Evidence that *CRABS CLAW* and *TOUSLED* have conserved their roles in carpel development since the ancestor of the extant angiosperms

Chloé Fourquin*, Marion Vinauger-Douard*, Bruno Fogliani†, Christian Dumas*, and Charles P. Scutt*‡

*Laboratoire de Reproduction et Développement des Plantes, Unité Mixte de Recherche 5667, Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique, Université Claude Bernard-Lyon, Institut Fédératif de Recherche 128-Biosciences-Lyon Gerland, 46 Allée d'Italie, Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07, France; and †Laboratoire de Biologie et Physiologie Végétales Appliquées, Université de la Nouvelle-Calédonie, BP 4477 Nouméa, New Caledonia

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The carpel is the female reproductive organ specific to flowering plants. We aim to define the genes that controlled carpel development in the common ancestor of this group as a step toward determining the molecular events that were responsible for the evolution of the carpel. *CRABS CLAW* (*CRC*) and *TOUSLED* (*TSL*) control important aspects of carpel development in the model plant, *Arabidopsis thaliana*. The basal angiosperm species *Amborella trichopoda* and *Cabomba aquatica* very likely represent the two most early diverging groups of flowering plants. We have identified putative orthologues of *CRC* and *TSL* from *A. trichopoda and C. aquatica*, respectively. We demonstrate the expression patterns of these genes in carpels to be very highly conserved, both spatially and temporally, with those of their *Arabidopsis* orthologues. We argue that *CRC* and *TSL* in *Arabidopsis* are likely to have conserved their respective roles in carpel development since the common ancestor of the living flowering plants. We conclude that a divergent role shown for the *CRC* orthologue in rice, *DROOPING LEAF*, most probably arose specifically in the monocot lineage. We show that, in addition to its expression in carpels, the *TSL* orthologue of *C. aquatica* is expressed in tissues that contribute to buoyancy and argue that its role in these tissues may have arisen later than its role in carpel development.

Amborella | *Cabomba* | ANITA | gynoecium | flower

The carpel is the female reproductive organ specific to the angiosperms, or flowering plants. In most species, the carpel is differentiated into stigma, style, and ovary tissues and may occur as a separate structure, or fused with other carpels in a syncarpic pistil. The carpel protects the ovules within its ovary and provides a location for pollen tube guidance and pollen incompatibility mechanisms. After fertilization, the ovary develops into a fruit that protects the seeds and may participate in their dissemination. For these reasons, the carpel was probably a major factor in the success of the angiosperms, which diversified from an unknown, presumably gymnosperm-like ancestor to form in excess of 300,000 species alive today.

To understand the molecular evolution events that led to the first carpels, we must first know what genes and mechanisms of carpel development were present in the earliest flowering plants. This information may be obtained by comparing the presence and functions of orthologous genes that control carpel development in present-day species whose evolutionary lineages diverged very early in flowering plant evolution. The two prerequisites of such an analysis are a robust molecular phylogeny of the flowering plants and an understanding of some of the genetic mechanisms of carpel development in model species.

The concordant results of five independent molecular phylogenetic studies, incorporating very widespread taxonomic sampling, have provided a more robust hypothesis for the evolutionary relationships between the major groups of seed plants than has ever before existed, as reviewed by Kuzoff and Gasser (1). These studies strongly suggest that the flowering plants and extant gymnosperms form two sister clades. Within the flowering plant clade, these studies support the view that seven extant families of dicots, collectively referred to as the ANITA grade, represent the first lineages to have diverged from the remaining lineage. The ANITA grade contains *Amborella*, Nymphaeales, and the ITA clade, this latter also being known as Austrobaileyales. Nymphaeales contains the two families Nymphaeaceae and Cabombaceae, whereas Austrobaileyales (the ITA clade) contains the four families Illiciaceae, Trimeniaceae, Austrobaileyaceae, and Schisandraceae. One issue still to be resolved concerns the order of divergence of the two most basal groups of angiosperms in the ANITA grade. The five initial studies (1) and a more recent reanalysis (2) concluded the earliest diverging lineage to be represented by a single species, *Amborella trichopoda*, which is unique to its order. According to these studies, the second-diverging lineage is represented by the Nymphaeales, an order of aquatic plants. An alternative hypothesis, derived from studies that corrected for long-branch attraction (3), proposed the earliest diverging angiosperm lineage to form a clade containing both *A. trichopoda* and the Nymphaeales. These two hypotheses seem too close to be conclusively resolved at present.

