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Functional Conservation between *CRABS CLAW* Orthologues from Widely Diverged Angiosperms

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• *Background and Aims* *CRABS CLAW* (*CRC*) encodes a transcription factor of the YABBY family that plays important roles in carpel and nectary development in *Arabidopsis thaliana.* Combined evolutionary and developmental studies suggest an ancestor of the *CRC* gene to have controlled carpel development in the last common ancestor of the angiosperms. Roles for *CRC* orthologues in leaf development and carpel specification in rice, and in nectary development in core eudicots, have accordingly been interpreted as derived. The aim of this study was to assess the capacity of *CRC* orthologues from a basal angiosperm and from rice to complement *CRC* mutants of arabidopsis. These experiments were designed to test the hypothesized ancestral role of *CRC* in the angiosperms, and to indicate whether putatively novel roles of various *CRC* orthologues resulted from changes to their encoded proteins, or from other molecular evolutionary events.
• *Methods* The *crc-1* mutant of arabidopsis was genetically transformed with the coding sequences of various *CRC* orthologues, and with paralogous YABBY coding sequences, under the control of the arabidopsis *CRC* promoter. The phenotypes of transformed plants were assessed to determine the degree of complementation of the *crc-1* mutant phenotype in carpel fusion, carpel form and nectary development.
• *Key Results* The *CRC* orthologue from the basal angiosperm *Amborella trichopoda* partially complemented the *crc-1* mutant phenotype in carpels, but not in nectaries. The *CRC* orthologue from rice partially complemented all aspects of the *crc-1* mutant phenotype. Though most non-*CRC* YABBY coding sequences did not complement *crc-1* mutant phenotypes, *YABBY2* (*YAB2*) proved to be an exception.
• *Conclusions* The data support a hypothesized ancestral role for *CRC* in carpel development and suggest that novel roles for *CRC* orthologues in monocots and in core eudicots resulted principally from molecular changes other than those affecting their coding sequences.

Key words: Carpel, gynoecium, nectary, *Amborella trichopoda*, *Arabidopsis thaliana*, *Oryza sativa*, *CRABS CLAW*, *DROOPING LEAF*, YABBY.

INTRODUCTION

CRABS CLAW (*CRC*) encodes one of six putative transcription factors of the YABBY family in *Arabidopsis thaliana*. This gene plays an important role in carpel development, and is also necessary for the development of nectaries (Alvarez and Smyth, 1999). Its precise expression pattern in carpel tissues (Bowman and Smyth, 1999) reflects the general role of YABBY genes in specifying the abaxial (facing away from the axis) side of plant lateral organs (Bowman, 2000; Bowman *et al.*, 2002). In the case of *CRABS CLAW*, this role is apparent in double mutant plants in which the strong *crc-1* mutation is combined with mutations in any one of the genes *KANADI*, *GYMNOS* and *AKETHE* (Eshed *et al.*, 1999), none of which belong to the YABBY family. Such double-mutants show a breakdown of abaxial–adaxial (adaxial = facing towards the axis) polarity at the margins of the two fused carpels in the arabidopsis gynoecium. Loss of developmental polarity in this case leads to the formation of placentas and ovules both inside and outside of the ovary. However, in single *crc-1* mutants, polarity defects are not apparent (Alvarez and Smyth, 1999). Instead, these mutants show defects in carpel fusion and in the overall size and shape of the gynoecium. In addition, nectaries are absent from *crc-1* mutants.

Strong evidence exists to suggest that the three 'ANA' (formerly 'ANITA') orders, Amborellales, Nymphaeales and Austrobaileyales, diverged from a remaining common lineage near the base of the phylogenetic tree of the extant angiosperms (Stevens, 2001 onwards). The remaining common lineage would later have diverged to form the two major angiosperm groups of the eudicots and monocots, in addition to several less species-rich clades. The expression pattern in carpel tissues of the putative orthologue of *CRC* from the ANA angiosperm *Amborella trichopoda* (Fourquin *et al.*, 2005) is remarkably similar to that of *CRC* in arabidopsis. This gene, *AmbCRC*, is expressed in an abaxial-specific manner in the ovary wall, and is not generally expressed in leaves and other non-reproductive organs. As *Amborella trichopoda* is the only member of what is generally regarded as the most basal angiosperm group (Amborellales), the correspondence in expression patterns between *CRC* and *AmbCRC* strongly suggests these genes to have conserved their expression patterns since the common ancestor of the extant angiosperms.

