Chapter 5

Flower Development: Open Questions and Future Directions

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Abstract

Almost three decades of genetic and molecular analyses have resulted in detailed insights into many of the processes that take place during flower development and in the identification of a large number of key regulatory genes that control these processes. Despite this impressive progress, many questions about how flower development is controlled in different angiosperm species remain unanswered. In this chapter, we discuss some of these open questions and the experimental strategies with which they could be addressed. Specifically, we focus on the areas of floral meristem development and patterning, floral organ specification and differentiation, as well as on the molecular mechanisms underlying the evolutionary changes that have led to the astounding variations in flower size and architecture among extant and extinct angiosperms.

Key words Flower development, Floral meristems, ABC model, Floral evolution

1 Introduction

Over two decades ago, the ABC model was proposed as a genetic framework for a key aspect of flower development, floral organ identity specification. Since then, extensive genetic and molecular analyses of this and other processes have resulted in tremendous progress in our understanding of flower development (as reviewed in Chapters 1–4). In particular, all of the floral organ identity genes, whose combinatorial activities are described in the ABC model, have been identified and detailed insights into their functions have been obtained. Similarly, key gene products and molecular mechanisms that underlie how stem cells in the flower are established, maintained and ultimately terminated have been characterized. While flower development has been studied mainly in

José Luis Riechmann and Frank Wellmer (eds.), *Flower Development: Methods and Protocols*, Methods in Molecular Biology, vol. 1110, DOI 10.1007/978-1-4614-9408-9_5,

the model plant *Arabidopsis thaliana*, as well as in a few other species (e.g., snapdragon, petunia, and rice), expanding molecular studies to additional species and new model organisms is providing a better understanding of flower evolution and diversity. Despite these advances, many unanswered questions or poorly understood topics remain. In this chapter, we discuss some of those open questions and, whenever pertinent, outline possible experimental strategies with which they could be addressed. While this list of questions is far from complete, we believe that the ones described in this chapter are among the most important problems that have to be solved in order to obtain a comprehensive view of the molecular processes underlying flower development and evolution.

2 Floral Meristem Development

The formation of flowers commences shortly after the so-called floral transition, which, in *Arabidopsis*, is controlled by a complex regulatory network of flowering time genes and pathways and depends on both internal and external cues (reviewed in: refs. 1–3). Floral transition involves the transformation of the shoot apical meristem (SAM) into an inflorescence meristem (IM). While the SAM initiates leaves that subtend branches formed by axillary meristems, during flowering, initially several cauline leaves and associated axillary meristems are formed before the IM generates floral meristems (FMs) on its flanks. FMs are typically subtended by bracts, although the outgrowth of these modified leaves is suppressed in several plant species, including members of the Brassicaceae (e.g., *Arabidopsis*) and the Poaceae [4]. FMs are therefore developmentally similar to axillary meristems as both types of meristems form a primordium consisting of a leaf-competent domain and a meristem domain, possibly as a consequence of a common developmental program [4, 5]. The fate of these two domains depends on the developmental stage of the plant, leading to rosette leaves with an axillary meristem during the vegetative phase and bracts with flowers during reproductive development. Notably, cauline leaves and the associated axillary secondary IMs display properties of both phases [6], suggesting that their formation is a direct result of the transition process, which leads from vegetative to reproductive development. A careful experiment using photoinduction of flowering further showed that newly formed primordia, which would otherwise develop into leaves with axillary meristems, can be induced into flowers or flower/leaf chimeric shoots, indicating that the fate of primordia can be modified after their initiation [7]. The genetic basis of the transitions between these different meristematic activities and the molecular mechanisms underlying primordia specification are largely

unknown, and future work will have to be directed towards unraveling these important questions.

The development of FMs can be divided into distinct phases [8]. The first phase is characterized by the formation of a region (the so-called floral *Anlage*) within an IM from which the FM ultimately arises. Subsequently, outgrowth of the floral meristem results in the formation of a floral primordium, which rapidly increases in size. At early floral stages, the formation of organ primordia commences and is followed by their differentiation and maturation. A key question that remains largely unanswered is how these 3 distinct phases, i.e., *Anlagen* formation, FM initiation and floral organ differentiation are interconnected. For instance, floral *Anlagen* are specified in a very precise position of the IM. It is known that a complex interplay between the plant hormones auxin and cytokinin is responsible for FM initiation and outgrowth. Auxin maxima are necessary for FM initiation, which is evidenced by the auxin efflux carrier *pin1* mutant, which develops a naked inflorescence without FMs [9, 10]. However, how auxin controls the definition of the floral *Anlagen* is still largely unknown although recent evidence suggests that the control of *LEAFY* (*LFY*) expression by the Auxin Response Factor MONOPTEROS (MP) plays an important role in this process [11]. *LFY* is one of the key genes controlling floral meristem identity, which is expressed in floral *Anlagen* [12]. *LFY* encodes a transcription factor specific to the plant kingdom, and in *lfy* mutants, inflorescence-like structures develop instead of flowers [13].

