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Carpel Development

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ABSTRACT

The carpel is the female reproductive organ that encloses the ovules in the flowering plants or angiosperms. The origin of the carpel and its subsequent morphological modifications were probably of vital importance to the evolution of the angiosperms, and the carpel is also very important as the precursor organ to the fruit. Here we describe the general attributes of the angiosperm carpel and several hypotheses for its evolutionary origin. As carpels share many developmental processes with leaves, we describe these processes in the leaf, and then detail the regulation of carpel and fruit development in the model angiosperm *Arabidopsis thaliana*. We also describe the relationship between carpel formation and the arrest of organ proliferation which occurs at the centre of the *Arabidopsis* floral meristem. We then provide a brief overview of carpel development in angiosperms occupying important phylogenetic

positions, including ANA grade angiosperms, monocots, basal eudicots and core eudicots, focussing on the probable ancestral state of the carpel in each case, and on the available molecular and genetic data. We end with a brief discussion of future research directions relating to carpel and fruit development.

I. INTRODUCTION

A. THE CARPEL AND GYNOECIUM

The carpel is the female reproductive organ that encloses the ovules in the flowering plants or angiosperms. By contrast, ovules in the remainder of the seed plants, or gymnosperms, occur as naked structures, often borne in the axils of leaf-like organs such as the cone scales of conifers. Indeed, the terms "angiosperm" and "gymnosperm" describe this difference, as they, respectively, refer to enclosed and naked seeds (from the Greek *angeion* = vessel, *gymnos* = naked and *sperma* = seed). Carpels typically occur in the fourth and innermost whorl of the angiosperm flower, which is termed the gynoecium. These organs may occur separately, in which case the gynoecium is said to be apocarpic, or may be fused together into a gynoecium which is then termed syncarpic. Both individual carpels and syncarpic gynoecia are divided longitudinally into tissues which perform distinct roles in reproduction. Thus, the stigma at the apex of these structures is specialised for the capture and germination of pollen grains, and below this the style conducts pollen tubes to the ovary, which houses the ovules and in which fertilisation takes place.

B. THE ADVANTAGE OF HAVING CARPELS

The carpel is thought to confer a number of major advantages on the flowering plants. Firstly, carpels protect the ovules within them, in part through the expression of genes associated with defence against insects and micro-organisms (Scutt *et al.*, 2003). Secondly, systems have evolved to enable pollen capture and pollen tube guidance in carpel tissues, which may represent considerable improvements over equivalent mechanisms operating in gymnosperms. Thirdly, during the phase of pollen germination and growth, the carpel provides a site for the operation of self- and inter-specific incompatibility mechanisms: self-incompatibility prevents close inbreeding and thereby conserves the capacity for evolutionary change, while inter-specific incompatibility prevents wide hybridisations that may lead to the production of unviable offspring.

Fourthly, carpel tissues undergo developmental changes after fertilisation to form fruits, which protect the developing seeds within them and, at maturity, contribute to the dissemination of these.

C. THE ADDED BENEFITS OF CARPEL FUSION

More than 80% of the angiosperm species are syncarpic: their carpels are fused into a single female structure in the centre of the flower and this trait has probably arisen over 20 times independently in the angiosperms (Armbruster *et al.*, 2002). Carpel fusion confers numerous advantages on syncarpic species (Armbruster *et al.*, 2002; Endress, 1982), of which one of the most important is the provision of a compitum: a tissue that acts as an interchange between the entire stigmatic surface and the ovary, thus allowing any pollen tube to access any ovule. Another potentially important advantage of syncarpy results from the enhanced competition that this produces between pollen tubes: an effect which may select for vigorous male parents. Syncarpy also allows for the production of larger fruits, with potentially more complex and efficient seed dispersal mechanisms. Finally, a syncarpic gynoecium may require a lesser energy input for cell wall production, compared to an apocarpic gynoecium of similar size.

Syncarpy can be divided into two types based on the timing of the fusion event involved: where carpels are fused from the earliest emergence of their primordia, the fusion is termed "congenital", whereas fusion that takes place during development is termed "post-genital". Congenital carpel fusion is the most common type, with post-genital fusion occurring in only a few families (Lolle and Pruitt, 1999). The molecular basis for congential carpel fusion has not been investigated in detail, though a large number of *Arabidopsis* mutants are known that disrupt congenital carpel fusion (Vialette-Guiraud and Scutt, 2010). Post-genital carpel fusion has been studied most fully in *Catharanthus roseus* (Apocyneaceae), in which two separate carpel primordia are initiated and then grow until their inner surfaces come into contact (Siegel and Verbeke, 1989; Verbeke, 1992; Walker, 1978). The already differentiated epidermal cells of these surfaces then begin to interlock and re-differentiate into parenchyma by a process which is dependent on diffusible, water-soluble agents produced by the carpels (Siegel and Verbeke, 1989). For all of the reason given above, both the origin of the carpel and the multiple origins of syncarpy were almost certainly the major factors in the evolutionary success of the angiosperms. This group arose from an unknown common ancestor, believed to have lived in the Lower Cretaceous Period, to generate an estimated 300,000 or more species alive today.

II. THE ORIGIN OF THE CARPEL

A. AN "ABOMINABLE MYSTERY"

This is how Charles Darwin famously referred to the recent apparition and rapid diversification of the angiosperms (see Friedman, 2009). The rapidity of early angiosperm diversification, as suggested by the fossil record, was in contradiction with Darwin's viewpoint as an evolutionary gradualist, and though this feature of angiosperm evolution may seem less mysterious to present-day biologists, we still lack understanding of many aspects of the origin of the flowering plants. For example, little is currently known of the molecular changes that were responsible for the highly novel anatomical features of the first flowers, including the carpel. Additionally, we lack a clear picture of the non-flowering progenitor of the flowering plants and knowledge of which fossil gymnosperms, such as Corystospermales, Caytoniales, Glossopteridales, Bennettitales or *Schmeissneria* (Taylor and Taylor, 2009; Wang *et al.*, 2007), might be sister or ancestral to the angiosperms. Further unanswered questions relate to where the flowering plants originated and to the date of this event: though a Lower Cretaceous origin is widely cited, the carpel and other key floral features may have originated earlier than that. Though much further work is thus necessary if we are to piece together the early evolution of the flower and carpel, the current lack of firm evidence has not prevented the construction of numerous hypotheses on this subject, as described below.

B. HYPOTHESES OF CARPEL ORIGIN

A hypothesis proposed by the writer and philosopher Goethe (von Goethe, 1790), and which is now well supported by molecular and genetic evidence (Honma and Goto, 2001), regards all plant lateral organs, including carpels, as variants of a basic leaf-type developmental ground plan. Though the carpel may thus be homologous to leaves, this floral reproductive organ is almost certainly more directly related to the leaf-like structures present in the reproductive axes of the angiosperms' sister group, the gymnosperms. On this subject, hypotheses for flower origin divide conceptually into two types, depending on whether they regard the carpel as derived by the modification of male or female structures in a presumed gymnosperm-like ancestor. The mostly male theory (MMT; Frohlich, 2003; Frohlich and Parker, 2000) postulates the flower to be mostly derived from the male strobili of a gymnosperm-like ancestor. According to this hypothesis (Fig. 1A), the ancestor of the flowering plants would first have generated ectopic ovules on male sporophylls,

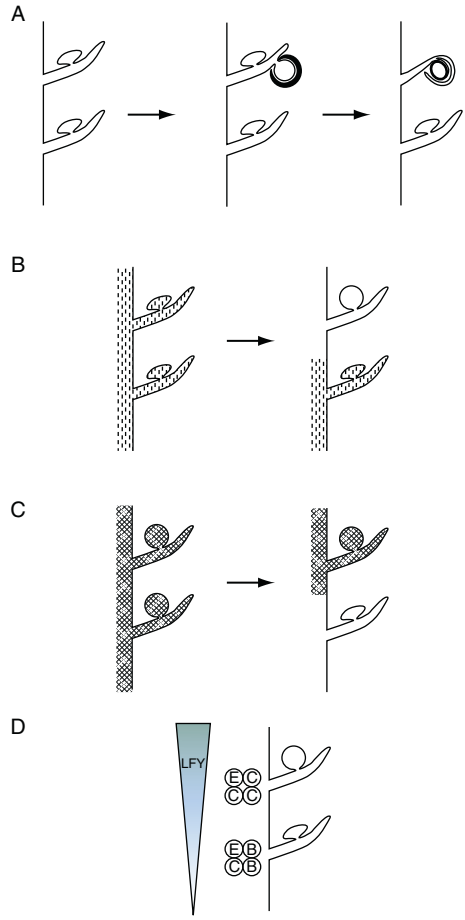

Fig. 1. Hypotheses for the origin of the flower and its carpel. (A) According to the Mostly-Male Theory (Frohlich, 2003), ectopic ovules formed on previously male sporophylls and, in a second step, these sporophylls lost their microsporangia and closed around the ovule to form the carpel. The outer integument of the angiosperm ovule (thick line) was formed from a pre-existing female cupule structure. (B) According to the Out-of-Male hypothesis (Theissen *et al.*, 2002), the basipetal movement of male-determining B-function MADS box gene expression (shaded area) in a male strobilus left female structures at the apex, which later became carpels. (C) According to the Out-of-Female hypothesis (Theissen *et al.*, 2002), the acropetal movement of B-sister MADS box gene expression (shaded area) in a female strobilus left male structures at the base, which later became stamens. Female structures at the apex became carpels. (D) According to the hypothesis of Baum and Hileman (2006), a temporal switch in the regulation by LFY of B- and C-function MADS box genes occurred in an ancestor of the flowering plants. This change generated high concentrations of C-function-rich MADS box complexes at late developmental stages, causing the patterning of the strobilus into apical female and basal male reproductive structures, and these later became carpels and stamens, respectively.

which would thereby have become bisexual. The MMT postulates that ectopic ovules were concentrated on sporophylls near the apex of the strobilus, and that these sporophylls subsequently lost their ability to produce microsporangia, thus becoming functionally female. The MMT goes on to postulate these newly female sporophylls to have closed around the ovules to form the first carpels. In subsequent evolutionary steps, the residual female strobili of these proto-flowering plants would have been lost, leaving only bisexual reproductive axes containing apical carpels and basal microsporophylls (later to become stamens).

The MMT is based on evidence from a number of sources, including data linked to *LEAFY* (*LFY*), which acts upstream of genes that specify the identities of floral organs in model angiosperms. In certain gymnosperms, a paralogue of *LFY* termed *NEEDLY* (*NLY*) has been shown to be expressed principally in female cones (Mouradov *et al.*, 1998). No direct orthologue of *NLY* has been found in any angiosperm, suggesting this gene lineage to have been lost from a common ancestor of the living angiosperms, subsequent to the separation of the angiosperm and gymnosperm lineages. The MMT postulates the loss of *NLY* to have been accompanied by the extensive loss of female-specific developmental programmes, and this loss to have contributed to the origin of the flower. Hence, the MMT regards the carpel as derived from male reproductive organs. The MMT even accounts for the origin of the outer integument of the ovule, which is also specific to the flowering plants, by proposing this to have arisen from a cupule that surrounded the ovules in their presumed gymnosperm-like ancestor. Indeed, the MMT cites Jurassic fossil Corystospermales as having cupules of a type which could have evolved to generate the outer integument. Though the MMT has been a very widely discussed and conceptually useful hypothesis for flower origin, it should be noted that several more recent studies have questioned the sex-specific expression of *LFY* and *NLY* in gymnosperms on which the MMT is partly based (Carlsbecker *et al.*, 2004; Dornelas and Rodriguez, 2005; Vazquez-Lobo *et al.*, 2007).

Several further hypotheses of flower origin have been proposed, which differ from the MMT in that they postulate the bisexuality of the flower to have arisen by a spatial or temporal change in factors controlling the sex of reproductive organs. Hence, these hypotheses do not, in contrast to the MMT, postulate the extensive loss of female developmental programmes during flowering plant evolution and consequently regard the carpel as homologous to female, rather than male, gymnosperm reproductive structures. The Out-of-Male (OOM) hypothesis (Theissen *et al.*, 2002) proposes the bisexual flower to have evolved by the basipetal movement of the expression of a male-promoting, B-class MADS box gene in a previously male

strobilus, resulting in the production of female structures at the apex (Fig. 1B). A sister hypothesis to the OOM hypothesis, termed the Out-of-Female (OOF) hypothesis (Theissen *et al.*, 2002), postulates a sex-determining role for B-sister MADS box genes, whose expression is proposed to have moved acropetally in a female strobilus to leave male structures in basal positions (Fig. 1C). In general, therefore, the OOM and OOF hypotheses focus below the level of *LFY* and *NLY* in the hierarchical control of gene expression, and postulate a spatial change in MADS box gene expression to form a boundary of B- or B-sister expression in a previously unisexual strobilus, thereby making this bisexual. It should be added that recent functional characterisation of B-sister genes in *Arabidopsis* has revealed roles in seed pigmentation (Nesi *et al.*, 2002) and outer integument development (de Folter *et al.*, 2006; Prasad *et al.*, 2010), rather than in carpel development *per se*. However, it cannot be excluded that B-sister genes may have played a role in carpel development in early flowering plants.

Baum and Hileman (2006) have formulated a further hypothesis, which will be termed here the BHH, to account for the evolution of the first flowers (Fig. 1D). Similar to the MMT, the BHH proposes a central role for *LFY* in the origin of the flower, but postulates that the origin of floral bisexuality was caused not by the loss of female-specific developmental programmes but by a temporally generated switch in responses to *LFY*. According to this hypothesis, LFY protein levels increase with time in the meristems of developing reproductive axes and, at a certain threshold, cause these meristems to switch from the production of (male) microsporophylls to (female) megasporophylls. This hypothesised switch may involve the action of *LFY* cofactors, such as *UNUSUAL FLORAL ORGANS* (*UFO*) and *WUSCHEL* (*WUS*). Whatever the precise mechanism, the BHH proposes that a change occurred during early flower evolution in the relative response to *LFY* of B- and C-class MADS genes. Accordingly, C-class proteins are proposed to have predominated at the high *LFY* concentrations encountered at the apex of the strobilus at late developmental stages, resulting in MADS box complexes that were rich in C-class proteins. These proteins would have formed C-rich complexes which would consequentially have specified the development of megasporophylls at the apex of the strobilus.

The above hypotheses may, to some extent, be tested. Baum and Hileman (2006), for example, propose a list of predictions of their hypothesis that could be tested in basal angiosperms and gymnosperms. The MMT stands out from the other hypotheses described here in proposing the extensive loss of female developmental programmes during early flower evolution. This prediction might provide a means to eliminate either the MMT or all other current contending hypotheses from consideration. Thus, if the predictions

of the MMT were correct, we would expect to find in gymnosperms numerous classes of genes with female-specific expression patterns, the orthologues of which had been lost from the angiosperms. Gymnosperm genes with male-specific expression patterns should not have been affected in this way. The testing of the MMT by this method has yet to be performed on a large scale. However, one question mark concerning such a test relates to the degree to which male and female developmental programmes in gymnosperms might be based on different sets of genes, rather than on subtle changes to the expression of a common set of genes. If the latter is predominately the case, such a relatively simple method of hypothesis testing may be unavailable.

C. A POSSIBLE ROLE FOR THE E-FUNCTION IN CARPEL EVOLUTION

The E-function MADS box genes may have played an important role in the origin of the flower and its carpel. In *Arabidopsis*, these genes encode the SEPALLATA1-4 (SEP1-4) transcription factors, which are hypothesised to act in quaternary complexes, together with combinations of A-, B- and C-function MADS box proteins, to specify organ identity in each whole of the flower (Honma and Goto, 2001; Pelaz *et al.*, 2000; Theissen and Saedler, 2001). Theissen and Melzer (2007) discuss the possibility that, before the flower, dimers of C-function genes may have specified the development of female reproductive organs, and that the evolution of quaternary MADS box complexes, incorporating both C- and E-function proteins, may have built on this mechanism to generate the carpel. More precisely, the evolution of MADS box quaternary complexes is hypothesised to have caused transcription factor binding to two distinct MADS box binding motifs, termed CArG boxes, in the *cis*-acting control regions of their target genes. According to this hypothesis, this newly evolved DNA-binding behaviour would have generated the necessary multiplicity of interactions to specify at least three novel organ types in early flowers: carpels, stamens and tepals.

SEP genes, encoding the E-function, appear to be specific to the angiosperms (Becker and Theissen, 2003). However, recent evidence suggests that the E-function may not be exclusively associated with the *SEP* clade: genes of the *AGAMOUS-LIKE6* (*AGL6*) clade have recently been demonstrated to contribute to the E-function in both *Petunia* (Rijpkema *et al.*, 2009) and Poaceae monocots (Li *et al.*, 2010; Ohmori *et al.*, 2009; Thompson *et al.*, 2009). The *AGL6* clade is sister to the *SEP* clade in angiosperms and is also present in gymnosperms (Becker *et al.*, 2000), thus leaving open the possibility that quaternary MADS box complexes involving AGL6 proteins might also form in gymnosperms. The presence of *AGL6* orthologues in gymnosperms tends to suggest the loss of the *SEP* clade from this group, rather than its

specific origin by duplication in the angiosperms, also casting some doubt on the idea that the genes responsible for the E-function arose specifically with the angiosperms. To resolve the question of the potential contribution of the E-function to the origin of the flower, and therefore of the carpel, attention is now being paid to the formation of higher order complexes of MADS box proteins in both angiosperms and gymnosperms (Melzer *et al.*, 2010).