Goremykin *et al.* (4, 5) have performed analyses using entire chloroplast genome data to derive the radically alternative phylogenetic hypothesis that the most basal division in the angiosperms lies between the monocots and dicots. However, it has been elegantly demonstrated that the incongruity between these results and those of other recent molecular phylogenetic studies results from limited taxon sampling in the work of Goremykin *et al.* and not from the smaller gene sets used in other studies. Critically, Goremykin *et al.* used only species of Poaceae (grasses), which are highly derived, to represent the monocots. If a single non-Poaceae monocot is added to phylogenetic analyses by using data sets composed of either several genes (6) or entire chloroplast genomes (7), the ANITA grade regains its basal position.

After the identification of the ANITA grade as the likely earliest diverging angiosperms, these have been reanalyzed (8) to derive a list of pleisiomorphic characters that most probably represent the ancestral state of the flower. According to these studies, the ancestor of the extant angiosperms would have possessed small, protogynous, bisexual flowers. Its flowers would have contained a gynoecium of separate, incompletely closed

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Abbreviations: *CRC*, *CRABS CLAW*; *TSL*, *TOUSLED*; *DL*, *DROOPING LEAF*; TLK, TOUSLED-like kinase; SEM, scanning electron microscopy.

Data deposition: The sequences reported in this paper have been deposited in the EMBL database [accession nos. AJ877257 (AmbCRC) and AJ877258 (CabTSL)].

‡To whom correspondence should be addressed. E-mail: charlie.scutt@ens-lyon.fr.

carpels with few ovules, a spiral phyllotaxy of floral organs, and a perianth that was not distinctly divided into petals and sepals.

The gynoecium of *Arabidopsis thaliana*, a highly evolved eudicot species, comprises two congenitally fused carpels. The development of this structure is specified by the expression of the MADS box C-function gene, *AGAMOUS*, according to the ABCE model (9). Putative C-function orthologues are expressed in reproductive organs of gymnosperms as in angiosperms, indicating aspects of C-function activity to have been conserved since the common ancestor of the seed plants (10, 11). The ancient origin of the C-function gene suggests that evolutionary changes in other genes may have been responsible for the more recent origin of the carpel. Candidates for these genes include the many sequences encoding transcription factors, protein kinases, and other developmental regulators, whose roles in carpel development have been investigated by the analysis of *Arabidopsis* mutants (12, 13).

We have begun to determine which genes of *Arabidopsis* carpel development may have conserved their functions since the ancestor of the living flowering plants. To perform this analysis, we have searched for putative orthologues of carpel development genes in representatives of the two most probably basal groups of flowering plants, *A. trichopoda* (Amborellales, Amborellaceae) and *Cabomba aquatica* (Nymphaeales, Cabombaceae). *A. trichopoda* is a scrambling dioecious tree of humid tropical forests, endemic to New Caledonia. *C. aquatica* is a small aquatic plant, native to Northern Brazil. Both of these species show the likely pleisiomorphic characters relating to carpel development (8). For certain classes of carpel development genes that are the subjects of continuing studies in our laboratory, orthology relationships have proved complex, or expression patterns have differed considerably between *Arabidopsis* and basal angiosperms. However, we present here the cases of *CRABS CLAW (CRC)* and *TOUSLED (TSL)*, two genes for which strong evidence has been obtained for a conservation of function in gynoecium development since the common ancestor of the living flowering plants.

CRC encodes a member of the small family of plant-specific YABBY putative transcription factors in *Arabidopsis*. It is expressed only in the gynoecium and nectaries and controls the development of these structures (14). *crc* mutants have abnormally wide gynoecia that are incompletely closed at the apex and show a defect in carpel fusion. *CRC* interacts genetically with three different classes of genes to specify abaxial cell fate in the ovary wall (15). This function is consistent with the specific expression of *CRC* in the abaxial cell layers of the ovary. *crc* mutations eliminate carpelloid structures in the first whorl of A/C-function double mutants (16), suggesting that *CRC* may control elements of a C-function-like pathway in a way that is masked by genetic redundancy in wild-type plants. A putative *CRC* orthologue, *DROOPING LEAF (DL)*, is known from rice (17). This gene, unlike *CRC*, seems to play a major role in the specification of carpel identity as *dl* mutants show homeotic conversion of carpels to stamens. Consistent with this function, *DL* is expressed in the presumptive zone, or anlagen, in the flower meristem from which the gynoecium develops. *DL*, also unlike *CRC*, controls the development of the mid-rib in leaves.

