

# Recruitment of *CRABS CLAW* to promote nectary development within the eudicot clade

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Accepted 26 August 2005

Development 132, 5021-5032

Published by The Company of Biologists 2005

doi:10.1242/dev.02067

## Summary

Nectaries are secretory organs that are widely present in flowering plants that function to attract floral pollinators. Owing to diversity in nectary positions and structures, they are thought to have originated multiple times during angiosperm evolution, with their potential contribution to the diversification of flowering plants and pollinating animals being considerable. We investigated the genetic basis of diverse nectary forms in eudicot angiosperm species using *CRABS CLAW* (*CRC*), a gene required for nectaries in *Arabidopsis*. *CRC* expression is conserved in morphologically different nectaries from several core eudicot species and is required for nectary development in both rosids and asterids, two major phylogenetic lineages of eudicots. However, in a basal eudicot species, no evidence

of *CRC* expression in nectaries was found. Considering the phylogenetic distribution of nectary positions and *CRC* expression analyses in eudicots, we propose that diverse nectaries in core eudicots share conserved *CRC* gene regulation, and that derived nectary positions in eudicots have altered regulation of *CRC*. As the ancestral function of *CRC* lies in the regulation of carpel development, it may have been co-opted as a regulator of nectary development within the eudicots, concomitant with the association of nectaries with reproductive organs in derived lineages.

Key words: Nectary, Carpel, *CRABS CLAW*, YABBY, Eudicot, *Arabidopsis*

## Introduction

A primary issue in biology concerns the origin of morphological diversity over evolutionary time. The convergence of evolutionary biology and developmental genetics provides a step toward understanding this complex issue. Approximately 90% of land plants are flowering plant species; despite their enormous diversity in morphology, flowers are recognizable owing to the regularity in their organization with whorls of floral organs in a stereotypical arrangement. Recent molecular genetic studies suggest underlying genetic programs directing flower development are conserved throughout angiosperms, consistent with a single origin of flowers (Ambrose et al., 2000; Ferrario et al., 2004; Kyojuka et al., 2000). By contrast, it is often difficult to identify a common ontology in structures highly modified within lineages over evolutionary time. Increasing genetic information about the development of such structures can provide clues to their origin.

With the rapid progress in developmental genetics and confidence in the establishment of the flowering plant phylogeny, a gene(s) involved in particular developmental processes in model species can be evaluated in an evolutionarily context by examining orthologs in divergent angiosperm species. Through comparative expression and functional studies, it is possible to gain insight into the relationship between the evolution of genes and morphology.

The nectary provides an interesting example with which to address this question owing to its diversity in both structure and ontogeny. Nectaries are highly variable in their morphologies, anatomies and locations, and are defined based on their shared function: the secretion of nectar (Fahn, 1979). Depending on location, nectaries either serve to attract pollinators or protect against herbivores. Although nectaries reportedly occur in ferns (Darwin, 1877) and Gnetales (Porsch, 1910), they are most widespread in angiosperms, predominantly developing or occurring within flowers, when compared with other parts of the angiosperm plant body. Many angiosperm flowers are animal pollinated (Eriksson and Bremer, 1992), with pollinators attracted to flowers to gather nectar or pollen as food sources. Fossil records of angiosperms and insects suggest that the timing of the radiations of angiosperms and certain insect classes were coincident (Crepet and Friis, 1987; Meeuse, 1978; Pellmyr, 1992). Therefore, along with the other floral organs, the innovation of nectaries may have played a major role in angiosperm and metazoan evolution.

Although locations of nectaries within flowers are constant at the family level, in broader taxonomic terms, their locations are highly variable (Brown, 1938). In basal angiosperms, nectaries tend to be associated with the perianth (the non-reproductive floral organs) (Endress, 2001), while in the eudicots, nectaries are usually associated with carpels and

stamens. Thus, Fahn (Fahn, 1953) argued that there is a trend in nectary position within flowers, shifting from peripheral perianth positions in basal taxa to central positions associated with reproductive organs in more derived taxa. In addition, extrafloral nectaries are currently known in 68 angiosperm families (Elias, 1983). Their structures and locations are also diverse across the families, although they occur most often 'on the upper half of the petiole at or near the base of the leaf blade than any other site' (Elias, 1983). The diversity of nectary forms and distributions within flowering plants suggest that they may have multiple independent origins. However, this does not preclude diverse nectaries from sharing developmental genetic machinery.

In *Arabidopsis*, *crabs claw* (*crc*) is the only known single mutant that lacks floral nectaries (Baum et al., 2001; Bowman and Smyth, 1999). *CRC* is expressed in nectaries, with expression commencing before the emergence of nectary glands and continuing until after anthesis. Although ectopic expression of *CRC* alone does not result in ectopic nectaries, ectopic expression of *CRC* in conjunction with other genes, such as *UFO*, or in specific mutant backgrounds, results in the development of ectopic nectaries at the bases of flower pedicels (Baum et al., 2001). Thus, *CRC* is required for nectary development and ectopic *CRC* expression in some contexts is sufficient for nectary formation (Baum et al., 2001). *CRC* activation in nectaries is mediated by a combination of florally expressed MADS box proteins, although the tissue-specific factors limiting expression to nectaries and carpels are unknown (Lee et al., 2005).

*CRC* encodes a putative transcription factor of the YABBY gene family (Bowman and Smyth, 1999). Several members of the YABBY gene family are expressed abaxially in developing leaf primordia and floral organs with their ectopic adaxial expression transforming the adaxial leaf surface into one with abaxial characteristics, implicating members of this gene family in establishing or interpreting leaf polarity (Sawa et al., 1999; Siegfried et al., 1999). Although *crc* single mutants do not affect carpel polarity, when combined with other mutations, such as *kanadi*, adaxial tissues develop in abaxial positions, indicating that *CRC* is required for proper carpel polarity (Eshed et al., 1999). Consistent with *CRC* promoting abaxial differentiation, *CRC* is expressed abaxially in the carpels (Bowman and Smyth, 1999). In addition, genetic studies with floral homeotic ABC mutants in *Arabidopsis* showed that *CRC* specifies carpel identity in parallel with *AGAMOUS* (Alvarez and Smyth, 1999). Studies of *CRC* orthologs in *Oryza* and *Amborella*, which together with *Arabidopsis* span the phylogenetic diversity of angiosperms, suggest that a role in carpel development is likely to be the ancestral function of *CRC* in angiosperms (Fourquin et al., 2005; Yamaguchi et al., 2004). In this study, we examined whether *CRC* has an ancestral function in the nectaries of angiosperms or whether *CRC* was recruited for a role in nectary development within the angiosperm lineage.

## Materials and methods

### Taxon sampling

Taxa were sampled to represent diverse nectary forms in representative eudicot lineages. For the analysis of *CRC* expression patterns from close relatives of *Arabidopsis*, *Lepidium africanum* and

*Cleome sparsifolia* [Cleomaceae based on work by Hall et al. (Hall et al., 2002)] were chosen because the two species show variation in the organization of floral nectaries. For the analysis in extrafloral nectaries, *Capparis flexuosa* in Capparaceae [sister to Brassicaceae/Cleomaceae (Hall et al., 2002)] and *Gossypium hirsutum* in Malvaceae were used. As representatives of asterids (APG, 1998), *CRC* homologues from *Nicotiana tabacum* and *Petunia hybrida* (both Solanaceae) were isolated, and the expression of *CRC* in *P. hybrida* was examined. For the analysis in flowers of basal eudicots (Soltis et al., 2000), *Aquilegia formosa* (Ranunculaceae) was chosen (Fig. 1).

### Cloning of *CRABS CLAW* orthologs

Total RNA was isolated from nectary or carpel tissue using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, USA). Partial fragments of *CRC* cDNAs were amplified by using degenerate primers dCRC-ZnF (5'-CDGTRACRGTGAAAT-GYGGYCATTYGRGYA-3') and dCRC-YB (5'-AIGCGATGGRA-GYCTSTGYTTCTTCTCRGG-3') (see Fig. S1 in the supplementary material). Polymerase chain reactions (PCR) were conducted with Takara ExTaq polymerase (Madison, USA) on a RoboCycler (Stratagene, La Jolla, USA) with the following protocol: 2 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 42-60°C and 30 seconds at 72°C, followed by one cycle of 2 minutes at 72°C. PCR products were separated on 0.7% TAE agarose gels, gel isolated (Qiagen, Hilden, Germany) and TOPO TA cloned (Invitrogen, Carlsbad, USA). Purified DNA was sequenced using an ABI PRISM 377 DNA sequencer. Following confirmation of *CRC* ortholog sequences, full-length cDNA sequences were obtained by 5' and 3' RACE using a SMART RACE cDNA amplification kit (Clontech, Palo Alto, USA).

