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

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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, Carpels, 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; Kyozuka 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 spinosa [Cleomeae 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/Cleomeae (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 (Solit 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 RNaseasy 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-ZaF (5'-CDGTRACRGGAATG- GYCTATTGGYA-AFG 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 (Quiagen, 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
Development

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 tetraoxide. Tissue was dehydrated and critical-point dried. After sputter coating with gold/palladium, tissue was observed on a Hitachi S-3500N scanning electron microscope.

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 (GGTTCCACAATCCGGACCATTT) and GhCRC-2 (CACAACGGATGATGCTGGAGAGAA) 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: GhaActin-F (CCCTTCAGCCTCTTTATC) and GhaActin-R (ATTCACTACTCACCTGGGA). 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 sepal glands, the sepal being places where adjacent walls of two carpels have not fused (Brown, 1938). Almost all monocots possessing nectaries have sepal 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 (Elías, 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),
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 involuce bracts, leaf-like organs that subtend flowers. Nectaries consist of multicellular secretory structures that proliforate 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 involucr e 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 allopolyploids, and the two nectarless loci mapped to syntenous regions of the two genomes in G. tomentosum, changes in a single gene may be responsible for the nectarless phenotype. To determine whether mutations in CRC could be responsible for the nectarless 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 nectarless individuals by RFLP analyses (data not shown). That the nectarless individuals did not predominantly have the G. tomentosum CRC allele indicates that alleles at this locus are not involved in the nectarless 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 nectarless 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 seedlings 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
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. 5L,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).
Development

CRC expression in extrafloral nectaries of *Gossypium* 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 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, pGhCRC 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 (pLaCRC, 4.3 kb) transcriptionally fused with GUS results in an expression pattern indistinguishable from *Arabidopsis* CRC and pLaCRC 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 pAtCRC::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*...
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...
species and the requirement of \textit{CRC} for nectary development in both a rosid (\textit{Arabidopsis}) and an asterid (\textit{Nicotiana}), representing the two major phylogenetic lineages within core eudicots, suggest that \textit{CRC} is a general regulator of nectary development in core eudicot species regardless of nectary position and morphology. Alternatively, independent recruitments of \textit{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 rosid and asterid species examined, in \textit{Aquilegia}, a basal eudicot species, \textit{CRC} is not expressed in nectar spurs, suggesting that \textit{CRC} is not required for nectary development in this species. We suggest that \textit{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 \textit{CRC}**

The \textit{CRC} ortholog in \textit{Oryza sativa} (rice) is a key regulator of carpel development (Yamaguchi et al., 2004) and our expression and functional analyses indicate that \textit{CRC} acts as a regulator of carpel development throughout the eudicot lineages. As the species examined in this study, along with \textit{Oryza} and \textit{Amborella} (Fourquin et al., 2005), span the phylogenetic diversity of flowering plants, this role of \textit{CRC} is probably conserved throughout angiosperms. As carpels evolved only once prior to the diversification of flowering plants, \textit{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 \textit{YABBY} gene family (Fig. 2). Based on genetic analyses in \textit{Arabidopsis}, \textit{CRC} is required for both proper establishment of carpel polarity and carpel identity, with \textit{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 trans-activation 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 cueing 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.

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