III. THE BASIC DEVELOPMENTAL PLAN OF LATERAL ORGANS

A. LEAVES AND CARPELS SHARE BASIC REGULATORY PATHWAYS

As mentioned in the previous section, leaves and floral organs most likely have a common evolutionary origin, or as Goethe memorably put it: *Alles ist Blatt* (All is Leaf; von Goethe, 1790). An increasing amount of evidence suggests that the pathways regulating the basic morphological outline of ancestral leaf-like organs have been recruited to the developmental programmes of both leaves and floral organs, including the carpels, of present-day plants, although in the latter case, these have been slightly modified and positioned downstream of genes determining floral organ identity. For instance, mutations in genes affecting carpel morphogenesis also result in defects in the basic morphogenesis of the leaves.

We will start by summarising what is known about the formation and patterning of leaves, in order to be able to use this information as a basis for a discussion of carpel and fruit morphogenesis in later sections. Impressive progress in our understanding of the genetic regulation of leaf initiation and morphogenesis has been made during the last decade, and this has been the subject recently of several in-depth reviews (Aida and Tasaka, 2006; Barkoulas *et al.*, 2007; Bowman and Floyd, 2008; Byrne, 2006; Husbands *et al.*, 2009; Kepinski, 2006; Pulido and Laufs, 2010; Shani *et al.*, 2006), though in contrast to these, we aim only to give an overview of the known regulatory networks.

B. INITIATION OF LATERAL ORGANS AT THE FLANKS OF THE SHOOT APICAL MERISTEM

In this section, we approach the following two basic questions: what regulatory events are required for lateral organ initiation at the peripheral zone (PZ) of the shoot apical meristem (SAM) and what are the events leading to

the formation of lateral organ primordia? Before the initiation of lateral organs, the cells of the SAM are maintained in an undifferentiated state by the activity of a number of regulators, including members of the class I KNOTTED1-like homeobox (KNOX) and WUSHEL-like homeobox (WOX) transcription factor families (Fig. 2A; Hake *et al.*, 2004; Laux *et al.*, 1996). Cell expansion is strongly correlated with differentiation, and the above-mentioned factors act in part by promoting a high ratio of cell division to cell expansion through the modulation of hormonal balances in the SAM (Jasinski *et al.*, 2005; Yanai *et al.*, 2005). Cell division is stimulated by cytokinin-induced activation of Cyclin D, and high cytokinin levels in the *Arabidopsis* SAM result, at least in part, from KNOX-induced activation of the cytokinin biosynthesis gene *ISOPENTENYL TRANSFERASE7* (*IPT7*; Yanai *et al.*, 2005). Not only cytokinin synthesis (Kurakawa *et al.*, 2007) but also cytokinin responsiveness is high in the central part in the meristem, and at least two members of the WOX family, WUS and STIMPY/WOX9, appear to stimulate cytokinin signalling in the SAM (Fig. 2A; Gordon *et al.*, 2009; Skylar *et al.*, 2010). Interestingly, recent data suggest that cytokinin stimulates *KNOX* and *WUS/WOX* activity, suggesting the presence of a positive feedback loop (Gordon *et al.*, 2009; Kurakawa *et al.*, 2007). At the same time, cell expansion is repressed by keeping the level of the hormone gibberellin low through the KNOX-mediated repression of the gibberellin biosynthesis gene *GA20-OXIDASE* (*ga20ox*) and by activation of the GA catabolism gene *ga2ox1* (Bolduc and Hake, 2009; Chen *et al.*, 2004; Hay *et al.*, 2002).

Lateral organ initiation in the PZ of the SAM requires the silencing of programmes that repress differentiation, such as the KNOX programme, and it has been suggested that the plant hormone auxin plays a major role in this process. Local auxin concentration maxima are formed at organ initiation sites by a directed auxin flux, which results from the action of auxin influx and efflux facilitators (Bainbridge *et al.*, 2008; Heisler *et al.*, 2005; Reinhardt *et al.*, 2000, 2003). In these "high auxin" organ initiation sites, *KNOX* gene activity is repressed by auxin action itself, as well as by the activity of a transcriptional repressor complex containing the ASYMMETRIC LEAVES1 (AS1) and AS2 proteins, resulting in the induction of leaf formation (Fig. 2A; Guo *et al.*, 2008; Hay *et al.*, 2006; Ikezaki *et al.*, 2010). The AS1/AS2 complex also maintains the silencing of *KNOX* throughout leaf development via the recruitment of the HIRA (Histone Regulation A) chromatin-remodelling factor to the *KNOX* gene (Guo *et al.*, 2008; Phelps-Durr *et al.*, 2005). Leaf initiation appears also to be regulated by a SQUAMOSA promoter-binding protein-like 9 (SPL9)-dependent, leaf-derived signal that may act via the modulation of auxin pathways (Wang *et al.*, 2008).

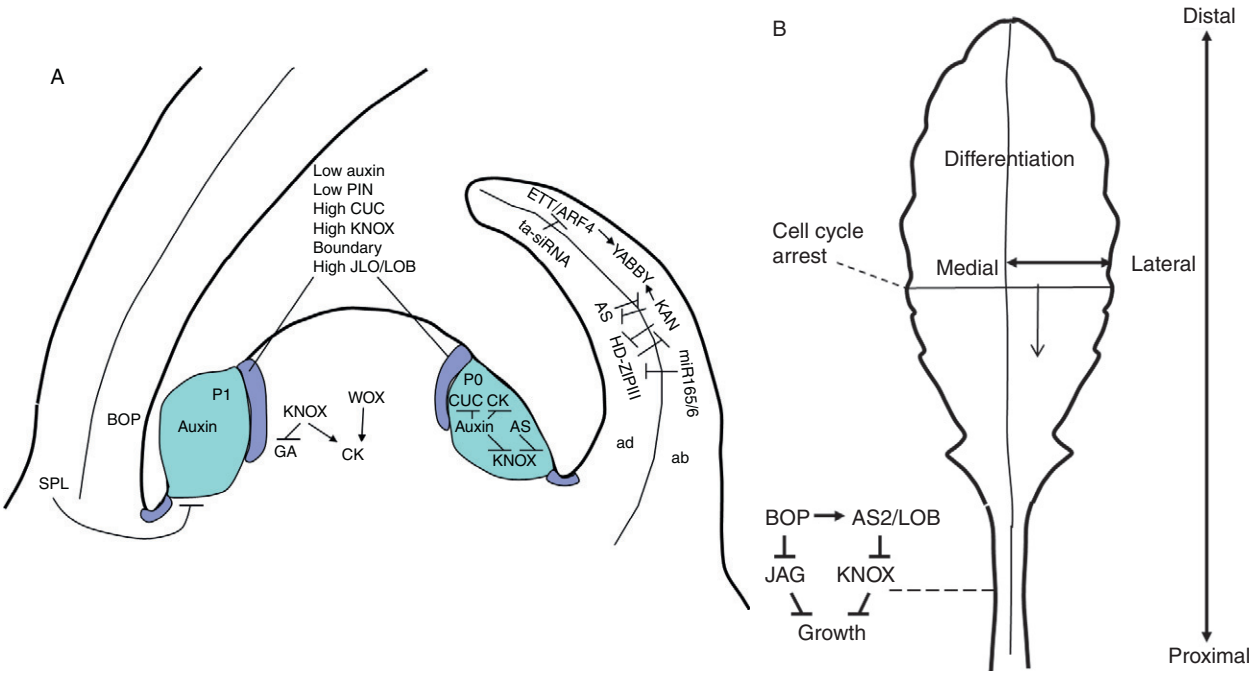

Fig. 2. Genetic networks controlling plant lateral organ development. (A) Interactions between hormones, transcription factors, and RNAs for lateral organ initiation, boundary formation, and establishment of adaxial–abaxial polarity in the leaf. (B) The establishment of distal–proximal polarity of the leaf.

C. ESTABLISHMENT OF BOUNDARIES

When a leaf primordium starts to grow, a boundary domain, in which cell expansion is reduced, becomes established to separate it from the neighbouring tissues. So, the next question is: what regulatory pathways position and establish boundaries between the SAM and newly initiated lateral organs? It has been suggested that a signal from the CZ of the meristem participates in the positioning of organ boundaries, and that activities in the meristem and the each new organ together establish the molecular changes required for boundary formation (Rast and Simon, 2008). As soon as one organ primordium has been initiated, a redirection of auxin flux to a new position in the PZ establishes the initiation of the next lateral organ (Heisler *et al.*, 2005). As a consequence, auxin is depleted and changes to gene expression occur in the cell layers surrounding the newly formed primordium, which creates a morphological boundary of distinct cell types with reduced cell division activity that separates the primordium from the rest of the meristem (Fig. 2A; Heisler *et al.*, 2005), as reviewed by Aida and Tasaka (2006). The NAC domain transcription factors CUP-SHAPED COTYLEDON (CUC1), CUC2 and CUC3 are expressed in the boundary domain, where their activity contributes to the repression of cell division and expansion (Hibara *et al.*, 2006; Sieber *et al.*, 2007; Vroemen *et al.*, 2003). The expression of the growth inhibiting *CUC* genes is restricted by members of the *miRNA164* family, indicating *CUC* genes to be central regulators of boundary size (Laufs *et al.*, 2004; Sieber *et al.*, 2007). The JAGGED LATERAL ORGANS (JGL) LBD domain protein is also expressed at the SAM/organ boundary, where it promotes the boundary function by repressing *PINFORMED* (*PIN*) activity, potentially resulting in low auxin concentrations at the boundary, and by activating *KNOX* genes (Borghi *et al.*, 2007; Husbands *et al.*, 2007; Shuai *et al.*, 2002). Recently, the BELL-type protein *ARABIDOPSIS THALIANA* HOMEOBOX GENE1 (ATH1) was also suggested to participate in boundary formation in a pathway parallel to that of the CUC proteins (Gomez-Mena and Sablowski, 2008).

D. REGULATION OF ADAXIAL–ABAXIAL POLARITY

Concomitant with the lateral outgrowth of the leaf primordium, a distinct polarity along the adaxial–abaxial axis is established. Anatomical features that optimise the leaf for photosynthesis are formed on the upper or adaxial side, adjacent to the SAM, whereas the lower or abaxial side differentiates to carry out gas exchange (for recent reviews see Chitwood *et al.*, 2007; Husbands

et al., 2009; Xu *et al.*, 2007). Members of the class III homeodomain-leucine zipper (HD-ZIPIII) gene family, such as *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), are expressed on the adaxial side of leaf primordia, where they play a major role in adaxial tissue specification (Fig. 2A; Emery *et al.*, 2003; McConnell and Barton, 1998; McConnell *et al.*, 2001; Prigge *et al.*, 2005). HD-ZIPIII genes are also expressed in the CZ of the meristem and have been suggested to coordinate communication between the SAM and the adaxial side of organ primordia (McConnell *et al.*, 2001). By contrast, *miRNA166* accumulates on the abaxial side of leaf primordial, where it represses the activity of HD-ZIPIII genes via post-transcriptional cleavage and/or chromatin modifications (Alvarez *et al.*, 2006; Bao *et al.*, 2004; Emery *et al.*, 2003; Kidner and Martienssen, 2004; Mallory *et al.*, 2004; Tang *et al.*, 2003; Williams *et al.*, 2005; Zhou *et al.*, 2007). The AS1 and AS2 proteins also support adaxial fate (Fu *et al.*, 2007; Lin *et al.*, 2003; Xu *et al.*, 2003). The abaxial side of leaves is specified by another set of regulators, acting antagonistically to the adaxial determinants, suggesting the mutually exclusive and opposing nature of adaxial and abaxial cell fates (Fig. 2A). Transcription factors belonging to the KANADI (KAN) subgroup of the GARP family and the auxin response factors AUXIN RESPONSE FACTOR3/ETTIN (ARF3/ETT) and ARF4 together specify abaxial identity (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001; Pekker *et al.*, 2005). It has recently been shown that KAN1 directly represses *AS2* transcription on the adaxial side of leaves, and other data suggest that KAN genes also act as negative regulators of HD-ZIPIII gene expression (Eshed *et al.*, 2004; Kerstetter *et al.*, 2001; Wu *et al.*, 2008). The activity of *ARF3* and *ARF4* is restricted to the abaxial side of the leaf primordium by the action of *TAS3* encoded ta-siRNAs that accumulate on the adaxial side (Adenot *et al.*, 2006; Fahlgren *et al.*, 2006; Garcia *et al.*, 2006; Hunter *et al.*, 2006; Nogueira *et al.*, 2007; Vazquez *et al.*, 2004; Williams *et al.*, 2005). Further data suggest the genes-encoding YABBY (YAB) transcription factors to act downstream of other polarity determinants, including the KANs and the ARFs, to direct leaf lamina expansion at the adaxial–abaxial boundary (Eshed *et al.*, 2001, 2004; Sawa *et al.*, 1999; Siegfried *et al.*, 1999). The antagonistic activity of the KAN and HD-ZIPIII genes has also been recruited to establish polarity during embryogenesis and vasculature formation, suggesting these to be important general regulators of polarity (Emery *et al.*, 2003; Eshed *et al.*, 2001; Ilegems *et al.*, 2010; Izhaki and Bowman, 2007).

One important question that remains to be answered concerns how polarity information is generated to induce the expression of fate-specific regulators. It has been suggested that the establishment of the adaxial–abaxial axis of leaves is dependent on the conversion of positional signals provided by the

SAM, and probably also by other surrounding areas, into the differential expression of the mutually antagonistic transcription factors mentioned above (Fig. 2A). Because the separation of the incipient leaf primordium from the CZ of the SAM through microsurgical sections results in radial abaxialised structures, it appears likely that adaxial identity is specified by a meristem-derived signal, continuously entering the primordium (Reinhardt *et al.*, 2005; Sussex, 1954). However, the nature of this signal is still not known. Because the START domain of HD-ZIPIII proteins appears capable of lipid/sterol binding, this was suggested as the potential target of an unknown SAM-derived lipid/sterol signal (McConnell *et al.*, 2001). Furthermore, as ta-siRNA-ARFs, encoded by *TAS3*, can move between cells, it has been suggested that these may play a role in SAM-to-primordia signalling (Garcia *et al.*, 2006). However, although the ta-siRNA-ARFs can move from below the SAM into the meristem proper, as well as from the adaxial to the abaxial side of the leaf blade, there is no evidence that these molecules act as messengers from the SAM to leaf primordia (Chitwood *et al.*, 2009). Instead, Chitwood *et al.* (2009) suggest that a gradient of these small RNAs is formed, which could define the expression boundary of their targets *ARF3* and *ARF4*. A signalling molecule often used for positional information is auxin, and Pekker *et al.* (2005) have suggested that auxin could act as an abaxially polarising signal, activating *ARF3* and *ARF4*. This is supported by the finding that the auxin influx facilitator AUX1 localises specifically in the abaxial epidermal layer, suggesting that auxin may flow into the abaxial half of the incipient primordium and that an auxin gradient may be established across the primordium (Reinhardt *et al.*, 2003).

E. LEAF DEVELOPMENT

Leaf development proceeds through various different steps during which cell proliferation, cell expansion and cell differentiation occur. One major question is: how is this process coordinated? Auxin gradients may contribute to leaf development by coordinating growth, and, for example, the differentiation and patterning of veins. An auxin maximum at the apical tip of the leaf primordium is established through auxin transport early in development (Reinhardt *et al.*, 2003), and is maintained by the induction of auxin biosynthesis at the tip, and later on also in the hydathodes at the margins of the leaf primordium. It has been suggested that this process allows the formation of a distal–proximal auxin gradient (Benkova *et al.*, 2003), which is important for controlled cell division and expansion, and gradients

formed by auxin transport from the leaf tip have been suggested to be important for midvein development (Mattsson *et al.*, 1999; Zgurski *et al.*, 2005).