### Phylogenetic analyses

To determine whether genes from various angiosperm groups represent orthologs, phylogenetic analyses were conducted employing the Bayesian method. We included *CRC* genes determined in this study, as well as sequences of YABBY gene family members from GenBank (the gene list is provided in Table S1 in supplementary material). Deduced amino acid sequences of the YABBY genes were aligned using ClustalX (Thompson et al., 1997) and manually adjusted using the program Se-Al (A. Rambaut, see <http://evolve.zoo.ox.ac.uk/>). The alignment confirmed the two domains recognized by Bowman and Smyth (Bowman and Smyth, 1999) (zinc finger and YABBY domains) are highly conserved across genes from angiosperms and gymnosperms (see amino acid alignment in Fig. S1 in the supplementary material). However, it is difficult to assume positional homology in the remaining regions of the genes owing to a higher level of variability. Thus, we analyzed only nucleotide sequences of the two conserved domains. The aligned data matrix was submitted to the TreeBase database (<http://www.treebase.org>) and is available upon request.

Bayesian phylogenetic analyses were performed with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) using the sequences from gymnosperms as outgroups. A Metropolis-coupled Markov chain Monte Carlo algorithm was employed for 2 million generations, sampling trees every 100 generations, with four independent chains running simultaneously. The general time-reversal model (Swofford et al., 1996) with six rate parameters and the gamma distribution, as determined by the hierarchical likelihood ratio test using Modeltest (Posada and Crandall, 1998), was used to estimate the likelihood values. All 20,001 resulting trees were imported into PAUP\* 4.0b10 (Swofford, 2002), and a 50% majority-rule consensus tree was generated after discarding the first 1001 trees (100,000 generations). These 'burn-in' generations, for which the log-likelihood values had not reached a plateau, were determined by plotting a graph of the log-likelihoods of each

generation versus generation numbers (Huelsenbeck and Ronquist, 2001).

### Microscopy

For Scanning Electron Microscopy (SEM), tissue was fixed overnight with 3% glutaraldehyde, phosphate buffered to pH 7, followed by the overnight fixation in 0.5% osmium tetroxide. Tissue was dehydrated and critical-point dried. After sputter coating with gold/palladium, tissue was observed on a Hitachi S-3500N scanning electron microscope. Anatomical analysis was carried out according to the method of Baum and Rost (Baum and Rost, 1996). Tissue sections were stained using the PAS reaction.

### In situ hybridizations and semi-quantitative RT-PCR

In situ RT-PCR was performed following Xoconostle-Cázares et al. (Xoconostle-Cázares et al., 1999) on thin hand-sectioned tissues from leaves and involucres of *Gossypium hirsutum*. Gene-specific primers GhCRC-1 (GGTTCCACAATCCGAGCATCTTT) and GhCRC-2 (CACAACGGATGATGCTGCTGGAGAA) were added to a RT-PCR mixture containing Oregon Green-labeled dUTP (Molecular Probes, Eugene, USA). Slides with fresh tissue and PCR mixture were sealed and the PCR was run at 60°C for 20 minutes followed by 10 cycles of 30 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C. As a negative control, RT-PCR was run in parallel without gene-specific primers. After washing out free dNTPs, fluorescent signals were observed using confocal laser scanning microscopy (CLSM).

For semi-quantitative RT-PCR, the above gene-specific primers were used along with actin primers as a quantitative control: Ghactin-F (CCTCTCCAGCCATCTTTCATC) and Ghactin-R (ATTCATCATACTACCCTTGGGA). Total RNA was extracted from various regions of plant tissue using a Qiagen RNeasy Plant Mini kit. Total RNA (2.5 µg) was used for cDNA synthesis and PCR was performed using Takara ExTaq polymerase. PCR was run at 94°C for 1 minute followed by 30 or 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C, and an extension of 5 minutes at 72°C.

Standard RNA in situ hybridization was as described by Vielle Calzada et al. (Vielle Calzada et al., 1999), except tissue in paraffin was sectioned at 10–15 µm and hybridization was carried out at 55°C for 36 hours.

### Virus induced gene silencing (VIGS)

A previously described Tobacco Rattle Virus (TRV)-based VIGS system was used to silence *CRC* in *Petunia* and *Nicotiana* (Liu et al., 2002; Chen et al., 2004). A 575 bp fragment of the *petunia CRC* gene corresponding to entire coding region was cloned into pTRV2 to form pTRV2 *CRC*. The constructs pTRV1 and pTRV2 or pTRV2 (see Liu et al., 2002) *CRC* were transformed into *Agrobacterium* strain GV3101 by electroporation. Virus infection was achieved by *Agrobacterium*-mediated infection of *Petunia hybrida* or *Nicotiana bethamiana* as described by Chen et al. (Chen et al., 2004). Flowers were examined for aberrant phenotypes 2–6 weeks post infection. Selection of *Petunia* flowers for examination was facilitated by the simultaneous VIGS mediated silencing of chalcone synthase (Chen et al., 2004).

## Results

### Phylogenetic distribution of angiosperm nectaries

Mapping of nectary positions onto the currently accepted phylogeny of flowering plants (Qiu et al., 1999; Soltis et al., 1999; Soltis et al., 2000) indicates that structural diversification of nectaries has occurred in the core eudicots, with nectaries primarily associated with reproductive organs in these species (Fig. 1). Brown (Brown, 1938) provided a thorough description on the distribution of floral nectary positions in angiosperms

based on observations in more than 3000 species. Focusing on their diverse nature, he stated, ‘Nectaries appear to have arisen independently in different lines of development and then to have undergone modifications characteristic of various groups’. However, mapping nectary positions on the currently accepted angiosperm phylogeny shows a pattern that supports Fahn’s (Fahn, 1953) argument of an evolutionary acrocentripetal movement of nectary position within angiosperms (Fig. 1). In general, nectaries are not well differentiated in basal angiosperms. Nymphaeaceae and Illiciaceae in the ANITA grade have nectaries on perianth parts, and *Amborella* generates liquid exudates to the carpel tip whose identity as nectar has not been characterized (Endress, 2001). A similar situation is found in most of the eumagnoliid lineage, except in the monocot clade. Floral nectaries in monocots are defined as septal glands, the septa being places where adjacent walls of two carpels have not fused (Brown, 1938). Almost all monocots possessing nectaries have septal glands. In eudicots, nectaries are more conspicuous, elaborate and usually associated with reproductive floral organs, with perianth nectaries being relatively rare (e.g. *Gossypium* sp.). At least 68 families are reported to have species with extrafloral nectaries (Elias, 1983) and the mapping of those species within the current angiosperm phylogeny indicates that extrafloral nectaries are mostly distributed in core eudicot species and monocots, and have independently evolved in several lineages (Fig. 1).