In addition to auxin, floral meristem outgrowth is also controlled by a complex network of transcription factors that specify meristem identity and prevent the precocious differentiation of floral organs. The genes encoding these factors are the so-called floral meristem identity genes, of which *LFY* is the central regulator. LFY is able to directly activate other floral meristem identity genes such as *LATE MERISTEM IDENTITY 1* (*LMI1*), *CAULIFLOWER* (*CAL*), and *APETALA1* (*AP1*) [14–18]. Subsequently, both AP1 and CAL are responsible for the maintenance of *LFY* expression in a positive regulatory loop [19, 20]. AP1 and CAL are MADS-domain transcription factors that are highly similar in sequence and partially redundant. In fact, the *ap1 cal* double mutant develops mainly IMs instead of flowers [21, 22]. Interestingly, in this background another MADS-box gene, closely related to both *AP1* and *CAL*, *FRUITFULL* (*FUL*), which is normally not expressed in wild-type FMs, appears to act as a floral meristem identity gene, since *ap1 cal ful* triple mutants show an enhanced phenotype when compared to an *ap1 cal* double mutant [19]. Thus, *FUL* is not a floral meristem identity gene but can act as such when it is ectopically expressed in the FM. These types of regulatory interactions can shroud the genetic interactions and render the genetic analyses

and the interpretations of the phenotypes of higher order mutants arduous.

Recently, two other MADS-box genes, *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE24* (*AGL24*), have been shown to be involved in conferring floral meristem identity, as *ap1 agl24 svp* triple mutants are phenocopies of the *ap1 cal* double mutant [23]. The role of *AGL24* and *SVP* in promoting FM identity was recently confirmed by demonstrating genetic interactions between these genes and *LFY* [24]. Moreover, they are part of the complex gene regulatory network that ensures floral meristem commitment and prevents floral reversion, and they are able to directly activate both *LFY* and *AP1* [24]. At stage 3 of flower development, when the formation of floral organs commences, *SVP* expression is lost and the expression of the *SEPALLATA* (*SEP*) genes is activated. Based on these and other observations, it has been suggested that the SEP proteins (which, like AP1, CAL, FUL, SVP, and AGL24, are MADS-domain transcription factors) compete with AGL24 and SVP for binding to AP1, and that AP1-SEP dimers eventually silence *AGL24* and *SVP* and promote the formation of floral organs [23, 25]. Although this working model is likely correct, at least in part, it is currently unclear what ultimately induces the differentiation of FMs. Is this switch triggered when FMs reach a certain size, or is it rather dependent on hormone levels or transcription factor concentrations? Answering these questions will be a major step forward in our understanding of the early processes of flower development.

One of the difficulties in studying the three phases of FM development and how they are interconnected is the extremely small size of these structures in *Arabidopsis*. However, the development of new technologies has already facilitated research in this area and will likely contribute to major breakthroughs in our understanding of FM development. For instance, it is now feasible to study the expression of key regulators of FM development and their protein–protein interactions with high spatial and temporal resolution using confocal microscopy (*see* Chapter 25). Furthermore, analysis of the transcriptomes of buds of different developmental stages, and of the regulatory processes that take place in these flowers, will be aided by techniques such as laser capture microdissection (*see* Chapter 19) in combination with RNA-Seq (*see* Chapter 23), or through the use of a floral induction system (*see* Chapter 16), which allows the collection of large quantities of early-stage floral buds for genomic and proteomic studies.

Another largely unanswered question regarding FMs is how the cells that give rise to individual floral organs (i.e., founder cells) are specified. Multiple lines of evidence demonstrate that the specification of organ founder cells precedes floral organ identity

determination. However, the number and position of floral organs that arise in each whorl, as well as the number of founder cells is variable in different parts of the flower. Although auxin and the gene *DORNRÖSCHEN-LIKE* (*DRNL*) are involved in specifying where floral organs will arise [10, 26–28], the precise interplay of how these factors establish a complex pattern of floral organ primordia from an undifferentiated FM is unclear. Unlike the SAM, which leads to a single organ type on its flanks, either leaves (during vegetative growth) or flowers (during reproductive growth), the FM generates a diversity of organ types that arise in very close proximity to one another. Founder cell specification is intrinsically linked to the establishment of boundaries between floral organs. While several genes involved in boundary formation have been identified [29–34], their precise functions at the molecular level remain to be elucidated.

