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REVIEW ARTICLE

An evolutionary perspective on the regulation of carpel development

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Abstract

The carpel, or female reproductive organ enclosing the ovules, is one of the major evolutionary innovations of the flowering plants. The control of carpel development has been intensively studied in the model eudicot species *Arabidopsis thaliana*. This review traces the evolutionary history of genes involved in carpel development by surveying orthologous genes in taxa whose lineages separated from that of *A. thaliana* at different levels of the phylogenetic tree of the seed plants. Some aspects of the control of female reproductive development are conserved between the flowering plants and their sister group, the gymnosperms, indicating the presence of these in the common ancestor of the extant seeds plants, some 300 million years ago. Gene duplications that took place in the pre-angiosperm lineage, before the evolution of the first flowering plants, provided novel gene clades of potential importance for the origin of the carpel. Subsequent to the appearance of the first flowering plants, further gene duplications have led to sub-functionalization events, in which pre-existing reproductive functions were shared between paralogous gene clades. In some cases, fluidity in gene function is evident, leading to similar functions in carpel development being controlled by non-orthologous genes in different taxa. In other cases, gene duplication events have created sequences that evolved novel functions by the process of neo-functionalization, thereby generating biodiversity in carpel and fruit structures.

Key words: Angiosperms, carpel, development, evolution, flower, flowering-plants, gynoecium, pistil.

The big cover up

In the gymnosperms, the most ancient group of living seed plants, ovules most frequently occur as naked structures that develop in the axils of leaf-like organs. By contrast, in the more recently evolved flowering plants or angiosperms, the ovules are enclosed and protected by a specialized female reproductive organ termed the carpel. Besides protecting the ovules, the carpel confers numerous further advantages on the flowering plants. Stigma tissues at the carpel apex are adapted in different species for the efficient capture of pollen carried by vectors including insects, mammals, birds, and the wind. In addition, the carpel provides a location for selective mechanisms that operate on pollen, such as self-incompatibility, which promotes out-breeding. Following pollination, compatible pollen tubes are guided with meticulous accuracy through the tissues of the carpel, specifically toward unfertilized ovules. After fertilization, the carpel tissues undergo further developmental changes to become the fruit, which protects the developing seeds and later contributes to the dissemination of these by a wide variety of mechanisms in different species. For all of these reasons, the carpel was undoubtedly a major factor in the evolutionary success of the angiosperms, which diversified from a common ancestor that is estimated to have lived in the Late Jurassic period, around 160 million years ago (MYA), to form approximately 300 000 species alive today.

The molecular control of carpel development has been investigated in several model species, although most thoroughly in *Arabidopsis thaliana* of the Brassicaceae. In parallel, molecular phylogenetic studies have now clarified the evolutionary relationships between the major groups of seed plants (Fig. 1), as reviewed by Kuzoff and Gasser (2000). The combination of developmental and

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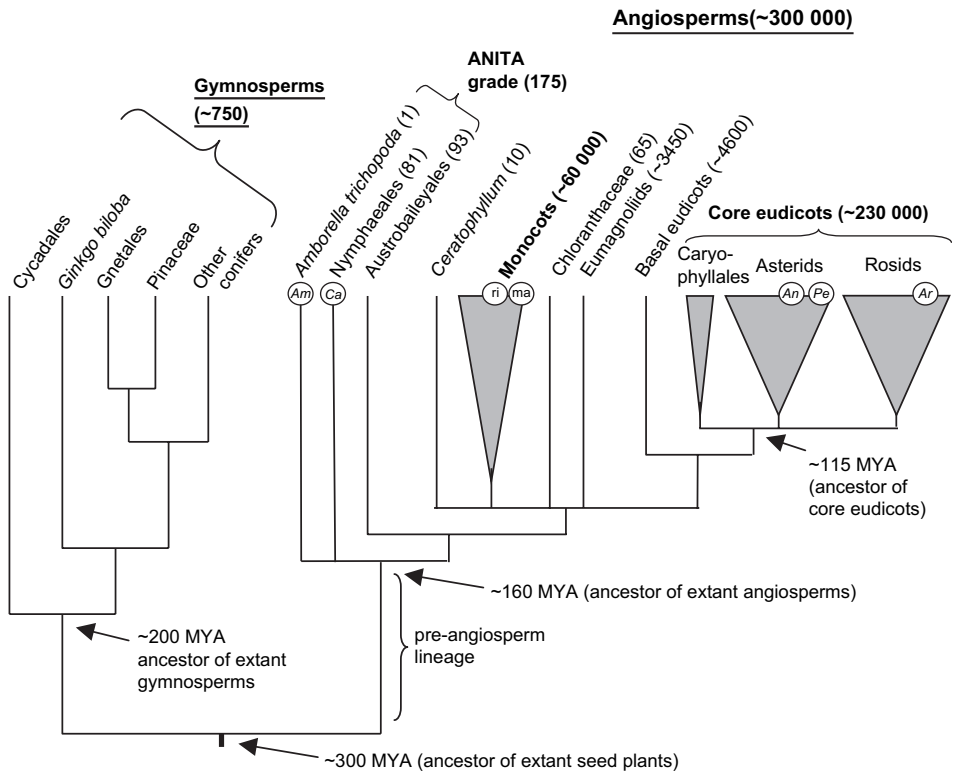