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The conservation of such a distinctive expression pattern is highly suggestive of a conserved role, and so it has been argued that *CRC* and *AmbCRC* have conserved a common role in the control of carpel development since the last common ancestor of the extant flowering plants (Fourquin *et al.*, 2005).

Yamaguchi *et al.* (2004) have identified a putative orthologue of *CRC* from rice (*Oryza sativa*), termed *DROOPING LEAF* (*DL*). In *dl* mutants, carpels are replaced by stamens in the fourth floral whorl, and leaf defects are also present, in contrast to the *crc* mutant phenotype of arabidopsis. The conservation of expression patterns of *CRC* orthologues between basal angiosperms such as *Amborella*, and eudicots such as arabidopsis, suggests the expression pattern and functions of *DL* in rice to be derived characters that originated after the divergence of the monocot and eudicot lineages (Fourquin *et al.*, 2005).

Similarly, the nectary development function associated with *CRC* in arabidopsis, and with *CRC* orthologues in other species of core eudicots (i.e. the crown group of the eudicots, excluding the most basal lineages of this clade), is not thought to be ancestral to the angiosperms (Lee *et al.*, 2005*b*). Angiosperm species that are external to the core eudicots do not show expression of *CRC* orthologues in nectary tissues, where these exist. Thus, *CRC* is hypothesized to have acquired a role in nectary specification at the base of the core eudicot clade. Floral nectaries are not present in most ANA grade angiosperms, including *Amborella* (Endress, 2001), or in rice.

Novel functions associated with *CRC* orthologues may have resulted from changes to their expression patterns, to their coding regions, or from changes to other genes acting together with, or downstream of, *CRC* orthologues in transcriptional control networks. In the present study, the aim is to assess to what extent the coding sequences of *CRC* orthologues from various species, and those of paralogous YABBY genes from arabidopsis, are able to functionally replace the wild-type *CRC* coding sequence in arabidopsis. Results from these analyses provide functional evidence for evolutionary hypotheses relating to the ancestral role of the *CRC* gene, and also indicate to what extent coding sequence changes, or other factors, may have been responsible for the hypothesized novel functions of *CRC* orthologues in monocots and in core eudicots.

MATERIALS AND METHODS

Transgene constructions

The plant transformation vector *pCAMBIA3300* was modified by the addition of the *nopaline synthase* gene terminator (*nos*), ligated in a $5'-3'$ orientation between unique *Sac*1 and *Eco*R1 restriction sites. The resulting plasmid was further modified by the ligation of a 'GATEWAY' (Invitrogen) *ccd*B selection cassette, bordered by *att*R1 and *att*R2 recombination sites, into a unique *Sma*1 restriction site immediately upstream of the *nos* terminator. A 3608-bp fragment from immediately upstream of the arabidopsis *CRC* start codon, taken to represent the *CRC* promoter (*proCRC*), was obtained by PCR amplification from genomic DNA of the Columbia ecotype of *Arabidopsis thaliana* using a proof-reading thermo-stable DNA polymerase. The resulting PCR product was cloned into a plasmid vector and fully sequenced. This *CRC* promoter fragment was subsequently excised from its cloning vector and ligated into the unique *Xba*1 restriction site of the modified *pCAMBIA* plasmid (above), adjacent to the *ccd*B cassette. Coding sequences of various YABBY genes from *Arabidopsis thaliana* (Columba ecotype), *Amborella* and *Orzya sativa* 'Nipponbare', were amplified from the appropriate full-length cDNAs by PCR to incorporate *att*B1 and *att*B2 GATEWAY recombination sites situated 2 bp upstream and 1 bp downstream, respectively, of their start and stop codons. The resulting coding sequences were inserted into *pDONR207* (Invitrogen) by GATEWAY 'BP' recombination reactions. The exact nucleotide sequences of the coding sequences were confirmed, and these were then transferred by GATEWAY 'LR' recombination reactions to the *proCRC*-containing plant transformation vector, thereby replacing its *ccd*B cassette. The YABBY coding sequences were thus finally situated in the plant transformation vector, downstream of the *CRC* promoter and upstream of the *nos* terminator, and immediately flanked by *att*B1 and *att*B2 recombination sites. GATEWAY-type plasmids containing β-glucuronidase (GUS) and improved green fluorescent protein (Heim *et al.*, 1994) reporter gene coding regions, flanked by *att*L1 and *att*L2 recombination sites, were obtained from Dr Vanessa Vernoud in our laboratory. These reporter genes were also transferred to the *proCRC*-containing plant transformation vector by GATEWAY 'LR' recombination reactions. Completed plant transformation vectors were transferred by electroporation to *Agrobacterium tumefaciens* strain C58pmp90 for plant transformation. Full nucleotide sequences of the constructions used for plant transformation will be made available on request.