To prevent the overgrowth of FMs, plants have evolved multiple mechanisms to terminate meristematic activity once floral organ development has been correctly initiated. A key regulator of one of these mechanisms in *Arabidopsis* is the floral organ identity MADS-domain factor AGAMOUS (AG), which integrates stem cell regulation with floral patterning events [35, 36]. *AG* expression is induced by the stem cell determinant WUSCHEL (WUS) in early floral primordia [37, 38]. AG then specifies reproductive organ identity and eventually terminates *WUS* expression. Precise temporal induction of the C2H2-type zinc finger repressor KNUCKLES (KNU) is essential to balance proliferation and differentiation in the AG-WUS feedback loop [39]. *KNU* is a direct target of AG and functions as an upstream repressor of *WUS* [39]. Although *knu* mutant flowers exhibit extra floral organs inside carpels [40], this indeterminacy is weaker than that typically observed in *ag* mutant flowers. Therefore, AG likely controls additional genes that control FM determinacy together or in parallel with *KNU*. One candidate for such a gene is *CRABS CLAW* (*CRC*), which encodes a YABBY-type transcription factor, and is involved in the regulation of carpel and nectary development [41]. *CRC* is a direct target of AG [42] and *knu crc* double-mutant flowers show stronger indeterminacy [43]. Furthermore, when *ag* mutants are combined with *superman (sup)* loss-of-function alleles, the *ag sup* double-mutant flowers continue to grow, reaching a size several times bigger than that of wild-type or *ag* single mutant flowers, often with a fasciated meristem in the center [44]. *SUP* starts to be expressed earlier than *KNU* and *CRC* in flower development [39–41, 45]. These results indicate that floral meristem activity is terminated by multiple genetic pathways functioning at different developmental stages. However, how many regulators act in this process, and when, where and through which mechanisms they function, are largely unanswered questions.

3 Understanding the Molecular Nature of the (A)BCE Model

The work to decipher the molecular mechanisms underlying flower development has been driven, at each stage, by advances in technology. The application of molecular biology to classical mutants, transgenic and other approaches to the analysis of gene function, methods to study protein–protein and protein–DNA interactions, and improvements in DNA sequencing and microscopy have all contributed to our current state of understanding. However, even if we just put to one side the small matter that we do not really understand exactly how the multiple actors in the (A)BCE model of floral organ identity specification (*see* Chapter 1) come to be present at the right levels and the right time and place, how much do we really know? There is accumulating evidence that the space between the model and reality is highly complex and that we are going to need several more technological advances to make the connections between the high-level regulators identified using the homeotic mutants and the proteins that ultimately make one cell different from another.

Focusing on the MADS-box organ identity genes highlights this challenge. We have known for several years that the MADS-domain transcription factors dimerize and heterodimerize [46, 47], form higher order complexes, especially when bound to DNA [48], and that they bind DNA as interacting heterodimer pairs to form tetramers, or quartets [49]. There have also been several reports in the literature, largely overlooked, of interactions between MADS-domain factors and other transcription factors or regulators (e.g., [25, 50–58]). These interactions clearly suggest that the (A)BCE MADS-domain factors do not work alone. Recently, a proteomics approach to study the composition of A, B, and C function complexes present in the developing meristem has shown that they are likely to be even more complicated than was imagined [59]. Whilst this novel approach validated previous MADS–MADS interactions, it also showed that the complexes are far larger than predicted; 670 kDa in the case of SEP3. The floral MADS-domain factor complexes include other transcription factors, co-repressors and, notably, several chromatin-associated factors such as nucleosome remodeling factors and histone demethylase. The composition of these complexes is likely to dictate the effect on the expression of their target genes (i.e., to repress or to activate), effects that will also be dynamic in development. For example, AP1, as one of the first of the (A)BCE genes to be expressed, acts to repress inflorescence identity genes such as *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and to promote floral meristem identity genes like *LFY* [20, 60]. Understanding the effects of the multiple possible (A)BCE-factor complexes, in terms of transcriptional regulation of their target genes, all in a dynamic developmental context, just became even more difficult.

Not knowing how a specific (A)BCE complex variant affects the expression of a specific target gene at a particular point in development leads on to a bigger and more intractable question: how do we understand and quantify the contribution of downstream targets to building a flower? Recently there has been tremendous progress in identifying targets of the floral organ identity factors, made possible by advances in the techniques for the genome-wide localization of transcription factor binding sites (*see* Chapter 24). Using this approach, targets have been identified for the MADS-domain proteins SEP3, AP1, APETALA3 (AP3), PISTILLATA (PI), FLOWERING LOCUS C (FLC) and SOC1 [20, 61–64]. These results have highlighted three serious obstacles to filling the space between the homeotic regulators and the ultimate effector proteins. Firstly, it is clear that targets of the (A)BCE-function transcription factors are more numerous than had been thought [60]. Secondly, the organ identity factors are not strictly hierarchical and act at all levels in the network, making network models highly complex. Thirdly, the genes that are directly, indirectly and in combination downstream of them are often redundant and do not lend themselves to genetic analysis by displaying the kind of informative mutant phenotypes observed for the homeotic regulators. Overcoming these challenges will require advances in target gene detection, verification and network modeling. More significantly, it will also necessitate new approaches to the analysis of gene function and phenotypic analyses to detect the diverse influences of the target genes on the development of the flower. These new approaches will require more sophisticated morphometric analyses to quantify subtle variation [65].