The mechanisms that establish the pattern of differentiation along the distal–proximal axis of the leaf have not yet been determined. It has been shown, however, that cell proliferation and differentiation/expansion occurs along a gradient from the (distal) leaf blade toward the (proximal) petiole, reflected by the gradual move of a front of cell cycle arrest from the tip to the base (Fig. 2B; Donnelly *et al.*, 1999; Nath *et al.*, 2003). Similarly, cell divisions in the mid-region decline slightly ahead of divisions at the leaf margins in a medio-lateral gradient (Byrne, 2005). Thus, it is quite clear that the control of cell division is an integral part of pattern formation, and most likely contributes to the multitude of leaf shapes found in nature. Transcriptional regulators of cell division include: JAGGED (JAG), AINTEGUMENTA (ANT), GRF-INTERACTING FACTORS (GIFS), GROWTH-REGULATING-FACTORS (GRF), PEAPOD (PPD), LEAFY PETIOLE (LEP) and class I TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP), all of which promote cell divisions in the leaf (Dinneny *et al.*, 2006; Horiguchi *et al.*, 2005; Hu *et al.*, 2003; Lee *et al.*, 2009; Li *et al.*, 2005; Mizukami and Fischer, 2000; van der Graaff *et al.*, 2000; White, 2006), as reviewed by Anastasiou and Lenhard (2007) and Ingram and Waites (2006). In addition, the final expression patterns of some of these regulators, such as the GRF and TCP genes, appear to be controlled by miRNAs (Palatnik *et al.*, 2003, 2007; Rodriguez *et al.*, 2010). By contrast, growth cessation is promoted by the bHLH protein SPATULA (SPT) and the class II TCP proteins, starting from the tip of the leaf and continuing to the base (Efroni *et al.*, 2008; Ichihashi *et al.*, 2010; Nath *et al.*, 2003; Ori *et al.*, 2007; Palatnik *et al.*, 2003). The distal–proximal polarity of the leaf also results in an asymmetry in the size of the leaf blade. The leaf is wide in its distal region, but at the proximal end, a petiole is formed which has a very narrow blade (Fig. 2B). Two BTB/POZ transcriptional co-activators, BLADE-ON-PETIOLE1 (BOP1) and BOP2, repress cell proliferation and growth of the petiole. These regulators are expressed at the base of the developing leaf, where they directly activate *AS2* transcription, establishing the conditions for the repression of *KNOX* gene expression that is necessary to correctly pattern the petiole tissues that form at the proximal end of the leaf (Ha *et al.*, 2003, 2004, 2007; Hepworth *et al.*, 2005; Jun *et al.*, 2010). Furthermore, the *BOP* genes also negatively affect cell proliferation in the proximal end of the leaf by repressing *JAG* and *NUBBIN* (*NUB*; Norberg *et al.*, 2005).

F. LEAF MARGINS

Controlled cell proliferation and elongation are also required for shaping the leaf margins (Dinneny *et al.*, 2004; Palatnik *et al.*, 2003) and interestingly, auxin appears to play an important role in establishing a part of this growth pattern. Auxin responsiveness is evenly distributed in the leaf margin of simple leaves that lack serrations (i.e. teeth; Aloni *et al.*, 2003), whereas in simple leaf primordia, which will eventually form serrations at their margins, high auxin responsiveness can be detected in one or two marginal epidermal cells before teeth are recognisable, suggesting that auxin localisation play an important role in patterning leaf margins (Kawamura *et al.*, 2010). Furthermore, the depth of the indentation between the teeth of serrated leaves is regulated by the meristem and boundary gene *CUC2* (Nikovics *et al.*, 2006), and *KNOX* meristem genes have also recently been implicated in leaf serration (Kawamura *et al.*, 2010). The process of leaf serration shows many similarities to that of leaflet formation in dissected leaves. For example, leaf dissection in *Cardamine hirsuta* also relies on the activity of *CUC* boundary-specifying genes in the area delimiting leaflet primordia and on the activity of KNOX genes at the leaflet initiation position, which cause a delay in cell differentiation relative to cell proliferation. This is followed by the production of auxin maxima at the leaflet tip (Canales *et al.*, 2010; Hay *et al.*, 2006). Interestingly, these modulations of leaf margins clearly provide evidence that processes related to meristem function are also used during lateral organ development.

IV. THE BASIC CONCEPTS OF THE *ARABIDOPSIS* GYNOECIUM AND FRUIT

A. THE *ARABIDOPSIS* GYNOECIUM

As described in Sections I and II, the *Arabidopsis* gynoecium is a complex syncarpic structure, most commonly interpreted as being composed of two congenitally fused carpels. The *Arabidospis* gynoecium is connected to the base of the flower by a short internode called the gynophore (Fig. 3). Above the gynophore is the ovary which contains between 50 and 80 ovules, and makes up most of the length of the gynoecium. The ovary is divided into two longitudinally by a septum which is formed post-genitally. The two ovary wall regions in the gynoecium are termed the valves and the external part of the septum is termed the replum. At the apical end of the ovary are the style and the stigma. The stigma consists of a single layer of specialised epidermal cells bearing elongated processes termed stigmatic papillae. This surface

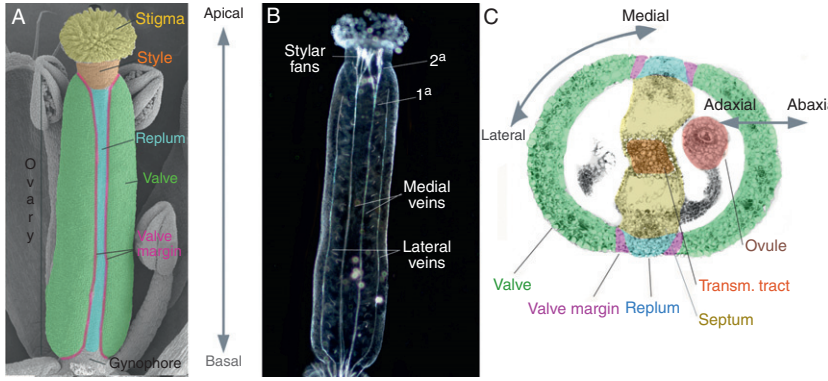

Fig. 3. Structure of the *Arabidopsis* gynoecium. (A) Scanning electron micrograph of the mature gynoecium at anthesis, artificially coloured to mark different functional domains. (B) The mature gynoecium at anthesis, cleared using chloral hydrate to reveal vascular patterning. Primary and secondary bifurcations of the medial veins are indicated with arrows. (C) Cross section of the ovary at anthesis, artificially coloured to mark different functional domain.

receives pollen grains and permits their germination. Following this, pollen tubes are guided by the transmitting tract present in the style and septum towards the ovules. After fertilization, the ovules develop into seeds and the *Arabidopsis* gynoecium is transformed into a two-chambered fruit called a silique. This structure opens at maturity to release its seeds along four dehiscence zones which consist of thin regions present at the valve margins on either side of the replum. The lignification of specific cells in these zones contributes to the dehiscence process by providing mechanical tension which stimulates the detachment of the two valves (Balanza *et al.*, 2006).

The gynoecial primordium arises in the centre of the floral meristem as a ring of cells enclosing a small depression, and then develops as an open-ended tube (Stages 6–8 of flower development, according to Smyth *et al.*, 1990). Two opposing meristematic ridges form in the internal medial regions of this cylinder and fuse together to form the septum. Placental tissues, which will give rise to the ovules, develop in the zones where the vertical septum and the gynoecial walls meet. At Stage 9, valve, placenta, septum, style and stigmatic cells begin to differentiate. At Stages 11 and 12, the apical part of the gynoecium closes, the stigmatic papillae complete their development and the style becomes distinct from the ovary. The gynoecium is mature at anthesis (Stage 13), when the flower opens and fertilization can take place. All the tissues required for fruit maturation and dehiscence are already present at this stage, and will complete their development after the fertilisation of the ovules (Bowman *et al.*, 1999; Roeder and Yanofsky, 2005).

B. FROM LEAF TO CARPEL: THE IDENTITY GENES

Two decades ago, the ABC model was formulated to explain the genetic interactions which lead to floral organ identity (Coen and Meyerowitz, 1991). Since then, extensive research has been carried out that has broadly validated the ABC model and provided evidence for a biochemical model for the action of the A-, B- and C-functions, termed the "floral quartet model" (Krizek and Fletcher, 2005; Theissen and Melzer, 2007). According to this model, a leaf, which corresponds to the "ground state" for lateral organs, can be transformed into a carpel by expressing the C-function MADS box gene *AG*, and at least one of the three E-function MADS box genes, *SEP1-3* (Honma and Goto, 2001). On the contrary, loss-of-function mutations in *AG* result in homeotic conversions of the carpel into a reiteration of the sequence sepals–petals–petals and the simultaneous loss of function of the redundant *SEP* genes results in a complete loss of carpel development programmes, transforming carpels into leaves (Ditta *et al.*, 2004; Pelaz *et al.*, 2000).

Despite its central role in specifying carpel identity, *AG* is not unique in providing carpelloid features. In fact, in the double mutant *apetala2* (*ap2*) *ag*, organs with carpel characteristics still develop in the first whorl of the flower (Bowman, 1991). This observation led to the conclusion that other genes involved in carpel identity were present and were, like *AG*, negatively regulated by the A-class gene *AP2*. These factors have been identified as two other highly related and entirely redundant MADS box genes *SHATTERPROOF1* (*SHP1*) and *SHP2*, which are principally involved in the specification of valve margin identity (Liljegren *et al.*, 2000; see Section IV.D). In the quadruple *ap2 ag shp1 shp2* mutant, all carpelloid structures disappear. Other complementary studies have demonstrated that the AG and SHP proteins are extremely similar at a functional level, but play distinct roles during carpel development, mostly due to their different expression patterns (Pinyopich *et al.*, 2001).

Two other putative transcription factors required for the development of carpel tissues are encoded by the bHLH gene *SPT* and the YAB gene *CRABS CLAW* (*CRC*), which seem to act downstream of AG/SHP. As in the *ap2 ag shp1 shp2* quadruple mutant, the loss of SPT or CRC function in an *ap2 ag* background results in the loss of all carpelloid features (Alvarez and Smyth, 1999), showing that these two genes are also necessary for carpel development. *SPT* is widely expressed in both vegetative and reproductive structures throughout development (Heisler *et al.*, 2001), although the main phenotypes associated with *spt* mutations are developmental defects in most of the marginal tissues of the carpel (Alvarez and Smyth, 1999). *CRC* is specifically expressed in nectaries and carpels; *crc* gynoecia are shorter and

wider than wild type and partially unfused at the apex (Bowman and Smyth, 1999). In the *crc spt* double mutant, the gynoecium is completely unfused and possesses a considerably reduced number of ovules, in addition to much less stigmatic and stylar tissue (Alvarez and Smyth, 1999). Supporting the idea that *CRC* and *SPT* mediate the *AG/SHP* carpel identity function, Alvarez and Smyth (1999) showed that *CRC* and *SPT* were also involved in other aspects of *AG* activity, such as the termination of the floral meristem. Recently, it has been shown that *CRC* acts in combination with three other genes, *REBELOTE* (*RBL*), *SQUINT* (*SQN*) and *ULTRAPETALA1* (*ULT1*), to control meristem determinacy (Prunet *et al.*, 2008). Interestingly, *DROOPING LEAF* (*DL*), the *CRC* orthologue from rice (Yamaguchi *et al.*, 2004) and *EcCRC*, the *CRC* ortholog from poppy (Orashakova *et al.*, 2009), have both been shown to play a role in the termination of the floral meristem, as discussed further in Section V. Moreover *DL*, like *AG*, plays a prominent role in the C-function.

What do we know about the chronology and the hierarchy of the molecular events leading to carpel identity? *AG* and *SEP* genes are expressed in carpel primordia, even before any morphological sign of differentiation can be observed (Hempel *et al.*, 1997; Savidge *et al.*, 1995; Yanofsky *et al.*, 1990). When the gynoecial primordium begins to form and develop as a cylinder, the expression of *AG* is uniform throughout this structure. Later however, *AG* is only expressed in specific cell types within the gynoecium, including the stigmatic papillae and ovules (Bowman *et al.*, 1991a). In the very early stages of floral meristem development, the expression of *AG* is activated by the joint action of the floral identity regulator LFY and the meristem maintenance factor WUS. Once present, the AG protein acts to down-regulate *WUS*, leading to a loss of floral meristem activity (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). It has been shown that the A-function gene *AP2* inhibits *AG* expression in the perianth whorls of the flower (Drews *et al.*, 1991). In *35S::SEP3* plants, *AG* is ectopically expressed, suggesting that SEP3 could participate in the early activation of *AG* (Castillejo *et al.*, 2005). Numerous studies have permitted the identification of many other factors involved in the regulation of *AG* activity, both at the transcriptional and post-transcriptional levels (Bao *et al.*, 2004; Chen *et al.*, 1999; Cheng *et al.*, 2003; Das *et al.*, 2009; Franks *et al.*, 2002; Gregis *et al.*, 2006; Krizek *et al.*, 2000; Liu and Meyerowitz, 1995; Sieburth and Meyerowitz, 1997; Yu *et al.*, 2009; see Section V for further details). In early stage of development, *SHP1* and *SHP2* are widely expressed in the *Arabidopsis* gynoecium. Later, their expression is restricted to the valve margins, the top of the gynoecium, the placental tissue and the ovules (Flanagan *et al.*, 1996; Savidge *et al.*, 1995). *SHP* genes seem to act downstream of *AG* and could therefore represent direct

AG-targets, though as demonstrated by *ap2 ag* mutants, these genes can also be activated by other factors. Recently, it has been demonstrated that the transcription factors FILAMENTOUS FLOWER (FIL), YAB3 and JAG, also involved in leaf development, jointly activate *SHP* expression in valve margins (Dinneny *et al.*, 2005; see Section IV.D for further details). Thus, both *SHP* and *AG* can be placed at the top of the carpel identity pathway, and could from this position directly or indirectly activate both *SPT* and *CRC*. *CRC* has been identified as a direct target of AG (Gomez-Mena *et al.*, 2005), though less is known about *SPT* activation.

C. PARTITIONING THE CARPEL: ADAXIAL–ABAXIAL AND MEDIO-LATERAL PATTERNING

Once organ identity has been specified, the gynoecial primordium is divided into different domains. In the first stages of development, abaxial–adaxial and medio-lateral patterning are specified, and later, as the primordium forms a cylinder, apical/basal polarity is defined. Abaxial–adaxial polarity refers to the differentiation between the outer (abaxial) and inner (adaxial) domains of the carpel (Fig. 3). These domains are, respectively, equivalent to the lower and upper sides of leaves, and the establishment of adaxial–abaxial polarity involves similar genetic mechanisms to those operating in leaves. Thus, the antagonistic interactions found in leaves between HD-ZIPIII genes, which direct adaxial fate, and KAN/YAB genes, which direct abaxial fate, also exist in the gynoecium (*cf.* Fig. 4A and B). Indeed, HD-ZIPIII genes are expressed, as would be expected, in the adaxial domain of the carpel. However, inactivation of these genes seems to have a milder effect on carpel than on leaf development, indicating that additional factors involved in promoting adaxial polarity might operate in the gynoecium (Dinneny *et al.*, 2006; McConnell and Barton, 1998). Recently, *JAG* and *NUB* have been identified as possible factors in carpel adaxialisation (Dinneny *et al.*, 2006). *NUB*, unlike *JAG*, which is expressed in a non-polar manner in all lateral organs, is expressed only in the adaxial zones of leaves, stamens and carpels. Single *nub* mutants do not present any obvious phenotype, but in the double *jag nub* mutant, floral organ growth is affected and carpels and stamens are abaxialised. In the abaxial domains of the carpel, as during leaf formation, the KAN genes, the auxin response factors *ETT* and *ARF4* and the YAB genes are all expressed from very early stages of development (Kerstetter *et al.*, 2001; Pekker *et al.*, 2005; Siegfried *et al.*, 1999). These genes are largely redundant in their abaxial activity, as single mutants of most of them only show a very weak adaxialisation phenotype. However, the combination of mutants can lead to severe polarity defects in which the

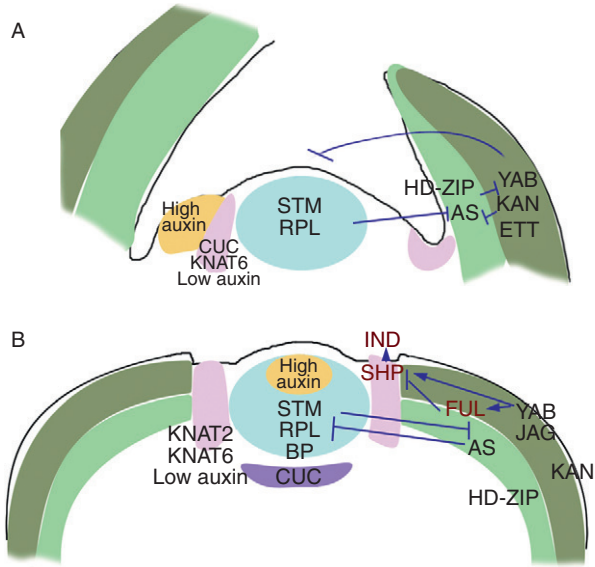

Fig. 4. Parallelism between genetic networks operating at the shoot apical meristem (SAM) and gynoecium in *Arabidopsis*. (A) Scheme of the SAM in longitudinal section. Meristem maintenance genes such as *STM* and *RPL* are expressed in meristematic cells (blue), antagonistically with AS, YAB, KAN and HD-ZIPIII genes which are expressed in developing leaf primordia (green). Auxin maxima (yellow) preceed the emergence of leaf primordia, and boundary genes *CUC* and *KNAT6* mark the outer boundary of the stem cell pool (pink). (B) Scheme of the *Arabidopsis* gynoecium in transverse section, indicating genetic pathways that direct medio-lateral patterning. Meristem-associated genes *STM*, *RPL* and *BP* are expressed in the medial region at the replum (blue), antagonistically to the valve-expressed AS, YAB, KAN and HD-ZIPIII genes (green). Boundary genes *KNAT2* and *KNAT6* are expressed at the valve margins (pink), where dehiscence zone genes *SHP* and *IND* also become activated. Auxin maxima occur in the replum, while auxin mimima occur at the valve margins.

gynoecium develops inside-out. Thus, in *crc kan1*, *kan1 kan2* or *ett arf4* double mutants, the transmitting tract and ovules form on the exterior of the gynoecium (Eshed *et al.*, 1999; Pekker *et al.*, 2005).