Fig. 1. The phylogeny of the seed plants, based on a consensus of molecular phylogenetic studies. The numbers of species in major clades are given in parentheses, while approximate dates of divergence are taken from Davies *et al.* (2004), based on a calibration of the molecular clock using fossil data. Very large clades are represented by shaded triangles. The positions of certain species referred to in the text are indicated as follows: *Am*, *Amborella trichopoda*; *An*, *Antirrhinum majus*; *Ar*, *Arabidopsis thaliana*; *Ca*, *Cabomba aquatica*; ma, maize; *Pe*, *Petunia hybrida*; ri, rice.

phylogenetic information provides a starting point to unravel the evolution of carpel development from the pre-angiosperm lineage through to present day model species such as *A. thaliana*. In addition, the comparison of carpel development mechanisms in different extant angiosperm groups should allow the identification of the molecular differences that underlie the diversity of carpel and fruit morphology throughout the flowering plants.

Before the carpel

The extant gymnosperms have been shown by molecular phylogenetic analyses to form a monophyletic group in a sister position to the angiosperms (Fig. 1). By the comparative analysis of reproductive development in gymnosperms and angiosperms, something may be deduced of the molecular mechanisms of female development that existed before the carpel. The ABC model for the development of a typical angiosperm flower (Coen and Meyerowitz, 1991), postulates a 'C-function' to specify carpel development in the fourth floral whorl (Fig. 2a). This model further postulates the combination of C-function activity with that of a 'B-function' to specify stamen development in the third whorl. The genes encoding the B- and C-functions have been identified from several model angiosperms and found to encode MADS box transcription factors of the Type II MIKC class (Parenicova *et al.*, 2003). Analyses of taxa from the major gymnosperm groups: Pinaceae (Tandre *et al.*, 1995), Gnetales (Becker *et al.*, 2000), Ginkgoales (Jager *et al.*, 2003), and Cycadales (Zhang *et al.*, 2004), clearly indicate the presence of both B- and C-function orthologues in gymnosperms. Male and female reproductive structures in gymnosperms develop on separate reproductive axes (cones etc), or even on separate individuals. C-function orthologues are expressed in both male and female reproductive axes in gymnosperms, whereas B-function orthologues are male-specific, mirroring the organ-specific expression of the equivalent B- and C-function genes in angiosperms (Fig. 2a, b). In addition, coding sequences of B- and C-function genes from gymnosperms show activities similar to those of the equivalent *A. thaliana* genes in transgenic *A. thaliana* (Tandre *et al.*, 1998; Winter *et al.*, 2002; Zhang *et al.*, 2004). It therefore appears that the last common ancestor of the extant seed plants, living some 300 MYA, possessed a C-function-like gene that played a role in the development of both male and female reproductive organs. The differentiation between the sexes in that ancestral seed plant would have depended on the male-specific expression of a B-function-like gene.