4 Floral Organ Formation

Flowers are highly complex organs that consist of many specialized tissues. Their development must therefore be tightly controlled in order to generate a functional structure that attracts pollinators, orchestrates both male and female gametogenesis, and generates and disperses the seeds of the subsequent generation. Yet the intercellular signaling landscape that mediates the coordination of floral organogenesis and the integration of flower-specific cellular activities is a largely uncharted territory that awaits discovery. Similarly, we know very little about the genes that control the size and shape of the floral organs. Genes involved in hormone biosynthesis and response are enriched in the transcriptome of early flower buds [66] and as targets of floral organ identity genes [20, 42, 62, 64, 67]. Some progress has been made in identifying roles for gibberellins, auxins, jasmonates, and brassinosteroids in regulating aspects of stamen and carpel development [68, 69], including anther differentiation [70], stamen maturation [71], and gynoecium

patterning [72]. Similarly, a variety of small polypeptide signaling genes are expressed in developing flowers [73, 74], and several components of ligand-mediated signal transduction pathways are known to control facets of anther and pollen formation [75–78]. However, these investigations represent only an initial foray into the functional analysis of these intercellular signals and the developmental events they direct and shape. Much work remains to characterize the intracellular constituents of each of the signaling pathways, to link the upstream signals with the downstream effectors that mediate specific cellular events, and to distinguish between direct and indirect regulatory interactions. It will be an even more ambitious undertaking to elucidate how gradients of signaling molecules are interpreted at the cellular level, and to integrate the inputs and outputs of the various signaling pathways into a comprehensive system that accurately reflects the intricacy of these beautiful but complex structures.

Despite the recent identification of target genes of the floral organ identity factors (*see* above), little is known about how these genes shape each floral organ and how they contribute to their modular structure by defining their specific functional compartments and tissues [79]. Many mutants related to floral organ patterning and tissue differentiation have been identified, but the connection of the genes affected by these mutations and the floral organ identity factors is poorly understood, and the genetic hierarchies of the network in the floral context are unclear as well. To further complicate things, it is frequently observed that these mutations cause defects in more than one whorl and are usually also affected in leaf morphology, supporting the long standing idea of the common basic developmental plan of the lateral organs, on top of which floral organ identity is laid [50, 80–82]. Thus, an open question remains on how these "general" factors acquire, within the floral context, new functions to direct the differentiation of highly specialized tissues. We can propose again a scenario based on the combinatorial nature of transcriptional regulators. The "leaf" factors could physically interact with ABC factors, or with the few floral-specific factors directly downstream of them, such as, for example, CRC [41], and thus generate new functional domains. In addition, specificity could be also given by the unique overlapping pattern of some of the general factors in the floral organs. In this sense, we could envision the existence of another floral combinatorial code, directing not organ specification but organ patterning. To explore this hypothesis, much work would be needed, beginning with the comprehensive identification of truly floral-specific factors, as well as a better definition of the protein interaction network among transcription factors in the flower.

A key aspect of floral organ formation is the development of the male and female reproductive structures of the plant. Male gametogenesis takes place in anthers, which usually have four similarly

structured portions called lobes. In most flowering plants, each lobe contains at least four layers of somatic cells: the epidermis, the endothecium, the middle layer, and the tapetum, which surrounds the male meiotic cells, or pollen mother cells (PMCs). Anther development starting from a primordium with cells from the three meristematic layers has been described for several plants, providing a morphological basis for understanding gene functions from mutant phenotypes [83–86]. Genetic studies of male sterile mutants and molecular analyses of the corresponding genes have identified regulators of early anther division and differentiation encoding likely cell-cell signaling components, such as putative receptor-like protein kinases and their ligands; additional genes essential for proper tapetum formation and function often encode putative transcription factors that are required for normal expression of thousands of genes, some of which are enzymes for synthesis of pollen wall components [83, 84].