TSL is a unique gene in *Arabidopsis* that has pleiotropic effects on flower and leaf development (18). It encodes a serine-threonine protein kinase containing an N-terminal regulatory domain in addition to a C-terminal kinase domain. The regulatory domain is necessary for the formation of homooligomers, upon which the catalytic activity of TSL depends (19). In *tsl* mutants, the number of floral organ primordia in whorls one to three is reduced, although the organs that develop from these are not greatly affected (18). Carpel fusion may be reduced by *tsl* mutations, probably due to uncoordinated growth of carpel primordia. Other than this effect, *TSL* intervenes only at a late stage in gynoecium development. In *tsl* mutants, the style and stigma develop incompletely, and the gynoecium remains open at its apex (20). This effect is consistent with the specific expression of *TSL* in the style and stigma at late stages of flower bud development. TOUSLED-like kinases (TLKs) are widely distributed in eukaryotes, including other plant species, *Drosophila*, *Caenorhabditis elegans*, and mammals, suggesting that these molecules play fundamental biochemical roles. In *C. elegans*, TLK is essential for transcription, and its inactivation leads to the complete arrest of development (21). Results from various animal systems, discussed by Ehsan *et al*. (22), show that TLK activity is linked to DNA replication and that TLKs may participate in the regulation of gene expression through chromatin modification. These authors have demonstrated links for TSL in *Arabidopsis* with both the cell cycle and with putative components of chromatin assembly (22), suggesting biochemical parallels with animal TLKs. Despite these similarities, the effect of *tsl* mutations in *Arabidopsis* remains less severe than that of TLK inactivation in *C. elegans*, suggesting some divergence in the roles of TLKs between plant and animal lineages.

Materials and Methods

Plant Material. Material of *A. trichopoda* Baill. was field-collected from locations near Col d'Amieu, New Caledonia (map IGN 4825). Material of *C. aquatica* Aublet was obtained from Anthias S.A., Les Chères 69, France. Seeds of *A. thaliana* Heyn. Landsberg *erecta* ecotype were obtained from the Nottingham *Arabidopsis* Stock Centre, Nottingham, U.K., and plants were grown to maturity in peat-based compost in a growth chamber at 20°C under 16 h light/8 h dark cycles.

RNA Preparation and RT-PCR. RNA was extracted from tissues of *A. trichopoda* and *C. aquatica* by the method of Chang *et al*. (23), and from *Arabidopsis* tissues by using TRIzol reagent (Invitrogen). Polyadenylated RNA for use in cDNA library construction and Northern blotting was purified from total RNA by using a PolyAtract kit (Promega). RT-PCR was performed on total RNA samples of *A. trichopoda* and *C. aquatica* by the CODEHOP method (24). Conserved regions of mRNAs homologous to *CRC* were amplified by using the partially degenerate primers 5′-TTGGACACAGTGACAGTGAAGTGYGGNCAYTG and 5′-AGCCCAATTCTTAGCAGCAGCASWRAANGCYTC. Those of mRNAs homologous to *TSL* were amplified by using the partially degenerate primers 5′-AATAAGAAGTCTCAGAAGATTATHCAYTAYGA and 5′-TTCAAAGCATTCTGGTGGCAAATACCARTANGT.

cDNA Library Construction and Screening. cDNA libraries were prepared by using a Bacteriophage λ Uni-Zap II kit (Strategene) from polyadenylated RNA of female flowers of *A. trichopoda* and of inflorescences of *C. aquatica* that included flower bud stages up to anthesis. Bacteriophage λ plaques were transferred onto nylon hybridization membranes and screened with radiolabeled RT-PCR products corresponding to fragments of *CRC*- and *TSL*-like cDNAs according to λ Zap prototcols (Stratagene). Positively hybridizing bacteriophage clones were purified through a round of secondary screening, and cDNAs were obtained from these in pBlueScript II plasmid vectors by *in vivo* excision using ExAssist (Stratagene) M13 helper phage.

Molecular Phylogenetic Analysis. Alignment of predicted amino acid sequences was performed by using CLUSTALW (25). Phylogenetic trees were constructed and bootstrapped by using the PHYLOWIN computer package (26), allowing comparison of results using neighbor joining, maximum likelihood, and maximum parsimony methods.

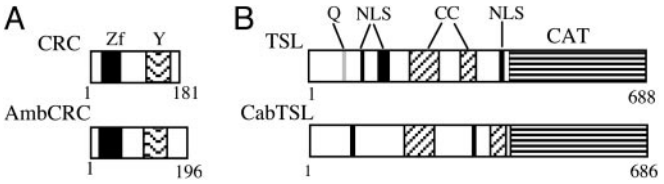

Fig. 1. Comparisons of predicted protein domains in CRC and AmbCRC (*A*) and TSL and CabTSL (*B*). CC, coiled-coil domain; CAT, protein kinase catalytic domain; NLS, nuclear localization signal; Q, glutamine-rich region; Y, YABBY (DNA-binding) domain; Zf, zinc-finger domain.