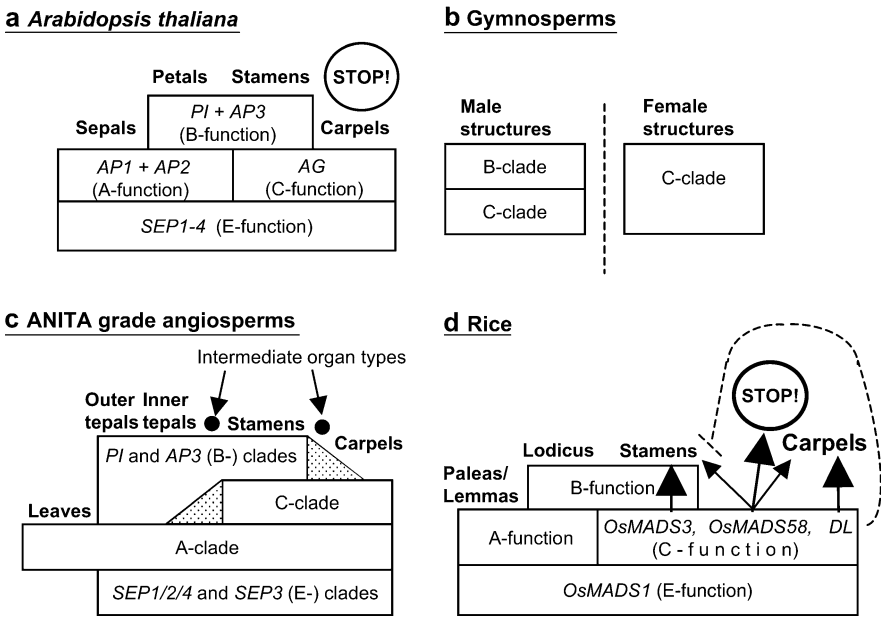

Fig. 2. The ABCE model of flower development in *A. thaliana*, and its derivatives in other taxa. (a) In *A. thaliana*, A-, B-, C-, and E-function floral homeotic genes, expressed in overlapping domains (horizontal bars) of the floral meristem, control the identities of floral organs in a combinatorial manner: A+E specifies sepal development in the first whorl, A+B+E specifies petal development in the second whorl, B+C+E specifies stamen development in the third whorl, and C+E specifies carpel development in the fourth whorl. In addition, the C-function causes an arrest of organ proliferation (the 'STOP' function) in the fourth whorl. (*AG*, *AGAMOUS*; *AP1*, *APETALA1*; *AP2*, *APETALA2*; *AP3*, *APETALA3*; *PI*, *PISTILLATA*; *SEP1–4*, *SEPALLATA1–4*.) (b) In gymnosperms, B- and C-clade MADS box genes are expressed in a combinatorial manner in male (B+C) and female (C alone) reproductive structures, resembling the expression of their *A. thaliana* orthologues in male and female floral organs. (c) In ANITA grade angiosperms, B- and C-clade MADS box gene expression resembles that of the respective *A. thaliana* orthologues, although with less well-defined boundaries (dotted areas). Strong B-clade gene expression is generally detected in the outer floral whorl of ANITA angiosperms, possibly reflecting an absence of developmental differentiation between whorls 1 and 2. A-clade MADS box gene expression differs radically between ANITA angiosperms and *A. thaliana*, extending throughout the flower and into leaves. (d) In rice flowers, typifying the Poaceae of the monocot clade, A-, B-, and E-function genes are expressed in similar patterns to those of their *A. thaliana* orthologues to specify specialized perianth organs (paleas, lemmas, and lodicules) and stamens. Two paralogous C-clade MADS box genes show a partial sub-functionalization between the third and fourth whorls, with one paralogue playing a major role in stamen development in the third whorl, while the other plays a major role in the 'stop' function in the fourth whorl (thick arrows, major roles; thin arrows, minor roles). The YABBY gene *DROOPING LEAF* (*DL*) plays a major role in carpel specification that is independent of C-clade MADS box gene expression. *DL* may act directly on carpel development (solid arrow), or indirectly by limiting the inner boundary of B-function gene expression (dashed arrow), or both of these.

In *A. thaliana*, B- and C-function genes have been shown to function together with a further class of MADS box genes encoding an 'E-function', thereby extending the ABC model to an ABCE model (Pelaz *et al.*, 2000; Honma and Goto, 2001). Accordingly, carpel development in *A. thaliana* requires a combination of activities of the C-function gene, *AGAMOUS* (*AG*), with that of the E-function, which is encoded by four genes, termed *SEPALLATA1–4* (*SEP1–4*), with extensively overlapping functions (Pelaz *et al.*, 2000; Ditta *et al.*, 2004). C- and E-function proteins are thought to act as hetero-tetramers (Theissen and Saedler, 2001) to control the transcription of their downstream target genes (Ito *et al.*, 2004; Gomez-Mena *et al.*, 2005) and thereby bring about carpel development.

E-clade genes have not been found in gymnosperms, but the closely related *AGAMOUS-LIKE6* (*AGL6*) MADS box clade is present in both angiosperms and gymnosperms (Carlsbecker *et al.*, 2004). These data suggest that a gene duplication event, generating the ancestors of the *AGL6* and *SEP* (E-clade) genes, occurred prior to the separation of the pre-angiosperm and gymnosperm lineages around 300 MYA (Becker and Theissen, 2003; Zahn *et al.*, 2005). If the E-clade did predate the ancestor of the extant seed plants as proposed, the flower (including the carpel) would not have evolved as a direct result of the origin of E-clade genes. However, the crucial mechanistic importance of the E-function for flower development in extant angiosperms implies that the recruitment, at least, of E-clade genes to these functions may have played a central role in the origin of this structure.

The extant angiosperms and gymnosperms both possess MADS box genes of a paralogous clade to the B-clade, termed B-sister genes (Becker *et al.*, 2002). Unlike the male-expressed B-function, B-sister genes seem to be expressed in female reproductive tissues and this characteristic is conserved between angiosperms and gymnosperms. The unique *A. thaliana* B-sister gene, *TRANSPARENT TESTA16*, plays a role in the pigmentation of the outer ovule integument (Nesi *et al.*, 2002), although it has been hypothesized that the widespread conservation of the

B-sister lineage is evidence of a more important ancestral role, probably in ovule development (Kaufmann *et al.*, 2005).

Theories for carpel origin

Carpels, along with the other principal floral organs, have for long been postulated to be modified from a leaf ground plan. Relatively recent experimental evidence supports this view: floral organs are converted to leaves in plants in which all of the A, B and C function genes (Coen and Meyerowitz, 1991), or the redundant E-function genes (Pelaz *et al.*, 2000), are inactivated. In addition, the ectopic expression of combinations of A, B or C with *SEP* (E-function) genes will convert leaves into floral organs (Honma and Goto, 2001).