Although it is certain that many genes important for anther development will continue to be uncovered by a combination of genetic, genomic, and biochemical approaches, greater challenges lie ahead in the comprehensive understanding of how these genes function at the molecular level to orchestrate the formation of the functional anther and to ensure proper pollen development. For example, the importance of cell-cell signaling has been strongly supported by the requirement for several genes encoding receptor-like protein kinases and intracellular kinases, but how they perceive extracellular signals and transmit them to regulate gene expression and other cellular processes are not known. At the same time, key transcription factors have been demonstrated to be important for tapetum function, with some evidence for genetic and physical interactions at multiple levels, yet how the tapetum transcriptome is precisely controlled spatially and temporally is far from understood. Furthermore, little is known about the earliest aspects of anther differentiation, even at the morphological or cellular levels, because of the lack of molecular and cellular markers that can distinguish the earliest cell types. Moreover, compared with the tapetum, the control of differentiation of other cell types is much less known.

As mentioned above, the male meiotic cells are formed interior of the tapetum in the anther locules, and they have been the source of most of the information regarding plant meiosis, including classic cytological studies that used plants with large genomes, such as maize and lily [87, 88]. *Arabidopsis* has been a recent member of this small club of plants [84, 88–91], yet its advantage in molecular genetics has allowed a relatively large number of genes to be studied using forward and reverse genetics, uncovering both conserved mechanisms and features unique to plants, from chromosome cohesion to meiotic recombination, from spindle function to meiotic cytokinesis. Functional homologs of well-known meiotic genes, such as *SPO11* and *RAD51*, first discovered in yeast and

other organisms, have been analyzed in *Arabidopsis* [90, 92–94]. Reverse genetic studies have also been very powerful in demonstrating the importance of gene functions that are also required for mitotic growth using hypomorphic alleles or meiosis-specific gene knock-down approaches. Perhaps more significantly, novel genes have been identified by forward genetics that have homologs in other organisms but were not recognized as having a meiotic function [84, 89, 95].

A central question of how germ line specification is controlled has recently seen a very exciting new development: the lack of oxygen, or hypoxia, has been implicated in promoting the male germ line fate [96]. How a low oxygen level is translated into molecular programs specifying the male meiocytes is not yet known, but there have been reports of genes involved in redox status of the cell being important for male fertility. Clearly, the molecular basis of homolog pairing is still largely a mystery, which might be solved with advances in both molecular cell biology and live imaging technology. Also not understood is how meiotic recombination is regulated at the chromosome level, where plants might offer new insights not possible from yeasts because of their very small genomes.

5 Evolution of Flower Development

Angiosperms exhibit astounding differences in floral architecture, size and color. While the molecular basis to these morphological variations is largely unknown, the recent advances in the technologies used for genome sequencing and transcriptome profiling will likely result in significant and rapid progress in the understanding of floral evolution. To date, evolutionary studies on flower development have focused mainly on the floral organ identity genes and their functions during organ specification and development. In order to obtain a more comprehensive understanding of the differences in the gene networks controlling flower development in different species, these studies will have to be expanded to genes involved processes such as floral patterning, growth and differentiation.

Analyses of phylogenetically diverse angiosperms showed that B and C functions specifying reproductive floral organ identity are highly conserved. For example, C class orthologs act to promote reproductive organ development in the monocot maize and eudicot *Antirrhinum*, although some subfunctionalization is evident in maize due to gene duplication [97, 98]. B function activity to specify petal identity also appears largely conserved across angiosperms from core eudicots to grasses, providing evidence that lodicules are homologous to petals [99–102]. Again, gene duplication events leading to subfunctionalization of B class activities have occurred in several taxa, for example in the Ranunculaceae and orchids [103, 104]. The roles of B and C class genes in specifying

reproductive organ identity likely predate angiosperms. For example, in gymnosperms (e.g., *Picea*, *Pinus*, *Gnetum*) C class genes are expressed in both male and female cones while B class genes are expressed in male cones [105–108]. Thus, a BC model of reproductive organ specification is likely ancestral for seed plants.

In contrast to B and C function, there has not been a consensus for a broadly conserved A class function, with models for non-Brassicaceae often consisting of a BC model [99, 100], and some authors arguing that A class is phylogenetically restricted to the Brassicaceae [109]. However, since outer perianth organs (sepals in many angiosperms) differ from leaves in many respects, there should exist an activity specifying them as such. In *Arabidopsis*, A class was originally defined as an activity that (1) promoted sepals and petal identity and (2) prevented C activity in the perianth whorls. The latter function is fulfilled by APETALA2 (AP2), which acts to repress *AG* transcription in first and second whorls [110]. Based on the floral phenotypes of weak *ap2* alleles and those in which *AP2* and all potential C class genes are eliminated, *AP2* also contributes to sepal identity [111, 112]. In contrast, the A class gene *AP1* does not act to restrict C class activity, but contributes to sepal and petal development [21, 113–116]. *AP1* and its close paralog, *CAL*, also act earlier in flower development in the specification of flower meristem identity [21, 22] (*see* above). It could be argued that the recent genome duplication within the Brassicaceae producing the *AP1* and *CAL* paralogs facilitated the identification of *AP1* as an A class gene. *AP1* promotes sepal identity, but how prominent a role *AP1* plays in petal specification is obscured by the loss of second whorl organs in the *ap1 cal* double mutant. Targeted loss-of-function of both paralogs in petals after their initiation would be useful to address that question.