### Single origin of *CRC* in angiosperms

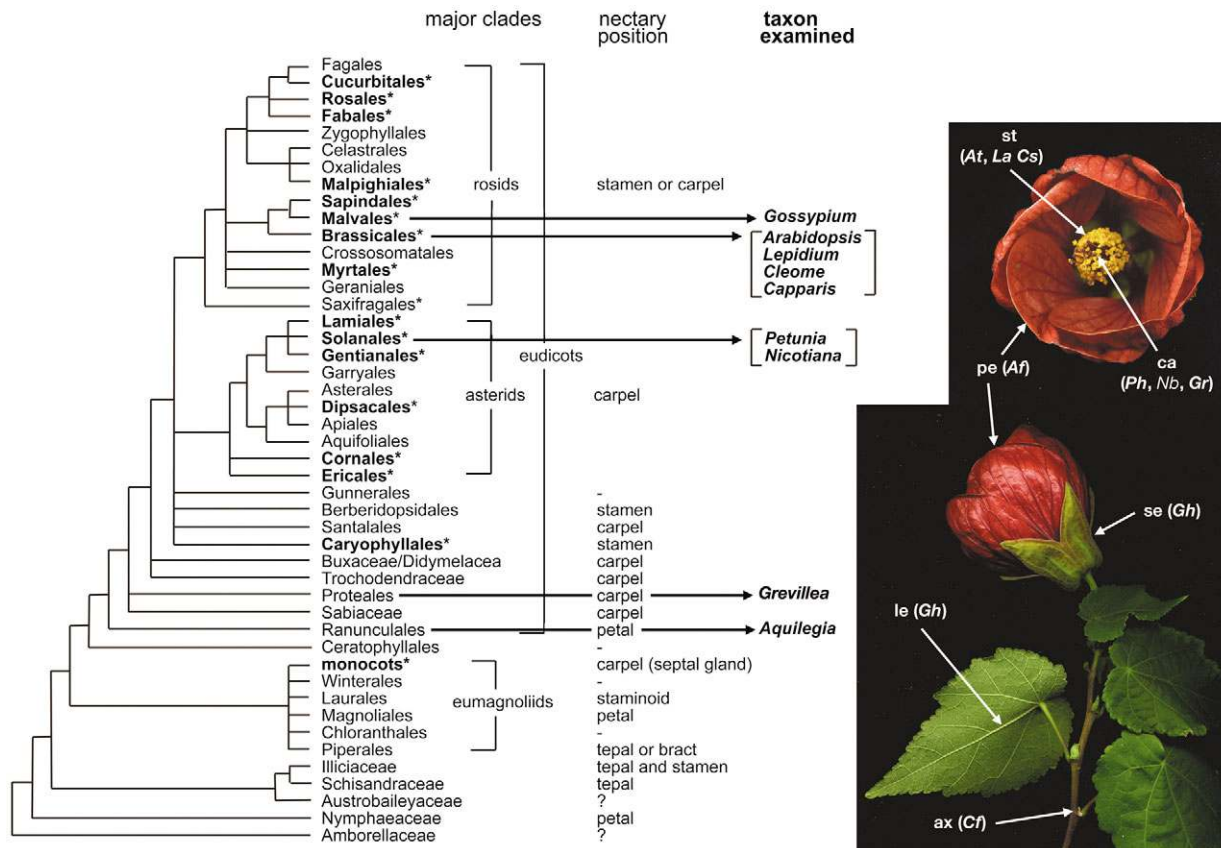
Bayesian phylogenetic analysis based on nucleotide sequences of two domains of several members of the *YABBY* family from a broad spectrum of angiosperm species and four gymnosperms resolved five major gene lineages, represented by *INO*, *CRC*, *YABBY2*, *FIL/YABBY3* and *YABBY5* (Fig. 2). *INO*, *CRC* and *YABBY5* genes formed strongly supported clades with the posterior probabilities (i.e. probability of the hypothesis given the data) of 100. *YABBY2* and *FIL/YABBY3* genes were, however, weakly supported as monophyletic lineages. Each clade contains members from basal angiosperms or monocots, suggesting the ancestral angiosperms had five *YABBY* family genes, consistent with the results of Yamada et al. (Yamada et al., 2004). Our gene trees weakly suggested that *INO* and *CRC* genes, both of which are involved in developmental processes in flowers, are derived from other *YABBY* genes that are expressed in all lateral organs (Siegfried et al., 1999). Further sampling in gymnosperms is required to ascertain the antiquity of the different clades of *YABBY* gene family members.

Our phylogenetic analysis of *YABBY* genes indicated that *CRC* genes originated once during the evolution of the *YABBY* genes. Thus, *CRC* genes in various diploid angiosperms are orthologs (Hillis, 1994) that have diversified via speciation not by gene duplication. The topology within the *CRC* gene clade is consistent with Soltis et al. (Soltis et al., 2000), even though our sampling of the genes is limited. The *CRC* genes from monocots and Brassicales both formed strongly supported clades, as did two cotton genes.

### *CRC* expression in floral nectaries is well conserved in Brassicaceae/Cleomaceae

In Brassicaceae/Cleomaceae species (Hall et al., 2002),





**Fig. 1.** Phylogenetic distribution of nectaries in angiosperms. Non-eudicot angiosperms have floral nectaries in the perianth, or nectary tissues are not conspicuously developed. In eudicots, floral nectaries are mostly associated with reproductive organs, although some examples of perianth nectaries exist. The angiosperm phylogeny is based on that of Soltis et al. (Soltis et al., 2000). Orders with extrafloral nectaries are marked bold with an asterisk. Extrafloral nectaries are found only within eudicots and monocots. The extrafloral nectary distribution is based on that of Elias (Elias, 1983) and locations of floral nectaries are primarily based on those of Brown (Brown, 1938) and others (Endress, 1986; Endress, 2001; Proctor et al., 1996; Ronse DeCraene, 2004; von Balthazar and Endress, 2002). Groups reported as not having nectaries are indicated (-). Austrobaileyaceae and Amborellaceae were marked with a question mark, as the possible nectar secretion on the tip of carpels has not been characterized. Taxa used in this study and the positions of nectaries in these taxa are indicated. ax, leaf axil (*Capparis flexuosa*); le, abaxial midvein of leaf (*Gossypium hirsutum*); se, sepal (*Gossypium hirsutum*); pe, petal (*Aquilegia formosa*); st, stamen (*Arabidopsis thaliana*, *Lepidium africanum*, *Cleome sparsifolia*); ca, carpel (*Petunia hybrida*, *Nicotiana benthamiana*, *Grevillea robusta*).

nectaries develop at the bases of stamen filaments (Fahn, 1979). Norris (Norris, 1941) classified Brassicaceae nectaries into three types: annular, four-nectary and two-nectary types. *Arabidopsis thaliana* is a four-nectary type with two medial and two lateral nectaries (Baum et al., 2001); *Lepidium africanum* is a two-nectary type with only two medial nectaries (Fig. 3A); and *Cleome sparsifolia* has annular type nectaries in which glands develop continuously around the circumference of the androecium (Fig. 3F). Although the sizes of nectary glands vary among these species, their external organizations are similar. Stomata develop at the abaxial tips of nectary glands (Fig. 3B), and epidermal cells of nectaries have distinctive reticulate cuticular thickenings (Baum et al., 2001). In these species, *CRC* expression commences at or before initiation of nectary development, increases during growth of the nectary, and decreases after anthesis (Fig. 3C,D,G). In carpels, *CRC* expression commences early, and is associated with abaxial regions of the carpel walls (data not shown), similar to *Arabidopsis* (Bowman and Smyth, 1999).

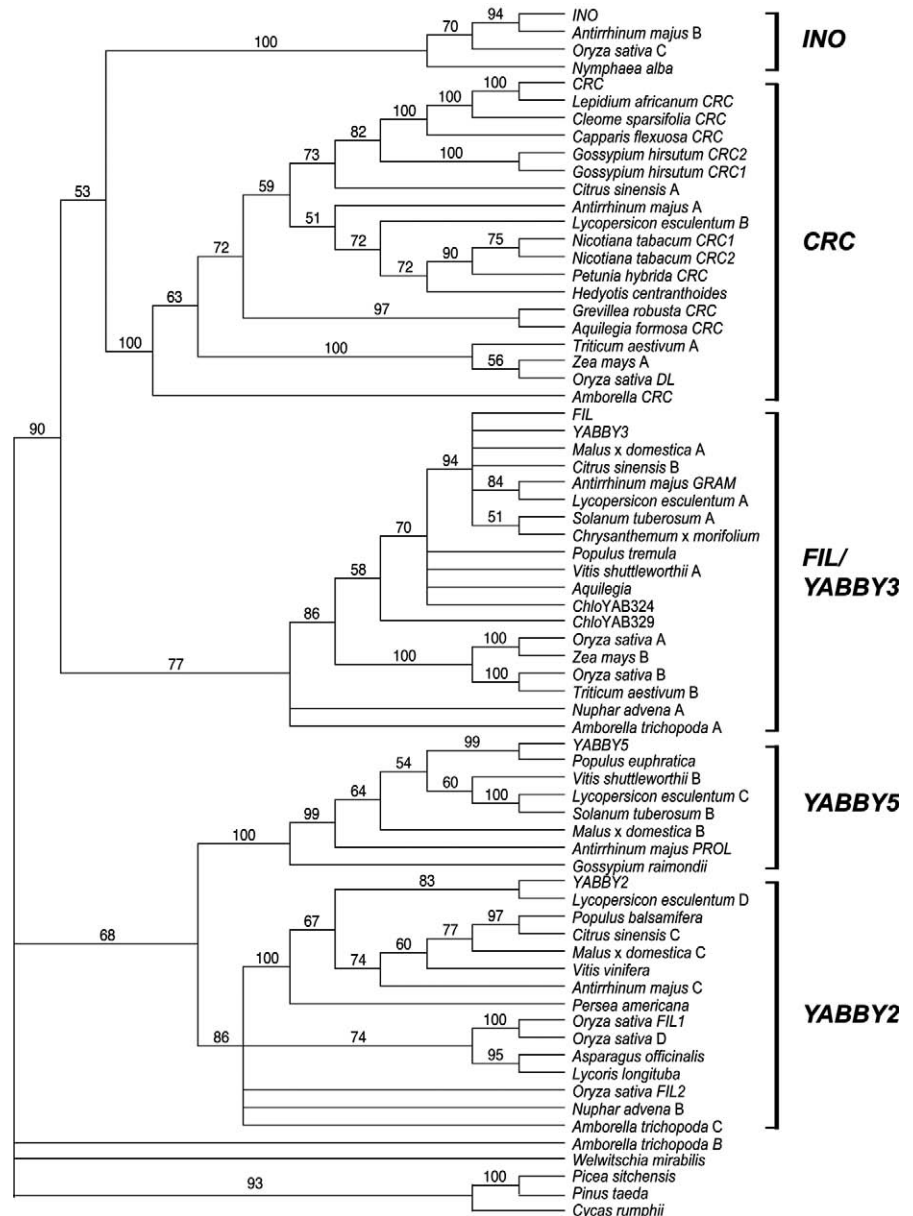
### Expression of *CRC* in extrafloral nectaries of rosids