Although the carpel appears to be a modified leaf, it may be more directly related to sporophylls, or leaves that carry spore-producing organs. As the carpel is female, it has traditionally been regarded as derived from megasporophylls that would have subtended ovules in the pre-angiosperm lineage. Accordingly, the carpel would be directly homologous to such gymnosperm organs as the female cone scales of conifers. A recent molecular explanation for the origin of the bisexual axis in the flowering plants, termed the Out-of-Male/Out-of-Female Theory (Theissen and Becker, 2004), is broadly consistent with this view of a female origin for the carpel. This theory proposes a pair of alternative mechanisms, based on the movement of a frontier of B-function gene expression in either a basipetal or acropetal direction along male or female reproductive axes, respectively, in the pre-angiosperm lineage. As a result, the axis affected is proposed to have become bisexual, with female organs at its tip and male organs at its base. Carpels would then have evolved by the closure of megasporophylls in the apical region of the bisexual axis.

Conversely, the 'Mostly Male Theory' (Frohlich and Parker, 2000; Frohlich, 2003) proposes the carpel to have been derived by the closure of (male) microsporophylls, around ovules that had developed ectopically on these. According to this view, all or most of the female-specific developmental pathways in the pre-angiosperm lineage, other than those required for ovule development, were lost during the evolution of the first angiosperms. One gene that was apparently lost prior to the radiation of the angiosperms is called *NEEDLY* (*NLY*). *NLY* is a gymnosperm-specific paralogue of *LEAFY* (*LFY*), which itself is present in all seed plants and is known to regulate positively B- and C-function genes in *A. thaliana*. Early studies suggested that *NLY* may specifically control female developmental programmes in gymnosperms, providing support for the Mostly Male Theory (Mouradov *et al.*, 1998). However, the sex-specific expression of *LFY* and *NLY* does not appear to be general in the gymnosperms (Carlsbecker *et al.*, 2004; Dornelas and Rodriguez, 2005). Although *LFY* and *NLY* may prove to be of lesser importance for the Mostly-Male Theory than was originally thought, it is possible that a systematic analysis of gene orthology and expression data between angiosperms and gymnosperms will provide other genes that could be used to test this and other theories that seek to explain the origin of the flower and carpel.

The ancestral carpel

Molecular phylogenetic analyses have clearly identified the first diverging lineages within the angiosperm clade (Mathews and Donoghue, 1999; Parkinson *et al.*, 1999; Qiu *et al.*, 1999; Soltis *et al.*, 1999; Barkman *et al.*, 2000). These are grouped into only three extant orders, Amborellales, Nymphaeales, and Austrobaileyales, collectively termed the ANITA grade. Amborellales contains the single species *Amborella trichopoda*, a small tree endemic to the tropical island of New Caledonia in the Southern Pacific. Nymphaeales is a cosmopolitan order containing two families of aquatic plants. Austrobaileyales contains four families, representing a mixture of endemic and more widely distributed groups. There is very good evidence that Amborellales and Nymphaeales diverged from the remaining angiosperm lineage before the divergence of Austrobaileyales (Aoki *et al.*, 2004; Stellari *et al.*, 2004). However, the relative order of divergence of the two most basal lineages, Amborellales and Nymphaeales, remains unclear. Most recent molecular phylogenies place Amborellales alone in the most basal position (Zanis *et al.*, 2002), while others group it together with Nymphaeales in a first-diverging clade (Qiu *et al.*, 2001).

Comparison of the features of ANITA angiosperms has enabled several important conclusions to be made on the likely state of the flower and carpel in the angiosperms' ancestor (Endress and Igersheim, 2000; Endress, 2001). According to these studies, the flowers of the ancestral angiosperm were probably small, bisexual, and protogynous. Its carpels were likely to have been simple (apocarpic) and incompletely closed by cellular structures, instead being sealed by substances secreted from the carpel margins. The stigmas of the angiosperms' ancestor were probably covered in muticellular protrusions and secretory. Its carpels are likely to have contained single ovules, which would probably have shown anatropous placentation, been covered by two integuments and possessed a large (crassinucellar) nucellus. It is furthermore likely that the embryo sac in the ancestral ovule was four-celled, rather than seven-celled as in most extant angiosperms (Williams and Friedman, 2002, 2004). Double fertilization would have been present in the ancestor of the angiosperms as in extant groups, leading to the production of an embryo and a biparental endosperm. However, this endosperm was most probably diploid, rather than triploid as in later-diverging groups (Williams and Friedman, 2002, 2004). Self-incompatibility (SI) systems operating between female

tissues and pollen grains are present in some ANITA angiosperms, including *Austrobaileya scandens* (Prakash and Alexander, 1984) and *Trimenia moorei* (Bernhardt *et al.*, 2003). However, it is uncertain whether homologous SI systems are to be found in any two lineages that separated at an early stage in angiosperm evolution, leaving open the question of SI as an ancestral trait in the angiosperms.