In the ancestor of the core eudicots, a gene duplication resulted in the *AP1* and *FUL* paralogs, whereas the basal eudicots and more basal angiosperms possess only a single paralog. The roles of *AP1* orthologs in floral meristem and perianth identity specification seem to be conserved in the core eudicots [117–119]. In poppies, a basal eudicot, the single paralog performs the combined functions that *AP1* and *FUL* perform in *Arabidopsis*, suggesting subfunctionalization within the core eudicots [120]. *AP1/FUL* orthologs have not been fully functionally examined in basal angiosperms. In contrast, *AP2* orthologs in other eudicots, such as *Antirrhinum*, have been shown to play a role in perianth development, but there is no evidence for a role in repression of C class activity [121]. However, in both *Antirrhinum* and *Petunia* the microRNA miR169 acts to restrict, at least in part, C function from the outer whorls via repression of a C function gene activator [122]. Thus, in the Brassicaceae (rosids) and in two asterids, different mechanisms have evolved to restrict C function from the perianth whorls.

MIKC^c gene family members are present in the genomes of all land plants [123, 124]. Several aspects of the evolutionary history of this gene family are revealed in a phylogenetic analysis of land plant MIKC^c genes (*see* Fig. 1). First, the common ancestor of land plants possessed a single MIKC^c gene. The presence of multiple genes in moss species is due to gene duplications with the moss lineage. Second, the common ancestor of vascular plants also likely possessed a single MIKC^c gene. As in the mosses, the presence to multiple gene family members in lycophyte species is due to gene duplications within the lycophyte lineage. Third, the common ancestor of the euphyllophytes possessed multiple (2–3?) MIKC^c genes. The tree topology suggests that the ancestors of the B and C genes might have diverged prior to the divergence of ferns from seed plants. Fourth, the common ancestor of seed plants possessed several (8–12?) MIKC^c genes. Several of these ancestral lineages expanded within the angiosperms, gymnosperms, or both. At least 10 MIKC^c genes are present in the *Amborella trichopoda* genome and most derived flowering plant genomes harbor 20 or more, suggesting they have been preferentially retained following genome/gene duplications. Fifth, orthologs for each of the A (*AP1/FUL*), B (*AP3/PI*), C (*AG*), and E (*SEP*) classes can be found in extant gymnosperms. The tree topology prompts a few questions posed below.

Knowledge of the functions of MIKC^c genes in other lineages of land plants might help elucidate the ancestral functions of the gene family and provide clues to the evolution of their more derived functions. For example, fern genes of two different clades, one more closely related to the B genes and the other to the A/C/E genes of seed plants, are broadly expressed in both vegetative and reproductive tissues, with some genes expressed in both sporophytic and gametophytic generations [125, 126]. What are the functions of these genes, and do they play any role in specification of tissues during reproductive development? Similar questions may be asked about the lycophyte genes, which are also broadly expressed during the sporophytic generation [127], and genes, such as the single MIKC^c gene in the liverwort *Marchantia* that is

Fig. 1 (continued) another and may be more closely related to different fern homologs. (3) There exist gymnosperm orthologs for each of the A, B, C, and E classes of angiosperm genes. Bayesian phylogenetic analysis was performed using Mr. Bayes 3.1 [140]. This version of the software conducts two independent analyses simultaneously. The mixed model option (aamodelpr = mixed) was used to estimate the appropriate amino acid fixed rate model. The analysis was run for 3,000,000 generations, which was sufficient for the standard deviation of the split frequencies to drop below 0.02. To allow for the burn in phase, the first 500 trees (10 % of the total number of saved trees) were discarded. The tree was rooted with the single *Marchantia* MIKC^c sequence resulting in a tree that broadly reflects land plant phylogeny. However, the positions of the fern genes relative to the seed plant genes are ambiguous. Directed analyses of well-supported clades or additional taxa and/or sequences may help resolve some aspects; however, this is not guaranteed [141, 142]