Medio-lateral polarity is also specified very early during gynoecium development. The lateral domains of the gynoecium will give rise to the valves, while its medial domains correspond to the fused carpel margins and will develop internally into the placentae, septum, apical style and stigma, and externally into the replum (Fig. 3). Along the medio-lateral axis, two opposite types of tissue develop: in medial zones a new meristem called the medial ridge forms, while lateral domains develop as differentiated tissues. Accordingly, several genes involved in SAM maintenance, such as the class I

KNOX factors and the "boundary genes" *CUC1* and *CUC2* are specifically expressed in the medial region, where they appear to be required for marginal tissue development. Ishida *et al.* (2000) analysed the gynoecium of *cuc1 cuc2* plants produced from calli, and therefore able to flower (grown from seed, these mutants would not progress beyond the seedling state). Interestingly, these plants showed defects in marginal tissue development and failed to develop a septum. Scofield *et al.* (2007) used inducible RNAi lines to study the effect of reduced activity of the KNOX I factor SHOOT MERISTEMLESS (STM) on gynoecium development. In some cases, the floral meristem aborted before forming any carpels, whereas in others a gynoecium was present, but this lacked a septum and showed reduced marginal tissue development, thus forming a completely unfused structure. Conversely, the expression of genes that repress the undifferentiated state, and thus promote the development of lateral primordial, is restricted to the lateral domains of the gynoecium. Thus, YAB genes, *JAG/NUB* and *AS1/2*, are specifically expressed in lateral regions of the young gynoecium (Alonso-Cantabrana *et al.*, 2007; Bowman and Smyth, 1999; Dinneny *et al.*, 2006; Siegfried *et al.*, 1999). The genetic networks that maintain SAM and lateral primordium boundaries appear to perform similar functions in medio-lateral patterning of the gynoecium (Alonso-Cantabrana *et al.*, 2007; Dinneny *et al.*, 2005; Ragni *et al.*, 2008). Dinneny *et al.* (2005) demonstrated *JAG*, *FIL* and *YAB3* to play a pivotal role in valve development in the gynoecium through the differential activation of valve and valve margin factors in the corresponding regions (Fig. 5). Alonso-Cantabrana *et al.* (2007) showed that later on in the development of the *as1* gynoecium, or in plants over-expressing *BP*, the replum is expanded and the valves are narrower. These authors concluded that AS1 plays a specific role in promoting valve initiation, and this action is likely to involve the repression of *KNOX I* and other factors directing replum development. A model was proposed in which both a gradient of FIL/YAB3/JAG with a lateral maximum, and AS1, are present in the valve, while a KNOX/RPL gradient with a medial maximum is present in the replum. According to this model, the two gradients would have opposite actions and would thus define valve margin development in their region of overlap.

Another factor that plays an important role in the patterning of the gynoecium is the phytohormone auxin. It has been proposed that a gradient of auxin established during carpel development controls tissue patterning along the apical–basal axis (Nemhauser *et al.*, 2000). From severe defects in apical–basal tissue distribution in *ett* mutants, the auxin response factor *ETT* has been proposed to be the principal integrator of auxin gradient information during apical–basal development in the ovary (Nemhauser *et al.*, 2000). In the same way, it is possible that auxin gradients also play important roles in the

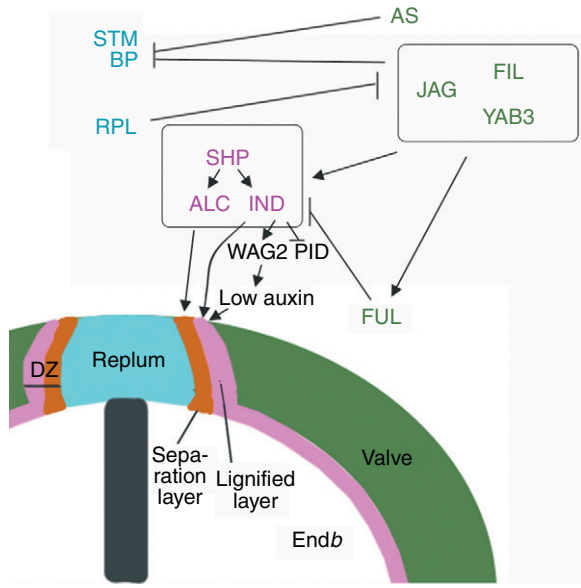

Fig. 5. A genetic model for medio-lateral pattening in the *Arabidopsis* gynoecium and the differentiation of fruit dehiscence zones.

establishment of abaxial–adaxial and medio-lateral polarity. We mentioned above that *ETT* and *ARF4* were involved in specifying abaxial fate, as seen from the "inside-out" gynoecium phenotype of the *ett arf4* double mutant. In addition, the inactivation of *YUCCA* (*YUC*) genes, which encode enzymes of the auxin biosynthesis pathway, or *PINOID* (*PID*), which encodes a regulator of auxin transport, transforms the gynoecium into a completely radial structure (Bennett *et al.*, 1995; Cheng *et al.*, 2006). Moreover, in plants in which polar auxin transport (PAT) is altered, the replum is expanded and the lateral valves are reduced (Bennett *et al.*, 1995; Nemhauser *et al.*, 2000). Very recently, it has also been demonstrated that a maximum of auxin is present in the replum, whereas a low level of this hormone is present in the valves (Sorefan *et al.*, 2009). Therefore, it seems that auxin, beyond its central role in the establishment of apical–basal polarity in the gynoecium, is also involved in patterning its abaxial–adaxial and medio-lateral axes.

D. DEVELOPMENT OF THE CARPEL LATERAL DOMAINS

The lateral domains of the gynoecial primordium give rise to the valves, which form the ovary wall, and the valve margins, which form at the valve/replum borders (Fig. 3). The valve margins later differentiate into the

dehiscence zones, where the *Arabidopis* fruit opens. *AG* has been proposed to specify valve identity, based on the phenotype of the *ap2 ag* double mutant, in which the carpeloid organs present in the first whorl of the flower do not show typical valve cell organisation (Bowman, 1991). Valve margin specification and dehiscence zone formation have been extensively studied over the last few years, and the major factors involved in this process (Fig. 5) have now been identified, as reviewed by Dinneny and Yanofsky (2005), Ferrandiz (2002) and Girin *et al.* (2009). Unlike *AG*, the closely related *SHP* genes direct valve margin identity. In the *shp1shp2* double mutant, the dehiscence zone fails to differentiate and the mature fruit does not open (Liljegren *et al.*, 2000). Similar phenotypes are observed in plants in which the *INDEHISCENT* (*IND*) or *ALCATRAZ* (*ALC*) genes, encoding bHLH transcription factors, have been inactivated (Liljegren *et al.*, 2004; Rajani and Sundaresan, 2001). *SHP* expression is restricted to the valve margins by the actions of the MADS box gene *FRUITFULL* (*FUL*) in the valve and of the homeodomain factor *RPL* in the replum (Ferrandiz *et al.*, 2000b; Gu *et al.*, 1998; Roeder *et al.*, 2003). Then, in a narrow domain between these tissues, which is composed of three to four cell layers, SHP activates *IND* and *ALC*, which are necessary for the formation of the dehiscence zone (Liljegren *et al.*, 2000, 2004; Rajani and Sundaresan, 2001). In *ful* mutants, *SHP*, *IND* and *ALC* are ectopically expressed in the valves and, as a result, small lignified cells, which are normally specific to the dehiscence zones, develop in these tissues. Consequently, *ful* fruits do not elongate, but instead break prematurely. In *rpl* mutants, the replum is reduced in width and the valve margins are expanded, as is expression of the corresponding valve margin genes. Based on genetic analyses, Dinneny *et al.* (2005) proposed a model in which the cooperative activity of FIL, YAB3 and JAG would activate the transcription of *FUL* and *SHP* genes in the valves and valve margins, respectively. According to this model, a high level of FIL/YAB/JAG activity would turn on *FUL* expression in the valves, while the activation of *SHP* in the valve margins would require a weaker activity of this same module. This model fits nicely with the observed phenotypes, but whether the activation of *FUL* and *SHP* lies in differences in YAB/JAG levels in different domains or in some other type of molecular interaction remains to be seen. In addition, it was shown in the replum that the homeodomain protein RPL represses the activity of the FIL/YAB/JAG module, therefore preventing SHP activation.

Auxin is also involved in the development of the lateral domains of the carpel. Sorefan *et al.* (2009) recently showed that a local auxin minimum is required for the differentiation of the dehiscence zones in the *Arabidopsis* fruit. These authors demonstrated that IND is involved in creating this auxin minimum by controlling the direction of auxin transport via PIN relocation.

SPT, which has been proposed to mediate auxin signalling in apical–basal development, is also expressed in the dehiscence zones of the developing fruit (Heisler *et al.*, 2001), where it appears to be regulated by IND (Groszmann *et al.*, 2008). Though *SPT* expression suggests this factor to play a role in the dehiscence zones, no such function has yet been discovered, raising the possibility of redundancy with other factors.

E. DEVELOPMENT OF CARPEL MARGINAL TISSUES

The carpel marginal tissues derive from the medial region of the gynoecial primordium, localised at the boundary between the two fused carpels (Fig. 3). A meristematic medial ridge of tissues develops along the adaxial side of the gynoecial tube and this gives rise to the placenta, septum, transmitting tract, style and stigma (Bowman *et al.*, 1999). As discussed above, meristem-associated genes are expressed in this region and seem to be involved in early marginal tissue development.

Many genes have been shown to play a role in marginal tissue development (Fig. 6). Most of these share functional redundancy, and strong phenotypes in carpel development can thus frequently only be seen in multiple mutants.

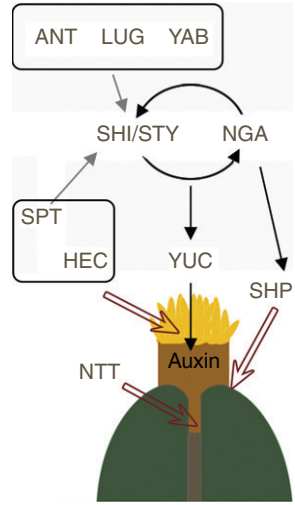

Fig. 6. Genetic networks directing marginal tissue differentiation at the apex of the *Arabidopsis* gynoecium. Black arrows indicate experimentally supported interactions, while grey arrows indicate possible interactions. Boxes indicate putative protein complexes.

Among these genes, *ANT*, *LEUNIG* (*LUG*), *SEUSS* (*SEU*) and *FIL* are the major players in the development of carpel marginal tissues. Whereas the single mutants corresponding to these genes display relatively mild effects on the medial gynoecial domain, the gynoecium of double mutant combinations almost completely lack marginal tissues. In *lug* mutants, the gynoecium is partially unfused at the apex and also presents defects in septum and ovule development (Chen *et al.*, 2000; Liu and Meyerowitz, 1995). The pistils of *ant* and *seu* single mutants present similar, though weaker, defects to those of *lug* mutants (Franks *et al.*, 2002; Krizek *et al.*, 2000). Strikingly, in the *ant lug* double mutant, the inner whorl of the flower consists of unfused valves-like structures which have style cells at their tips, but lack placenta, ovule, septum and stigma tissues (Liu *et al.*, 2000). Similar carpel growth defects have been described in *lug seu*, *ant seu*, *fil ant* and *fil lug* double mutants (Azhakanandam *et al.*, 2008; Chen *et al.*, 1999; Franks *et al.*, 2002; Nole-Wilson and Krizek, 2006). These four genes have been shown to repress *AG* in the first two whorls of the flower (Chen *et al.*, 1999; Franks *et al.*, 2002; Krizek *et al.*, 2000; Liu and Meyerowitz, 1995). *LUG* and *SEU* encode transcriptional co-repressors that can interact to form a regulatory complex (Elliott *et al.*, 1996; Siegfried *et al.*, 1999; Sridhar *et al.*, 2004). From all these information, it has been suggested that ANT, LUG, SEU and FIL form a multimeric complex involved in marginal tissue development in the gynoecium (Azhakanandam *et al.*, 2008; Nole-Wilson and Krizek, 2006).

SPT, which has been mentioned above for its role in other aspects of gynoecium morphogenesis, is also a major factor in the formation of carpel marginal tissues. In *spt* mutants, the stigma and the style are reduced, the septum is distorted and the transmitting tract is not properly formed (Alvarez and Smyth, 1999). As this phenotype can be partially rescued by the chemical inhibition of PAT, it has been proposed that *SPT* mediates auxin signalling (Heisler *et al.*, 2001). Some other factors have also been specifically related to transmitting tissue development. The three closely related bHLH genes *HECATE1* (*HEC1*), *HEC2* and *HEC3* have been shown to redundantly specify stigma and transmitting tract development (Gremski *et al.*, 2007). HEC proteins are able to physically interact with SPT in yeast two hybrid assays, so it has been suggested that HEC and SPT function together in this process. Crawford *et al.* (2007) described the first gene specifically required for *Arabidopsis* transmitting tract development: *NO TRANSMITTING TRACT* (*NTT*). In *ntt* mutants, pollen tubes are unable to migrate efficiently due to the lack of transmitting tissues and fertility is consequently much reduced.

Another set of factors that promote marginal tissue development in the apical domain of the *Arabidopsis* gynoecium are the members of the SHI/STY gene family, which encode zinc-finger transcriptional activators.

STYLISH1 (*STY1*) is the only such factor showing a phenotype as a single mutant: *sty1* presents subtle defects in style development (Kuusk *et al.*, 2002), though this phenotype is gradually enhanced in combination with mutations in further SHI/STY family members. Accordingly, multiple SHI/STY mutants show radically reduced style and stigma tissues, an abnormal septum and an incomplete closure of the gynoecium apex. This phenotype is similar to that observed in *lug* mutants; moreover, it has been shown that the *lug* mutation is epistatic over *sty1*, and that *STY* expression is reduced in *lug* mutants. Thus, STY factors may act downstream of *LUG* to mediate marginal tissue formation (Kuusk *et al.*, 2006). STY1 activity has been linked to auxin as STY1 is a direct activator of *YUC4*- an auxin biosynthesis gene (Eklund *et al.*, 2010; Sohlberg *et al.*, 2006; see Section IV.F for further details). Recently, the small NGATHA (NGA) gene family, encoding B3-domain transcription factors, has been described. The *NGA* genes are redundantly involved in style development (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009). In the quadruple *nga* mutant, the style and stigma are completely absent, similar to *shi/sty* multiple mutants. In addition to showing similar mutant phenotypes, *NGA* and *STY* genes share similar expression patterns and it has been shown that *YUC2* and *YUC4* expression is radically reduced in the *nga* quadruple mutant. Moreover, simultaneous over-expression of *NGA* and *STY* transforms the ovary into style tissue. Accordingly, Trigueros et al. (2009) have suggested that *NGA* and *STY* may act cooperatively in style development, at least partially by promoting YUC-mediated auxin biosynthesis in the apical region of the gynoecium. While the precise regulatory hierarchy of NGA and SHI/STY factors has not yet been elucidated, several pieces of evidence point to a positive feedback loop acting between *STY* and *NGA* (Alvarez *et al.*, 2009; Trigueros *et al.*, 2009).

The *SHP* genes have been mentioned above for their important role in carpel identity and valve margin differentiation. In addition however, a recent study has indicated that these genes to be also involved in style and stigma development (Colombo *et al.*, 2010). Gynoecia of the *ant crc shp1 shp2* quadruple mutant almost completely lack marginal tissues, resembling those of *ant lug* or *fil ant* double mutants. Surprisingly however, *STY* and *NGA* expression was not reduced in this quadruple mutant, while *SHP* expression was absent in the apical part of the carpel in *nga* quadruple mutants, and expanded when *NGA* genes were over-expressed (Alvarez *et al.*, 2009; Colombo *et al.*, 2010). These data suggest that *NGA* activity acts upstream of *SHP* expression in the style. Thus, *SHP* genes seem to act in a complicated and not fully understood regulatory network that controls most of the events which direct patterning and tissue specification in the gynoecium.