Using molecular techniques to compare ANITA angiosperms with model plants, the mechanisms likely to have controlled carpel development in the ancestral angiosperm can now be analysed. Phylogenetic analyses of the MADS box family in ANITA angiosperms and gymnosperms clearly indicate that duplication events took place in at least three MADS box lineages, the B-, C- and E-function lineages, prior to the common ancestor of the living flowering plants. These duplications may have been caused by a large-scale genomic duplication in the pre-angiosperm lineage, evidence of which is present in the *A. thaliana* genome, as reviewed by De Bodt *et al.* (2005). The pre-angiosperm C-function duplication generated two clades, respectively containing the clade-defining genes *AG* from *A. thaliana*, and *FLORAL BINDING PROTEIN7* and *11* (*FBP7/11*) from *Petunia hybrida* (reviewed by Kramer *et al.*, 2004). The *AG* clade contains angiosperm C-function genes, while the *FBP7* clade contains genes involved in ovule development in both *P. hybrida* and *A. thaliana*. The role of *FBP7*-like genes in ovule development has been defined as a new floral genetic function, the D-function (Angenent *et al.*, 1995; Colombo *et al.*, 1995), although it is not clear how widely the D-function concept applies within the flowering plants. The *FBP7* clade may have been lost from some angiosperm groups, including the Ranunculales of the basal eudicots (Kramer *et al.*, 2004).

A further duplication occurred in the ancestral E-function gene to generate two distinct E-function sub-clades in the pre-angiosperm lineage. *SEP1*, *SEP2*, and *SEP4* from *A. thaliana* appear to be descended from one of the paralogues generated by this ancient duplication, while *SEP3* appears to be descended from the other (Zahn *et al.*, 2005). As these two *SEP* sub-clades play largely redundant roles in *A. thaliana*, the functional significance of the proposed pre-angiosperm E-function duplication is not yet entirely clear.

The expression patterns of C- and E-function genes in basal angiosperms have recently been analysed (Kim *et al.*, 2005), as summarized in Fig. 2c. Expression of C-function genes is mostly limited to the third and fourth floral whorls in ANITA taxa, while E-function genes are expressed in all floral organs. These expression patterns closely resemble those of C- and E-function genes in *A. thaliana*, suggesting that important elements of the control of carpel identity may have been conserved throughout angiosperm evolution. Despite the apparent conservation of C-function expression, Kim *et al.* (2005) noted some expression of C-function genes in the perianth organs of two ANITA taxa, *Amborella* (Amborellales) and *Illicium* (Austrobaileyales). However, this observation may be related to the rather gradual transition of floral organ types that is frequently apparent in ANITA angiosperms, with intermediate forms of floral organs present at whorl boundaries (Kim *et al.*, 2005; Fig. 2c).

In addition to MADS box floral homeotic genes, the expression patterns of two further carpel development genes have recently been analysed in basal angiosperms. One of these, *CRABS CLAW* (*CRC*), encodes a transcription factor of the YABBY class. YABBY genes play roles in the specification of abaxial cellular identity of plant lateral organs by defining the side of these organs that faces away from the developmental axis (Bowman, 2000). *CRC* is expressed in the abaxial tissues of the *A. thaliana* gynoecium and in nectaries (Bowman and Smyth, 1999). A putative orthologue from the ANITA angiosperm *Amborella trichopoda* shows a similar pattern of expression in carpels to that of *CRC* from *A. thaliana* (Fourquin *et al.*, 2005), suggesting these two genes to have conserved a common developmental role since the speciation event that separated their lineages at the base of the flowering plants. Similarly, *TOUSLED* (*TSL*), encoding a serine-threonine protein kinase, shows conserved expression patterns between *A. thaliana* and the ANITA angiosperm *Cabomba aquatica* (Nymphaeales, Cabombaceae). *TSL* is necessary for normal development of the carpel apex in *A. thaliana* and shows a peak of expression in that tissue (Roe *et al.*, 1997). The orthologue of *TSL* from *C. aquatica* is also expressed at a high level in the carpel apex (Fourquin *et al.*, 2005), suggesting a conservation of function since the common ancestor of the flowering plants.

The control of carpel identity in monocots

The monocots form a monophyletic group of angiosperms whose lineage diverged later those of the ANITA grade, perhaps around 145 MYA (Davies *et al.*, 2004; Fig. 1). This group has undergone considerable evolutionary divergence to form over 60 000 extant species. Genes controlling floral organ identity have been analysed principally in two monocot models, rice and maize, both from the Poaceae or grass family. Phylogenetic analyses suggest at least one major gene duplication event to have occurred in the MADS box C-clade prior to the separation of the rice and maize lineages, with an additional subsequent duplication in one of the two sub-clades generated, specifically in the maize lineage. Accordingly, the rice C-clade gene *OsMADS58* appears orthologous to the maize gene *ZAG1*, while *OsMADS3* from rice is putatively orthologous to the two paralogous maize genes, *ZMM2* and *ZMM23* (Mena *et al.*, 1996; Yamaguchi *et al.*, 2005).

