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 API. 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 F2 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 API (pYK65; Mandel et al., 1992) and crc, placing CRC 0.2 cM distal to API. The proximal to distal order of markers is thus CLV2 API CRC CLV1.

Establishment of YAC, cosmid, and cDNA maps

Yeast artificial chromosome (YAC) clones of genomic fragments covering API were obtained from Usha Vijayraghavan (Vijayraghavan et al., 1995). A single recombinant was detected between the left end of one of these, YAC EW5H4 (pSH4-L), and crc in the crc clv1 mapping population. As API 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 (Oziewski et al., 1988) (clone N3-5), and a second was constructed from EW5H4 itself using the pOCA18 vector (Oziewski 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 subcloning the 5’ portion. In cdNA 36 (CRC), a 470 base pair subclone containing the 5’ end of the cdNA to the HindIII site in the fifth exon was used to synthesize anti-sense RNA probes. The in situ hybridization procedure was performed using 35S-labelled antisense probes as previously described (Drews et al., 1991).

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

Complementation of the crc-1 mutant

An 11 kb EcoRI fragment of cosmId J12 containing the CRC gene was initially sub-cloned into Bluescript (p12RI). A 7 kb Xbal-EcoRI 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.

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 E5WH4 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 2×10^5 inflorescence clones. Fifty one of these corresponded to AP1, while the other 29 comprised 14 non-cross-hybridizing clones. 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 C2CZ 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
The result in a G to A change in the splice site acceptor in each case.

First, sixth and fourth introns of the (Fig. 3). In all cases, the change is from AGG to AAG, and

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estimate that the family comprises 6-7 members in YABBY2 as a link to the founding member of the family, this the ‘yabby’ family after the Australian fresh water crayfish domains and thus define a new gene family. We have named close similarity in both the zinc finger and helix-loop-helix CRC

apparatus.

Thus, sequence analysis basic amino acids that could potentially serve as a nuclear localization signal (Raikhel, 1992). Thus, sequence analysis inverted triangles. The zinc finger-like domain (double underline), a domain has been aligned with the consensus sequence of five zinc finger-like domain (above), and the yabby helix-loop-helix

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 (Baxesvans 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 mutations involve single base pair changes that results in a stop ( crc-2, crc-4, crc-5, crc-6 ).

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 gyroecal 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.
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 receptive 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 occurs in 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 (Bowman 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, sepal 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
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 
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...
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 carpelloid organs of a stage 3 flower (3). Later, epidermal and internal expression occurs in the developing first whorl carpels (left), paralleling 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) Expression in the ag-1 single mutants. Expression patterns in the amalgamated third and fourth whorl carpels parallel those in the wild-type carpels. (M,N) Expression in the ag-1 triple mutant, showing a longitudinal section of 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-1 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).
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 C2C2 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 groove 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 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.

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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 radically 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.

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