smyth, 1999). To test if *CRC* expression remains in the absence of AG activity, in situ experiments were carried out. Both *ap2-2 ag-1* and *ap2-2 pi-1 ag-1* flowers were used. In the latter, all organs are carpelloid, and stamens and stamen mosaics that occur in the former genotype are absent. In addition, flowers of the quadruple mutant *ap2-2 pi-1 ag-1 spt-2* were examined because in these all organs are almost completely leaf- and sepal-like (Alvarez and Smyth, 1999), and it is of interest to see if *CRC* expression remains.

*CRC* mRNA levels are reduced in all three genotypes. Transcripts are initially detectable during stage 3, when the first whorl organ primordia arise (e.g. Fig. 6J,M). This is slightly later than *CRC* expression in the A class single mutants, *ap2-2* (Fig. 6D) and *lug-3* (Fig. 6G,H). In *ap2-2 ag-1* and *ap2-2 pi-1 ag-1* flowers, *CRC* expression is observed in those whorls in which the organs develop into carpelloid leaves (first, fourth, seventh, etc. whorls for *ap2-2 ag-1* flowers, and all whorls for

*ap2-2 pi-1 ag-1* flowers). Expression is primarily in two domains, at the margins of the organs and in an internal domain that is closer to the adaxial side (Fig. 6J,M,N). The marginal domain persists for longer than the marginal domain (Fig. 6N). *CRC* expression is also observed in a similar pattern in the leaf-like organs of the quadruple mutant *ap2-2 pi-1 ag-1 spt-2* (Fig. 6O,P), indicating that CRC by itself is unable to specify any significant carpelloid properties. Expression is relatively more intense in the quadruple than in the *ap2-2 pi-1 ag-1* triple mutant, showing that AG function is not required for the derepression of *CRC* expression that occurs in *spt* mutant plants.

In conclusion, it seems that *CRC* expression can occur independently of AG, but that if AG function is absent, *CRC* expression is weaker and its spatial pattern is modified.

## DISCUSSION

### *CRC* encodes a protein with a zinc finger and a new domain resembling part of an HMG box

*CRC* likely encodes a new class of transcription factor with two strongly conserved domains, a zinc finger domain and a helix-loop-helix domain. The same combination of domains has been found in five other genes in *Arabidopsis*, and in several ESTs in rice (Kellee R. Siegfried and J. L. B., unpublished), and we have named them the yabby family. Database searches indicate that the yabby family is plant specific.

The putative zinc finger in the yabby family is of the $C_2C_2$ type, with a spacing of 20 amino acids between the pairs of cysteines (Fig. 4). Several conserved hydrophobic residues and a conserved proline residue near the center of the spacer region could provide secondary structure to the finger (Klug and Schwabe, 1995). While many zinc fingers have been implicated in DNA binding, primarily in the major groove, others have been shown to mediate protein-protein interactions (Mackay and Crossley, 1998), and the role of this region in the yabby family awaits further study.

The remarkable conservation of the 48 amino acid helix-loop-helix domain in the yabby family (Fig. 4) has led us in turn to designate it the 'yabby' domain. It exhibits some sequence similarity to the first two helices of the HMG box, a conserved ~80 amino acid DNA-binding domain of three alpha helices found in a large family of eukaryotic proteins (Baxevanis and Landsman, 1995). In some HMG proteins, including the SRY protein involved in sex determination in mammals, the HMG box binds to the minor grove of DNA and bends the double helix at this point. The solution structure of SRY in association with its specific DNA target has been determined (Werner et al., 1995). The first two alpha helices of SRY fold back on each other as a consequence of the proline present in the intervening loop. This proline is absolutely conserved in HMG boxes (Baxevanis and Landsman, 1995), and is also present in all members of the yabby family (Pro-29, Fig. 4). In SRY, a T-shaped wedge of five amino acids in the first two helices is responsible for bending the DNA double helix. In the hydrophobic cross-bar of this wedge, two highly conserved aromatic amino acids are located at positions that correspond exactly in spacing to Tyr-13 and Trp-44 in the yabby family (Fig. 4). Another strongly conserved aromatic amino acid is found three residues C terminal to position 13 in both HMG boxes and yabby domains (Phe-16, Fig. 4). Finally, positively charged amino acids are frequent in HMG boxes, and four in the SRY protein form salt bridges with the phosphate backbone of the sense strand. Three of these correspond to the highly conserved Lys-5, Arg-8 and Lys-18 positions in the yabby domain (Fig. 4).

Despite these strong similarities, the HMG box contains a third helix that forms one side of the V-shaped domain that also contacts the DNA target (Werner et al., 1985). This is absent in the yabby domain, suggesting that if it interacts with DNA (as seems likely), it does so in a manner somewhat different from the HMG-DNA association. The yabby domain could represent part of an ancestral HMG1/2 gene, related to those still present in *Arabidopsis* (Fig. 4; Stemmer et al., 1997), that was coopted and combined with a zinc finger gene early in the evolution of plants.