Phenotypes associated with mutations in C-clade genes have been investigated in both rice and maize, although more thoroughly in the former of these species. The

inactivation of *OsMADS58* in rice leads to defects in, though does not eliminate, carpel development (Yamaguchi *et al.*, 2005; Fig. 2d). In addition, *osmads58* mutants show reduced floral determinacy, indicating a major contribution of this gene to the 'stop' function. The inactivation of *OsMADS3* eliminates stamen development, but has little or no effect on either carpel development or flower determinacy (Kang *et al.*, 1998; Yamaguchi *et al.*, 2005). Rice plants in which both *OsMADS3* and *OsMADS58* have been inactivated produce aberrant carpels, similar to those of *osmads58* single mutants, indicating *OsMADS3* to make no significant contribution to carpel development (Yamaguchi *et al.*, 2005). In maize, *zag1* mutants show a defect in floral determinacy, indicating functional conservation of *ZAG1* with its rice orthologoue *OsMADS58*. In addition, further genes that have yet to be identified are also required for female flower determinacy in maize (Laudencia-Chingcuanco and Hake, 2002).

Data from rice and maize therefore indicate the past occurrence of sub-functionalization events between two C-function gene clades in the monocots. By comparison with *A. thaliana*, a partial separation of male- and female-acting components of the C-function is apparent in grasses, with one sub-clade acting principally in stamens and the other in the fourth floral whorl to arrest organ proliferation. Interestingly, the persistence of carpel development in rice plants that lack any active C-clade MADS box genes indicates a potentially important difference in the mechanism of carpel specification between grasses and *A. thaliana*.

In contrast to the effect of inactivating C-clade MADS box genes, carpels are entirely replaced by ectopic stamens in rice plants in which the YABBY family gene *DROOPING LEAF* (*DL*) has been inactivated (Yamaguchi *et al.*, 2004; Fig. 2d). DL is also required for normal leaf development. *DL* expression has been shown to be maintained in the carpels of rice plants in which both *OsMADS3* and *OsMAD58* have been inactivated (Yamaguchi *et al.*, 2005), demonstrating its action to be independent of these. It is not yet clear whether carpel development depends on *DL* expression *per se*, or whether *DL* is mainly responsible for preventing B-function gene expression in the fourth whorl. Experiments that combine B-clade, C-clade, and *dl* mutations in rice will be needed to evaluate the relative contributions of MADS box genes and *DL* to the specification of carpel identity. *DL* appears to be orthologues to *CRC* from *A. thaliana*. The conservation of expression patterns of *CRC* orthologues between *A. thaliana* and very basal angiosperms (Fourquin *et al.*, 2005), as discussed above, suggests that the distinct roles of *DL* in carpel identity and leaf development (Yamaguchi *et al.*, 2004) arose specifically in the monocot lineage.

SEP-like genes, necessary for carpel development in eudicots, are also known from monocots. *OsMADS1* from rice, corresponding to the *LEAFY HULL STERILE1* locus, groups within the same clade as *SEP1*, *2*, and *4* from *A. thaliana* (Zahn *et al.*, 2005). Outer whorl floral organs in *osmads1* loss-of-function mutants take on a leaf-like appearance, whereas inner whorl floral organs are partially converted to paleas and lemmas, which are normally found in the first whorl of rice flowers (Agrawal *et al.*, 2005). These results suggest *OsMADS1* to be a principal component of the E-function in rice (Fig. 2d), while the functions of four other rice *SEP* clade genes, *OsMADS5*, *OsMADS7*, *OsMADS8*, and *RMADS217* (Zahn *et al.*, 2005), remain to be fully investigated.

Gene duplication and carpel evolution in the core eudicots

The core eudicots form a monophyletic group that is estimated to have diverged from the more basal lineages of eudicots around 110 MYA (Davies *et al.*, 2004; Fig. 1). The core eudicot clade includes all of the well-known dicot model taxa such as *Arabidopsis*, *Petunia*, and *Antirrhinum*. Analysis of the *A. thaliana* complete genome sequence has provided evidence of a large-scale duplication event that may have occurred at around the time of the ancestor of the core eudicots (De Bodt *et al.*, 2005). Evidence of this duplication can also be found in the MADS box families present in extant taxa. The comparison of diverse core eudicot groups has provided an excellent opportunity to study evolutionary events such as sub-functionalization and neo-functionalization (Moore and Purugganan, 2005), several of which are evident in eudicot genes controlling carpel, fruit, and ovule development and floral determinacy.

In the core eudicots, two gene lineages are present in place of an ancestral C-function lineage whose single descendant is present in basal eudicots. In *A. thaliana*, one of the novel lineages, the *AG* lineage, contains the *AG* gene itself, while the other, the *PLENA* (*PLE*) lineage (Fig. 3), contains a pair of paralogous genes termed *SHATTERPROOF1* and *2* (*SHP1/2*). In *Antirrhinum majus*, the probable orthologue of *AG* is termed *FARINELLI* (*FAR*), while that of *SHP1/2* is the clade-defining gene *PLENA* (*PLE*). Interestingly, the non-orthologous genes *AG* and *PLE* are responsible for specifying the C-function in *A. thaliana* and *A. majus*, respectively (Davies *et al.*, 1999; Kramer *et al.*, 2004; Fig. 3). *FAR*, by contrast, is redundantly involved in stamen development and is also required for pollen fertility in *A. majus*, while *SHP1* and *SHP2* redundantly play a novel role in *A. thaliana* fruit development (Liljegren *et al.*, 2000). In *Petunia hybrida*, which is more closely related to *Antirrhinum* than to *Arabidopsis* (Fig. 1), a further case of sub-functionalization is apparent, where the *AG* orthologue *PMADS3* is principally responsible for stamen development (Kapoor *et al.*, 2002), but probably also plays redundant roles with the *PLE* orthologue *FLORAL BINDING PROTEIN6* (*FBP6*) in both carpel development and floral determinacy (Kramer *et al.*, 2004).