Extrafloral nectaries develop in leaf axils in *Capparis flexuosa* (Zimmerman, 1932). Nectaries are covered with sheaths and consist of cytoplasmically dense cells innervated with phloem extending from the stem (Fig. 3H-L). Stomata present in the epidermis of the nectary might be involved in nectar secretion (Fig. 3K). As with floral nectaries, *CRC* expression correlates with the proliferation of nectariferous cells underneath the sheaths with *CfCRC* RNA levels increasing in developing nectaries and decreasing in mature nectaries (Fig. 3M-P).

To determine whether *CRC* expression correlates with extrafloral nectary development in phylogenetically distant species, *GhCRC* expression in extrafloral nectaries of *Gossypium hirsutum* (cotton) was analyzed. In *G. hirsutum*, extrafloral nectaries develop from the midvein on the abaxial sides of leaves and cotyledons, and on involucre bracts, leaf-like organs that subtend flowers. Nectaries consist of multicellular secretory structures that proliferate in a concave indentation on the abaxial midvein, positioned approximately one-third of the distance to the distal tip of the leaf (Fig. 4A-

D). Owing to high concentrations of secondary metabolites, RNA in situ hybridization was difficult in this species and our experimental results were equivocal. Thus, *GhCRC* expression was analyzed by RT-PCR and in situ RT-PCR, which detected *GhCRC* expression in the nectariferous cells of the leaf and bract (Fig. 4E,F). To evaluate the specificity and levels of *GhCRC* expression during nectary development, semi-quantitative RT PCR was performed using actin as a quantitative control (Holland et al., 2000). *GhCRC* is not only expressed in developing nectaries but also in other regions of the leaves, albeit at lower levels. *GhCRC* expression commences at an early stage of leaf development before any visible signs of secretory cells and is more abundant in the region of developing extrafloral nectaries. This expression pattern is also observed in extrafloral nectaries on involucre bracts. At a stage when nectaries are morphologically visible, levels of *GhCRC* mRNA are clearly higher than in other regions within the bract (Fig. 4G). Although not evident at the cellular level, RNA analysis suggests that *GhCRC* is correlated with extrafloral nectary development in *G. hirsutum*.

*Gossypium tomentosum*, a native Hawaiian species closely related to *G. hirsutum*, does not develop extrafloral nectaries, a derived condition within the genus (Small et al., 1998). Previous genetic analysis of the nectariless phenotype suggested that two loci are involved, with the *G. tomentosum* alleles being recessive (Meyer and Meyer, 1961). As these species are AD genome allotetraploids, and the two nectariless loci mapped to syntenous regions of the two genomes in *G. tomentosum*, changes in a single gene may be responsible for the nectariless phenotype. To determine whether mutations in *CRC* could be responsible for the nectariless phenotype, two experiments were conducted. First, we analyzed F2 plants derived from an F1 hybrid between *G. hirsutum* and *G. tomentosum*, and examined the segregation of *CRC* orthologs in nectariless individuals by RFLP analyses (data not shown). That the nectariless individuals did not predominantly have the *G. tomentosum* *CRC* allele indicates that alleles at this locus are not involved in the nectariless phenotype. In addition, mapping studies using *CRC* RFLP markers positioned the *CRC* orthologs on chromosomes 6 and 14, which does not correspond to the map positions of the nectariless phenotype [chromosomes 5 and 9 according to Meyer and Meyer (Meyer and Meyer, 1961)]. In *G. tomentosum*, *CRC* expression is reduced in seedlings when compared with *G. hirsutum*. In the



**Fig. 2.** Phylogenetic analysis of YABBY genes. Bayesian phylogenetic tree based on the nucleotide sequences of the zinc finger and YABBY domains of the YABBY family gene members. Posterior probabilities for clades are shown above branches.

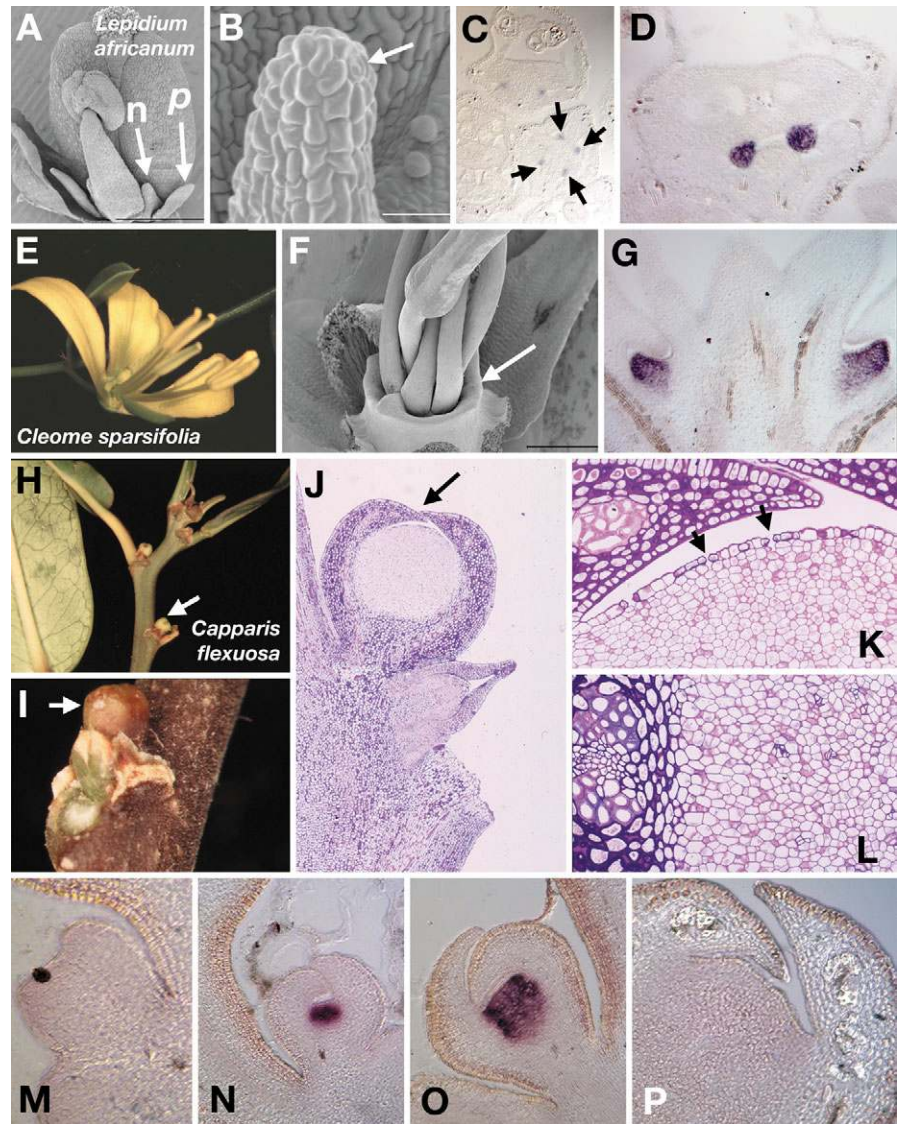
apices of seedlings at the stage when cotyledons have expanded, *CRC* is expressed at high level in *G. hirsutum*, whereas there is no expression detected in *G. tomentosum* (compare lane 29 with lane 32 in Fig. 4G).