F. ESTABLISHMENT OF APICAL–BASAL POLARITY IN THE GYNOECIUM

Auxin is clearly one of the major morphogens involved in apical–basal patterning of the gynoecium: mutations in genes such as *PIN* and *PID*, which mediate PAT, result in defects in apical–basal patterning, producing enlarged apical and basal regions concomitantly with drastically reduced ovaries (Bennett *et al.*, 1995; Okada *et al.*, 1991). Similar defects are also found in mutants which lack functional auxin response factors MONOPTEROS (MP/ARF5) or ETT/ARF3 (Przemeck *et al.*, 1996; Sessions and Zambryski, 1995, Sessions *et al.*, 1997). Nemhauser *et al.* (2000) have accordingly proposed that an auxin gradient spans the gynoecial primordium and controls apical–basal patterning. Their model predicts maximum auxin levels at the apex, which induce the differentiation and proliferation of the stigma and style, and intermediate and low auxin levels lower down the gynocium, which, respectively, specify the development of the ovary and gynophore (Fig. 7). In accordance with this model, the regulators

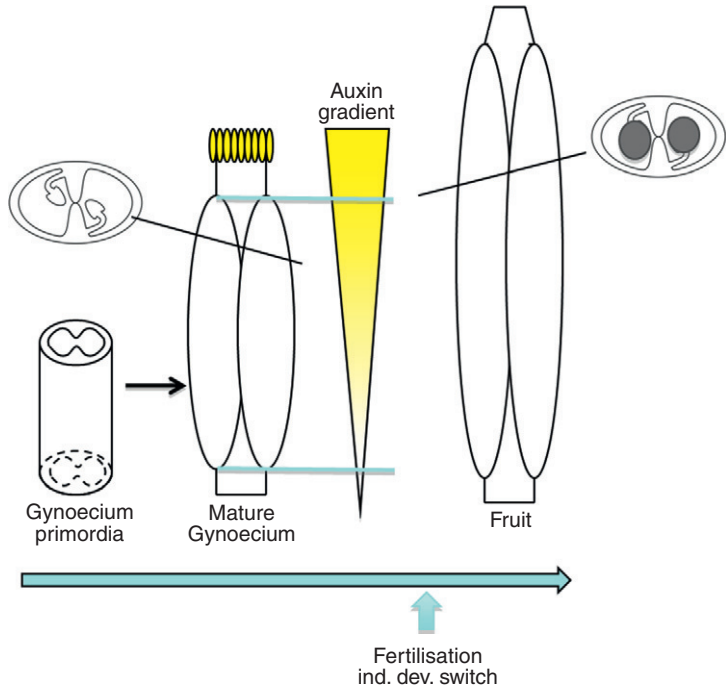

Fig. 7. Apical–basal patterning in the *Arabidopsis* gynoecium. An apical–basal auxin gradient has been suggested to participate in the apical–basal patterning of the gyneocium by positioning the borders between the apical style and stigma, the centrally placed ovary, and the short stem or gynophores at the base. After fertilisation, the ovary continues to elongate.

of auxin biosynthesis *SHI/STY* and *NGA*, in addition to their downstream targets, the auxin biosynthesis genes *YUC4* and *YUC2*, have recently been shown to be expressed in the apex of the young gynoecium (Alvarez *et al.*, 2009; Eklund *et al.*, 2010; Kuusk *et al.*, 2002, 2006; Sohlberg *et al.*, 2006; Trigueros *et al.*, 2009) as is also the auxin response reporter construct *ProDR5:GFP* (Aloni *et al.*, 2003; Benkova *et al.*, 2003). According to this model, auxin is trapped apically at the biosynthesis site under condition in which PAT is reduced, causing shifts in the boundaries between the different tissues which form along the apical–basal axis (Fig. 7). Interestingly, PAT inhibition, as well as over-expression of the auxin biosynthesis activator STY1, can restore style and stigma proliferation in mutant lines including: *lug, seu, ant*, *sty1*, *spt, crc* and *jag*, which are affected in the development of apical tissues that are derived from marginal regions of the gynoecium. These observations suggest that auxin may act downstream of, or in parallel to, corresponding apical tissue-promoting factors during style and stigma development (Chen *et al.*, 2000; Nemhauser *et al.*, 2000; Sohlberg *et al.*, 2006; Staldal and Sundberg, 2009; Staldal *et al.*, 2008). An exception to this is the *nga* quadruple mutant, suggesting *NGA* genes to act not only upstream but also downstream of auxin (Alvarez *et al.*, 2009).

The repression of the apical programme in the zone of the gynoecium corresponding to intermediate auxin levels may be required for the specification of the ovary. It has been suggested that ovary size may be controlled via a specific response of ETT/ARF3 to intermediate auxin levels (Heisler *et al.*, 2001; Sessions and Zambryski, 1995; Sessions *et al.*, 1997). As ETT/ARF3 represses the activity of *SPT* and the *HEC* genes, which promote stigma, style and transmitting tract differentiation (Alvarez and Smyth, 1999; Gremski *et al.*, 2007; Heisler *et al.*, 2001), ETT activity in the intermediate zone locally represses the apical developmental programme. Gynophore development may be restricted to the basal end of the gynoecium by the activity of the zinc-finger protein KNUCKLES (KNU), via the establishment or maintenance of a tissue boundary at this location (Payne *et al.*, 2004).

Though the auxin gradient model can be used to explain apical–basal patterning in the gynoecium, there is at presence no direct evidence of the existence of such a gradient other than the presence of an auxin maximum at the gynoecium apex. Auxin level measurements along the length of the developing gynoecial cylinder have not yet been made, and so the hypothesised intermediate and low auxin levels in the ovary and gynophore have yet to be demonstrated. In addition, it has recently been suggested that the basal end of the gynoecium may be distinguished from the ovary by an opposing cytokinin gradient (Ostergaard, 2009). We also need to know more about how a potential auxin gradient could be interpreted. Lines carrying

mutations in some of the genes involved in the promotion of apical tissues, such as *LUG*, *SEU* and *STY*, are hyper-responsive to the chemical or genetic inhibition of PAT (Pfluger and Zambryski, 2004; Sohlberg *et al.*, 2006; Staldal *et al.*, 2008), as are auxin biosynthesis mutants (*yuc1*, *yuc4*) and auxin response mutants (*axr1-3*, *ett/arf3* and *tir1*) (Cheng *et al.*, 2007, Nemhauser *et al.*, 2000, Staldal *et al.*, 2008), suggesting that this group of genes could promote either auxin gradients or response pathways. Conversely, *jag* and *ant* mutants respond to PAT reductions to the same extent as wild type (Staldal and Sundberg, 2009), suggesting that JAG and ANT may not be involved in the establishment or responses to the hypothesised auxin gradient. Interestingly, *spt*, *nga* and *crc* mutants are less sensitive to PAT inhibition (Alvarez *et al.*, 2009; Nemhauser *et al.*, 2000; Staldal *et al.*, 2008), which may suggest that these genes could participate in the modulation of PAT or in the sensing of parts of the auxin gradient.

G. POST-FERTILISATION CARPEL GROWTH

Upon fertilisation, gynoecium and ovule developmental programmes are switched to those of fruit and seed development (Fig. 7). In *Arabidopsis*, fruit development is characterised by a dramatic elongation of the ovary, concomitant with the differentiation of specific tissues along the carpel margins. Unfertilised gynoecia fail to elongate and develop seeds, and will eventually undergo senescence. Interestingly, the switch to fruit development after fertilisation appears to rely on a hormone-induced signal evoked in the fertilised ovules, and some data suggest that auxin signalling participates in this process (Dorcey *et al.*, 2009; Goetz *et al.*, 2006; Vivian-Smith *et al.*, 2001). One piece of evidence supporting this suggestion is that a knockout of the auxin response factor *ARF8* gene results in parthenocarpy, or fertilisation-independent fruit development (Goetz *et al.*, 2006; Vivian-Smith *et al.*, 2001).

The major external parts of the *Arabidopsis* ovary comprise the pod walls, the replum, which extends along the length of the fruit, and the carpel margins, which form at the carpel/replum border where fruit opening will occur. During fruit development, the carpel margins differentiate into narrow strips consisting of a separation layer and a lignified layer, both of which contribute to the process of fruit opening (see Section IV.E). The key regulators of valve margin specification have been identified (Ferrandiz *et al.*, 2000a; Liljegren *et al.*, 2004; Roeder *et al.*, 2003), and it was recently shown that a local auxin minimum, generated by the valve margin identity factor IND, is required for separation layer development (Figs. 4 and 5; Sorefan *et al.*, 2009). Thus, both auxin maxima and minima appear to contribute to the activation of specific developmental programmes during fruit development.

V. FLORAL MERISTEM TERMINATION IN THE CENTRAL ZONE OF THE *ARABIDOPSIS* GYNOECIUM

At the time when carpel primordia are initiated, another essential process takes place in the centre of the *Arabidopsis* flower, in a domain that will later correspond to the base of the gynoecium. Stem cells in this domain, which had previously been maintained within the flower bud, and whose divisions had generated the different floral organs, cease to be maintained as the carpel primordial form. This disruption of stem cell maintenance makes the flower determinate and assures its future fertility by blocking the development of floral organs, in the place of ovules, within the gynoecium.

A. *AGAMOUS* (*AG*), THE MAIN GENE RESPONSIBLE FOR CARPEL DEVELOPMENT, ALSO PLAYS A CENTRAL ROLE IN FLORAL MERISTEM TERMINATION

AG function is not restricted to the control of stamen and carpel identity. It also promotes floral meristem termination (Fig. 8): in strong *ag* mutants (*ag-1* to *ag-3*), stamens are transformed into petals and carpels are replaced by a new flower bud, which turns into a new, abnormal flower (Bowman *et al.*, 1991b, Yanofsky *et al.*, 1990). Stem cells are thus indefinitely maintained within the FM and allow for the endless production of floral organs. Interestingly, FM termination is the most sensitive role of AG: weaker *ag* alleles (e.g. *ag-4* and *AG-Met-205*) trigger a strong loss of FM termination, but fewer organ identity defects (Sieburth *et al.*, 1995), while indeterminacy is the first phenotypic flaw associated with reduced levels of AG (Chuang and Meyerowitz, 2000; Mizukami and Ma, 1995).

A delay or loss of FM termination has also been observed in various mutant backgrounds or transgenic plants (Alvarez and Smyth, 1999; Bowman *et al.*, 1992; Chen *et al.*, 2004; Clark *et al.*, 1993, 1995; Das *et al.*, 2009; Fletcher, 2001; Jacobsen *et al.*, 1999; Kayes and Clark, 1998; Liu *et al.*, 2010; Maier *et al.*, 2009; Payne *et al.*, 2004; Prunet *et al.*, 2008; Schultz *et al.*, 1991; Sun *et al.*, 2009; Zhao *et al.*, 2007). However, unlike *AG*, none of the genes corresponding to these mutations, with the notable exception of *KNU*, are strictly required for the arrest of stem cell maintenance within the FM. Moreover, most of these genes appear to act upstream of *AG*, and thus control FM termination through this factor. For example, mutations to *ULT1*, *CLAVATA1* (*CLV1*) or *PERIANTHIA* (*PAN*) can trigger a defect in *AG* expression (Clark *et al.*, 1993; Das *et al.*, 2009; Fletcher, 2001; Maier *et al.*, 2009). *DICER-LIKE1/CARPEL FACTORY* (*DCL1/CAF*) and *HUA*

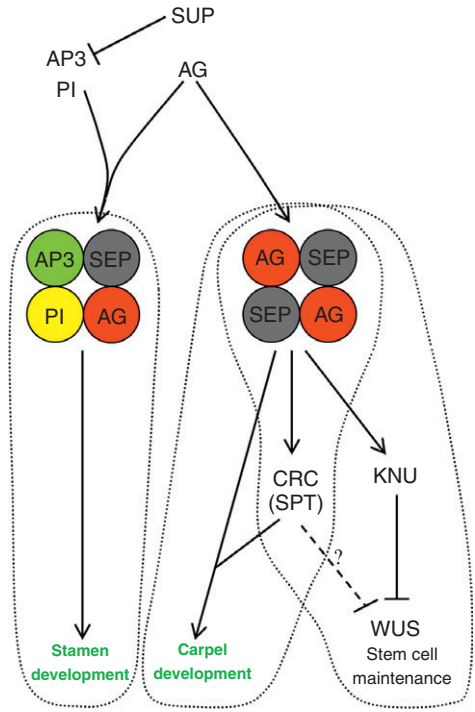

Fig. 8. Genetic pathways controlling floral meristem (FM) termination. AG is the main switch towards FM termination, but triggers different developmental programmes depending on its protein partners: when AG interacts with B-class proteins AP3 and PI, it promotes stamen development, and without B-class proteins it promotes both carpel development and FM termination. SUP, which excludes B-class gene expression from the fourth whorl, promotes these two latter functions. In whorl 4, AG activates several targets, among which *KNU* plays a central role in switching off *WUS,* and thus stem cell termination. *CRC* and *SPT*, acting downstream of AG, also contribute stem cell termination, showing this process to involve signalling by the developing carpels to the FM.

ENHANCER1 (*HEN1*) encode proteins that are required for the proper accumulation of *miR172* (Chen *et al.*, 2002; Jacobsen *et al.*, 1999; Park *et al.*, 2002), which promotes *AG* expression by down-regulating the *AG* repressor *AP2* (Aukerman and Sakai, 2003; Chen *et al.*, 2002, 2004). Other proteins that participate in the control of FM termination control *AG* expression at the post-transcriptional level: HUA1, HUA2, HEN2 and HEN4 are required for proper splicing of *AG* pre-mRNA (Cheng *et al.*, 2003), while the interaction of AG with SEP proteins is necessary for AG to perform its function (Ditta *et al.*, 2004; Goto *et al.*, 2001; Pelaz *et al.*, 2001).

B. THE B-CLASS GENES *APETALA3* (*AP3*) AND *PISTILLATA* (*PI*) PROMOTE THE MALE DEVELOPMENTAL PROGRAMME AND ANTAGONISE FM TERMINATION

The various functions of AG rely on its interactions with different protein partners. In the third whorl, AG participates in a protein complex with AP3 and PISTILLATA (PI) which promotes stamen identity (Fig. 8). AP3 and PI are excluded from the fourth whorl and cannot therefore interact there with AG, and this results in carpel identity and FM termination (Goto *et al.*, 2001; Krizek and Fletcher, 2005; Theissen, 2001; Theissen and Saedler, 2001). Indeed, the loss of function of *AP3* or *PI* results in overdeterminate flowers with stamens transformed into carpels, and a strongly reduced number of floral organs within whorls three and four, compared to wild type (Fig. 8; Bowman *et al.*, 1991b). Overdeterminacy is even more striking in *Antirrhinum majus* plants which are mutant for *DEFICIENS* or *GLOBOSA*: the respective orthologues of *AP3* and *PI*, the flowers of which entirely lack a fourth whorl (Sommer *et al.*, 1990; Trobner *et al.*, 1992). Conversely, the over-expression of *AP3* alone (*p35S::AP3*) or together with *PI* (*p35S::AP3/PI*) and the over-expression of their activator *UFO* (*p35S::UFO*), delays FM termination: flowers of *p35S::AP3*, *p35S::AP3/PI* and *p35S::UFO* plants exhibit several extra whorls of stamens, while carpels in these plants are often staminoid or absent (Jack *et al.*, 1994; Krizek and Meyerowitz, 1996; Lee *et al.*, 1997). B-class genes thus appear to antagonise AG's fourth whorl functions, including the arrest of stem cell maintenance.

Plants that are mutant for the C2H2 zinc-finger transcription repressor *SUPERMAN* (*SUP*) exhibit a phenotype similar to that of plants over-expressing B-class genes: *sup* mutant flowers exhibit extra stamens which usually develop at the expense of carpels (Bowman *et al.*, 1992; Schultz *et al.*, 1991), although some alleles also trigger a moderate increase in carpel number (Jacobsen and Meyerowitz, 1997; Rohde *et al.*, 1999). The extra stamens in *sup* mutants are indeed associated with an expansion of B-class gene expression within the fourth whorl (Bowman *et al.*, 1992; Goto and Meyerowitz, 1994). Two models have been proposed to explain the origin of the extra organs in *sup* mutant flowers. One of these proposes the expansion of B-class gene expression observed in *sup* flowers to be responsible for prolonging the developmental state of the FM which normally precedes carpel initiation and stem cell termination. According to this model, SUP thus promotes flower determinacy indirectly (Bowman *et al.*, 1992; Schultz *et al.*, 1991). Conversely, the other alternative model proposes SUP to directly repress cell division within the inner part of the third whorl, in which it is expressed together with B-class genes (Sakai *et al.*, 1995, 2000). According to this second model, extra stamens would then be generated by

increased cell division in the inner part of the third whorl, and the expansion of B-class gene expression would thus be a consequence, rather than a cause, of this increased rate of division. More recently, ectopic expression, using various promoters of *SUP* and its orthologues in several species, has shown these genes to be able to repress both the expression of B-class genes and cell proliferation (Bereterbide *et al.*, 2001; Hiratsu *et al.*, 2002; Nakagawa *et al.*, 2004; Nandi *et al.*, 2000; Yun *et al.*, 2002). Neither model can thus be excluded so far, though ectopic expression of B-class genes is likely to at least participate in the formation of extra organs in *sup* mutant flowers, as reviewed in more detail by Prunet *et al.* (2009).