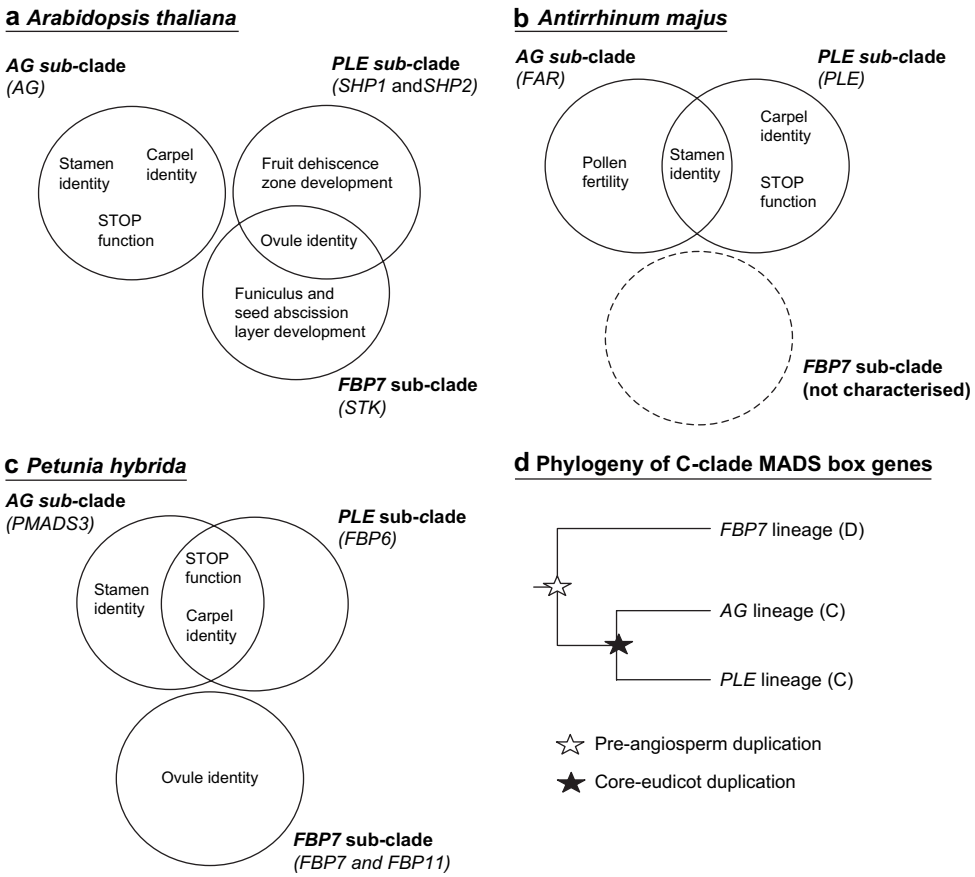

Fig. 3. Fluidity in the functionalization of C/D-clade MADS box genes in the core eudicots. (a–c) Venn diagrams representing the known functions of three C/D-function MADS box sub-clades (*AG*, *PLE*, and *FBP7*) in wild-type plants of three species from the core eudicots. Overlapping regions represent functional redundancy between genes from different sub-clades in wild-type genetic backgrounds (*AG*, *AGAMOUS*; *FAR*, *FARINELLI*; *FBP*, *FLORAL BINDING PROTEIN*; *PLE*, *PLENA*; *SHP*, *SHATTERPROOF*). (d) The phylogeny of the eudicot C/D-MADS box gene clade.

Although sub-functionalization between the paralogous *AG* and *PLE* clades in *A. thaliana* (respectively represented by the genes *AG* and *SHP1/2*) has left *AG* playing the major C-function role, elegant experiments involving multiple mutants show that the *SHP* genes have retained a capacity for C-function activity. Ectopic carpelloid organs may develop in the first floral whorl of plants lacking an active *AG* gene if the *APETALA2* (*AP2*) A-function gene is additionally inactivated (Bowman *et al.*, 1991). This effect is thought to occur because *AP2* is responsible for down-regulating C-clade genes in the outer floral whorls of wild-type plants. In the case of *ag/ap2* double mutants, the C-function activity responsible for specifying ectopic carpel development in the first whorl is provided by *SHP1* and *SHP2*, evidenced by the fact that first whorl organs of *ap2/ag/shp1/shp2* quadruple mutants are devoid of carpelloid features (Pinyopich *et al.*, 2003). These data indicate a subtle effect of functional overlap between paralogous gene clades that does not equate to simple genetic redundancy.