Plant material and genetic transformation

Seeds of the Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh., and of the *crc-1* mutant in the Landsberg *erecta* genetic background, were obtained from the Nottingham Arabidopsis Stock Centre (UK). *Agrobacterium tumefaciens*-mediated genetic transformation of arabidopsis plants was performed by the floral dip method (Clough and Bent, 1998), to generate transformants carrying YABBY or reporter gene coding sequences under the control of the *CRC* promoter. Seeds harvested from dipped plants (T_1 seeds) were sown initially onto sand and selected by watering with ammonium glufosinate (BASTA) herbicide (7·5 mg dm^{-3}). BASTA-resistant seedlings were transferred to potting compost. These plants were then grown under 8-h day-length conditions in a growth chamber for 4 weeks and then transferred to 18-h day-length conditions to induce flowering.

Northern blot hybridization

Northern blot hybridization was performed as previously described (Fourquin *et al.*, 2005).

Microscopy

Plant tissues were photographed at low magnification using a binocular dissecting microscope (Leica MZFIII) fitted with a digital camera Leica (DC 300F). GUS assays were performed as described by Nakayama *et al.* (2005). Excitation of GFP was performed using a UV light source fitted with a band-pass filter of 450–490 nm. The presence or absence of nectaries in arabidopsis flowers was recorded in fresh plant material using an environmental scanning electron microscope (Hitachi S800).

RESULTS

A 3·6-kb DNA fragment immediately upstream of the CRC coding region shows CRC promoter activity

A genomic DNA fragment of 3608 bp, from immediately upstream of the arabidopsis *CRC* start codon, was taken to represent the *CRC* promoter in these studies. The promoter activity of this fragment was initially assayed using *GUS* and *GFP* reporter genes in populations of 40 transgenic *Arabidopsis thaliana* plants of the Columbia ecotype. Sixty per cent of plants transformed with *GUS*, and 5 % of those transformed with *GFP* reporter genes, showed moderate reporter gene activities that were largely restricted to carpels and nectaries, similar to the wild-type expression pattern of *CRC* (Fig. 1A, B). However, very strong *GUS* expression in leaves (Fig. 1C) and floral organs (Fig. 1D) was noted in 25 % of T_1 plants containing the *proCRC::GUS* transgene (Fig. 1C). The presence of high levels of reporter gene mRNAs in leaves and inflorescences in 37·5 % and 25 % of two further T_1 populations of 16 *GUS* and 16 *GFP* transformants, respectively, was subsequently demonstrated by northern blotting (results not presented).

Subsequent to the start of this study, a detailed analysis of sequences upstream of the *CRC* coding region, performed by comparison between three species of Brassicaceae, has indicated the presence of five conserved 'modules' of functional significance within the *CRC* promoter (Lee *et al.*, 2005*a*). A synthetic promoter containing only these five modules was found to be capable of correctly directing reporter gene expression, as did an intact *CRC* promoter fragment of 3·8 kb. The 3·6-kb *CRC* promoter fragment used in the experiments described here contained all five of the conserved modules identified by Lee *et al.* (*2005a*), terminating some 548 bp upstream of the most distal (to the *CRC* coding sequence) of these modules. Therefore, the expression of reporter genes outside of the normal *CRC* expression domain, noted in a proportion of transformed plants in the present study, probably does not reflect the absence of important *cis*-acting regulatory sequences in the 3·6-kb promoter fragment used. As a proportion of T_1 transformants in each population examined showed reporter gene expression that was largely limited to the domain of *CRC* expression in wild-type plants (Fig. 1A, B), the 3·6-kb promoter fragment used in these experiments was considered to have generated the expected *CRC* expression profile in at least a proportion of transgenic plants. The ectopic reporter gene expression noted here, in the leaves and other organs of a further group of T_1 transformants (Fig. 1C, D), was concluded to have been produced by some position-dependent mechanism associated with the sites of transgene insertion.