### ***CRC*: a general regulator of nectary development in the core eudicots**

The correlation of *CRC* expression with developing extrafloral nectaries of *G. hirsutum* and *C. flexuosa* raises the question of the genetic independence of ontogenetically unrelated nectaries. As *CRC* expression is observed in structurally different nectaries in closely related species, we examined *CRC* in nectaries in a broader range of eudicot species. In *Petunia hybrida*, a core asterid species, nectaries develop as a disc at



**Fig. 3.** Nectaries of the Brassicales. Nectaries and expression of CRC orthologs in *Lepidium africanum* (A-D), *Cleome sparsifolia* (E-G) and *Capparis flexuosa* (H-P). (A) Floral nectaries (n) of *Lepidium africanum* are located between stamens and petals (p). Nectary glands flank each of the two stamens, such that each flower has four glands. (B) Nectary glands have apical stomata (arrow). (C,D) *LaCRC* is expressed in all cells of the nectary glands (arrow in C), from inception beyond anthesis. (E,F) *Cleome sparsifolia* floral nectaries (arrow) form a ring at the abaxial bases of the stamens. (G) *CsCRC* is expressed at high levels throughout nectaries. (H,I) Extrafloral nectaries of *Capparis flexuosa* (arrows) are located in leaf axils and are covered with scales, dissected away in I. (J) Longitudinal section of an extrafloral nectary reveals the internal anatomy (arrow). An axillary bud is adjacent and below the extrafloral nectary. (K) The covering sheath is composed mostly of thickened collenchyma cells and fibers, and the nectary has stomatal openings on its surface (arrows). (L) Nectaries comprise mostly parenchyma cells with phloem traces containing sieve tube members and companion cells. (M-P) Before nectary tissue develops inside the sheath, *CfCRC* expression is not observed (M, the dark spot is not signal). As nectary tissue initiates development, *CfCRC* expression increases dramatically and levels remain high throughout the nectary (N,O). As nectary growth ceases *CfCRC* expression decreases and only a minimal expression level remains (P).

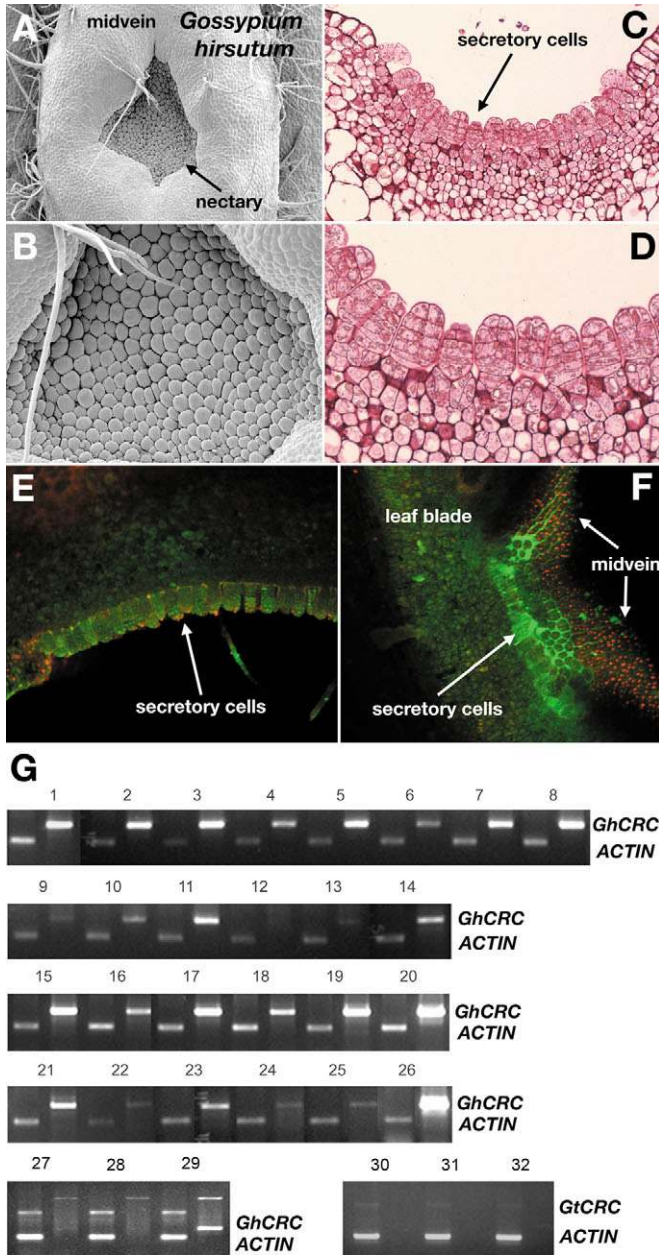


the base of the ovary (Fig. 5A), which is typical for asterid species, and *PhCRC* expression is associated with the development of carpels and nectaries. *PhCRC* is expressed in carpel primordia at initiation, abaxially in ovary walls, and subsequently abaxially in placental tissue developing interior to the ovary walls (Fig. 5B-E). In addition, *CRC* is expressed at a high level in the developing style and stigma (Fig. 5D,F). As carpel development progresses, abaxial expression of *CRC* abates and remains only in the basal regions of the ovary walls from where nectaries arise (Fig. 5F-H). In a stage 2 flower (Izhaki et al., 2002), nectary development is conspicuous as outgrowths on opposite sides of the septa and *PhCRC* expression is specific to the growing nectary tissue (Fig. 5I). Ovary walls adjacent to the nectary do not show detectable *PhCRC* expression (Fig. 5J). By stage 4, nectary tissue surrounds the base of the ovary walls (Fig. 5I,J) and *PhCRC* expression in nectaries continues through anthesis (Fig. 5K).

A virus-induced gene-silencing (VIGS) system based on tobacco rattle virus (TRV) was employed to determine the function of *CRC* in *Petunia hybrida* and *Nicotiana benthamiana* flowers (Chen et al., 2004). Nectaries in most

Solanaceae species are easily distinguished by their orange color owing to carotenoid accumulation (Fig. 5A,L). VIGS using the *PhCRC* gene resulted in a loss of nectaries, loss of carpel fusion and reduced determinacy in *N. benthamiana* flowers (Fig. 5L), all characteristics of *crc* mutations in *Arabidopsis* (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). In all infected plants, ~80% of *N. benthamiana* flowers exhibited a severe phenotype, as depicted in Fig. 5. In *Petunia*, concomitant downregulation of chalcone synthase allows one to select flowers exhibiting VIGS. The most severe phenotype observed was a loss of floral meristem determinacy, with the cells normally giving rise to the placenta instead developing into another flower (Fig. 5M). However, in *Petunia hybrida*, downregulation of *PhCRC* via VIGS was less severe, with a conspicuous phenotype in only two plants out of eight examined, and less than 50% of white flowers exhibiting indeterminacy. We interpret these results as a partial loss of *CRC* function induced by VIGS in *Petunia* and a more severe loss of *CRC* function in *N. benthamiana*, perhaps owing to *N. benthamiana* being more susceptible to virus infection (Yang et al., 2004).