Several pieces of evidence suggest that stamen and carpel identity, as well as FM termination, are very sensitive to the ratio of AG to AP3/PI proteins. Firstly, increased ectopic expression of B-class genes correlates with an increased indeterminacy phenotype, as shown by the greater number of stamens in *p35S::AP3*, *sup-1* and *p35S::AP3 sup-1* flowers (Jack *et al.*, 1994) and *p35S::AP3*, *sup-1*, *p35S::AP3/PI* and *p35S::AP3/PI sup-1* flowers (Krizek and Meyerowitz, 1996). Similarly, a reduction in the dose or activity of AG strongly enhances the indeterminacy phenotype of *sup*: *ag-1/AG sup-1* and *ag-4 sup-1* flowers are fully indeterminate (Prunet *et al.*, 2008; Schultz *et al.*, 1991). Conversely, increased expression of *AG* within the third whorl, in which *AG* is normally expressed together with *AP3* and *PI*, is sufficient to trigger a partial transformation of stamens into carpels (Lohmann *et al.*, 2001). Also, stamen identity is less sensitive to a reduced dose of AG than its carpel identity or FM termination (Chuang and Meyerowitz, 2000; Mizukami and Ma, 1995). Given that AG participates in several different transcription factors complexes, together with AP3 and PI in whorl 3 and without them in whorl 4, the importance of the relative dose of AG to AP3/PI hints that B-class genes may oppose AG's fourth whorl functions, including FM termination, by competing with AG in the formation of these complexes.

C. *CRABS CLAW* (*CRC*) AND *SPATULA* (*SPT*), TWO GENES INVOLVED IN CARPEL DEVELOPMENT, ALSO PARTICIPATE TO FM TERMINATION

AG is not the only gene involved in the female developmental programme to promote FM termination. *CRC* and, to a lesser extent, *SPT* encode two other transcription factors that control carpel growth, polarity and congenital fusion, and which also participate in floral determinacy (Fig. 8; Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Heisler *et al.*, 2001). Flowers of *crc* and *spt* single mutants are normally determinate, but those of the *crc spt* double mutants, and more often those of *crc AG/ag-1* plants, are indeterminate and possess extra whorls of stamens, and secondary carpels

that developing within the gyoecium (Alvarez and Smyth, 1999). A strong loss of FM termination is also seen when *crc* is combined with mutations in genes such as *ULT1*, *RBL* or *SQN*, which do not show an extensive indeterminacy phenotype as single mutants (Berardini *et al.*, 2001; Fletcher, 2001; Prunet *et al.*, 2008). Interestingly, in several other angiosperm species, a loss of *CRC* function is sufficient to cause strong floral indeterminacy (Lee *et al.*, 2005; Nagasawa *et al.*, 2003; Orashakova *et al.*, 2009; Yamaguchi *et al.*, 2004), suggesting that *CRC*'s ancestral role in FM termination may be more important than its current role in that process in *Arabidopsis*.

AG promotes the expression of *CRC* and *SPT*, which occurs through direct transcriptional activation in the case of *CRC* (Bowman and Smyth, 1999; Gomez-Mena *et al.*, 2005; Heisler *et al.*, 2001). CRC and SPT may thus mediate a proportion AG's functions in carpel development and flower determinacy. Most genes known to control this latter process act upstream of *AG*, as described above, but no positive feedback loop between *CRC*, *SPT* and *AG* has yet been shown. Indeed, the function of both CRC and SPT is at least partly independent of *AG* (Bowman and Smyth, 1999; Heisler *et al.*, 2001). How CRC and SPT influence stem cell termination is thus currently unclear. However, the fact that genes controlling female development other than *AG* share its role in stem cell termination confirms the close link between these two processes. It is particularly interesting to note that *CRC*'s role in floral determinacy is non-cell-autonomous: *CRC* is expressed in the emerging carpel primordial, but not at the base of the gynoecium where indeterminacy phenotypes first become apparent (Bowman and Smyth, 1999). This observation clearly suggests primordia to signal back to the FM to oppose stem cell maintenance.

D. AG IS REQUIRED IN THE CENTRE OF THE FM TO TRIGGER FM TERMINATION

AG's three functions, in stamen and carpel specification and in FM termination, are dose-dependent (Chuang and Meyerowitz, 2000; Mizukami and Ma, 1995), but can also be separated on a spatial basis. *pAP3::AG ag-3* plants express functional *AG* in the third whorl, but not in the fourth, and produce completely indeterminate flowers which lack carpels, but have normal stamens (Jack *et al.*, 1997). AG is therefore required specifically in the fourth whorl to promote carpel development and FM termination. While this phenotype tends to confirm the close association between female development and stem cell termination, these two programmes also can be spatially separated. Indeed, numerous mutants suggest that FM termination requires the expression of *AG* in an even more restricted domain within the

fourth whorl. In the flowers of *clv*, *ult1* and *pan* single mutants and of *crc sqn* and *crc ult1* double mutants, stamens and carpels are correctly specified, suggesting that AG is still active within whorls 3 and 4 of their flowers. However, AG fails to terminate stem cell maintenance in these mutants, which all exhibit a delay in or loss of flower determinacy (Clark *et al.*, 1993; Das *et al.*, 2009; Fletcher, 2001; Maier *et al.*, 2009; Prunet *et al.*, 2008). Indeed, depending on allelic strengths, the indeterminacy phenotype of these mutants is associated with a transient or persistent defect in *AG* transcription in an inner, intercarpellary domain within the fourth whorl (Clark *et al.*, 1993; Das *et al.*, 2009; Fletcher, 2001; Maier *et al.*, 2009; Prunet *et al.*, 2008). Interestingly, a similar defect in *AG* expression is seen in the flowers of plants over-expressing a modified version of *AG*'s repressor *AP2* which has been made resistant to *miR172* (*35S::AP2m3*): these flowers are fully indeterminate, with numerous supernumerary stamens (Chen *et al.*, 2004; Zhao *et al.*, 2007). AG is thus specifically required at the base of the gynoecium to promote FM termination.

E. TO TERMINATE STEM CELL MAINTENANCE, AG PROMOTES A SPECIFIC DEVELOPMENTAL PROGRAMME AT THE BASE OF THE GYNOECIUM

The subdomain of whorl 4 at the base of the gynoecium corresponds to the centre of the FM and contains the floral stem cells. AG disrupts the maintenance of these cells by switching off the expression of the stem cell-promoting gene *WUS* at the moment of emergence of the carpel primordial. Accordingly, *WUS* mRNA becomes undetectable at Stage 6 (Smyth *et al.*, 1990) of determinate, wild-type flower development (Mayer *et al.*, 1998), but persists until later stages in indeterminate, *ag* mutant flowers (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). Such a maintenance of *WUS* expression beyond Stage 6 has been described in most mutant or transgenic plants with indeterminate flowers (Carles *et al.*, 2004; Das *et al.*, 2009; Maier *et al.*, 2009; Prunet *et al.*, 2008; Schoof *et al.*, 2000; Sun *et al.*, 2009; Zhao *et al.*, 2007) and appears both necessary and sufficient to maintain stem cells within the flower, unlike another pro-meristematic gene, *STM* (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001).

AG is required in L2 cells, in which *WUS* is not expressed, to disrupt stem cell maintenance (Mayer *et al.*, 1998; Sieburth *et al.*, 1998), suggesting that, despite its role as a transcription factor, AG does not directly repress *WUS* transcription. Indeed, recent data suggest that KNU, a C2H2 zinc-finger protein (Payne *et al.*, 2004), is the main intermediate between AG and *WUS* (Fig. 8; Sun *et al.*, 2009). Accordingly, AG is required for the expression of *KNU*, and directly binds to its promoter, and in turn, KNU appears both

necessary and sufficient to turn off the expression of *WUS* and therefore stem cell maintenance within the flower. Interestingly, *KNU* expression starts at Stage 6 and then becomes restricted to precisely the domain at the base of the gynoecium in which AG expression is specifically required for FM termination, as described above (Payne *et al.*, 2004; Sun *et al.*, 2009). Later on, *KNU* expression is also detected in anthers, though it remains very strong at the basis of the gynoecium. The importance of KNU in FM termination, together with its expression pattern, confirms AG to activate a specific developmental programme in the centre of the FM, resulting in the disruption of stem cell maintenance at Stage 6 of flower development.

F. CONCLUSIONS

The data discussed above clearly demonstrate FM termination to be closely associated with the development of the gynoecium. FM termination relies on a genetic network centred on *AG*, which is responsible for both male and female developmental programmes. However, AG is able to promote carpel development and stem cell termination only if the male-promoting B-class genes are excluded from the centre of the flower. The AG-induced disruption of stem cell maintenance mainly depends on a cascade of transcription factors in a subdomain of whorl 4, at the base of the gynoecium: AG activates *KNU* specifically at this location and KNU in turn switches off the expression of *WUS*. However, FM termination also involves signals from the developing carpels: another target of AG, *CRC*, is expressed only in carpel primordia, and not at the base of the gynoecium, but nonetheless participates by an as yet unknown mechanism in FM termination.

VI. CARPEL DIVERSIFICATION IN THE ANGIOSPERMS

A. A PHYLOGENETIC BACKGROUND

To reconstruct the different paths that carpel evolution has taken in distinct angiosperm groups, it is first necessary to gain some insight into phylogenetic relationships within the angiosperms. Several independent studies published around 10 years ago (reviewed by Kuzoff and Gasser, 2000) provided an early outline of angiosperm phylogeny, and this has been expanded and updated ever since by the Angiosperm Phylogeny Group (Bremer *et al.*, 2009). These phylogenetic studies indicate the angiosperms to form a monophyletic clade in which three extant groups, Amborellales, Nymphaeales and

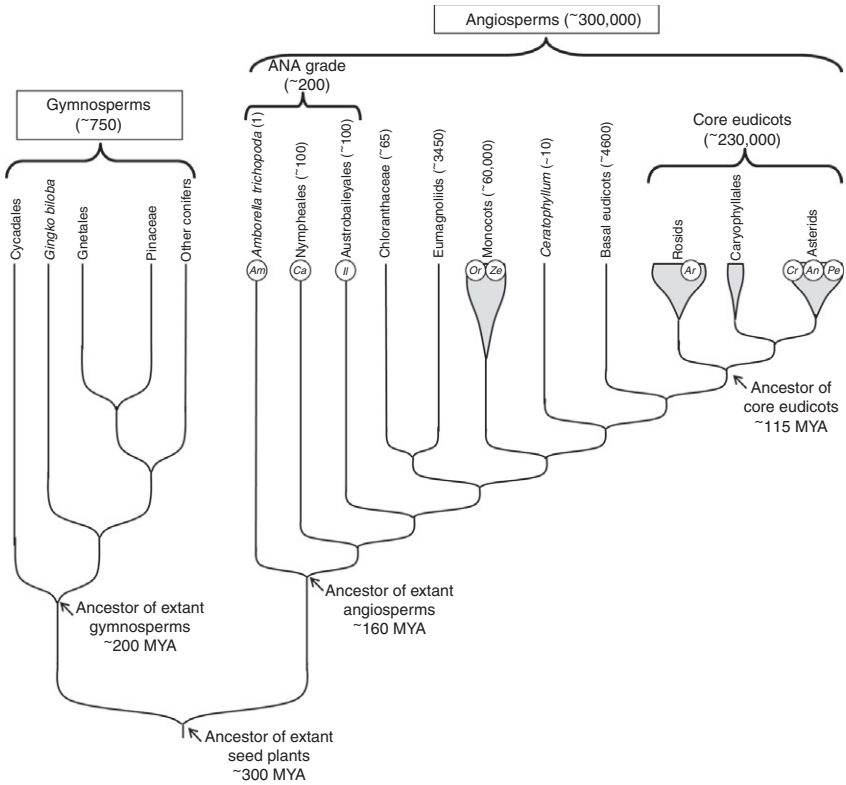

Fig. 9. Seed plant phylogeny. The numbers of species in major clades are given in parentheses, while approximate dates of divergence within the angiosperms, based on a calibration of the molecular clock using fossil data, are estimated from Davies *et al.* (2004). The positions of some of the taxa referred to in the text are indicated as follows: *Am*, *Amborella trichopoda*; *An*, *Antirrhinum majus*; *Ar*, *Arabidopsis thaliana*; *Ca*, *Cabomba*; *Cr*, *Catharanthus roseus*; *Il*, *Illicium*; *Ze*, *Zea mays*; *Pe*, *Petunia hybrida*; *Or*, *Oryza sativa*.

Austrobaileyales, collectively known as the ANA grade, diverged in a series of basal bifurcations to leave a remaining lineage from which all other extant angiosperms are descended (Fig. 9). Amborellales contains the single species *Amborella trichopoda*, a shrub endemic to the South Pacific island of New Caledonia. Nymphaeales contains the three families of herbaceous aquatic plants Nymphaeaceae, Cabombaceae, and Hydatellaceae, which include a total of around 100 species and Austrobaileyales contains Austrobaileyaceae, Schisandraceae (incorporating Illiciaceae) and Trimeniaceae, which are composed of a total of around 100 woody species. Following the divergence of the ANA lineages, the remaining angiosperm lineage later diversified to form the five further extant angiosperm groups of: eudicots, monocots,

eumagnoliids, Chloranthanceae and *Cerratophyllum*. Of these, the eudicots and monocots together account for over 95% of the estimated 300,000 or more extant angiosperm species.

The resolution of angiosperm phylogenetic relationships provides an excellent framework to analyse the processes through which carpel development has diversified throughout the flowering plants. However, a further finding of molecular phylogenetic studies provides less encouraging news for the analysis of carpel evolution. To understand how the carpel first appeared, it would be very useful to have available for study one or more living groups whose lineages had diverged from that of the flowering plants shortly before the origin of the carpel. However, molecular phylogenetic analyses indicate the remaining seed plants, or gymnosperms, to form a sister clade to the angiosperms (Fig. 9), meaning that no individual group of living gymnosperms appears to be more closely related to the angiosperms than any other. Molecular clock estimates suggest a date for the divergence of the angiosperm and gymnosperm lineages of around 300 MYA (Goremykin *et al.*, 1997; Savard *et al.*, 1994), whereas the earliest known angiosperm macrofossils date from around 125 MYA (Sun *et al.*, 1998). Thus, the extant flowering plants and gymnosperm lineages may have diverged up to 175 MYA before the origin of the flower and carpel.

As will be apparent from Section II, lack of evidence concerning the origin of the angiosperms has not prevented the construction of numerous hypotheses for the evolution of the flower and carpel. However, given the absence of a living, non-flowering, close relative of the flowering plants, it seems likely that a full understanding of the evolutionary origin of these structures will require the inclusion of fossil data. In this respect, further evidence from mesofossils may prove extremely valuable. These are small fossils, of up to a few millimetres in diameter, many of which are ''coalified'' specimens that appear to have been generated by forest fires. Numerous early angiosperm mesofossils containing considerable anatomical detail have already been discovered (Friis *et al.*, 2001, 2010) raising hopes that further discoveries may provide important new insights into early flower evolution (Frohlich and Chase, 2007).

B. CARPEL MORPHOLOGY AND FUNCTION IN ANA GRADE ANGIOSPERMS

Morphological comparisons of the three extant lineages of ANA grade angiosperms (Fig. 9) have enabled a number of conclusions to be made on the likely state of the gynoecium in the last common ancestor of the living flowering plants (Endress, 2001; Endress and Igersheim, 2000). The gynoecium in all extant ANA grade taxa is apocarpic, except Nymphaeaceae, which are syncarpic.

Apocarpy is thus present throughout Amborellales and Austrobaileyales, and is also the basal condition in Nymphaeales, clearly implying this to be an ancestral trait of the living angiosperms. The carpels of apocarpic ANA grade angiosperms are ascidiate (bottle-shaped) rather than plicate (folded), as in many later-diverging groups, implying the carpel to have been ascidiate in early angiosperms.

The carpels of many ANA grade species are not only separated from each other (apocarpic) but also incompletely closed by cellular structures at maturity, leaving a secretion-filled aperture or canal through which pollen tubes grow to bring about fertilisation (Fig. 10A). The only exceptions to this are *Illicium* (Austrobaileyales, Schisandraceae) and Nymphaeales, in which closure of the carpel margins occurs, at least in part, through post-genital cell divisions (Endress and Igersheim, 2000). Thus, comparative analysis of ANA grade species clearly indicates the basal condition of the angiosperms to have been carpels that were closed at the apex by substances secreted from their margins, rather than by post-genital cell division.

In some ANA grade angiosperms, the stigmatic surface is covered by multicellular protrusions, rather than by the unicellular papillae present in most later-diverging groups. This is the case in *Amborella* (Amborellales) and *Trimenia* (Austrobaileyales), both of which have stigmatic surfaces containing multicellular ridge-like structures, and in Cabombaceae and Hydatellaceae (Nymphaeales), which possess bi- or multicellular stigmatic hairs (Endress, 2005; Endress and Igersheim, 2000; Rudall *et al.*, 2007). However, all other Austrobaileyales and most Nymphaeaceae have stigmatic surfaces covered with unicellular papillae (Endress, 2001). Thus, multicellular protrusions on stigmatic surfaces are present in all three ANA grade lineages, leaving open the possibility that this may represent the ancestral condition in the angiosperms.