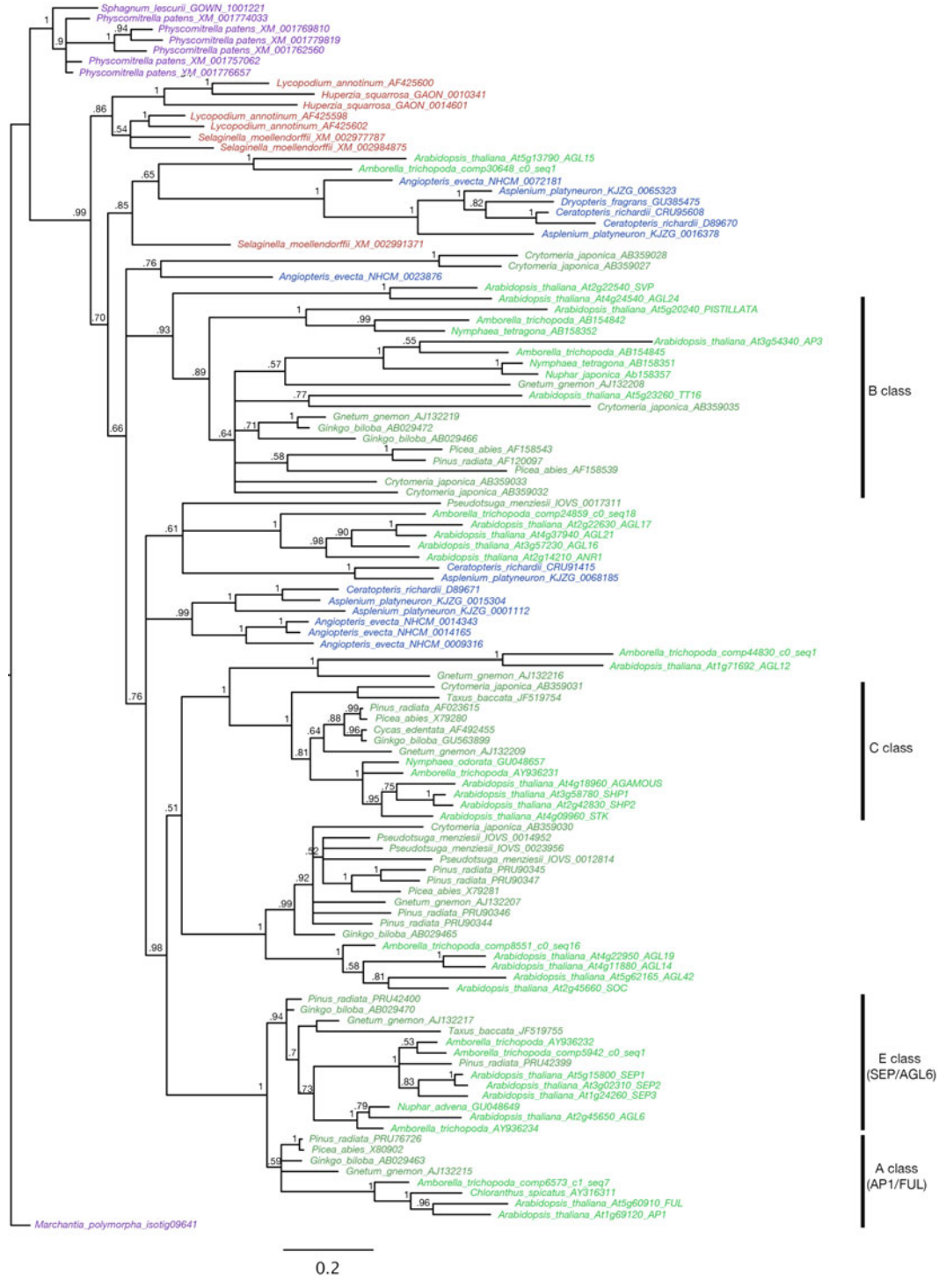

Fig. 1 Bayesian phylogram of land plant MIKC^c genes based on alignment of 163 characters (amino acids). Numbers above the branches indicate posterior probability values. While there is a lack of resolution in many branches of the tree, three aspects are relevant to the evolution of the ABC genes. (1) There are no one-to one orthologs of the ABC genes in non-seed plant taxa. (2) The B and C class genes are only distantly related to one

expressed in the gametophyte. In contrast to the broad expression patterns of MIKC^c genes in basal lineages of land plants, where it has been investigated, expression of MIKC genes in Charophytes is restricted to haploid reproductive tissues [128]. However, the Charophycean genes may have their origin prior the gene duplication producing MIKC^c and MIKC* classes of MADS-box genes in land plants [128], and if so, represent orthologs to both classes of land plant genes. Functional characterization of the MIKC^c genes in non-seed plant lineages of land plants has the potential to uncover ancestral gene functions and possibly provide insight into how this obscure gene family, from a non-seed plant perspective, came to dominate the reproductive developmental programs in seed plants, most strikingly in angiosperms. Furthermore, since gymnosperms have orthologs of each of the A, B, C, and E classes of MIKC^c genes, it may be that changes in how these genes are regulated in angiosperms as compared to gymnosperms facilitated the evolution of the flower.