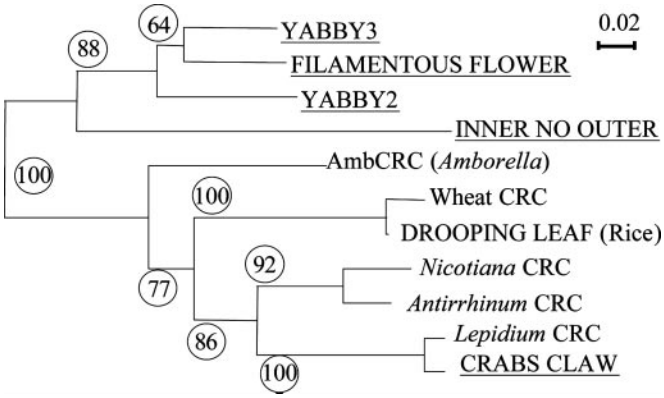

Fig. 2. Phylogenetic analysis of AmbCRC with other predicted YABBY proteins. The tree shown was constructed by the neighbor joining method (37) from an alignment corresponding to amino acid residues 19–55 (the zinc-finger domain) and 110–157 (the YABBY domain) of *Arabidopsis* CRC. *Arabidopsis* sequences are underlined, and percentage support for nodes in 500 bootstrap replicates are encircled. Unpublished sequence accession numbers are as follows: AY451399 (*Antirrhinum*), AY703987 (*Lepidium*), AY071845 (*Nicotiana*), and AF545436 (wheat).

Northern and Virtual Northern Blot Hybridizations. Northern blots of *C. aquatica* and *A. thaliana* were prepared containing 2.5 μg per track of polyadenylated RNA samples. Virtual Northern blots, which are cDNA blots giving a sensitivity of detection equivalent to polyadenylated RNA Northern blots, were prepared from *A. trichopoda* total RNA samples as described by Teakle *et al.* (27). Blot hybridizations were performed by using radio-labeled probes corresponding to full-length cDNAs as described (28), although using a hybridization buffer containing 1% (wt/vol) bovine serum albumen, 0.2 M sodium phosphate (pH 7.2), 1 mM EDTA, 7% (wt/vol) SDS, and 15% (vol/vol) formamide.

***In Situ* Hybridization.** Nonradioactive *in situ* hybridization to tissue sections of antisense and sense (control) strand riboprobes derived from *AmbCRC* and *CabTSL* cDNAs was performed as described by Ferrandiz and Sessions (29). *In situ* hybridization images were captured under bright-field illumination by using a Zeiss Axiovert 125 inverted microscope.

Scanning Electron Microscopy (SEM). Plant material was fixed and stored in FAA (3.7% formaldehyde/5% acetic acid/50% ethanol). Samples were rehydrated and examined by using a Hitachi (Tokyo) S800 environmental scanning electron microscope.

Results

Putative Orthologues of *TSL* and *CRC* Are Expressed in Flower Tissues of the Most Early Diverging Groups of Angiosperms. RT-PCR was used to amplify PCR products homologous to *CRC* and *TSL* from flower RNAs of the basal angiosperms *A. trichopoda* and *C. aquatica*. The PCR products obtained were ligated into plasmid vectors for DNA sequencing and reexcised for use as probes to screen flower cDNA libraries. The predicted amino acid sequences of full-length cDNAs obtained from library screens were aligned with known homologous sequences, and phylogenetic trees were constructed to infer gene orthology relationships.

AmbCRC, a *CRC*-like cDNA from *A. trichopoda*, encoded a protein containing zinc-finger and YABBY domains, in a similar arrangement to CRC and other YABBY putative transcription factors, as shown in Fig. 1*A*. In phylogenetic analyses, *AmbCRC* grouped closely with *CRC* and its putative orthologues, as shown in Fig. 2. This grouping proved very robust and was maintained by using different combinations of amino acid or nucleic acid data sets and different analysis methods. *AmbCRC* was therefore concluded to represent a putative orthologue of *CRC*. By contrast, a full-length cDNA obtained from *C. aquatica* grouped most closely to *YABBY3* in phylogenetic analyses (results not presented) and was concluded to not represent an orthologue of *CRC*. Further screens of a *C. aquatica* cDNA library at a reduced stringency of hybridization using *CRC* and *AmbCRC* probes identified several further *YABBY*-like cDNAs, although none of these grouped with *CRC* in phylogenetic analyses. *C. aquatica* flower-expressed *YABBY* cDNAs will require further study.