### *CRC* expression patterns in relation to the *crc* carpel mutant phenotype

The expression of *CRC* in carpels is complex, dynamic, and ephemeral. It is first expressed in lateral sectors of the carpel primordium from their inception at stage 6. The *crc* mutant phenotype is also first visible at this stage. The gynoecial primordium is radially expanded (J. Alvarez and D. R. S., unpublished), and the wild-type CRC function may keep this expansion in check. In the mutant, the increase in size could be jointly responsible for the slight increase in the mean number of carpels per flower seen in *crc* mutants, and for the much stronger increase in floral meristem indeterminacy seen when AG function is also reduced (Alvarez and Smyth, 1999). In each case the extra tissue may allow additional organs to be generated, either in the fourth whorl itself or in further internal whorls.

Later, two distinct expression domains, epidermal and internal, are evident within the developing carpels. These correspond in time with the rapid elongation of the gynoecium, and CRC's role here may be switched to one promoting growth. In particular, it seems the internal strips of expression are responsible for growth promotion. Evidence for this comes from the weak allele *crc-2*. This results from a rearrangement that truncates the *CRC* promoter, abolishing *CRC* expression except for these internal strips, and allowing the length of the *crc-2* gynoecium to approach that of wild type. It is interesting that similar twin zones of internal expression are seen within elongating leaves, sepals and petals for the growth-promoting gene *AINTEGUMENTA* (Elliott et al., 1996).

The role of the epidermal expression domains of *CRC* remains to be established. Epidermal cells of *crc* mutant gynoecia are somewhat larger on average than in wild type (J. Alvarez and D. R. S., unpublished), so their expansion may be held in check by the CRC product. However, their larger size might be an indirect consequence of reduced rates of cell division in mutant gynoecia.

One aspect of the *crc* mutant phenotype not yet discussed is the reduced fusion of carpels seen at the apex of the gynoecium. This is first apparent early in development (stage 7), when growth of the medial regions of the elongating gynoecial tube falls behind the lateral regions (J. Alvarez and D. R. S., unpublished). *CRC* expression is present from this stage, at least in the epidermis. However the expression is continuous around the gynoecium, and the products of other

patterning genes may constrain its growth promotion to those regions where the carpels adjoin. Later in development, *CRC* expression ceases in the replum region where carpels are unfused in *crc* mutants. A non-autonomous effect may occur here if, for example, CRC is responsible for the transcriptional activation of components of cell to cell signalling pathways (Roe et al., 1997).

### Transcriptional regulation of *CRC* by the ABC floral organ identity genes

The general conclusion from expression studies in ABC mutant plants is that *CRC* is expressed in floral organs with carpelloid properties wherever they arise.

In A function mutants *ap2* and *lug*, the first whorls are carpelloid. Expression of the C function gene *AG* is no longer repressed (Drews et al., 1991), and the same applies to *CRC* as well. In B function mutants *pi* and *ap3*, the third whorl organs become carpelloid because C function is no longer accompanied by B function. *CRC* expression now occurs in the third whorl carpels, and the inference is that *CRC* expression is also normally suppressed by B class function. This is consistent with genetic data (Alvarez and Smyth, 1999), and with the lack of *CRC* expression in the second and third whorls of *ap2-2 ag-1* double mutant flowers where B function alone remains.

The relationship between *CRC* expression and C function is more complex. Genetic observations indicate that *CRC* can function independently of *AG* (Alvarez and Smyth, 1999), and we have confirmed this. Both *AG* (Drews et al., 1991) and *CRC* are ectopically expressed in first whorl organs of *ap2-2* mutants, but *CRC* expression continues even when AG function is removed, as in *ap2-2 ag-1* double mutants. Thus *CRC* is not simply a downstream target gene of *AG*, but it can be activated in parallel with *AG* to influence the fate of cells in developing floral organs (Alvarez and Smyth, 1999).

There is a further situation where *AG* and *CRC* expression are regulated independently. In the incomplete A function mutant *ap2-1*, the first whorl organs are not carpelloid and *AG* expression is absent until late and even then it is patchy (Drews et al., 1991). *CRC* expression, however, is seen much earlier (stage 3) and is more consistently present. Thus the A function remaining in *ap2-1* mutants is able to repress *AG* expression much more effectively than *CRC* expression.

*AG* and *CRC* expression are not fully independent, however, as *AG* may positively regulate *CRC* expression to some degree. This is the conclusion reached from the reduced levels of *CRC* expression, and its later induction, seen in the first whorl organs of *ap2-2 ag-1* double mutants compared with *ap2-2* controls. Gene dosage studies gave no hint of regulation in this direction, although they did suggest that *CRC* may enhance AG function to some extent (Alvarez and Smyth, 1999). Spatially, too, there is evidence that AG may influence *CRC* expression. In the carpelloid leaf-like organs of *ap2-2 ag-1* (and *ap2-2 pi-1 ag-1*) flowers, epidermal expression is absent, but two other domains occur, one at the margins and an internal domain nearer the adaxial side. The marginal domain seems new, but the relationship of the internal domain to that seen inside wild-type carpels is not clear. In general, all changes in *CRC* expression seen in *ag* mutants may be the consequence of the loss of carpelloid identity of the valve regions (Alvarez and Smyth, 1999), and AG may normally provide an appropriate cellular milieu for the epidermal and internal expression domains of *CRC* to arise.