The fluidity of functions among duplicated genes is further illustrated by an exchange of function between C- and D-clade MADS box genes in the eudicots. Two paralogous D-function genes in *P. hybrida*, *FBP7* and *FBP11*, are redundantly essential for ovule development (Angenent *et al.*, 1995). The probable *A. thaliana* orthologue of these two genes, *SEEDSTICK* (*STK*), is also involved in ovule development, but in this case the redundancy relationship extends beyond the D-clade to include the genes *SHP1* and *SHP2* of the *PLE* sub-clade (Fig. 3). Accordingly, the *fpb7/fpb11* double mutant of *P. hybrida* (Angenent *et al.*, 1995) is phenotypically similar to the *stk/shp1/shp2* triple mutant of *A. thaliana* (Pinyopich *et al.*, 2003). Both of these mutants possess supernumerary carpels in the place of ovules within the gynoecium. In addition to its redundant role in ovule specification, *STK* plays non-redundant roles in the development of the funiculus and in seed abscission in *A. thaliana* (Pinyopich *et al.*, 2003). The C/D-function gene clade in the eudicots therefore represents a complex situation, where evolutionary processes including sub-functionalization, exchanges of function between paralogous genes, exchanges of function between non-paralogous genes, and neo-functionalization, have all taken place (Fig. 3).

The A-function gets into carpel development

A further likely consequence of the hypothesized genome duplication at the base of the core eudicots was the generation of a second sub-clade of MADS box genes within the A-function clade (Litt and Irish, 2003). The A-function MADS box gene *APETALA1* (*AP1*) plays roles in floral meristem patterning and the specification of perianth (petal and sepal) organ identity in *A. thaliana*. This latter role corresponds to the A-function, as defined by the ABCE model. However, gene (or genome) duplication in the core eudicots has provided further A-clade sequences, one of which appears to have been recruited to carpel and fruit development somewhere along the *A. thaliana* lineage. The A-clade gene *FRUITFULL* (*FUL*) is involved in the patterning of the gynoecium and fruit wall in *A. thaliana* (Gu *et al.*, 1998). *FUL* is known to act in a network involving a large number of genes (Roeder *et al.*, 2003; Liljegren *et al.*, 2004), including the MADS box genes *SHP1* and *2* (Ferrandiz *et al.*, 2000) that also function redundantly with *STK* in ovule development. Gene duplication in the A-function clade of MADS box genes, possibly caused by a whole genome duplication event, has thus resulted in novel fruit shattering mechanisms in the Brassicaceae by the process of neo-functionalization.

An interesting feature of gene-duplication in the A-clade is the evolution of a distinct C-terminal protein motif in the *AP1* sub-clade, apparently produced by a frame-shift mutation that occurred towards the 3′-extremity of the coding sequence (Litt and Irish, 2003). This frame-shift created a farnesylation site that is known to be post-translationally modified *in vivo* in *A. thaliana* and which is required for wild-type *AP1* protein activity (Yalovsky *et al.*, 2000). Other frame-shift mutations in duplicated genes are present in the B- and C-function MADS box clades of the eudicots (Vandenbussche *et al.*, 2003). However, the conserved motifs generated in these cases are distinct from that of the *AP1* lineage and do not contain farnesylation sites. The novel C-terminal motifs present in certain lineages within the eudicot A, B, and C MADS box clades have been conserved over a very long period, clearly indicating their functional significance. However, it is not yet known whether the functions of these novel motifs are connected with biochemical processes in common, such as the higher-order assembly or sequestration of MADS box transcription factor complexes (Vandenbussche *et al.*, 2003).

The carpel of the future

Many of the key questions of carpel evolution remain to be answered. For example, it is not known to which organ in gymnosperms the carpel is homologous. The mechanism of carpel closure and the potentially diverse mechanisms of fusion between carpels have yet to be discovered in the more highly evolved syncarpic species (Armbruster *et al.*, 2002). Little is known of how stigma, style, and ovary differentiation occurs in model plants, and certainly there is no information on how these processes first evolved. Although very good progress has been made to unravel the mechanisms of fruit development in *A. thaliana*, many other forms of angiosperm fruits have not yet been investigated at a molecular level.

Future research aimed at understanding carpel evolution will undoubtedly be helped by the extension of functional genetic approaches to non-model taxa. Such technological advances will depend to some extent on the development of plant transformation procedures in non-model plants, permitting the use of such techniques as RNAi (Smith *et al.*, 2000) or the directed mis-expression of transgenes. However, apart from the eudicots and monocots, many of the key taxa to be studied are of a woody habit and take several years before flowering. One technique that may help to overcome this practical difficulty is that of Virus Induced Gene Silencing (VIGS), reviewed by Burch-Smith *et al.* (2004). The VIGS technique involves the use of a transgenic virus that can inactivate a given plant gene through an RNAi-related mechanism. Several different VIGS vectors have been developed and recent studies show that at least one of these (Liu *et al.*, 2004) can infect relatively basal groups of angiosperms (Hileman *et al.*, 2005).

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