A *TSL*-like cDNA identified from *C. aquatica*, *CabTSL*, encoded a protein containing a coiled-coil putative regulatory domain, nuclear localization signals, and a putative serine-threonine protein kinase domain, thereby showing overall structural similarity to TSL, as shown in Fig. 1*B*. CabTSL did not contain an N-terminal glutamine-rich domain of unknown function that is present in TSL, although it is absent from TSL orthologues of rice and maize (accession numbers AC091811 and AY644701). Because *TSL* is a unique gene in *Arabidopsis*, *CabTSL* may be regarded as its putative orthologue. A *TSL*-related cDNA identified from our other basal angiosperm model, *A. trichopoda*, was found to be interrupted by a stop codon upstream of its predicted kinase domain. Furthermore, no in-frame start codon was present upstream of the *TSL*-like reading frame in this molecule. These features, inconsistent with the synthesis of an active TSL-like kinase, were found to be conserved between several independent cDNAs and their analysis was not continued. The present study has, therefore, identified two cDNAs encoding putative CRC- and TSL-orthologous proteins, from *A. trichopoda* and *C. aquatica*, respectively.

***AmbCRC* Is Expressed Abaxially in the *A. trichopoda* Carpel Wall, Closely Resembling *CRC* Expression in *Arabidopsis*.** Virtual Northern blot hybridizations, shown in Fig. 3, indicated *AmbCRC* to be

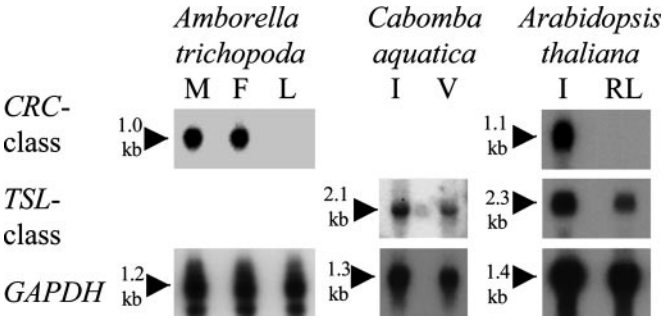

Fig. 3. Northern and virtual Northern blot hybridizations of *AmbCRC*, *CabTSL*, and their *Arabidopsis* orthologues. Hybridizations are to virtual Northern blots of *A. trichopoda* and polyadenylated RNA Northern blots of *C. aquatica* and *A. thaliana*. Hybridizations to a cDNA encoding GAPDH from each species are included to demonstrate equivalent loading of tracks. F, female flowers; I, inflorescences; L, leaves; M, male flowers; RL, rosette leaves; and V, vegetative tissues (submerged leaves and stems).

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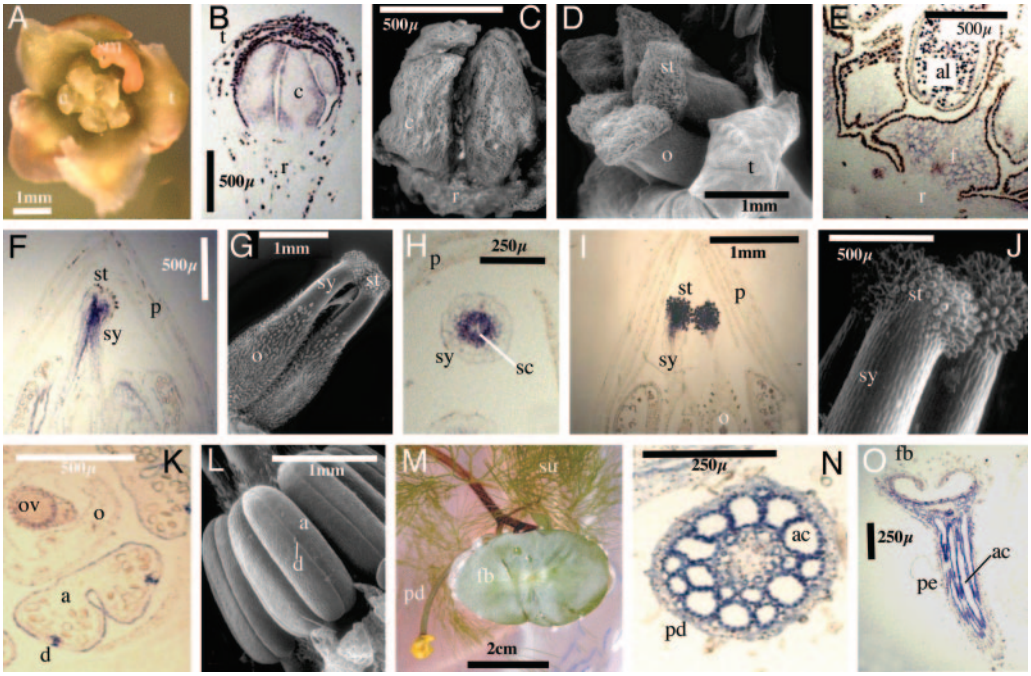