The CRC orthologues from Amborella and rice show both quantitative and qualitative differences in their functional conservation with CRC from arabidopsis

Compared with wild-type plants (Fig. 2A–C), *crc-1* mutants produce carpels that are only partially fused together (Fig. 2D) to form gynoecia, and later siliques, that are considerably shorter and wider than wild-type (Fig. 2E). These mutants also lack floral nectaries (Fig. 2F). In this study, a *proCRC::CRC* transgene was found to almost completely restore the wild-type phenotype to 65 % of transformed *crc-1* mutants in a T_1 population of 40 plants. Carpels and siliques of these tranformants were completely closed (Fig. 2G), and were close to wild-type length and width (Fig. 2H). The flowers of these transformants possessed nectaries (Fig. 2I). The remainder of the transformed plants from a T_1 population containing the *proCRC::CRC* transgene showed lesser degrees of complementation, or no difference from the *crc-1* mutant phenotype, possibly reflecting lesser levels of protein expression due to position-specific effects associated with the insertion of transgenes. These results form a positive control for the

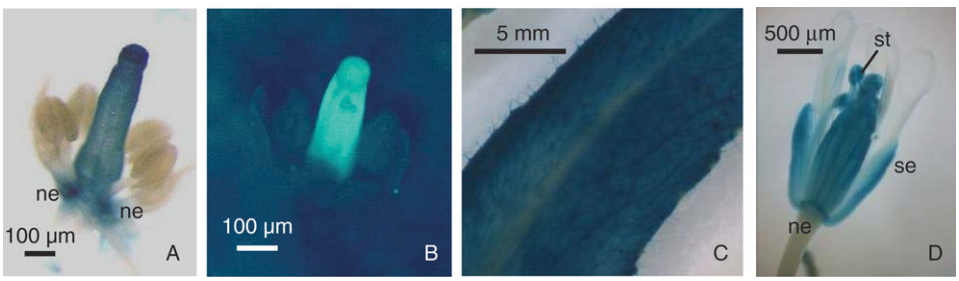

FIG. 1. *GUS* and *GFP* reporter gene expression under the control of the *CRC* gene promoter. (A) GUS staining in the gynoecium and nectaries (ne) of a flower from a *proCRC::GUS* transformant at developmental stage 11–12 (Smyth *et al.*, 1990), showing the expected *CRC*-like pattern of reporter gene expression. (B) GFP localization in the gynoecium of a flower from a *proCRC::GFP* transformant at developmental stage 10–11 (Smyth *et al.*, 1990), showing the expected *CRC*-like pattern of reporter gene expression. (C) GUS staining in leaf tissue of a *proCRC::GUS* transformant showing reporter gene activity outside of the normal zone of *CRC* expression. (D) GUS staining in mature flower tissues (se = sepal, st = stamen) in a *proCRC::GUS* transformant showing reporter gene activity outside of the normal zone of *CRC* expression.