Self-incompatibility (SI) systems operating between carpel tissues and pollen grains are present in the Austrobaileyales species: *Illicium floridanum* (Thien *et al.*, 1983), *Austrobaileya scandens* (Prakash and Alexander, 1984) and *Trimenia moorei* (Bernhardt *et al.*, 2003). It is not yet clear whether these SI systems are homologous, which would make SI an ancestral feature of Austrobaileyales. *Amborella*, the only representative of the likely most basally diverging angiosperm lineage Amborellales, avoids inbreeding by dioecy, rather than through an SI mechanism. However, male *Amborella* flowers contain a structure which may be a relictual gynoecium, and female flowers of this species contain staminodes, or non-functional stamens (Endress, 2001), strongly suggesting *Amborella* to be descended from a bisexual ancestor. Hydatellaceae (Nymphaeales) are either dioecious or possess bisexual reproductive units that may be derived from unisexual flowers (Rudall *et al.*,

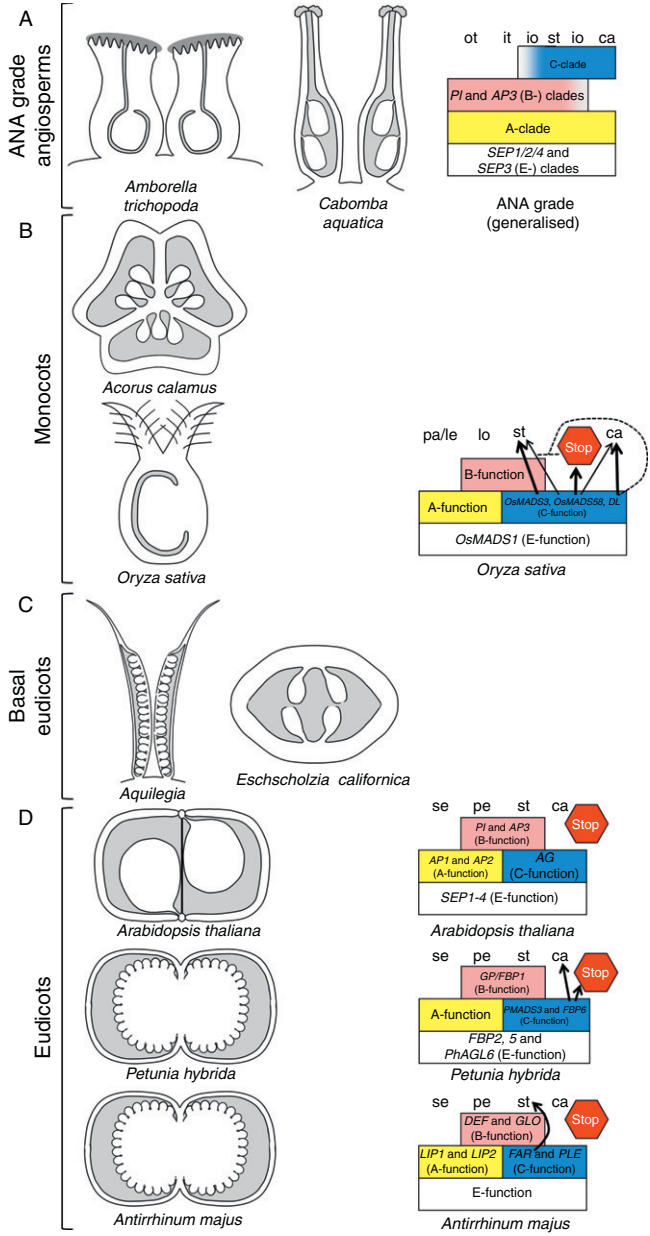

Fig. 10. Gynoecium structure and mechanisms specifying carpel development in the angiosperms. (A) Typical gynoecium structures in ANA grade angiosperms are apocarpic, as in *Amborella trichopoda* and *Cabomba aquatica* (represented schematically in longitudinal section), with carpels that contain one or a few ovules. A secretion-filled canal leading from the carpel apex to the ovary provides a route for pollen tube growth. C- and E-clade MADS box gene expression

2007), suggesting their possible descent from a monecious ancestor. Most other outbreeding members of the ANA grade are protogynous, while *Barclaya* and *Euryale* (Nymphaeceae) are partially cleistogamous and inbreeding (Endress, 2001). Protogyny, and possibly even SI, may thus have been present to promote outbreeding in the last common ancestor of the living angiosperms. By contrast, it seems clear that outbreeding by dioecy and inbreeding mechanisms, which are sporadically present in other ANA groups, have arisen secondarily.

Ovules in all ANA grade angiosperms are anatropous, except in *Amborella* and *Barclaya* (Nymphaeaceae) which have orthotropous ovules (Endress, 1986; Schneider, 1978). Of these, the *Amborella* ovule shows a residual curvature near its point of attachment, suggesting a previously anatropous structure (Endress and Igersheim, 2000). Thus, the ovule of the last common ancestor of the extant angiosperms was probably anatropous. Similar comparisons (Endress and Igersheim, 2000) indicate that this ancestral ovule was probably covered by two integuments and crassinucellar (containing a large nucellus tissue).

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correlates with carpel identity in ANA grade angiosperms, as in core eudicot models. MADS box gene expression is, however, less tightly controlled in ANA grade angiosperms (graded colouring of gene expression boundaries), frequently producing intermediate floral organ types in boundary zones. (B) The basal monocot *Acorus calamus* (shown in transverse section) has a trimerous, syncarpic gynoecium, which probably represents the pleisomorphic condition in monocots. However, the gynoecium in the model monocot *Oryza sativa* (rice; shown in longitudinal section), as in other Poaceae, is reduced to a single carpel containing one ovule. Paralogous C-clade MADS box genes show partial sub-functionalisation between the third and fourth whorls of the rice flower: *OsMADS3* plays a major role in stamen development, while *OsMADS58* functions principally in floral determinacy (thick arrows = major roles; thin arrows = minor roles; "STOP" = floral determinacy function). The YAB gene *DL* plays a major role in carpel development either directly (solid arrow) or indirectly by limiting the inner boundary of B-function gene expression (dashed arrow). (C) Basal eudicots of Ranunculales include both apocapic and syncarpic taxa, such as *Aquilegia* (shown in longitudinal section) and *Eschscholzia californica* (shown in transverse section), respectively, though apocarpy is believed to be the pleisiomorphic condition in eudicots. (D) The last common ancestor of the core eudicots is believed to have possessed a dimerous, syncarpic gynoecium, as is the case in its present-day model plant descendents *Arabidopsis thaliana*, *Petunia hybrida* and *Antirrhinum majus* (all shown in transverse section). However, the dimerous gynoecium of *Arabidopsis* is divided into two loculi by the secondary development of a false septum, whereas those of *Petunia* and *Antirrhimum* are divided by the common wall of their congenitally fused carpels. Carpel and stamen identity, and floral determinism ("STOP"), are controlled by MADS box C-clade genes, though sub-functionalisation has occurred to differently partition these roles among C-clade paralogues in *Arabidopsis* (*AG*), *Petunia* (*PMADS3* and *FBP6)* and *Antirrhinum* (*FAR* and *PLE*). (ca = carpels; io = intermediate organs; it = inner tepals; ot = outer tepals; pa/le = paleas/lemmas; pe = petals; se = sepals; st = stamens).

Double fertilisation, leading to the production of an embryo and a bi-parental endosperm, also appears to be a pleisiomorphic feature of the angiosperms. In the majority of flowering plants, the embryo sac arrangement is that of the *Polygonum* type, which contains seven cells of which the central cell is binucleate (Fahn, 1982). The two nuclei of this central cell combine with one sperm nucleus following fertilisation to generate a triploid endosperm. However, in the ANA grade taxa *Nuphar* and *Trithuria* (incorporating *Hydatella*) of Nymphaeales, and *Illicium* of Austobaileyales, the embryo sac contains only four cells, including a uninucleate central cell (Friedman, 2008; Williams and Friedman, 2002, 2004). Double fertilisation in these taxa generates an embryo and endosperm which are both diploid. However, the likely most basal ANA grade lineage, Amborellales, does not fit this pattern: the *Amborella* embryo sac contains eight cells, including a binucleate central cell that produces a triploid endosperm after fertilisation (Friedman, 2006). The *Amborella* embryo sac arrangement is thus more similar to that of *Polygonum* and the majority of later-diverging angiosperms than to other members of the ANA grade, though it contains one extra cell in its egg apparatus compared to the *Polygonum* type. The major difference in embryo sac arrangement between Amborellales and other ANA lineages renders the ancestral state of the embryo sac and endosperm ploidy ambiguous in the extant angiosperms. Interestingly, a perisperm is present in *Trithuria*, in addition to its diploid endosperm (Friedman, 2008). Similar to the endosperm, the perisperm is an embryo-nourishing tissue, though one which is derived exclusively from maternal cells. The presence of a perisperm is mainly associated with gymnosperms and the occurrence of such a tissue in *Trithuria* has been suggested to form a link with the hypothesised gymnosperm-like ancestor of the flowering plants (Friedman, 2008).

C. THE MOLECULAR CONTROL OF CARPEL DEVELOPMENT IN THE ANA GRADE

Phylogenetic analyses of MADS box genes in ANA grade angiosperms and gymnosperms suggest a duplication event to have taken place in the C-function lineage prior to the last common ancestor of the living flowering plants (Kim *et al.*, 2005). As a result of this duplication, the ancestors of the clade-defining genes *AG* from *A. thaliana*, and *FLORAL BINDING PROTEIN7* (*FBP7*) from *Petunia hybrida* (reviewed by Kramer *et al.*, 2004) were generated, and both of these lineages were thus present in the last common ancestor of the flowering plants. The *AG* clade contains angiosperm C-function genes, whereas the *FBP7* clade contains genes involved in ovule development in diverse later-diverging angiosperm groups (Angenent

et al., 1995; Colombo *et al.*, 1995; Dreni *et al.*, 2007). In addition to the C- and D-clades, two clades of *SEP* genes, encoding E-function MADS box proteins, have been found in basal angiosperms. The genes *SEP1*, *SEP2* and *SEP4* from *Arabidopsis* appear to be orthologous to one of these ANA grade *SEP* clades, while *SEP3* appears to be orthologous to the other (Zahn *et al.*, 2005).

The expression of C-function genes in ANA grade angiosperms is mostly limited to the third and fourth floral whorls, while the *SEP*-like E-function genes of these species are expressed in all floral organs (Fig. 10A; Kim *et al.*, 2005). These expression patterns closely resemble those of the corresponding genes in *Arabidopsis*, suggesting important elements of the control of carpel identity to have been conserved in both the *Arabidopsis* and ANA grade lineages throughout angiosperm evolution. Despite this broad conservation, Kim *et al.* (2005) noted some expression of C-function genes in the perianth organs of the ANA grade angiosperms *Amborella* (Amborellales) and *Illicium* (Austrobaileyales), in contrast to the expression patterns of C-function genes in model eudicots. However, as pointed out by these authors, such imprecise boundaries of gene expression may reflect the gradual transition of floral organ types that is apparent in ANA grade angiosperms, with intermediate organ types often present.

The expression patterns of several YAB transcription factors have also been analysed in ANA grade angiosperms. As discussed in Sections III and IV, studies of *Arabidopsis* indicate YAB factors to participate in the specification of abaxial cellular identity in lateral organs by defining the side of these organs that face away from the developmental axis. The YAB gene *AmbCRC* (Fourquin *et al.*, 2005), from the ANA grade angiosperm *Amborella*, shows a similar pattern of expression to that of its *Arabidopsis* orthologue *CRC* (Bowman and Smyth, 1999), suggesting these genes to have conserved a common developmental role in abaxial tissue specification in the carpel since the last common ancestor of the flowering plants. *INNER NO OUTER* (*INO*) represents a further YAB gene with a very specific role in female reproductive development. In *Arabidopsis*, *INO* is specifically expressed in the outer ovule integument and is necessary for the development of this structure (Villanneva *et al.*, 1999). A putative *INO* orthologue from the ANA grade angiosperm *Nymphaea alba* is specifically expressed in both ovule integuments and the suspensor (Yamada *et al.*, 2003), suggesting this gene to have functioned in integument development since the last common ancestor of the flowering plants. Thus, the carpel and outer integument, which are both pleisiomorphic features of the angiosperms, may have been associated with the expression of distinct YAB gene lineages throughout flowering plant evolution.

In general, the study of carpel development genes in ANA grade angiosperms has highlighted several instances of the broad conservation of gene function since the common ancestor of the last flowering plants. However, much work remains to be done in this field, as many families of transcription factors, and other regulatory proteins of known importance to carpel development, have yet to be analysed in ANA grade angiosperms. Furthermore, molecular studies of ANA grade angiosperms have, to date, relied principally on expression data to infer gene function. This approach may be tenable in the case of genes with highly characteristic and specific expression patterns, such as the MADS box and YAB genes discussed above. However, further substantial evolutionary-developmental advances in ANA grade angiosperms will surely require the development of methods for the direct study of gene function in these species.

D. CARPEL DEVELOPMENT IN MONOCOTS

The monocots form a monophyletic group of angiosperms numbering some 60,000 species. Phylogenetic studies have identified the small genus *Acorus* as sister to all other monocots, with the moderately large Alismatales as the next-earliest diverging group. *Acorus* contains a syncarpic gynoecium of three fused carpels (Fig. 10B), and comparison with Alismatales indicates a trimerous syncarpic arrangement to be the probable ancestral condition in the monocots (Igersheim *et al.*, 2001). From similar comparisons, the presence of more than two ovules per carpel can also be concluded as a probable ancestral feature of the monocots. Other characteristics of the gynoecium in the last common ancestor of the monocots are, however, more difficult to infer, partly due to differences between *Acorus* and other basal monocot lineages. For example, the carpels of *Acorus* are completely closed by post-genital cell divisions, whereas post-genital fusion is absent or partial in other early-diverging monocots (Igersheim *et al.*, 2001), with the exception of Tofeldiaceae (Alismatales). Similarly, ovules are pendent in *Acorus* but ascendant in other basal monocot groups, rendering the ancestral state of ovule orientation undetermined in monocots. Most molecular-developmental studies of monocots have, to date, been performed in two models from Poaceae (the grass family): *Oryza sativa* (rice) and *Zea mays* (maize). Poaceae are highly derived monocots in which the gynoecium, which contains a single ovule, appears also to have been reduced to a single carpel (discussed by Kellogg, 2001 and Rudall *et al.*, 2005).

Phylogenetic analyses of carpel development genes in Poaceae suggest at least one major duplication event to have occurred in the MADS box C-clade, prior to the separation of the rice and maize lineages, with a further additional

duplication in the maize lineage. Accordingly, the rice C-clade gene *OsMADS58* is orthologous to *ZAG1* in maize, while *OsMADS3* from rice is orthologous to both *ZMM2* and *ZMM23* (Yamaguchi *et al.*, 2006). The inactivation of *OsMADS58* in rice leads to defects in carpel development, though it does not completely eliminate carpels (Fig. 10B; Yamaguchi *et al.*, 2006). In addition to abnormal carpels, *osmads58* mutants show a reduced level of determinacy in the spikelet (or Poaceae-type flower). Whereas *OSMADS58* appears to act mainly in the fourth whorl, the inactivation of its paralogue *OsMADS3* has little or no effect on either carpel development or floral determinacy. Instead, stamen development is eliminated in *osmads3* mutants (Kang *et al.*, 1998; Yamaguchi *et al.*, 2006). Rice plants in which both *OsMADS3* and *OsMADS58* have been inactivated produce aberrant carpels similar to those of *osmads58* mutants, suggesting *OsMADS3* to make no specific contribution to carpel development (Yamaguchi *et al.*, 2006). In maize, *zag1* mutants show a defect in floral determinacy, indicating the functional conservation of *ZAG1* with its rice orthologue *OsMADS58*. It appears, therefore, that C-clade MADS box genes in the grass family have undergone significant sub-functionalisation following a monocot-specific gene duplication. The well-known C-clade functions of carpel development, stamen development and floral determinacy are thus shared in rice and maize in a whorl specific manner between two and three C-clade genes, respectively. The functions of a paralogous pair of D-clade MADS box genes, *OsMADS13* and *OsMADS21*, have also been investigated in rice (Dreni *et al.*, 2007). Of these, *OsMADS13* is ovule-specifically expressed, and its inactivation accordingly results in the ectopic conversion of ovules into internal carpelloid organs similar to the D-function knockout phenotype observed in *Petunia* (Angenent *et al.*, 1995; Colombo *et al.*, 1995). *OsMADS21*, by contrast, appears to make no significant contribution to the D-function and is expressed more widely than its paralogue in female reproductive tissues (Dreni *et al.*, 2007).

Carpels are entirely replaced by stamens in rice mutants in which the YAB gene *DL* has been inactivated (Fig. 10B; Yamaguchi *et al.*, 2004), which also causes the loss of the leaf mid-rib. In agreement with these functions, *DL* is expressed throughout the carpel anlagen (presumptive primordium), and in leaves, and its orthologues share similar expression patterns in other Poaceae (Ishikawa *et al.*, 2009). It is not yet clear whether carpel development in rice depends on *DL* expression *per se*, or whether *DL* is responsible for preventing B-function gene expression, and thus ectopic stamen development in the fourth floral whorl. *DL* is the rice orthologue of *CRC* in *Arabidopsis*, and its coding sequence, when expressed from the *CRC* promoter, is able to fully rescue *Arabidopsis crc* mutants (Fourquin *et al.*, 2007). It therefore seems that the functional differences between *DL* and *CRC* may reside principally outside

their coding sequences. *DL* expression is maintained in the carpels of rice plants in which both *OsMADS3* and *OsMAD58* have been inactivated (Yamaguchi *et al.*, 2006), demonstrating its action to be independent of these C-clade MADS box genes. This characteristic may represent a further difference between *DL* in rice and *CRC* in *Arabidopsis*, as the latter is known to be a direct target of the C-function transcription factor AG (Gomez-Mena *et al.*, 2005).