**Fig. 4.** The extrafloral nectaries of *Gossypium*. (A) Extrafloral nectaries are located on midveins on the abaxial sides of the leaves. (B) The secretory structure is composed of trichomes that densely populate the concave region of the midvein. (C,D) Secretory trichomes are multicellular and small cytoplasmically dense parenchyma cells are located below the trichomes. (E) Expression of *GhCRC* in an involucre bract nectary localized by in situ PCR with a green signal representing the fluorescence signal from in situ PCR products of *GhCRC*. (F) *GhCRC* is highly expressed in the papillar cells and subtending cells in a leaf nectary. (G) Expression of *GhCRC* during extrafloral nectary development as monitored by semi-quantitative RT-PCR. 1-29, *G. hirsutum*; 30-32, *G. tomentosum*; left lane, *actin*; right lane, *GhCRC* (or *GtCRC*) amplified from same cDNA for *actin* under the same PCR condition. The upper bands in lanes 27-29 are from genomic DNA; the lower bands represent cDNA. 1, 3 mm whole leaf; 2, 5 mm whole leaf; 3, 1 cm whole leaf; 4, 1.4 cm leaf blade; 5, 1.4 cm leaf vein; 6, 2.8 cm leaf blade; 7, 2.8 cm leaf vein; 8, 2.8 cm leaf nectary; 9, 7 cm leaf blade; 10, 7 cm leaf vein; 11, 7 cm leaf nectary; 12, 12 cm leaf blade; 13, 12 cm leaf vein; 14, 12 cm leaf nectary; 15, bract nectary of 1.5 cm flower; 16, rest of bracts; 17, sepals; 18, petals; 19, stamens; 20, carpels; 21, bract nectary of 6 cm long flower; 22, rest of bracts; 23, sepals; 24, petals; 25, stamens; 26, carpels; 27, root of seedlings; 28, hypocotyl of seedlings; 29, apex of seedlings; 30, root of seedlings; 31, hypocotyl of seedlings; 32, apex of seedlings.

nectaries of *Arabidopsis*, though floral expression is detected in the pedicel and receptacle, as well as the style and stigma of the gynoecium (Fig. 6D,E). In contrast to the dramatic differences in the *GhCRC* promoter, a *GhCRC* cDNA is fully functional in *Arabidopsis*, rescuing the phenotypic defects of *crc-1* mutants when expressed under control of the *Arabidopsis CRC* promoter (Fig. 6G). Consistent with the change in expression domain, p*GhCRC* 8kb::*GhCRC* never rescued nectary development in *crc-1* mutants, although it frequently rescued carpel fusion defects (data not shown), suggesting that promoter elements directing carpel expression might be partially functionally conserved. As a positive control, the heterologous *Lepidium* promoter (p*LaCRC*, 4.3 kb) transcriptionally fused with GUS results in an expression pattern indistinguishable from *Arabidopsis CRC* and p*LaCRC* fused to the *LaCRC*-coding region can fully rescue *crc-1* mutants (Fig. 6F).

The dramatic alterations in *CRC* regulation are not due to the greater evolutionary distance between *Arabidopsis* and *Gossypium* than between *Arabidopsis* and *Lepidium*, as a p*AtCRC*::GUS transgene in *Nicotiana tabacum* (an asterid, whereas the other three species are rosids) drives GUS expression in carpels, primarily in style and stigma tissues, and later at the base of the ovary where the nectary develops (Fig. 6H). This pattern is reminiscent of *CRC* expression pattern in *Petunia* (Fig. 5), another member of the Solanaceae, suggesting conservation of *CRC* regulation in distantly related eudicot species. Thus, the lineage-specific changes in regulatory regions of cotton *CRC* probably contributed to the alterations in nectary position.

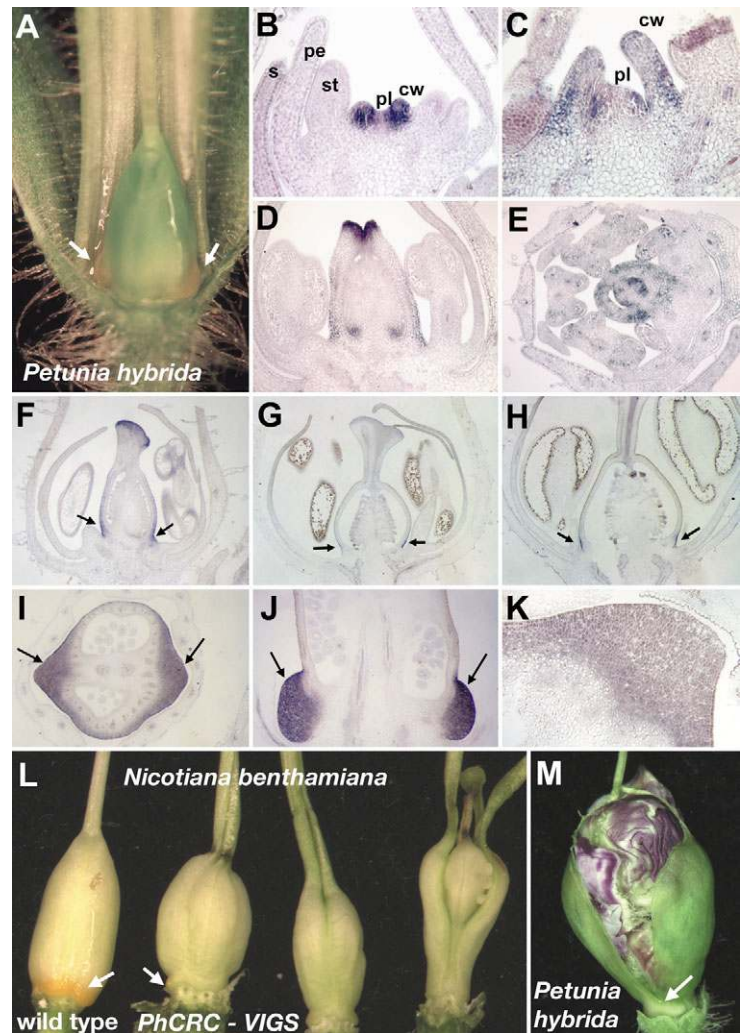
#### ***CRC* expression in *Aquilegia* flowers (Ranunculaceae): a basal eudicot**

Nectaries in *Aquilegia* flowers develop at the tips of petal spurs, the length of which is pollinator specific. Although there has been some debate on the identity of petal spurs of *Aquilegia*

#### **Conservation and changes in regulating *CRC*: mechanisms to retain *CRC* as a nectary regulator?**

*CRC* expression in extrafloral nectaries of *Gossypium hirsutum* implies alterations in spatial and temporal control of *CRC* gene regulation relative to that of *Arabidopsis*. Thus, we examined whether changes have occurred in the *GhCRC* promoter itself by examining its activity in *Arabidopsis*. The GUS reporter gene was transcriptionally fused with 8 kb of sequence 5' to the coding region of *GhCRC* and transformed into *Arabidopsis*. Whereas the *Arabidopsis CRC* promoter drives expression in nectaries and carpels, the *Gossypium CRC* promoter drives GUS expression in a pattern reminiscent of *CRC* expression in *Gossypium* (Fig. 6A-E). Expression initiates in seedlings, primarily in the vasculature, with maximum expression proximally in the midvein where nectaries normally develop in cotton (Fig. 6A-C). GUS expression was never found in floral

**Fig. 5.** Floral nectaries of *Petunia hybrida* and *Nicotiana benthamiana*. (A) The *Petunia hybrida* nectary develops as a disc at the base of the ovary but the gland (arrows) is more pronounced where the carpels are fused. (B-D,F-H,J) Longitudinal sections; (E,I,K) transverse sections. (B) *PhCRC* expression (blue-purple color) initiates in carpel primordia, specifically in carpel walls. s, sepal; pe, petal; st, stamen; pl, placenta; cw, carpel wall. (C) *PhCRC* expression is abaxial in both carpel walls and placenta. (D) Abaxial *PhCRC* expression in the carpel wall and placenta is stronger in the basal regions. *PhCRC* expression is also observed at the tips of the ovary walls, from which the style and stigma will develop. (E) Abaxial expression is present in the carpel wall and placenta, but no expression is detected in margins of fusion of the two carpels, similar to *CRC* expression in *Arabidopsis*. (F) As the style and stigma begin to develop *PhCRC* is expressed at the tip of the ovary. Abaxial expression in the basal region of carpel walls persists (arrows) but expression in the placenta has diminished. (G) *PhCRC* expression has ceased except at the base of the carpel walls (arrow) where the nectary will develop in this stage 0 flower (Izhaki et al., 2002). (H) Expression patterns in stage 1 flowers are similar to that of stage 0 flowers with higher expression at the base of ovary wall (arrow). (I) *PhCRC* expression is present throughout developing nectaries in stage 4 flowers. The two lobes of the nectary are indicated by arrows. (J) *PhCRC* expression is very strong and specific to the nectary (arrows) in stage 6 flowers. (K) *PhCRC* expression levels decrease after anthesis but persist throughout the nectary in stage 8 flowers. (L) VIGS-mediated silencing of *CRC* results in a loss of nectaries in *Nicotiana benthamiana*. In wild type (flower on left), nectaries are clearly visible as an orange ring of tissue at the base of the ovary (arrow). When *CRC* function is reduced, a progression of severity in phenotype is observed, with mildly affected flowers having some nectary tissue (second flower from left, arrow) and severely affected flowers having little or no nectary tissue (two flowers on right). (M) VIGS-mediated silencing of *CRC* results in a loss of flower meristem determinacy in *Petunia hybrida* flowers, but little visible effect on nectary development (arrow).