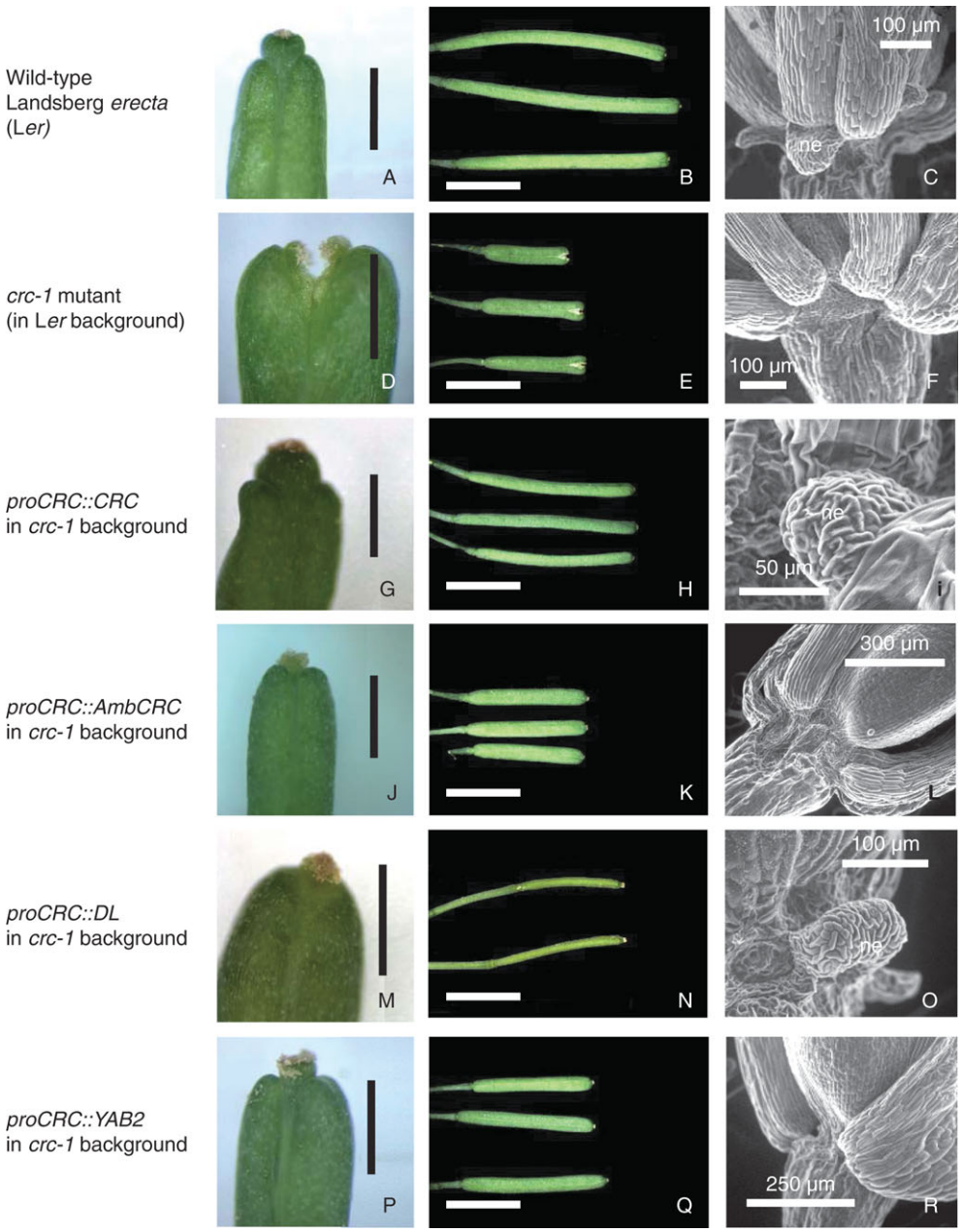

FIG. 2. Complementation of the *crc-1* mutation by transformation with various YABBY coding sequences under the control of the *CRC* promoter. (A, D, G, J, M, P) Silique apices showing degrees of carpel fusion in wild-type plants (A), *crc-1* mutants (D) and *crc-1* mutants transformed with the YABBY coding sequences *CRC* (G), *AmbCRC* (J), *DL* (M) and *YAB2* (P). Scale bars = 1 mm. (B, E, H, K, N, Q) Fully elongated siliques in wild-type plants (B), *crc-1* mutants (E) and *crc-1* mutants transformed with the YABBY coding sequences *CRC* (H), *AmbCRC* (K), *DL* (N) and *YAB2* (Q). Scale bars = 5 mm. (C, F, I, L, O, R) The base of the third floral whorl, showing the presence or absence of nectaries (ne), in mature flowers of wild-type plants (C), *crc-1* mutants (F) and *crc-1* mutants transformed with the YABBY coding sequences *CRC* (I), *AmbCRC* (L), *DL* (O) and *YAB2* (R).

present study, providing a near-perfect complementation of the *crc-1* mutation in a high proportion of transformants, against which the effects of other YABBY coding sequences can be compared.

To test the conservation of *CRC* function between distantly related angiosperms, the degree of complementation of the *crc-1* mutation was assessed in arabidopsis plants transformed with constructions containing the probable orthologues of *CRC* from the basal angiosperm *Amborella*, Amb*CRC*, and from rice, *DL*. Transformation of the *crc-1* mutant with a *proCRC::AmbCRC* construction resulted in a partial restoration of the wild-type phenotype in 55 % of transformants from a T_1 population of 40 plants. These plants showed complete carpel closure (Fig. 2J), and slightly increased silique length, compared with that of *crc-1* mutants (Fig. 2K). No floral nectaries were present in any of the *proCRC::AmbCRC* transformants examined (Fig. 2L). These results indicate the *AmbCRC*

coding sequence to be partially capable of substituting for *CRC* in the control of carpel development, but incapable of such a substitution in the control of nectary development.

Transformation of arabidopsis *crc-1* mutants with a *proCRC::DL* transgene produced a range of distinct phenotypes. In 15 % of a T_1 population of 40 plants, the gynoecium apex was completely closed (Fig. 2M) and gynoecium and silique dimensions were intermediate between those of *crc-1* mutants and wild-type plants (Fig. 2N). These transformants possessed floral nectaries (Fig. 2O), indicating the *DL* coding sequence to be capable of replacing *CRC* functions in nectary development, in addition to carpel development, when correctly expressed under the control of the arabidopsis *CRC* promoter. A proportion of plants from the T_1 population transformed with *proCRC::DL* exhibited a range of partially complemented phenotypes (25 %), or were apparently not complemented (20 %), closely resembling *crc-1* mutants.