OsMADS1 from rice corresponds to the *LEAFY HULL STERILE1* locus, and groups within the *SEP1* clade of E-function MADS box genes which contain the genes *SEP1*, *SEP2* and *SEP4* from *Arabidopsis* (Zahn *et al.*, 2005). Outer whorl floral organs in *osmads1* loss-of-function mutants take on a leaf-like appearance, whereas the inner whorl floral organs of these mutants are partially converted to paleas and lemmas, which are normally found in the outer two whorls of the Poaceae spikelet (Agrawal *et al.*, 2005). These results suggest *OsMADS1* to contribute to the E-function in rice (Fig. 10B), while the functions of four remaining rice *SEP*-clade genes, *OsMADS5*, *OsMADS7*, *OsMADS8* and *RMADS217* (Zahn *et al.*, 2005), remain to be determined. Interestingly, the inactivation of *AGL6*-clade MADS box genes in both rice and maize has also recently been shown to generate E-function-like mutant phenotypes (Li *et al.*, 2010; Ohmori *et al.*, 2009; Thompson *et al.*, 2009). As discussed above, the *AGL6* and *SEP* clades occur in sister positions in MADS box phylogeny and similar *SEP*-like phenotypes for an *AGL6* orthologue have also recently been demonstrated in *Petunia* (Rijpkema *et al.*, 2009).

In general, carpel and ovule development in the highly derived Poaceae monocots seem to depend on the orthologues of regulatory genes that are known to play key roles in these processes in *Arabidopsis* and other eudicots. However, specific duplications have taken place in several MADS box lineages in Poaceae, including those of the C-, D- and E-functions, in some cases leading to sub-functionalisation events between paralogous genes. The precise limits of this sub-functionalisation have not yet been defined, which might explain the currently unclear contribution made by MADS box genes to the specification of carpel identity in Poaceae models. Further work is also required in other monocot groups, and perhaps particularly in basal monocots, to establish the extent to which conclusions arising from molecular data of Poaceae models can be applied outside this highly derived family.

E. CARPEL DEVELOPMENT IN BASAL EUDICOTS

The eudicots form the largest group of angiosperms and are characterised by the single synaptomorphic character of tricolpate pollen (containing three apertures in the pollen wall). Phylogenetic analyses clearly indicate

Ranunculales to be sister to all other eudicots (reviewed by Judd and Olmstead, 2004), and the comparison of this and other basally diverging groups provides insight into the structure of the carpel in the last common ancestor of the eudicots (Endress and Doyle, 2009; Endress and Igersheim, 1999). These comparative studies suggest the gynoecium in the ancestral eudicot to have been apocarpic, and to have contained no extragynoecial compitum: a system that allows for the exchange of pollen tubes between the carpels of an apocarpous gynoecium. Its ovules were probably pendant, though no firm conclusions can be made of the number of ovules in each carpel and on the mechanism of carpel closure (i.e. secretion vs. post-genital cell division) in the last common ancestor of the eudicots. The presumed ancestral features of the ancestral eudicot gynoecium are illustrated by the Ranunculaceae genus *Aquilegia* (Fig. 10C).

Numerous Ranunculales are being developed as molecular-genetic models, though most of the work on these species has so far been focussed on perianth structure, rather than on the gynoecium. An exception to this is *Eschscholzia californica* (California poppy) of Papaveraceae, though in contrast to the presumed state of the eudicot ancestor, this species possesses a gynoecium of two fused carpels (Fig. 10C). Placentation in *Eschscholzia* is parietal, with two rows of ovules forming in the single loculus of its syncarpic ovary. Molecular studies of *Eschscholzia*, with relevance to the gynoecium, have focussed on orthologues of *LFY/FLORICAULA* (*LFY/FLO*) and of *CRC*, which are, respectively, termed *EcFLO* and *EcCRC*. *EcFLO* expression was found to be absent from the centre of the *Eschscholzia* floral meristem (Becker *et al.*, 2005), which represents a considerable difference from *Arabidopsis*, in which *LFY* expression is required for the expression of the C-function gene *AG* in the inner floral whorls (Hong *et al.*, 2003). The functional analysis of *EcCRC* in *Eschscholzia* (Orashakova *et al.*, 2009), performed using virus-induced gene silencing (VIGS; Wege *et al.*, 2007), demonstrates a significant overlap of function of this gene with *CRC* in *Arabidopsis* (Alvarez and Smyth, 1999). Accordingly, both *CRC* and its *Eschscholzia* orthologue appear to contribute to abaxial tissue identity in the carpel, floral determinacy, and the growth of tissues that develop from the carpel margins. However, unlike its *Arabidopsis* orthologue, *EcCRC* appears to have acquired novel functions in placenta development and ovule initiation (Orashakova *et al.*, 2009). Further molecular analyses in basal eudicots have focussed on the MADS box family, and comparison of this family in basal and core (later-diverging) eudicots has provided evidence of numerous duplications in MADS box lineages of relevance to carpel development, as discussed in the section below.

F. THE ROLE OF GENE DUPLICATIONS IN CORE EUDICOT CARPEL EVOLUTION

The core eudicots form a monophyletic crown group of eudicots. This group includes the major clades of the rosids, asterids and Caryophyllales, which together account for the majority of extant angiosperm species. Several well-known molecular-genetic models occur in the core eudicots (Fig. 10D), including *A. thaliana* (rosids, Brassicales, Brassicaceae), *A. majus* (asterids, Plantaginales, Plantaginaceae) and *P. hybrida* (asterids, Solanales, Solanaceae). Comparison of the major clades making up the core eudicots indicates the presence of numerous novel floral features, as compared to the earlier common ancestor of all eudicots. Perhaps, the most notable change in carpel structure between these two successive stages in eudicot evolution was the origin of syncarpy, which appears to be a pleisiomorphic feature of the core eudicots (Armbruster *et al.*, 2002; Endress and Doyle, 2009).

Analysis of the *Arabidopsis* genome sequence has provided evidence of a large-scale duplication event that probably occurred not long before the last common ancestor of the core eudicots (De Bodt *et al.*, 2005). Accordingly, single genes in basal eudicots are frequently found to be orthologous to pairs of genes in core eudicots and this is the case for several classes of MADS box genes that control carpel development. For example, two C-clade lineages are present the core eudicots in place of a single *paleoAG* lineage in basally diverging eudicots. Thus in *Arabidopsis*, the *euAG* clade contains the *AG* gene itself, while the *PLENA* (*PLE*) clade, contains the paralogous genes *SHP1* and *SHP2*, which probably resulted from a further duplication within or near Brassicaceae (Fig. 11). In *A. majus*, the probable orthologue of *AG* is termed *FARINELLI* (*FAR*), and that of *SHP1/2* is the clade-defining gene *PLE*. Interestingly, the non-orthologous C-clade genes *AG* and *PLE* are responsible for specifying the C-function in *Arabidopsis* and *Antirrhinum,* respectively (Fig. 11; Davies *et al.*, 1999; Kramer *et al.*, 2004). *FAR*, by contrast, is redundantly involved in stamen development and contributes to pollen fertility. In an example of neo-functionalisation, *SHP1* and *2* have acquired a novel role in *Arabidopsis* fruit development (Liljegren *et al.*, 2000). In *P. hybrida*, which, as a member of the asterids, is more closely related to *Antirrhinum* than to *Arabidopsis* (Fig. 9), a further case of sub-functionalisation is to be found. The *Petunia AG* orthologue *PMADS3* is principally responsible for stamen development (Kapoor *et al.*, 2002), though also plays a redundant role with the *PLE* orthologue *FBP6* in both carpel development and floral determinacy (Kramer *et al.*, 2004).

Though sub-functionalisation between the paralogous *AG* and *PLE* clades in the *Arabidopsis* lineage has left *AG* playing the major C-function role, elegant experiments involving multiple mutants demonstrate the *SHP* genes

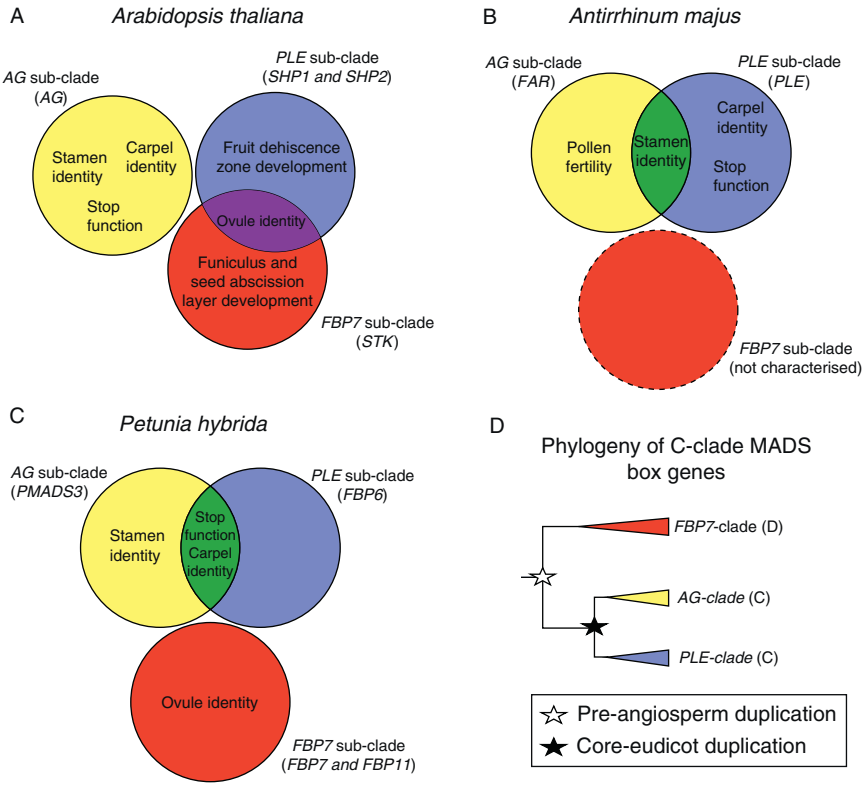

Fig. 11. Fluidity in the functionalisation of C- and D-function MADS box genes in core eudicots. (A–C) Venn diagrams representing the functions of genes from the *AG*, *PLE* and *FBP7* MADS box clades in three species of core eudicots. Overlapping regions represent functional redundancy between genes in wild-type genetic backgrounds. (D) The sequence of duplications that generated of the eudicot *AG*, *PLE* and *FBP7* MADS box gene clades.

also to have retained a capacity for C-function activity. Ectopic carpelloid organs are formed in the first floral whorl of *Arabidopsis ag* mutants, conditionally on the inactivation of *AP2*, which is known mainly for its contribution to the A-function (Bowman *et al.*, 1991b). This effect is thought to occur because, in wild-type *Arabidopsis* plants, *AP2* is responsible for down-regulating C-clade genes in the outer floral whorls. 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*, as demonstrated by the complete lack of carpelloid features to be observed in the first whorl organs of quadruple *ap2/ag/shp1/shp2* mutants (Pinyopich *et al.*, 2003). These data indicate a subtle effect of functional

overlap between paralogous gene clades, which does not equate to simple genetic redundancy: the *SHP* genes adopt a novel C-function role in *Arabidopsis*, conditionally on the inactivation of *AP2* and *AG*.

The fluidity of functions among duplicated genes in the core eudicots is further illustrated by an exchange of function between MADS box genes of the C- and D-clades. Two paralogous D-function genes in *Petunia*, *FBP7* and *FBP11*, are redundantly required for ovule development (Angenent *et al.*, 1995). The *Arabidopsis* orthologue of these genes, *SEEDSTICK* (*STK*), is also involved in ovule development, though *STK* shares this role redundantly with the C-clade genes *SHP1* and *SHP2* (Fig. 11). Accordingly, the *Arabidopsis stk/shp1/shp2* triple mutant (Pinyopich *et al.*, 2003), like the *Petunia fpb7/fpb11* double mutant (Angenent *et al.*, 1995), produces 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 (Pinyopich *et al.*, 2003). The combined C + D-clade in the eudicots, whose different lineages were separated by duplication events that occurred both before and after the radiation of the angiosperms, therefore represents a complex situation in which diverse evolutionary processes have taken place. Examples can be found in this clade of: the repartition of multiple pre-existing functions between paralogues genes (sub-functionalisation), the generation of novel functions associated with one or both genes of a pair of paralogues (neo-functionalisation), and exchanges in function, both between paralogues and non-paralogous genes (Fig. 11).

A further likely consequence of the genome duplication that appears to have occurred at the base of the core eudicots is the generation of a second sub-clade of MADS box genes within the A-clade (Litt and Irish, 2003). The *Arabidopsis* A-function MADS box gene *AP1* plays roles in floral meristem patterning and in the specification of perianth organ identity. However, gene duplications in the core eudicots have provided further A-clade sequences, of which *FUL* has acquired a role in the patterning of the gynoecium wall in *Arabidopsis* (Gu *et al.*, 1998). *FUL* is known to act in a network involving a large number of genes (Liljegren *et al.*, 2004; Roeder *et al.*, 2003), including the MADS box genes *SHP1* and *SHP2* (Ferrandiz *et al.*, 2000b) that also function redundantly with *STK* in ovule development. Gene duplication in the MADS box A-clade, followed by neo-functionalisation, has thus resulted in the evolution of novel fruit shattering mechanisms in Brassicaceae.

An interesting feature of gene duplication in the A-clade is the evolution of a distinct C-terminal protein motif in AP1, which was apparently produced by a frame-shift that occurred near the 3′-extremity of the *AP1* coding sequence in a common ancestor of the core eudicots (Litt and Irish, 2003).

This frame-shift created a farnesylsation site in the encoded protein, which is known from studies of *Arabidopsis* to be post-translationally modified and required for wild-type *AP1* protein activity (Yalovsky *et al.*, 2000). Other frame-shift mutations in duplicated genes are also present in the B- and C-function MADS box clades in the core eudicots (Vandenbussche *et al.*, 2003), though the 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-clades of MADS box genes have been conserved over a long evolutionary timescale, clearly indicating their functional significance. However, it is not known whether the functions of these novel motifs are connected with biochemical processes in common, such as the higher order assembly of MADS box complexes, or the sequestration of transcription factors through membrane attachment (Vandenbussche *et al.*, 2003).

VII. GENERAL CONCLUSIONS

In this chapter, our treatment of carpel and gynoecium development has been the most thorough in *Arabidopsis*, reflecting both our own research interests and the relative wealth of data available in this model species. Until recently, most molecular-genetic studies of gynoecium development have focussed on individual genes and interactions, though the development of large-sale and modelling approaches means that more ambitious goals can now be set. Accordingly, a major challenge for the future will be to construct integrated models of gynoecium development using a system biology approach. A central factor in such an analysis will surely be the hormone auxin. As described in Section IV, we already know that genes involved in auxin synthesis, transport and responsiveness have many important effects on *Arabidopsis* gynoecium development. A systems biology approach should permit the construction of integrated models of gynoecium development by using a dynamic map of auxin distribution and transport to link together auxin-related genetic elements. Further experimental and modelling approaches can be used to link the many known gynoecium development transcription factors to the proximal causes of development, such as cell division and differentiation.

Though much still remains to be done on *Arabidopsis* carpel development, even more work is required in other species if we are to better understand comparative aspects of carpel development and evolution. Such comparative studies will require in some cases the development of new angiosperm models to fill gaps that currently exist at key phylogenetic positions. The advent of a new generation of sequencing technology (e.g. Eid *et al.*, 2009) is expected to

revolutionise many aspects of evolutionary-developmental biology, and should provide complete genome sequences from species occupying phylogenetic positions of relevance to flower and carpel evolution (Cyranoski, 2010; Soltis *et al.*, 2008). However, attention must also be paid to the development of functional genetics approaches in new plant models, especially if the expected wealth of genome sequence data is to be exploited to its full potential.

One aspect of carpel development that has only briefly been touched on in this chapter concerns what becomes of the carpel after fertilisation. Indeed, the greater part of carpel-related biodiversity becomes apparent only at this later stage, as the gynoecium transforms into a fruit (Ostergaard, 2010). Fruits are, of course, plant parts of major economic interest, and the contribution of fruits to seed dispersal by air, wind, water, explosion, mammals, birds, reptiles, insects and so on (Willson and Traveset, 2002) must also represent one of the major reasons why the flowering plants have been so successful. To accomplish their role in seed dispersal, fruits undergo considerable post-fertilisation changes, though many of the mechanisms that bring these about are established at earlier developmental stages. Thus, the basis for fruit development is laid down during the formation of the carpel (Roeder and Yanofsky, 2005; Sorefan *et al.*, 2009) and involves many of the genetic programmes described in Section IV of this chapter. An increasingly important goal of research in this area will be to understand how carpel genetic networks have been modified through evolution to generate the enormous diversity of fruit forms found in nature, and indeed how knowledge of these networks can help us to further modify fruit characteristics to meet our agricultural needs (Doebley *et al.*, 2006; Ostergaard *et al.*, 2006).

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