(e.g. Erbar et al., 1998), the simplest interpretation is that spurs are extensions of petals (Fig. 1; Fig. 7A). As with petals in most eudicot species, *Aquilegia* petals develop slowly compared with the other floral organs. Initial growth is in the lateral dimension, which causes the organs to be flattened. Subsequently, anticlinal cell divisions in the central region of the organs result in a furrow and from this furrow the spur will develop such that it extends from the abaxial side of the petal. Spur-like structures have been characterized in *Antirrhinum* mutants where ectopic expression of KNOX genes in the petals may be responsible for spur-like growth (Golz et al., 2002). Whether a similar mechanism directs spur growth in *Aquilegia* is unknown. Anatomical observations of *Aquilegia* nectar spurs reveal small cytoplasmically dense parenchyma cells on the adaxial side of the spur tip. These cells appear to comprise the nectary tissue (Fig. 7H-I).

*Aquilegia* flowers consist of two outer whorls of sepals and petals, followed by six to nine whorls of stamens, a single whorl of staminodes and an inner whorl of carpels. Each whorl contains five organs. *AfCRC* is expressed in regions of the floral meristem from which carpel primordia will emerge and subsequent expression in carpel primordia is abaxial (Fig. 7B-D). *AfCRC* expression in Fig. 7D-F is clearly in carpels, but it is possible that expression in primordia in Fig. 7B is in

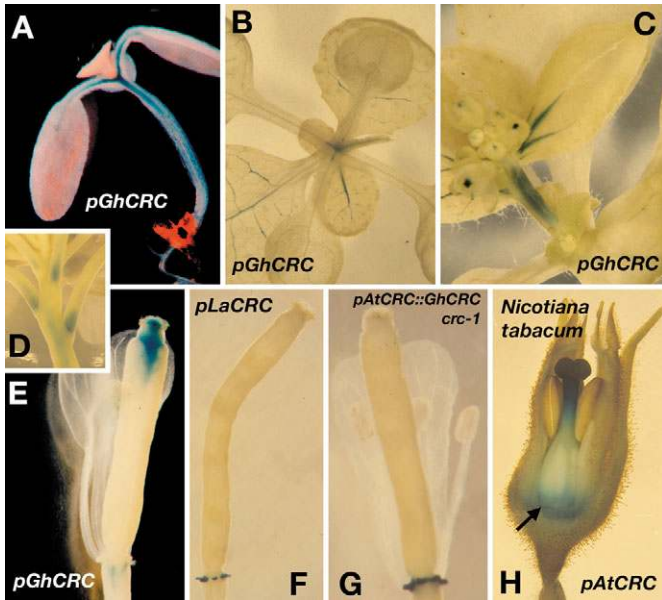
staminodes. Later during carpel development, *AfCRC* is expressed in another domain surrounding the central vascular bundle of the carpel, but excluded from the region from which the vasculature will differentiate (Fig. 7E-G) resulting in a ring-shaped expression pattern. However, unlike other core eudicot species examined, we did not detect expression of *AfCRC* in the nectaries of the spur (Fig. 7J).

## Discussion

### Role of *CRC* in nectary ontogeny

As developmental genetic studies in *Arabidopsis* indicate that *CRC* is one of the key genes for nectary development (Baum et al., 2001; Bowman and Smyth, 1999; Lee et al., 2005), we analyzed *CRC* expression in nectaries of exemplary eudicots. Owing to the diverse nature of nectary development in terms of both location and morphology, the current analysis represents only a small fraction of nectary types that exist in eudicots. However, as nectaries of Brassicaceae, Solanaceae and Malvaceae differ dramatically in their external features and locations, it is likely that common gene expression patterns reflect shared developmental genetics. A similar argument can be made for floral and extrafloral nectaries. The common expression of *CRC* in nectaries of phylogenetically diverse



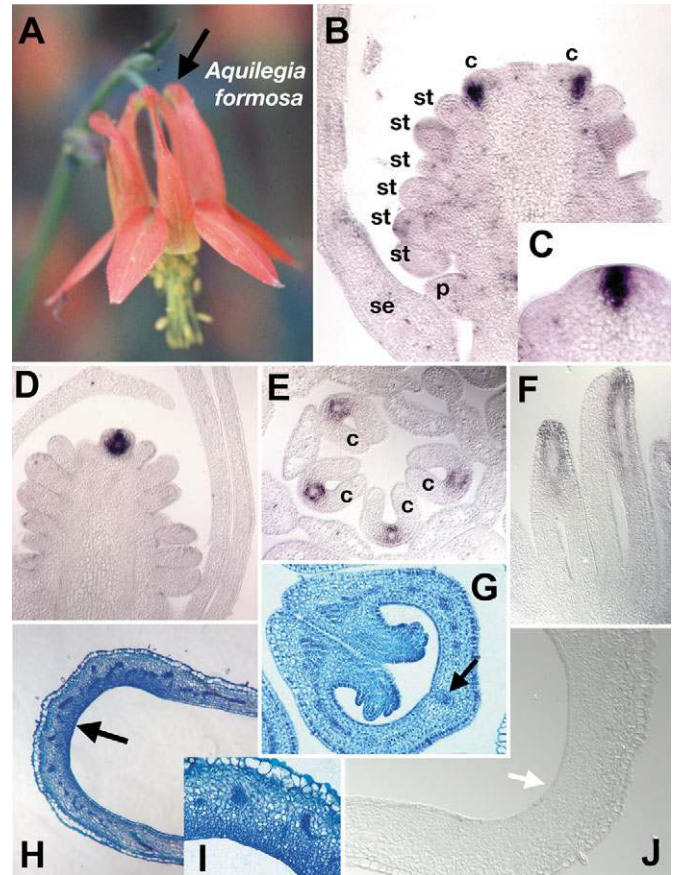


**Fig. 6.** Change versus conservation of *CRC* regulation. (A-E) Expression of *pGhCRC(8kb)::GUS* in *Arabidopsis*. *GUS* expression is detected in the vasculature of the hypocotyl and cotyledons in 1-week-old seedlings (A), in rosette leaves (B) and in cauline leaves (C) of older plants. *GUS* expression is also detected in the pedicels and receptacles of flowers (D), and the styles and stigma of gynoecia (E). (F) *pLaCRC::GUS* in the *Arabidopsis* nectary in a stage 15 flower. (G) *pAtCRC::GhCRC* is sufficient to rescue the nectary and carpel phenotypes in a *crc-1*. *GUS* expression in the nectary is provided by a *pAtCRC::GUS* transgene. (H) *pAtCRC::GUS* in *Nicotiana tabacum* flower. *GUS* expression is in the nectary disc (arrow) and style of the gynoecium.

species and the requirement of *CRC* for nectary development in both a rosoid (*Arabidopsis*) and an asterid (*Nicotiana*), representing the two major phylogenetic lineages within core eudicots, suggest that *CRC* is a general regulator of nectary development in core eudicot species regardless of nectary position and morphology. Alternatively, independent recruitments of *CRC* orthologs in each lineage with different types of nectaries could have occurred; although not impossible, such a scenario is less parsimonious as it would require many additional changes in gene regulation, and may be considered less likely by Occam's razor. Unlike the rosoid and asterid species examined, in *Aquilegia*, a basal eudicot species, *CRC* is not expressed in nectar spurs, suggesting that *CRC* is not required for nectary development in this species. We suggest that *CRC* was recruited to be a regulator of nectary development within the eudicots, concomitant with the phylogenetic association of nectaries with the floral reproductive organs.