The *proCRC::DL* transformants described above presented no striking differences to wild-type plants in leaf development or in general plant architecture (Fig. 3A). However, a further proportion (40 %) of *proCRC::DL* transformants from the same T_1 population exhibited aberrant development in all above-ground organs (Figs 3B–D), suggesting the generalized overexpression of the *DL* transgene. Similar phenotypes were noted in plants in which the *CRC* coding sequence was overexpressed under the control of the cauliflower mosaic virus 35S promoter (Fig. 3E). The leaves in *proCRC::DL* transformants that showed leaf development phenotypes, and in all *pro35S::CRC* transformants examined, were somewhat narrowed, and contorted out of their normal plane of growth by irregular blade expansion (Fig. 3B–F). Approximately half of the highly aberrant plants transformed with a *proCRC::DL* transgene eventually produced flowers (e.g. Fig. 3C), though these were also developmentally abnormal (data not presented) and almost completely sterile. *ProCRC::DL* transformants showing aberrant leaf development also showed a lack of internode extension in their reproductive phase (Fig. 3C), unlike *p35S::CRC* transformants (Fig. 3E). The presence of high levels of expression of *DL* in the leaves and inflorescences (where produced) of *proCRC::DL* transformants that showed altered leaf development was confirmed by northern blotting. High levels of *DL* transcripts in leaf tissue correlated with abnormal leaf phenotypes in a sample of six *proCRC::DL* transformants, three of which had abnormal leaves (Fig. 3G). The generalized transgene expression observed in a proportion of *proCRC::DL* transformants seems, therefore, to be similar to that observed in a proportion of *proCRC::GUS* (Figs 1C and D) and *proCRC::GFP* (data not presented) transformants.

The YABBY2 coding sequence shows partially conserved functions with that of CRC

The capacities of two further YABBY coding sequences from arabidopsis, *FILAMENTOUS FLOWER* (*FIL*) and *YABBY2* (*YAB2*), to replace the functions of *CRC* were tested. A *proCRC::FIL* transgene showed no capacity to complement the *crc-1* mutation. All *crc-1* transformants examined from a T_1 population containing a *proCRC::FIL* transgene showed a phenotype identical to that of *crc-1* mutants (data not presented). However, 70 % of *crc-1* plants from a T_1 population transformed with a *proCRC::YAB2* construction showed a partial restoration of the wild-type phenotype. The most highly complemented individuals from this population showed complete carpel closure (Fig. 2P) and silique dimensions that were