Fig. 4. Nonradioactive *in situ* hybridizations showing expression of *AmbCRC* in *A. trichopoda* and *CabTSL* in *C. aquatica*, with accompanying images. *In situ* hybridization signals appear blue or violet. Very dark material in tepals of *A. trichopoda* is natural coloration. All gene expression signals referred to were specific to antisense-strand riboprobes and were not observed by using negative control sense-strand riboprobes on serial sections from the same tissue blocks (results not presented). (*A*) A female flower of *A. trichopoda*. (*B*) *In situ* hybridization to a longitudinal section (l.s.) of an *A. trichopoda* female flower bud showing *AmbCRC* expression in the carpel wall. (*C*) SEM of a female *A. trichopoda* flower bud with the perianth removed (stage as for *B*). (*D*) SEM of a mature *A. trichopoda* female flower. (*E*) *In situ* hybridization to an l.s. of an *A. trichopoda* male flower bud showing *AmbCRC* expression in the stamen filaments. (*F*) *In situ* hybridization to an l.s. of a *C. aquatica* flower bud showing expression of *CabTSL* in the style and stigma. (*G*) SEM of the *C. aquatica* gynoecium (stage as for *F*). (*H*) *In situ* hybridization to a transverse section (t.s.) of a 3-mm-long *C. aquatica* flower bud showing expression of *CabTSL* in the style and stigma. (*I*) *In situ* hybridization to an l.s. of a 4-mm-long *C. aquatica* flower bud showing expression of *CabTSL* in the stigma. (*J*) SEM of the stigma and upper style of *C. aquatica* (stage as for *I*). (*K*) *In situ* hybridization to a t.s. of a *C. aquatica* flower bud showing expression of *CabTSL* in the anther wall. (*L*) SEM of a *C. aquatica* flower bud showing the stamens (stage as for *K*). (*M*) A flower and dimorphic leaves of *C. aquatica*. (*N*) *In situ* hybridization to a t.s. of a *C. aquatica* flower peduncle showing expression of *CabTSL* in cells surrounding air canals. (*O*) *In situ* hybridization to an l.s. of a *C. aquatica* floating leaf showing expression of *CabTSL* in cells surrounding air canals in the petiole. a, anther; ac, air canal; al, anther loculus; c, carpel; d, dehiscence zone; f, stamen filament; fb, floating leaf blade; o, ovary; ov, ovule; p, perianth; pd, peduncle; pe, petiole; r, receptacle; sc, stylar canal; sm, staminode; st, stigma; su, submerged leaves; sy, style; t, tepal.

expressed in flowers but not in leaves of *A. trichopoda*, similarly to its orthologue *CRC* in *Arabidopsis*. To precisely localize the expression of *AmbCRC* in flower tissues, *in situ* hybridizations were performed, shown in Fig. 4. Female flowers of *A. trichopoda* (Fig. 4*A*) typically contain a perianth of seven to eight tepals, five separate carpels, and one to two staminodes (sterile stamens) that may be relics of a bisexual ancestor (30). *In situ* hybridization indicated *AmbCRC* to be expressed specifically in the carpel wall at early to mid-developmental stages (Fig. 4 *B* and *C*). This expression seemed stronger toward the abaxial (outer) surface of the carpel wall, mirroring the expression of *CRC* in the *Arabidopsis* gynoecium (14). Later in female flower development, a wet, secretory stigma forms at each carpel apex (Fig. 4*D*). These wet stigmas may possibly provide a reward for pollinating insects (8), in addition to a receptive surface for pollen grains. By this stage, *AmbCRC* expression could no longer be detected (results not presented).

Virtual Northern hybridizations demonstrated *AmbCRC* to be expressed in both male and female flowers of *A. trichopoda* (Fig. 3). Male flower buds of *A. trichopoda* contain a perianth of 9–11 tepals that encloses 12–21 stamens. Each stamen consists of a four-loculate anther supported on a short, wide filament (30). Expression of *AmbCRC* in male flowers was localized by *in situ* hybridization to the stamen filaments (Fig. 4*E*). By contrast, *CRC* expression has not been shown in the stamen filaments of *Arabidopsis* (14), and *crc* mutants are not affected in stamen development (16).