#### Ancestral and derived functions of *CRC*

The *CRC* ortholog in *Oryza sativa* (rice) is a key regulator of carpel development (Yamaguchi et al., 2004) and our expression and functional analyses indicate that *CRC* acts as a regulator of carpel development throughout the eudicot lineages. As the species examined in this study, along with *Oryza* and *Amborella* (Fourquin et al., 2005), span the phylogenetic diversity of flowering plants, this role of *CRC* is



**Fig. 7.** Nectaries and carpels of *Aquilegia formosa* flowers. (A) Owing to growth in the pedicel, the nectar spurs of *Aquilegia formosa* flowers, which develop from the abaxial side of the petals, point upwards (arrow). (B) As carpel (c) primordia emerge, *AfCRC* expression (B-F) is restricted to the abaxial side as seen in this flower. st, stamen; p, petal; se, sepal. (C) *AfCRC* expression precedes the emergence of carpels. As carpel primordia begin to elongate, *AfCRC* expression is excluded from the vasculature and restricted to cells adjacent to the vasculature (D,E). In developing carpels, *AfCRC* expression is specifically localized to a ring of cells surrounding the vasculature in the abaxial regions (E,F). (G) Anatomy of the carpel. Each of the five carpels is separate with ovules initiating from the margins of the carpels. *AfCRC* expression is excluded from the vasculature (arrow) but is expressed in cells surrounding it. (H,I) Anatomy of the nectar spur. The tip of the developing nectar spur consists of densely cytoplasmic parenchymatous cells, characteristic of nectary tissue (arrow in H). (J) No detectable *AfCRC* expression was observed in developing nectar spurs (arrow).

probably conserved throughout angiosperms. As carpels evolved only once prior to the diversification of flowering plants, *CRC* might have evolved or have been recruited as a regulator of carpel development in the last common ancestor of angiosperms, a scenario consistent with the phylogeny of the YABBY gene family (Fig. 2). Based on genetic analyses in *Arabidopsis*, *CRC* is required for both proper establishment of carpel polarity and carpel identity, with *CRC* expressed abaxially in the valves and in placental tissues (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Eshed et al., 1999). The abaxial expression pattern is conserved among species examined in this study and a similar abaxial expression pattern

of *CRC* is observed in *Amborella* (Fourquin et al., 2005), suggesting this domain of function may be ancestral for angiosperms. In *Petunia*, *CRC* is also expressed abaxially in the placenta, a domain that may be related to the placental expression domain in *Arabidopsis*. Other expression domains such as the tips of the carpel in *Petunia*, and regions surrounding the vascular bundles in *Aquilegia* may represent independent recruitments of *CRC* for specific functions, similar to the recruitment of *CRC* to leaf midvein development in at least some grasses (Yamaguchi et al., 2004).

By contrast, expression analyses suggest that the role of *CRC* as a regulator of nectary development is restricted to a clade within the eudicots. Although nectaries within core eudicots differ enormously in their structures, a commonality is their association with the floral reproductive organs. In *Arabidopsis*, *CRC* is activated in nectaries by a combination of MADS box proteins encoded by the B and C class floral homeotic genes and *SEPALLATA* genes (Baum et al., 2001; Lee et al., 2005). Because the B, C and *SEPALLATA* genes are intimately involved in the specification of reproductive floral organ identity, the recruitment of *CRC* as a nectary regulator might have played a role in localizing floral nectaries near stamens and carpels in core eudicots. Based on the phylogenetic distributions of nectary locations (Fig. 1), we speculate that this could have occurred in the common ancestor of the core eudicots and the Proteales/Sabiaceae. Analyses of *CRC* in additional basal eudicots, such as *Grevillea robusta* (Fig. 1), are required to lend further support to this hypothesis.

That the *Arabidopsis* *CRC* promoter drives expression in nectaries and carpels in tobacco is consistent with the idea that *CRC* is regulated similarly in these two distantly related eudicot species. The regulation of other genes specifically expressed in nectaries, which are apparently involved in biochemical pathways in later stages of nectary development, is conserved between *Brassica* and *Petunia*, suggesting that gene regulation at later stages of nectary development might also be conserved among eudicots (Ge et al., 2000; Song et al., 2000). To better understand the genetic homology and diversity of nectaries, additional genes acting in conjunction and downstream of *CRC* need to be surveyed across angiosperm taxa.

### Derived nectary positions within the eudicots

Although nectaries in most eudicots are associated with reproductive organs, some taxa, such as *Capparis* and *Gossypium*, exhibit extrafloral or perianth associated nectaries. Mutant analyses in *Arabidopsis* have shown that in some genetic backgrounds, e.g. *leafy* and *apetala1* mutants, nectaries develop extraflorally. In these mutants, ectopic nectary structures expressing *CRC* develop at the pedicel base or along the pedicel (Baum et al., 2001). However, ectopic expression of *CRC* is not sufficient to generate ectopic nectaries, implying that additional genes are required for nectary development and that multiple changes in gene regulation, including that of *CRC*, might be required to alter nectary positions in eudicot lineages. When introduced into *Arabidopsis*, the *Gossypium* *CRC* promoter directs expression of a reporter gene in a pattern reminiscent of *CRC* expression in *Gossypium*, rather than the endogenous *Arabidopsis* *CRC* pattern, suggesting that the trans-acting factors directing the respective *CRC* expression patterns in the two species are conserved, but are largely non-

overlapping. This is in contrast to the conservation of gene regulation observed with *pAtCRC::GUS* in *Nicotiana* where both cis- and trans-acting elements may be functionally conserved. Thus, while activation of *CRC* in nectaries associated with reproductive tissues in core eudicots could be conserved at the level of trans-acting factors, activation of *CRC* in nectaries in derived positions required the recruitment of different transcriptional networks.

### Conclusions

Based on expression and functional analyses in a phylogenetic context, we propose the following scenario. Near the base of the eudicots, *CRC* was recruited to play a role in nectary development. This recruitment might have been concomitant with nectaries being associated with floral reproductive organs in the earliest branching eudicot lineages, i.e. possibly just subsequent to the divergence of the Ranunculids from the other eudicots (Fig. 1). As promoting carpel identity or polarity was probably the ancestral function of *CRC* in angiosperms, the recruitment of *CRC* to nectary development may have entailed *CRC* expression being brought under control of additional trans-acting factors together with an expansion of the downstream targets of *CRC* itself. Derived nectary positions within the eudicots appear to result from changes in cis-acting sequences controlling *CRC* expression rather than alterations in the encoded protein. Thus, the recruitment of *CRC* to a role in nectary development probably entailed similar molecular changes as proposed for other transcription factors where changes in gene expression patterns, rather than alterations in protein activity, were responsible for driving changes in morphological evolution (Doebley and Lukens, 1998).

Shared *CRC* gene expression in diverse nectaries of core eudicots is in some ways comparable with shared gene regulation during eye development. Specification of the eye field requires homologous members of a retinal determination gene network, including *PAX6*; the network is conserved for different types of eyes in Bilateria (Silver and Rebay, 2004). This striking conservation of master regulators of eye development challenged the long-standing view of multiple origins of eye evolution (Abouheif, 1997). However, developmental and physiological differences between insect and human eyes are enormous, suggesting differential recruitment of downstream programs of eye formation in each lineage under the same master regulators (Pichaud et al., 2001; Nilsson, 2004). Such genetic conservation of master regulation is explained as a result of evolutionary constraints of regulatory circuits for cuing some developmental processes (Hodin, 2000). Identifying where genetic differences occur in each eye development pathway will shed light on understanding the developmental differences arising during evolution. Likewise, identification of additional nectary regulators is required to gain insight into the conservation and divergence of nectaries within angiosperms.

We thank Dr Beatriz Xoconostle-Cazares and Dr Roberto Ruiz-Medrano for providing in situ RT-PCR techniques, Ravit Eshed for transforming *pAtCRC::GUS* into tobacco, Dr Jonathan Wendel at Texas A&M University for providing F2 seeds of *G. hirsutum* × *G. tomentosum*, Dr Andrew Patterson at University of Georgia for *GhCRC* mapping, and Fairchild Tropical Botanical Garden (FL, USA) for providing *Capparis flexuosa*. We thank Sandy Floyd, Chris



Zalewski and other members of the Bowman laboratory for comments and discussions. This work was made possible with funding from the Department of Energy, Division of Biosciences: DE-FG03-97ER20272 (J.L.B.).

### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/132/22/5021/DC1>

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