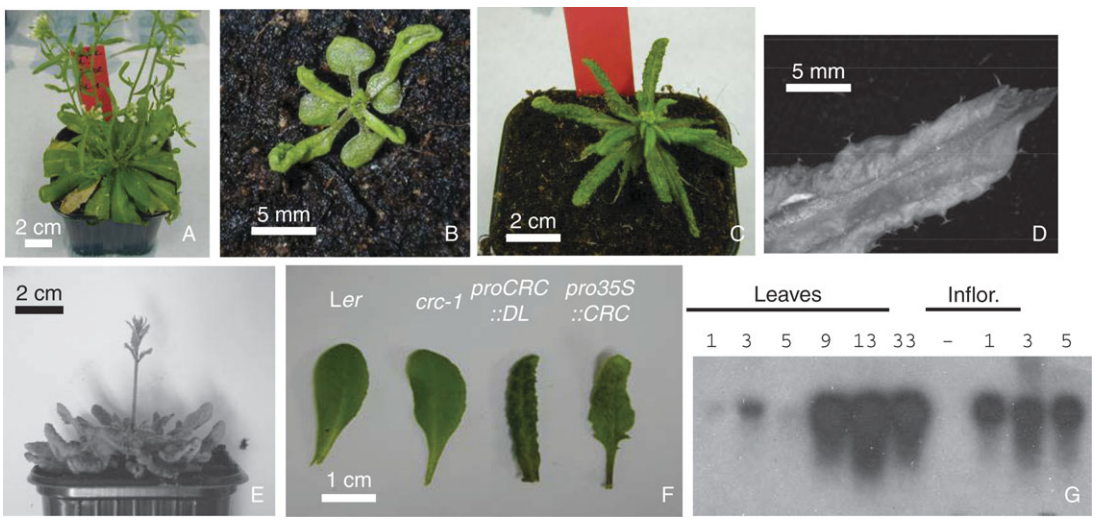

FIG. 3. Phenotypic effects and expression of *DL* in leaves and other organs in a proportion of *proCRC::DL* transformants. (A) A mature plant transformed with *proCRC::DL* (corresponding to plant 1 in Fig. 3G), showing normal development of leaves and inflorescence architecture. (B) A seedling transformed with *proCRC::DL*, showing aberrant leaf development. (C) A mature plant transformed with *proCRC::DL* (corresponding to plant 9 in Fig. 3G), showing aberrant leaf development and plant architecture. (D) The underside of a leaf from the *proCRC::DL* transformant shown in (C). (E) A pro35S::*CRC* transformant showing a leaf phenotype similar to that of some *proCRC::DL* transformants. (F) Mature rosette leaves, showing the effect of overexpression of *CRC* and *DL* transgenes in leaf tissue. (G) Northern hybridization of a *DL* cDNA probe to RNA from leaves and inflorescences of three *proCRC::DL* transformants (plants 1, 3 and 5) that showed normal leaf development, and to RNA from leaves of three *proCRC::DL* transformants (plants 9, 13 and 33) that showed aberrant leaf development. High levels of *DL* expression in leaves correlate with aberrant leaf phenotypes.

intermediate between those of *crc-1* mutants and wild-type plants (Fig. 2Q). Silique length was more completely restored to wild-type dimensions by transformation with this construction than with a *proCRC::AmbCRC* construction (Fig. 2K). No floral nectaries were present in *proCRC::YAB2* transformants (Fig. 2R), indicating the YAB2 coding sequence to be incapable of replacing the nectary development function of *CRC*.

DISCUSSION

Studies of functional conservation between orthologous coding sequences support a role for CRC in carpel development in the last common ancestor of the flowering plants

A principal aim of this study was to obtain functional data concerning the ancestral role of *CRC* in the angiosperms. Previous comparative studies, performed between the putatively most-basal angiosperm, *Amborella*, and the model angiosperm, arabidopsis, had already shown the likely orthologous genes from these two species, *AmbCRC* and *CRC*, to share a common expression pattern in carpel tissues. This result was highly suggestive of a conserved role in carpel development, implying that the ancestral *CRC* gene would have already played this role in the last common ancestor of the flowering plants. The present study aimed to determine the extent to which the *AmbCRC* coding sequence could compensate for the lack of a functional *CRC* gene in arabidopsis mutants, and thereby provide some functional evidence for the hypothesized conserved role of the *CRC* gene.

AmbCRC has been shown to be capable of partially restoring a wild-type phenotype to the strong *crc-1* mutant of arabidopsis. *AmbCRC* was fully able to restore carpel fusion, and slightly increased carpel and silique lengths, though not completely to wild-type dimensions. *AmbCRC* was not, however, able to restore nectary development to *crc-1* mutants. These results lend some support to the hypothesis that the carpel development role of *CRC*, as determined by genetic studies in arabidopsis (Alvarez and Smyth, 1999), has been conserved since the common ancestor of the living flowering plants, some 160 Mya (Davies *et al.*, 2004).

Novel functions of DL in rice may principally have resulted from changes in gene expression, rather than from changes to the DL coding sequence

The phenotype associated with the *dl* mutation in rice (Yamaguchi *et al.*, 2004) is strikingly different to the *crc* mutant phenotype in arabidopsis (Alvarez and Smyth, 1999), despite the probable orthology between *DL* and *CRC*. *dl* mutants show a homeotic replacement of carpels by stamens and their leaves lack a mid-rib, contrasting with the effects on carpel form and nectary specification of *crc* mutants. As the role played by *CRC* in arabidopsis carpel development has been interpreted as the ancestral role of this gene in the angiosperms (Fourquin *et al.*, 2005), the roles of *DL* in carpel specification and leaf development may be considered as derived.

The results of the present study, showing that the *DL* coding sequence is able to largely replace that of *CRC* in the control of carpel development, suggest the differences between the phenotypes of *dl* and *crc* mutants to be principally due to factors other than physical differences between the DL and CRC proteins. The complementary experiment of transforming rice *dl* mutants with a *proDL::CRC* construction would be useful to provide a fuller answer to this question. At least one of the factors contributing to the novel roles of *DL* in rice must be the control of its expression, as *DL* and *CRC* expression patterns differ markedly (Bowman and Smyth, 1999; Yamaguchi *et al.*, 2004). The novel functions of *DL* may thus have arisen in part by evolutionary changes to its *cis*-acting control regions, or to *trans*-acting factors controlling its expression.