***CabTSL* Is Expressed in *C. aquatica* Style and Stigma Tissues, Closely Resembling *TSL* Expression in *Arabidopsis*.** Northern blot hybridizations (Fig. 3) demonstrated expression of *CabTSL* in both inflorescence and vegetative tissues of *C. aquatica*, suggesting that *CabTSL*, like its *Arabidopsis* orthologue, plays roles in both reproductive and vegetative development. Flowers of *C. aquatica* typically contain three sepals, three petals, six stamens, and a gynoecium of three separate carpels. *In situ* hybridization to flower buds demonstrated strong *CabTSL* expression in style and stigma tissues of carpels at mid to late developmental stages. In buds of 3-mm length, *CabTSL* was strongly expressed in the internal cell layers of the style and stigma (Fig. 4 *F* and *G*). *CabTSL* was expressed in the cells surrounding a secretion-filled canal running longitudinally through the style (Fig. 4*H*). This feature is considered an important pleisiomorphic character in the angiosperms (8). In slightly later carpel development corresponding to flower buds of 4 mm length, *CabTSL* expression showed a reduction in the style and an increase in the stigma (Fig. 4*I*). This developmental stage correlated with a phase of rapid elongation of the stigma papillae (Fig. 4*J*). Expression of *TSL* in *Arabidopsis* has also been demonstrated in the style and stigma during later stages of flower bud development (20). Expression of *CabTSL* in gynoecium tissues therefore strongly resembles that of its *Arabidopsis* orthologue. In addition to its expression in female tissues, *in situ* hybridization revealed *CabTSL* expression in stamens. Expression of *CabTSL* was apparent in the outer epidermis of the anther wall in buds of

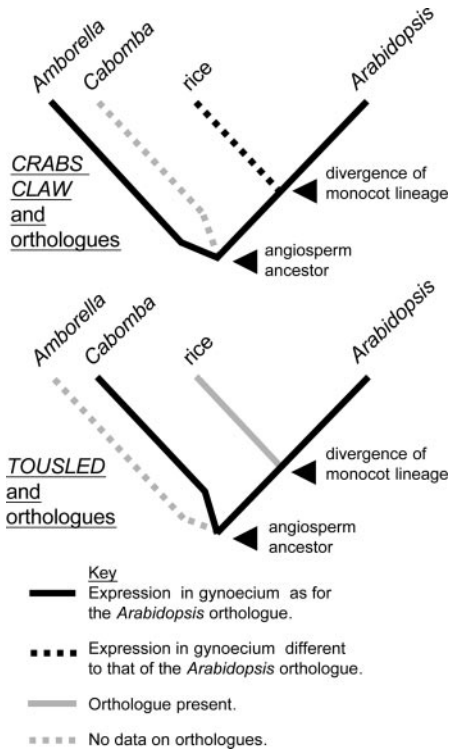

Fig. 5. Mapping of *CRC* and *TSL* expression in gynoecium tissues onto the phylogenetic tree of the angiosperms. The *Amborella* and *Cabomba* (Nymphaeales) lineages are placed in equally basal positions to reflect uncertainty in their order of branching (2).

2–3.5 mm in length, most strongly in the developing dehiscence zones (Fig. 4 K and 4L). By contrast, expression of *TSL* in *Arabidopsis* has not been reported in the anther epidermis.

***CabTSL* Is Highly Expressed in Tissues Specialized for Buoyancy in *C. aquatica*.** Flowers of *C. aquatica* are supported at the water's surface by specialized leaves that are round and flattened in shape, by comparison with the highly dissected, submerged leaves of this species (Fig. 3*M*). *In situ* hybridization detected strong expression of *CabTSL* in both the peduncles of flowers and the petioles of floating leaves. This expression was localized to single layers of parenchyma cells surrounding air canals that run the entire length of the peduncles and petioles and function to provide buoyancy to these structures (Fig. 4 *N* and *O*).

Discussion

***CRC* and *TSL* Play Roles in Gynoecium Development That Seem to Have Been Conserved Since the Common Ancestor of the Extant Angiosperms.** The present study demonstrates that putative orthologues of *CRC* and *TSL* are expressed in gynoecium tissues in one or other of the species *A. trichopoda* and *C. aquatica* in a near identical manner, both spacially and temporally, to the corresponding *Arabidopsis* genes. We argue such similar expression patterns in orthologous genes to be strongly indicative of the conservation of functions. *A. trichopoda* and *C. aquatica* represent the two earliest diverging groups of angiosperms (1, 2). The ancestor shared between these groups and the remaining lineage, containing *Arabidopsis*, may be regarded as the common ancestor of the living flowering plants. We therefore conclude that the ancestral sequences of *CRC* and *TSL* are likely to have played roles in carpel development in the ancestor of the extant angiosperms that are similar or identical to the present-day roles of these genes in *Arabidopsis*. We have mapped our expression data onto a phylogeny of some of the angiosperm species for which *CRC* and *TSL* orthologues are known, shown in Fig. 5. Our results fall short of proof of conservation of gene function for three main reasons. First, we cannot discount the possibility that similarity in gene expression may have resulted from the parallel recruitment of orthologous genes to similar roles after the evolutionary divergence of their respective plant lineages. Second, evidence of gene expression is not direct functional evidence. Such evidence will depend on technical advances that allow, for example, the disruption of gene expression by RNA interference (31) in basal angiosperms. Third, our conclusions are based on the hypothesis that ANITA angiosperms are basal in the flowering plant clade.