It is of interest to note that the A function gene *LUG* also influences spatial aspects of *CRC* expression. In addition to its role as an A class gene where it represses *AG* and *CRC* in the outer whorl of the flower, *LUG* activity is required for several aspects of carpel morphogenesis (Liu et al., 1995). In *lug* mutant carpels, whether arising ectopically in the first whorl or in the centre of the flower, the epidermal expression domain of *CRC* is present but the internal domain is absent, or weak and patchy. This suggests either that *LUG* normally supports the activation of *CRC* in its internal carpel domain, or alternatively, it is usually required for the formation of internal tissue that will express *CRC*. That is, *LUG* acts as both a negative and a positive regulator of *CRC* depending on the cellular context.

If C function is not directly involved, which other genes activate *CRC*? C function itself is activated by the floral meristem identity genes, *LEAFY* and *APETALA1* (Weigel and Meyerowitz, 1993), but carpelloid organs nevertheless arise in their absence. One hypothesis is that carpelloid properties result from the activation of *CRC* (and another *AG*-independent carpel gene, *SPT*) by factors that mediate floral induction (J. Alvarez, unpublished), and direct tests of this are now possible.

One significant finding was that *CRC* expression is elevated in *spt-2* mutants, and that this is independent of AG function. This suggests that SPT normally represses *CRC* expression to some extent. This was unexpected given that the *spt-2* mutant phenotype is the same in *crc-1*/+ heterozygotes as in +/+ controls (Alvarez and Smyth, 1999). On the other hand, the observation that the *crc-1* mutant phenotype is strengthened in *spt-2*/+ heterozygotes suggested the converse, that *CRC* supports SPT function to some degree (Alvarez and Smyth, 1999). Further study is required to uncover the basis of these genetic interactions.

In conclusion, *CRC* expression is suppressed by A and B functions but can occur independently of C function, confirming proposals from mutant studies (Alvarez and Smyth, 1999). On the other hand, both A and C function may influence the spatial and temporal patterns of *CRC* mRNA accumulation. It remains to be established whether these regulatory steps are direct or downstream.

### *CRC* is required for nectary development

The function of *CRC* in nectary development is unequivocal. No signs of nectaries are observed in *crc* mutants, and *CRC* function is thus necessary for initiation of their growth at stage 9. In fact, *CRC* expression is detectable much earlier in the zone where nectaries will arise, almost encircling the receptacle between the perianth and stamen primordia from stage 6. This suggests that CRC plays a role in the early specification of cells that will eventually become nectaries. Later stages may also require CRC activity in that *CRC* mRNA continues to be present at high levels in nectaries throughout their growth, differentiation and maturity.

Although *CRC* is necessary for nectary development, constitutive expression of *CRC* is not sufficient to induce ectopic nectary development in transgenic plants (J. L. B., unpublished). Presumably *CRC* acts in concert with other, as yet unidentified, genes. One gene known to be expressed in mature nectaries is *AG* (S. W. Baum and J. L. B., unpublished). However, *AG* is not required for nectary development as

nectaries continue to arise in the normal position in *ag* mutant flowers, albeit more sporadically (Davis, 1994b), and *CRC* continues to be expressed in them. Indeed, the same is true for all floral organ identity mutants examined here. This demonstrates that the specification of nectary development by CRC is dependent upon position within the floral receptacle, not the identity of surrounding floral organs. A corollary of this is that the regulation of CRC expression in nectaries is independent of its expression in carpels.

Identification of genes that activate CRC, proteins that collaborate with it, and its downstream target genes, will ultimately allow us to place CRC within the separate morphogenetic pathways that control development of these two important components of the angiosperm flower.

We thank Nancy Garavelas for providing *crc-2*, Jo Putterill for *crc-3*, and Marcus Heisler for *crc-4* and for valuable comments on the manuscript; Assunta Pelosi for RFLP mapping data; Usha Vijayraghavan, Kumuda Kushalappa, and Sai Murali Raj for mapping information and clones; Kellee Siegfried for RNA blot analyses and Amit V. Bhakta and Amita Patel for help with sequencing. John Alvarez generously provided strains, advice and ideas throughout this study. We thank the Rice Genome Research Program (NIAR/STAFF, Tsukuba, Japan) for the gift of EST2174. This work was supported by an Australian Research Council Post-Doctoral Fellowship and an NSF grant (96-31458) to J. L. B., and an ARC Grant (A19230662) to D. R. S.

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