The acquisition by CRC of a role in nectary development in the core eudicots may have arisen independently of changes to the CRC coding sequence

The *DL* coding sequence from rice was able to restore nectary development to *crc-1* mutants of arabidopsis, even though the role of *CRC* in nectaries is thought to have evolved within the eudicot clade, after the last common ancestor shared between this group and the monocots (Lee *et al.*, 2005*b*). This result suggests that the acquisition of a role in the control of nectary development by *CRC* did not occur as a direct result of evolutionary changes to the *CRC* coding sequence. The novel role of *CRC* in nectary development may instead have resulted from one or more changes affecting, for example, the expression of *CRC* in nectaries, its repertoire of target genes, or aspects of its potential partner proteins.

Seven *crc* mutants are known, which form an allelic series showing a range of carpel phenotypes, though a uniformly complete absence of nectaries (Bowman and Smyth, 1999). In the present study, only the *CRC* and *DL* coding sequences were able to restore both wild-type carpel and nectary development to *crc-1* mutants, whereas the more distantly related coding sequences *AmbCRC* and *YAB2* only restored carpel phenotypes. Taken together, these data suggest nectary development to be more sensitive than carpel development in arabidopsis to changes to the *CRC* coding sequence.

YAB2 shows a level of functional conservation with CRC that cannot be explained by its phylogenetic position within the YABBY family

It was shown that *YAB2* from arabidopsis was able to replace *CRC* functions in transgenic plants to a somewhat greater extent than was *AmbCRC* from *Amborella*. Thus, a *proCRC::YAB2* construction completely restored carpel fusion and showed a marked effect on gynoecium and silique length, but failed to restore nectary development to *crc-1* mutants. Of the six YABBY coding sequences from arabidopsis, only *YAB5* has yet to be tested for its capacity to compensate for a lack of *CRC* activity in transgenic plants. Y. Eshed and J. L. Bowman (unpubl. res., cited in Meister *et al.*, 2005) found that neither *FIL*, nor

its paralogue *YABBY3* (*YAB3*), was capable of restoring wild-type phenotypes to *crc-1* mutants. In the case of *FIL*, these findings have been confirmed in the present study. Meister *et al.* (2005) found that *INNER NO OUTER* (*INO*) was unable to replace *CRC* when expressed under the control of the *CRC* promoter. *CRC* is only distantly related to the other YABBY genes present in arabidopsis, having arisen as a distinct member of the YABBY family by a gene duplication event that occurred before the radiation of the flowering plants (Fourquin *et al.*, 2005; Lee *et al.*, 2005*b*). Globally, the results of the present and previous studies indicate the different members of the YABBY family to exhibit distinct protein activities, and the partial *CRC* activity of which *YAB2* is capable represents an exception to this rule. Meister *et al.* (2005) found, by constructing chimeric *CRC*/*INO* proteins, that the three domains of *CRC*: the zinc-finger, central, and YABBY (DNA binding) domains, contributed equally to *CRC* activity. Further experiments will be required to determine which domains of the *YAB2* coding sequence confer its capacity to partially replace *CRC* in carpel development.

A 3·6-kb fragment of the CRC promoter shows erratic position effects in transgenic plants

A currently unexplained phenomenon, noted in the present study, led to the very high level of expression of *DL*, *GUS* and *GFP* coding sequences under the control of a 3·6-kb promoter fragment from the *CRC* gene in leaves and other organs in a proportion of transgenic plants. As this effect was only present in some transformants from each T_1 population, with other plants showing the expected *CRC*-like pattern of transgene expression, it would seem to be a position-related effect. Further experiments are in progress to investigate the erratic expression of reporter genes noted in the present study with the use of the 3·6-kb *CRC* promoter fragment. However, the main conclusions of the present work were not affected by this gene expression phenomenon as they were based on transformed plants in which developmental changes were limited to tissues in which *CRC* is expressed in wild-type plants.

CONCLUSIONS

It is concluded from the present study that putative orthologues of *CRC* from very widely diverged angiosperm groups show a conserved activity in carpel tissues. These data support the hypothesis of an ancestral role for *CRC* in the control of carpel development. The present data suggest the putatively derived roles of *CRC* orthologues in the control of nectary development in the core eudicots, and in carpel determination and leaf development in monocots, to have been acquired by molecular evolution events that did not principally affect the coding sequences of the *CRC* orthologues present in these plant groups. These conclusions have been reached by studying the coding regions of genes taken from a few widely diverged taxa (*Amborella*, arabidopsis and rice). Studies of intermediate taxa would be useful to confirm our conclusions.

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