A recently published study (32) has shown a putative orthologue of *YABBY2 (YAB2)* to be expressed in adaxial, rather than abaxial, tissues of lateral organs in *A. trichopoda*. These authors postulate that a switch in polarity must have occurred for the *YAB2* orthologue in either the *Arabidopsis* or *A. trichopoda* lineages. The present study suggests that such a polarity switch has not taken place for at least one other pair of YABBY orthologues, *CRC*/*AmbCRC*.

The Role of *DL* in Rice Was Acquired After the Divergence of the Monocot Lineage. *DL*, the *CRC* orthologue from rice, has been shown to specify carpel identity and controls leaf development, unlike *CRC* in *Arabidopsis*. The expression pattern of *DL* in rice flowers is consistent with its role in determining carpel identity and does not resemble the expression patterns of *CRC* in *Arabidopsis* (14) and *AmbCRC* in *A. trichopoda*. Our results, combined with those of Yamaguchi *et al.* (17), suggest that the novel functions and expression pattern of *DL* in rice were acquired after the divergence of the monocot lineage, as shown in Fig. 5. This and other changes in gene function may have been important for the evolution of the highly specialized flowers and leaves of monocots.

The Role of *CRC* in the Development of Secretory Tissues Was Acquired Later Than Its Role in Gynoecium Development. *CRC* controls nectary development in *Arabidopsis*, in addition to its role in gynoecium development. Specialized nectaries are absent from the flowers of ANITA grade angiosperms (8), suggesting that these organs evolved after the separation of the ANITA clades from the remaining lineage. In female *A. trichopoda* flowers, the stigmas are highly secretory, although *AmbCRC* expression was not detected in these tissues. Studies within later-diverging angiosperm groups may indicate the level at which the ancestor of *CRC* was recruited to a role in the development of floral secretory tissues.

***CabTSL* May Play a Role in the Development of Structures Specialized for Buoyancy That Is More Recent Than Its Role in Gynoecium Development.** We have shown that *CabTSL* is highly expressed in cells surrounding the air canals in petioles and peduncles that function to carry floating leaves and flowers of *C. aquatica* to the water's surface. Fossil evidence indicates that an aquatic growth habit has been present in the Nymphaeales for at least 115 million years (33). Feild *et al.* (34) have argued from a comparison of basal angiosperm groups, however, that the ancestor of the extant angiosperms was a woody, terrestrial species. If this interpretation is correct, the potential role of *CabTSL* in developmental specialization for buoyancy may have been acquired subsequently to its role in gynoecium development. It will be interesting to discover whether *TSL* orthologues from other species of the Nymphaeales are expressed in buoyancy-related tissues.

What Can *CRC* and *TSL* Tell Us About the Evolution of the Carpel? Traditionally, the carpel has been thought to have evolved by the closure of a female organ, homologous to the ovule-bearing scales of gymnosperms. This closure may have proceeded by means of a partially closed cupule, such as those known from *Caytonia* and several other fossil gymnosperms whose relationships to the angiosperms are uncertain (35). Conversely, the more recent "mostly male theory" (36) proposes that the carpel evolved by the closure of a male organ, the microsporophyll, around ovules that had developed ectopically. We have shown that the ancestral sequences of *CRC* and *TSL* are likely to have controlled carpel development in the ancestor of the extant angiosperms. It will now be interesting to discover whether orthologues of these genes are also expressed in the reproductive structures of the angiosperms' nearest living relatives, the gymnosperms. If orthologues of *CRC* and *TSL* are not expressed in gymnosperm reproductive structures, it may be that these genes were newly recruited to carpel development in the angiosperm lineage and may therefore have played important roles in carpel evolution. If, by contrast, we find that orthologues of *CRC* and *TSL* are expressed in gymnosperm reproductive structures, it will be interesting to know whether their precise expression patterns support a male, or a female, origin for the carpel.

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