The outer perianth of most angiosperm flowers is morphologically distinct from leaves suggesting that their identity as such must be specified by some means. Despite some initial skepticism, it is becoming apparent that *AP1* orthologs have a relatively conserved function with respect to floral organ identity in eudicots: loss of AP1 ortholog function results in conversion of sepals into bract- or leaf-like organs and a loss or partial loss of flower meristem identity. However, as mentioned above, the role of *AP1* orthologs in petal development requires further examination, with loss-of-function alleles resulting in a conversion of petals into a more foliar identity, the nature of which is not clear, but is not conspicuously carpelloid in most cases. Furthermore, the function of *AP1* orthologs as A class genes must be investigated in basal angiosperms.

One idea to reconcile the ABC model proposed for *Arabidopsis* [129] with the BC models [99, 100] proposed for *Antirrhinum* is that the specification of floral identity and that of sepals are intimately linked, that is, the default state of organs produced by a floral meristem is a sepal-like organ, whose identity can be modified by the additional expression of B and C class genes. For example, one BC scenario that could account for floral organ specification without the requirement for A class if E class genes have some role in organ identity, with B + E class promoting petal identity and E class alone outer perianth identity. Such possibilities should be considered when searching for the "missing" A class in other angiosperms. The status of the other aspect of *Arabidopsis* A class, that responsible for repression of C class in the perianth, in other angiosperms is not clear. Given the disparity in regulatory networks excluding C function from the perianth in *Arabidopsis* (*AP2*) [110] as compared to *Antirrhinum* and Petunia (*miR169*) [122], and the potential for regulatory mechanisms to evolve quickly, functional analyses are required to determine which, if either, is found more broadly throughout the angiosperms.

6 Further Considerations and Conclusions

Degeneracy, a partial functional overlap of structurally distinct elements, is a common feature of biological systems [130–132]. This is quite in contrast to systems designed by human engineers that seek to minimize degenerate functions. Degeneracy supports robustness within biological processes and thus is a key feature of evolvable systems. Theoretical treatments of evolvability suggest that degeneracy is a key source of robustness and is required for a system to be both robust and yet adaptive [131, 132]. Future explorations into the role of degenerate functional modules during development will provide insight into the evolution of developmental structures, as well as the ability of plants to develop properly under fluctuating environmental conditions. Genome-wide analyses of degenerate functional elements offer an opportunity to study the functional integration and independence within the complex hierarchy that supports the development of critical structures such as flowers. Also, modeling approaches that consider "imperfectly engineered" biological solutions will likely provide additional insight into the emergent properties of complex adaptive systems.

The application of current methods—RNA-seq, ChIP-seq, and TRAP-seq (*see* Chapters 18, 23 and 24)—will allow, after considerable effort, an outline of the gene regulatory networks in operation in each cell type of the developing flower. This in turn will enable a much more detailed understanding of the evolution of the regulatory networks that control organ specification and organ growth. It seems likely, as well, that continued application of methods such as live imaging (*see* Chapter 25) and computational modeling (*see* Chapter 26) will result in satisfactory models for the developmental origin of organ numbers and positions, much as such models have been developed for phyllotaxis (e.g., [133, 134]). What current methods and experiments will not supply is the link between gene expression networks and the size and shape of floral organs and of flowers, and in this area—predicting morphological phenotype from genotype—lies a great challenge for the future.

To make this connection we need a better understanding of the consequences of gene network activation on cellular activities, including cell expansion, anisotropy of cell expansion, and cell division. We also need an understanding of the communication between cells that allows the coordination of their expansion and division across tissues, and that limits the direction and amount of these activities to create final size and shape of organs. Recent evidence ties together the chemical signaling that controls domains of gene activity in meristems (e.g., [135, 136]) with the physical interactions of cells as they push on their neighbors due to anisotropic cellular expansion and division (e.g., [133, 137, 138]).

Chemical signals, such as auxin, control cell expansion, and cell expansion controls levels and directions of auxin transport. In the center of this set of feedbacks is the cell wall, as it creates the anisotropy that controls direction of expansion, and therefore, future signaling. This in turn indicates that another critical control is the cytoskeleton, as the microtubule cytoskeleton controls the pattern of cell wall deposition (e.g., [139]).

The path to the future, and to a full understanding of flower development, thus lies in achieving a new level of understanding of cytoskeletal dynamics and cell wall synthesis, and in relating cellular function and cell division to the feedback control of cellular processes through chemical and physical signaling in groups of cells. This new synthesis will require a far more dynamic view of cellular behavior than we have now, and development of computational modeling environments in which the detailed activities of genes, cells, and tissues can interact at multiple levels. If this challenge is met, we will not only understand flower development, but also have a toolbox with which we can understand all of development, plant and animal.

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